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Violacein targets the cytoplasmic membrane of bacteria

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Violacein is a tryptophan-derived purple pigment produced by environmental bacteria which displays multiple biological activities, including strong inhibition of Gram-positive pathogens. Here we applied a combination of experimental approaches to identify the mechanism by which violacein kills Gram positive bacteria. Fluorescence microscopy showed that violacein quickly and dramatically permeabilizes *B. subtilis* and *S. aureus* cells. Cell permeabilization was accompanied by the appearance of visible discontinuities or rips in the cytoplasmic membrane, but it did not affect the cell wall. Using in vitro experiments, we showed that violacein binds directly to liposomes made with commercial and bacterial phospholipids and perturbs their structure and permeability. Furthermore, molecular dynamics simulations were employed to reveal how violacein inserts itself into lipid bilayers. Thus, our combined results demonstrate that the cytoplasmic membrane is the primary target of violacein in bacteria. The implications of this finding for the development of violacein as a therapeutic agent are discussed.

Keywords: violacein, mechanism of action, membrane disruption, membrane-active agent, daptomycin, *Staphylococcus aureus*.

Infectious diseases caused by antimicrobial-multiresistant bacteria are difficult to eradicate and represent an increasing health problem worldwide. Resistance to antibiotics may be (1) intrinsic, as in the case of many Gram-negative bacteria, which carry an outer membrane [1], (2) can occur through the acquisition of genetic elements carrying resistance genes or (3) by mutations in the bacterial genome [2]. Resistance to a new antibiotic is inevitable, but its spread has been favored by poor and excessive use of antibiotics, both in

human health and in agriculture [3]. The antibiotic crisis is also exacerbated by the fact that most of the currently used antibiotics were discovered between 1940 and 1960 [4] and are directed against a limited set of targets. Therefore, the development of new antimicrobials with novel mechanisms of action is an urgent need (World Health Organization – Who/Emp/Iau/2017.12).

Violacein (Figure 1) is a blue-purple pigment, a bisindole formed by the condensation of two molecules of tryptophan. This substance is produced as a secondary metabolite by several phylogenetically distinct bacteria found in diverse environments, such as oceans, glaciers, rivers and soil [5]. Violacein production has been reported in *Collimonas* sp. [6], *Pseudoalteromonas* [7], *Alteromonas* [8], *Duganella* sp. [9] and *Janthinobacterium* [10]. However, the first bacterium described as violacein producer and the most studied thus far is *Chromobacterium violaceum* [11–13]. In *C. violaceum*, violacein production is under the control of quorum sensing mechanisms [5] and the biosynthetic pathway involves a five genes cluster (*vioA, vioB, vioC, vioD and vioE*) transcribed in the same direction [5]. The entire synthesis process consists of five enzymatic (VioA-E proteins) and one non-enzymatic step, the latter corresponding to the conversion of violaceinic acid into violacein by oxidative decarboxylation [5,14].

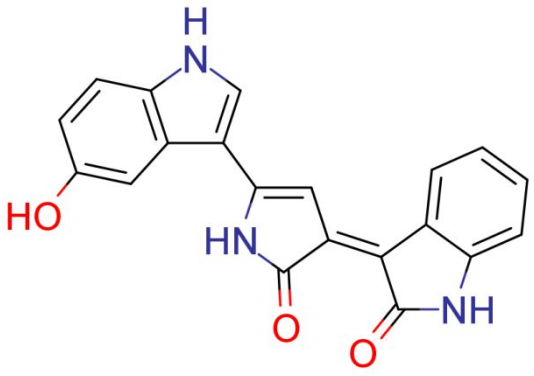


Figure 1. Violacein structure.

Violacein has attracted attention because of its wide spectrum of biological activities. It is a potent antibacterial, the first report of this activity dating back more than 70 years [15]. In addition, violacein displays antifungal [16], antiprotozoal [13,17], antiviral [18], antitumoral [19] and antioxidant [20] activities.

The antibacterial potential of violacein has become more widely explored in recent years. Violacein displays activity against different Gram-positive bacteria, with MICs for *S. aureus* ranging from 1.6 (this work) to 25 μM [21–23]. This large variability in MIC values is likely due to the use of different sources of violacein by different laboratories, some of which were produced in house while others were purchased commercially. Despite this variability, violacein has been shown to be highly active against antibiotic resistant pathogens, such as methicillin-resistant and multidrug-resistant *S. aureus* [24,25].

Violacein seems to be significantly less active against Gram-negative bacteria [21,22,26], but there are at least two reports showing that this molecule can sensitize Gram-negative pathogens of the clinically important ESKAPE group to currently used antibiotics [27], including carbapenemase producers [28]. Thus, violacein could help circumvent the threat of widespread antibiotic resistance. Nevertheless, a crucial step before the real therapeutic potential of violacein can be assessed is the determination of its mechanism of action.

The study of the cell biological effects of antibiotic compounds on bacteria has emerged as a simple and powerful approach to determine their mechanism of action [29]. Here, we applied such an approach to violacein and found that this molecule quickly and dramatically permeabilizes Gram-positive bacterial cells. Cell permeabilization was accompanied by the appearance of visible discontinuities or rips in the cytoplasmic membrane, but it did not affect the cell wall. Using in vitro experiments, we showed that violacein binds directly to liposomes made with commercial and bacterial phospholipids and perturbs their structure and permeability. Furthermore, molecular dynamics simulations were employed to reveal how violacein inserts itself into lipid bilayers. Thus, our combined results demonstrate that the cytoplasmic membrane is the primary target of violacein in bacteria. The implications of this finding for the development of violacein as a therapeutic agent are discussed.

Results

Violacein disrupts the bacterial cytoplasmic membrane

We used fluorescence microscopy and a set of indicator dyes as a way to determine how violacein kills Gram-positive bacteria. Because the microscopy experiments are carried out under cell density conditions different from MIC assays (10^7 cells/ml instead of 5 x 10^5 cells/ml) we first confirmed that the MICs we measured for violacein (0.8 μM for *B. subtilis* and 1.6 μM for *S. aureus*) were also sufficient to inhibit growth of denser cultures (Supplemental Figure S1). Because growth is fully inhibited at higher cell density, we chose to maintain the MIC as the reference concentration in both microscopy and ATP release experiments that will be reported later.

When *Bacillus subtilis* wild-type (PY79) cells were treated with violacein at 0.8 μM, approximately 20% of the cells became permeable to propidium iodide (PI) as early as 30 minutes after treatment. The fluorescence intensity and the number of permeabilized cells further increased after 60 minutes of treatment, with approximately 50% of cells being stained with PI. Interestingly, visualization of the membranes of treated cells, by means of the membrane specific dye FM1-43, showed remarkable alterations, with permeable (PI+) cells often exhibiting patches of stain discontinuity of different sizes, which we called membrane holes (see arrowheads in Figures 2 and 4). Some holes were barely visible at the resolution of the light microscope whereas others were as large as a third of a cell´s length. To confirm that the membrane staining discontinuities were indeed holes we also treated a strain of *B. subtilis* expressing GFP with violacein and observed that the presence of

membrane staining discontinuities perfectly correlated with loss of GFP content (Figure 2B).

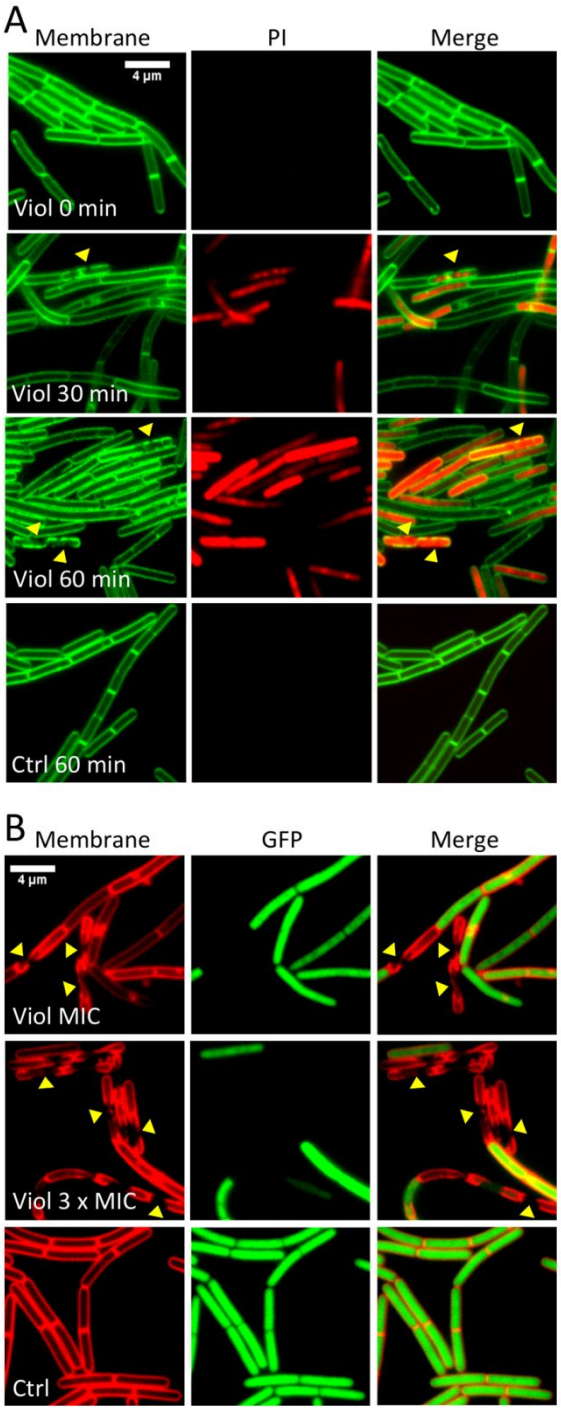


Figure 2. Violacein disrupts the membrane of *B. subtilis*. A. Representative fluorescence microscopy images of wild-type (PY79) *B. subtilis* cells treated at the minimal inhibitory concentration (MIC) of violacein (0.8 µM) and stained with a membrane dye (FM1-43) and propidium iodide (PI). Yellow arrowheads point to holes in the membranes. B. Treatment of a *B. subtilis* strain that expresses cytoplasmic GFP (FG897) shows that cells that have membrane stain discontinuities have lost their GFP content. Note that the membrane stain used in B was FM-4-64, which is red and compatible with GFP. Experiments were repeated at least three times.

Treatment of wild-type *Staphylococcus aureus* (ATCC 29213) cells with violacein at the MIC for this species (1.6 μM) produced results similar to those observed for *B. subtilis*. After 30 minutes of treatment, approximately 25% of the cells were stained with PI, and after 60 minutes this percentage was greater than 50% (Figure 3). However, a difference between the two bacterial species was the absence of visible holes in the membranes of *S. aureus* samples. This may be due to the morphological differences between these two bacteria, since the *B. subtilis* cells are larger (4 to 10 μm in length) and rod-shaped and those of *S. aureus* are smaller (0.5 to 1 μm) and round. For both *B. subtilis* and *S. aureus*, cells from untreated control cultures remained intact for the duration of the assays (less than 1% cells were stained with PI).

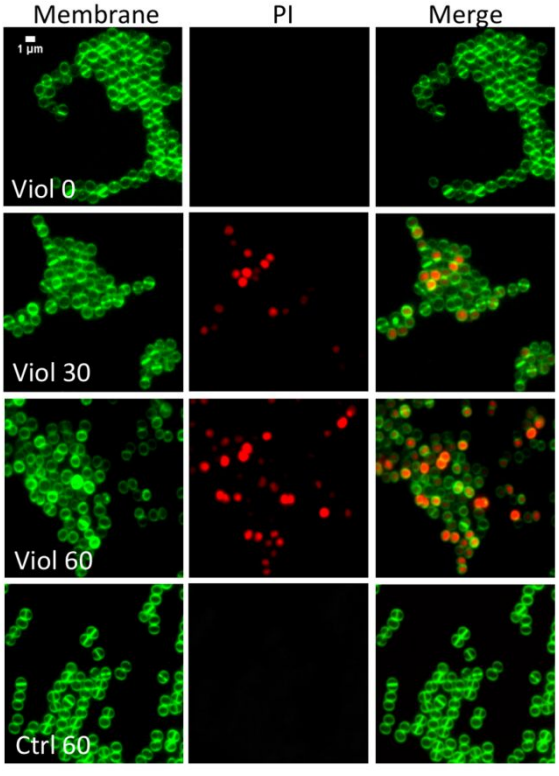
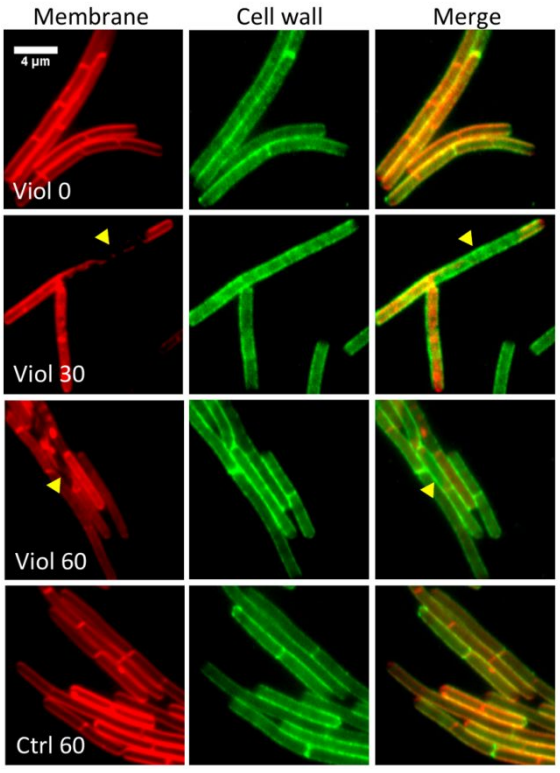


Figure 3. Violacein disrupts the membrane of *S. aureus*. Representative fluorescence microscopy images of wild-type (ATCC 29213) *S. aureus* cells treated at the minimal inhibitory concentration (MIC) of violacein (1.6 μM) and stained with a membrane dye (FM1-43) and propidium iodide (PI). The experiment was repeated three times.

We also treated *B. subtilis* and *S. aureus* with violacein concentrations equivalent to 5-fold their MICs, and, as expected, observed stronger membrane perturbation effects (Supplementary Figure S2 A and B). Under these conditions, PI permeability and membrane damage revealed by FM1-43 were apparent immediately after the treatment and after 30 minutes practically all cells were stained with PI and membrane fluorescence was much dimmer, suggesting a direct effect on this structure. This effect on the membrane stain increased further with time and after one hour of treatment the FM1-43 stain was almost undetectable, indicating that membranes were completely destroyed or dissolved.

An important observation made from the images above is that cells did not lose their normal shape even when fully permeabilized by the violacein treatment, suggesting that this compound does not directly affect the cell wall. To check for more subtle cell wall defects we also stained violacein-treated cells with a cell-wall specific marker (WGA-Alexa Fluor 488), which showed that all cells, including those with heavily damaged membranes, exhibited a pattern of WGA staining that was indistinguishable from untreated cells (Figure 4). These experiments cannot rule out that violacein, by affecting the membrane, will have an indirect effect on cell wall synthesis. However, the fact that violacein treated cells maintain both their normal morphology and their WGA staining pattern is most consistent with the cell wall not being the primary target of violacein.

Figure 4. Cell shape and cell wall staining are not significantly affected by violacein. Representative fluorescence microscopy images of *B. subtilis* cells treated at the minimal inhibitory concentration (MIC) (0.8 μM) of violacein and stained with the red membrane dye (FM4-64) and a cell wall binding lectin (WGA- Alexa Fluor 488). Yellow arrowheads point to cells with damaged membranes but whose cell walls are intact. The experiment was repeated three times.



Violacein promotes fast leakage of ATP from cells

If violacein disrupts the cytoplasmic membrane and makes cells permeable to external dyes, it should also promote the leakage of cellular contents. We tested this possibility by measuring the appearance of ATP in the culture medium at different times after violacein treatment. We used as controls daptomycin, a well-established membrane agent, and ampicillin, whose effect on membrane integrity should be slower, because it depends on growth induced weakening of the cell wall. When *B. subtilis* cells were treated with violacein at 5x its MIC (4 μM) large amounts of ATP became detectable in the

medium almost instantly, and as early as five minutes post-treatment essentially all the ATP from cells was found extracellularly (Figure 5, black line). The ATP leakage effect of violacein was not an artifact of high antibiotic doses because treatment of cells at 1 x MIC also promoted extensive release of ATP, although with a slightly slower kinetics (Figure S3 A). Daptomycin under similar conditions (5 x and 1 x MIC) also promoted fast leakage of ATP from cells (Figure 5, grey line), although, surprisingly, its effect was significantly slower than violacein´s, which could indicate that the two compounds affect the membrane in different ways. Leakage of ATP occurred at an even slower rate upon treatment with ampicillin (Figure 5, red line), as expected from the mode of action of this beta lactam. The kinetics of ATP release by the different antibiotics correlated well with the speed in which they inhibited growth and caused lysis, something that can be inferred from the measure of total ATP in the bacterial cultures in the same experiment (Figure S3, B).

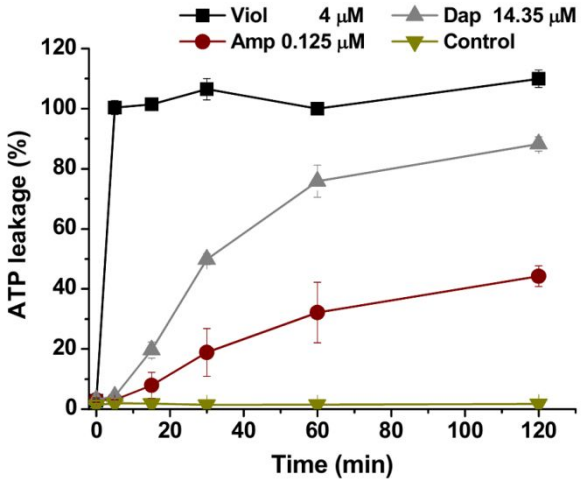


Figure 5. ATP leakage from *B. subtilis* cells after treatment with violacein, daptomycin and ampicillin at concentrations equivalent to 5 x MIC (4 μM, 14.4 μM and 0.125 μM, respectively). A.U.: Arbitrary units. Each point is the mean (± standard deviation, SD) of three different replicate experiments, each performed in triplicate.

Violacein kills stationary cells

Drugs and peptides known to perturb the bacterial membrane effectively kill stationary or dormant cells [30]. This is because nongrowing cells still depend on ATP production and ion transport to maintain their viability. We investigated the effect of violacein on stationary cells and found that treatment with 1 x MIC for 120 minutes caused a 100 fold reduction in viability for *S. aureus* and 1000 fold for *B. subtilis* (Figure 6 A and B). In contrast, ampicillin, had essentially no effect on the viability of stationary cultures of the same bacteria (Figure 6 A and B), as expected of a drug that preferentially kills growing cells. These results demonstrate that violacein´s activity is independent of the metabolic state of the cell, a finding which is also consistent with the hypothesis that it affects the bacterial membrane.

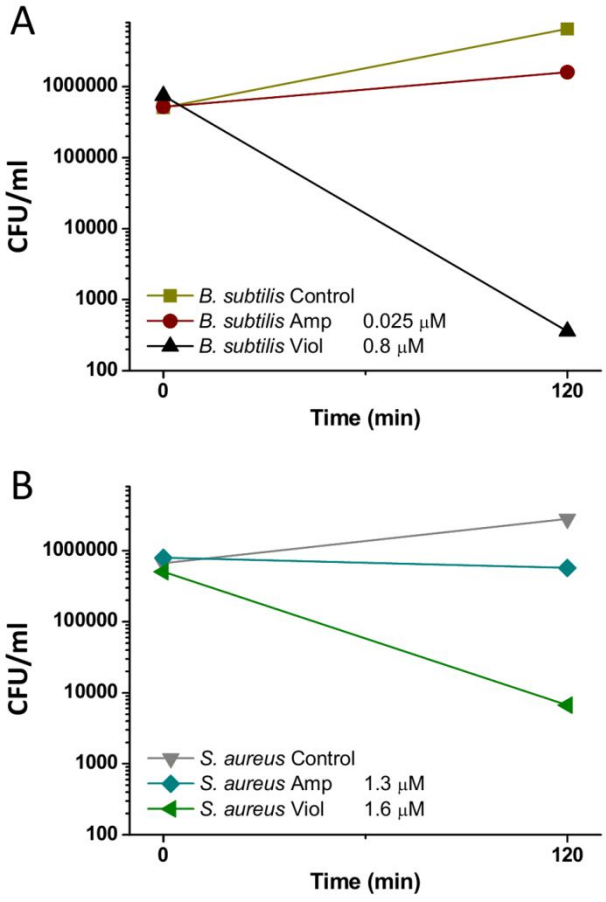


Figure 6. Violacein kills stationary cells. Stationary cells (5 x 10^5/ml) of *B. subtilis* (A) or *S. aureus* (B) were treated with 1 x MIC of violacein or ampicillin for 120 min and viability was assessed by plating.

Violacein binds to LUVs and release their content

The ability of violacein to disrupt the membranes of *B. subtilis* and *S. aureus* could be due to effects on both the lipid and/or the protein components of the bilayer. To distinguish between these possibilities, we evaluated the action of this antimicrobial on large unilamellar vesicles (LUVs) of different lipid compositions.

LUVs were produced with commercial lipids, using mono-component systems or a combination of them: POPC (phosphatidylcholine), POPG (phosphatidylglycerol), POPC: POPG (70:30) and POPC: POPG (50:50). These different compositions were used to explore whether the effect of violacein was related to lipid head-group charge since phosphatidylcholine (POPC) is zwitterionic and phosphatidylglycerol (POPG) has a net negative charge. The effect of violacein on the different LUVs was monitored by dye leakage assays, in which LUVs were previously loaded with carboxyfluorescein (CF).

In preliminary experiments using 20 μM of POPC LUVs we found that 39 μM of violacein produced leakage of 50% of the dye (Supplementary Figure S4A). However, we noticed that the effect of violacein was quite sensitive to the LUV concentration in each experiment. For example, if we used 10 or 40 μM of LUVs the C_{50} of violacein varied to 19 μM and 77 μM, respectively (Supplementary Figure S4A). This is because the effect of membrane perturbing agents generally depends on the accumulation of a threshold level of the damaging agent on target membranes, and this, in turn, is directly affected by the ratio of drug to membrane in each experiment. Thus, we chose to express the effect of violacein in terms of the violacein:lipid molar ratio that led to 50% of CF leakage (V/L_{50}), since this is a normalized measure of activity that does not vary with the initial LUV concentration (Supplementary Figure S4B).

According to the data presented in Figure 7A, violacein has the ability to permeabilize all types of LUVs tested. The V/L_{50} of liposomes of different compositions were quite similar, varying just 2-fold between LUVs made with 100% PG (V/L_{50} = 1.5) and liposomes made with 100% PC (V/L_{50} = 3.1). LUVs made with a mixture of both phospholipids had V/L_{50} values of 1.9 (PC:PG 30:70), 2.3 (PC:PG 50:50) and 1.7 (PC:PG

70:30). This demonstrates that violacein can directly disturb phospholipid membranes. In addition, the similar V/L_{50} values for LUVs of different compositions suggest that the interaction of violacein with membrane lipids is not highly dependent on headgroup charge. For the sake of comparison, cationic antimicrobial peptides such as Magainin exhibit marked preference (10-100 fold lower V/L_{50}) for negatively-charged membranes [31].

We also measured the binding affinity of violacein for the different LUVs by monitoring changes in its intrinsic fluorescence in the presence of lipids (Supplementary Figure S5 and Supplementary Table 1). These experiments showed that violacein bound with highest affinity to liposomes made of 100% PC (K_b of 1,295 M^{-1} in 0 mM salt and 1,381 M^{-1} in 300 mM salt) and with lower affinity to liposomes with a net negative charge (PC:PG 70:30) (K_b of 343 M^{-1} in 0 mM salt and 628 M^{-1} in 300 mM salt). Interestingly, there was no correlation between the leakage activity and binding affinities against the two types of LUVs: although violacein presented higher binding affinity for membranes containing only PC, this antimicrobial presented a greater destabilizing effect on membranes containing anionic phospholipids. This lack of correlation has been observed before and can be explained by LUV permeability being affected not only by the amount of drug bound, but also by factors such as the fluidity and packaging of the bilayer, which vary with different charged headgroups [32].

Tests were also carried out with LUVs made with phospholipids extracted from bacteria, in order to better mimic the membranes of these microorganisms (Figure 7B). In addition to *B. subtilis* and *S. aureus*, we tested LUVs made with *E. coli* lipids, even though this Gram-negative bacterium is resistant to violacein. As shown in Fig. 7B, the LUVs made with bacterial lipids were as affected by violacein as the commercial liposomes, with

V/L_{50} values of 0.9 (*B. subtilis*), 1.4 (*S. aureus*) and 1.5 (*E. coli*). This suggests that violacein does not require non-phospholipid components such as lipid II, to recognize and perturb bacterial membranes. The observation that LUVs produced with *E. coli* phospholipids presented similar values of V/L_{50} as LUVs produced with phospholipids from Gram-positive bacteria also suggests that the outer membrane of Gram-negative bacteria is what makes them insensitive to violacein.

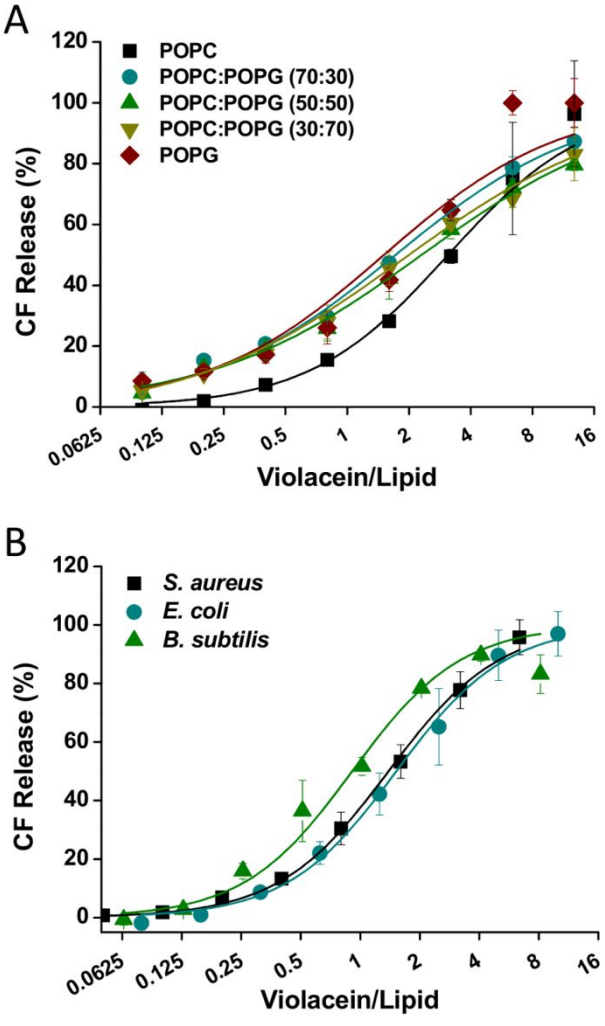


Figure 7. Violacein permeabilizes LUVs. A. Release of CF from LUVS of different composition after 60 minutes of violacein treatment (concentrations ranging from 1 to 256 μM) using LUVs composed of POPC; POPC:POPG (70:30); POPC:POPG (50:50); POPC:POPG (30:70) and POPG. Experiments were performed in 10 mM Tris-HCl buffer, pH 7.4, 300 mM NaCl and lipid concentration of 20 μM, at 37°C. B. Release of CF from LUVs prepared with lipids extracted from different bacterial species (S. aureus ATCC 29213, E. coli ATCC 25922 and B. subtilis PY79) after 60 minutes of violacein treatment. Experiments were performed in

10 mM Tris-HCl buffer, pH 7.4, 300 mM NaCl and lipid concentration of 20 μM, at 37°C..POPC: phosphatidylcholine; POPG: phosphatidylglycerol. Each point is the mean (± standard deviation, SD) of three different replicate experiments, each performed in triplicate..

Simulations of violacein-membrane interactions

To gain further insight into how violacein interacts with the bacterial membrane, molecular dynamics (MD) simulations were performed in which we followed the insertion of a single violacein molecule into membrane models with different lipid headgroups (POPC, POPG and POPC:POPG 5:5). In 200 ns of simulation, we observed that violacein penetrates lipid bilayers and becomes predominantly inserted in the interfacial area between the lipid head group and the aliphatic chains (Figures 8 A-E and movies S1, S2 and S3). This can be clearly seen in plots of the location of violacein and landmark atoms of the phospholipid molecules integrated over the duration of the MD trajectories, which showed that violacein exhibits a mass number density peak (meaning a high probability of finding the molecule in that position) that matches the location of the first carbon in the acyl chains of the phospholipids and is also close to the phosphate peak (Figure 8 B-E). Importantly, the position of violacein did not change substantially in membranes of different composition. However, the violacein peak was broader in POPG, probably because fluidity is higher due to electric repulsion of the headgroups, leading to higher mobility of violacein within this membrane.

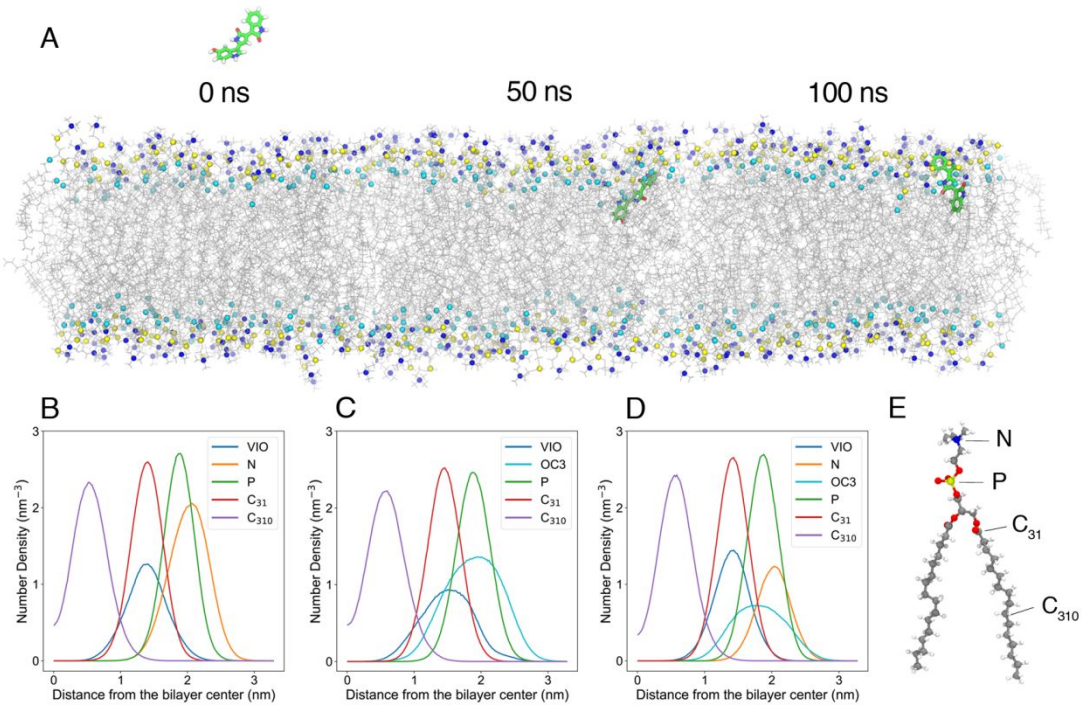


Figure 8. Molecular dynamics (MD) simulations of violacein interacting with bilayers of different lipid compositions. A. Three snapshots at different time points (0, 50, 100 ns) of violacein interacting with a 100% POPC bilayer. Violacein is represented in spacefill (green). Silver: POPC acyl chains; Cyan: carbonyl carbon from sn1 chain; Yellow: phosphate phosphorus; Blue: choline nitrogen. Movies of the complete simulation, and for the simulations with POPG, are available in the Supporting Information. B. Number density plot of the distribution of violacein in a 100% POPC bilayer. C. Number density plot of the distribution of violacein in a 100% POPG (phosphatidylglycerol) bilayer. D. Number density plot of the distribution of violacein in a POPC: POPG (50-50%) bilayer. E. POPC molecule highlighting the reference atoms of this phospholipid tracked in the number density plots. OC3 refers to the POPG glycerol oxygen and is not present in POPC.

Discussion

The urgent need for therapeutic alternatives capable of fighting multiresistant bacteria has triggered renewed interest in the identification of new antimicrobial compounds. Here we focused on violacein, a natural product whose antimicrobial activity was first described long time ago [15], but that was only explored extensively as an antimicrobial in the last decade [21,27].

Previous studies have described violacein´s spectrum of antibacterial activity and synergy with other antibiotics [21,27]. In contrast, the goal of our work was to identify the target and mode of action of violacein. By applying a combination of cytological, biochemical and biophysical assays we generated results that demonstrate unequivocally that the primary target of violacein in bacteria is the cytoplasmic membrane.

Fluorescence microscopy showed that violacein disrupts the membrane of *B. subtilis* and *S. aureus* when used at MIC concentrations. The effect was quite fast (30 minutes) and became essentially instantaneous at higher violacein concentrations (5 x MIC) (Supplementary Figure S2). Using a specific membrane dye (FM1-43), we could clearly see that violacein produces “holes” or “rips” in the membranes of *B. subtilis* at 1 x MIC (Figure 2). The fact that this effect could not be observed in *S. aureus* is probably due the differences in the shape and/or size of cells, which make membrane holes easier to visualize in *B. subtilis*. At higher violacein concentrations (5 x MIC), the membranes of both *B. subtilis* and *S. aureus* were essentially dissolved (Supplementary Figure S2). Another way to demonstrate membrane permeabilization by violacein was to measure ATP leakage out of treated cells. Violacein promoted massive leakage of ATP from cells and this happened

as fast as the membrane permeabilization seen by microscopy (Figure 5). Finally, as expected from a membrane damaging agent [30,33], violacein was equally effective against growing and nongrowing cells (Figure 6). Therefore, violacein belongs to a heterogenous group of antimicrobials that target the bacterial membrane such as nisin [34], daptomycin [35], the vancomycin derivatives telavancin [36] and oritavancin [37], and DCAP [33], among others.

Antibiotics that disrupt the bacterial cytoplasmic membrane are generally lipophilic, being able to directly interact with the membrane, causing changes in its functions and physical structure [30]. Simply inspecting violacein´s structure and solubility (predicted LogP of 2.75 – http://zinc.docking.org/substances/ZINC000014829611/) suggests that it should indeed interact with membranes. To confirm this and to better characterize the specificity of violacein-membrane interaction we carried out in vitro experiments with model membranes. These experiments showed that violacein binds and affects the permeability of LUVs of different composition with similar efficiency. Violacein exhibited somewhat higher affinity to zwitterionic than to anionic LUVs (Table S1), probably because violacein itself is a neutral molecule, but this higher affinity for neutral LUVs does not translate into higher permeabilizing activity, since LUVs made with 100% anionic phospholipids were slightly easier to disrupt than neutral LUVs, requiring two-fold less violacein to release half of their content (Figure 7A). In contrast, well-studied antibacterial membrane damaging agents, such as classic antimicrobial peptides, nisin and daptomycin, exhibit marked preference for anionic membranes [38,39]. Another important observation of the in vitro experiments was that violacein affected LUVs made with bacterial lipids as well as it affected those made with commercial lipids. This suggests that violacein does not recognize non-phospholipid components of the bacterial membrane, like lipid II, which significantly

increases the binding of molecules such as nisin and oritavancin to the bacterial membrane [40,41]. Finally, molecular dynamics simulations showed that violacein inserts itself in the interface between the polar headgroups and the hydrophobic core of lipid bilayers, a finding supported by experimental observations with indole and other tryptophan analogs structurally similar to violacein, which were shown to occupy the same location within the membrane as violacein in our simulations[42] . Because the simulations also showed that violacein insertion was not affected by the lipid headgroup (Figure 8 and movies S1, S2 and S3), the overall conclusion from the in vitro and computational experiments is that violacein is a relatively non-selective membrane damaging agent. This is in agreement with the broad biological activities of this natural product, which has been described to affect a variety of prokaryotic and eukaryotic cells [21,27,43,44].

The results from the in vitro experiments and MD simulations also allowed us to propose a mechanism for how violacein perturbs membranes. By using equation 2 and the binding constants determined by fluorescence experiments we estimated that under the typical conditions of the CF release experiments (20 μM of total POPC and 32 μM of violacein) ca. 1.9 % of the total violacein is bound to the bilayers, which translates to a ratio of one violacein for every 35 POPC molecules. This low ratio, together with violacein's low solubility in water and low amphiphilicity, suggests that the mechanism of LUV permeabilization does not consist in a detergent-like membrane solubilization. Instead, we propose that the interaction of violacein with the interfacial region of the bilayer changes the distances and alters the packing of the phospholipids, causing defects and discontinuities (thinning, transient holes and/or phase separation) that allow the passage of molecules across the membrane. This mechanism is supported by experimental work with

other hydrophobic or amphiphilic small molecules that, similarly to violacein, were shown to occupy the interfacial region when partitioned within bilayers[45–49]. In vivo, the turgor pressure of cells may further stress the bilayer and cause it to rupture and produce the holes documented in our microscopy experiments. However, it must be noted that such drastic alterations of membrane structure are not necessary for violacein to exert its effects, as cell permeabilization often happens in the absence of visible membrane holes.

While this work was under way, de Souza *et al.* [50] showed that violacein was able to interact with Langmuir monolayers made of dipalmitoylphosphatidylserine (DPPS) and dipalmitoylphosphatidylcholine (DPPC), representing tumorigenic and healthy cells, respectively. However, in this study, violacein was not able to permeabilize LUVs composed of DPPC, DPPS and POPC. This apparent discrepancy with our results is likely due to differences in the experimental conditions employed by the two groups. For example, the highest violacein to lipid ratio used by de Souza *et al.* [50] was 0.5, a ratio which we also found insufficient to significantly permeabilize LUVs (Figures 7A and B). Therefore, we predict that if de Souza *et al*. [50] had done their experiments at higher violacein to lipid concentration they too would have concluded that violacein can permeabilize membranes.

Recently, Aruldass *et al.* [25] published results claiming that violacein acts on the *S. aureus* membrane. However, this paper analyzed violacein effects 12 and 48 hours after treatment. At such late time points it is impossible to determine if effects on membrane structure and permeability are direct or indirect. In fact, Aruldass *et al.* [25] observed very similar results for violacein and vancomycin in all their assays and it is well known that vancomycin does not directly permeabilize membranes. Moreover, Aruldass *et al.* [25] did not

provide any evidence, in vitro or otherwise, of a direct interaction of violacein and phospholipids. Thus, this paper´s conclusion is not supported by careful interpretation of its results.

The discovery that violacein is a membrane targeting compound should set the stage for future research on the usefulness of this natural product. The membrane is an attractive and underexploited target of antimicrobials. In contrast to the antibiotics currently used such as β-lactams, aminoglycosides, tetracyclines, and quinolones, which are effective only against actively growing cells, membrane perturbation can kill slow growing and persister cells, and eradicate biofilms, all of which are important sources of treatment failure [30,51]. Violacein is a molecule that conforms to Lipinski rules and is as potent against some clinically relevant strains of Gram positive bacteria as daptomycin and, thus, could expand the limited arsenal of membrane targeting antibiotics. However, the observation that violacein seems to bind bacterial membranes rather non-specifically has important implications for its development as a therapeutic. In fact, the multiple biological activities of violacein, from antiviral to antitumor, and its reported hemolytic activity [17,44], are strong indications that this natural product is capable of disrupting membranes of eukaryotic cells as well. Thus, the challenge going forward with the development of violacein as an antibiotic would be to more systematically determine if it can display selectivity against bacterial cells. The question of selectivity is not as simple as it may seem: as explained above, the activity of membrane perturbing agents like violacein is strongly affected by the ratio of drug to lipids (or drug to cells) employed in the experiments, and these ratios are not usually taken into account in the literature (see Matsuzaki [52] for an insightful discussion of selectivity with focus on antimicrobial peptides). Thus, even though the activity of

violacein has been measured against a variety of bacteria and eukaryotic cells, these results are not comparable because the experiments were likely carried out with widely different drug to lipid ratios.

Despite the lack of systematic toxicity studies, there is at least one report of the therapeutic use of violacein in vivo, e.g., in the mouse model of *Plasmodium* infection[17], suggesting that violacein is not overly toxic. Even if violacein is found to be too toxic in its natural form to be of therapeutic value, it should be possible to use medicinal chemistry to modify it into a more discriminating membrane targeting agent. In support of this idea, a recent publication showed that deoxyviolacein is much more selective than violacein against protozoa, when compared with mammalian host cells [44]. Another way to circumvent toxicity to the host would be by employing adequate formulation and delivery strategies. In either case, knowing that violacein targets membranes should help direct and interpret these experiments.

Methods

Bacterial strains and reagents

The bacterial strains used were *B. subtilis* PY79 [53], *S. aureus* ATCC 25923, a methicillin-sensitive strain (MSSA), and *E. coli* ATCC 25922, both obtained from the American Type Culture Collection. Violacein and ampicillin were purchased from Sigma, FM1-43, FM4-64, WGA-Alexa Fluor 488 and propidium iodide were purchased from ThermoFisher and daptomycin was purchased from Selleck Chemicals. Violacein was dissolved in DMSO to create 20mM or 2 mM stock solutions, depending on the experiment.

Fluorescence microscopy

Sample preparation for fluorescence microscopy analysis was performed according to [54]. Bacterial cultures were grown until OD_{600} = 0.1 and violacein (Sigma Aldrich, St. Louis, MO, USA) was added at the desired concentration. At appropriate times after violacein treatment, cells were concentrated 10-fold by centrifugation and stained for 1 minute with 1 μg/ml of FM1-43 and 10 μg/ml of propidium iodide to evaluate membrane damage and permeability. For cell wall integrity, cells were stained with 1 μg/ml of FM4-64 and 10 μg/ml of WGA-Alexa Fluor 488. Stained cells were imaged using a Nikon Eclipse TiE microscope equipped with a Plan APO VC Nikon 100X objective (NA=1.4), a 25-mm Smart Shutter and Andor EM CCD i-Xon camera.. Each assay was repeated at least three times.

MIC determinations

MICs were determined by twofold broth microdilution according to CLSI guidelines [55]. Bacteria were grown in Muller Hinton broth or cation adjusted Muller Hinton broth in the case of daptomycin. For violacein and ampicillin, similar MIC values were obtained whether bacteria were grown in Muller Hinton or in LB broth. Inoculum density was approximately 5 x 10^5 CFU/mL and the MIC was determined as the lowest concentration capable of inhibiting visual growth of the tested strains after 18-24 hours incubation at 37°C. The values of the MICs measured for the different antibiotics against *B. subtilis* and *S. aureus* are shown in Table 1.

Table 1. MICs (μM) measured in this work. ND, not determined.

	ampicillin	Daptomycin	violacein
B. subtilis	0.025	2.9	0.8
S. aureus	1.3	ND	1.6

Time-kill of stationary cells

Cultures were grown for 20 hours in Mueller Hinton broth to reach stationary phase, washed twice in PBS (pH 7.4), pelleted (4000 x g for 15 minutes at 4ºC) and diluted to a density of approximately 5 x 10^5 CFU/ mL in nutrient-depleted medium (depMH). This spent medium was prepared by removing the cells of a 48-hour bacterial growth in Mueller Hinton broth using sequential centrifugation (5000 x g, 10 minutes at 4°C) and filtration (0.45 μm-pore-size) steps.

Diluted stationary cells were treated with MIC concentrations of antibiotics: (1.6 and 0.8 μM of violacein and 1.3 and 0.025 μM of ampicillin for *S. aureus* and *B. subtilis*, respectively). Bacterial cultures without treatment were used as negative controls. Aliquots of 0.1 mL were withdrawn immediately after (0 min) and 120 minutes after antibiotic addition, serially diluted in saline solution and plated (25 μL) on MH Agar. MH plates were incubated overnight at 37°C for determination of the CFU/mL. The experiment was repeated three times.

Measurement of ATP leakage from cells

Leakage of ATP from cells was evaluated using the BacTiter – Glo Microbial Cell Viability Assay Reagent (Promega, Madison, WI). *B. subtilis* cells were grown in LB until the OD_{600} reached 0.5, diluted to OD_{600} 0.1 (approximately 10^7 cells/ml) and treated with antibiotics in concentrations equivalent to 1 x and 5 x the MIC. ATP determination in culture supernatant is usually done by separating cells from the medium by centrifugation[56] but control experiments in our laboratory showed that standard centrifugation (16,200 x g for one minute) caused significant leakage of ATP from cells. Therefore, we chose to use filtration through 0.22 μm –pore-size filters as the way to separate cells from medium because our controls showed that this procedure caused no leakage of ATP from cells. For each condition, we sampled an aliquot of the culture before filtration to measure total ATP and filtered another aliquot through the 0.22 μm filter to measure ATP in the supernatant. 50 μL of the total culture or filtered supernatant were then mixed with 50 μL of BacTiter – Glo Reagent directly in a 96-well opaque plate (Costar, Corning) and incubated for five minutes before reading in a SpectraMax M2 plate reader (Molecular Devices) at 560 nm. ATP leakage was expressed as a percentage of total ATP measured at the same condition

and time point (ATP leakage = 100 x ATP in supernatant/total ATP). The experiment was performed in triplicate and repeated three times.

Large Unilamellar Vesicle (LUV) Preparation

Lipids used to prepare LUVs were from two different sources: commercial lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Lipids extracted from bacterial cultures were obtained as described previously [57,58]. In brief, cells were grown in LB until OD_{600} = 0.1 and harvested by centrifugation at 4800 x g for 15 minutes at 4°C. The pellet was re-suspended with 50 mL of chloroform, methanol and water (2:1:0.8, v/v) and samples were shaken vigorously for approximately 1 minute and maintained for at least 24 hours at -20°C with occasional shaking. Solvent was separated from bacterial mass by filtration with filter paper and the bottom (organic) phase of the biphasic system was collected. The solvent was evaporated under nitrogen until completely dried.

LUVs composed of 100% of phosphatidylcholine, 30:70, 50:50 and 70:30 phosphatidylcholine:phosphatidylglycerol and 100% phosphatidylglycerol were made as described previously [59]. Initially, the lipids were homogenized in chloroform, which was evaporated with nitrogen for complete removal of the solvent and subjected to vacuum for two hours. Then, a 50 mM solution of carboxyfluorescein (CF) in Tris HCl 10 mM and NaCl 300 mM (pH 7.4) was added to the lipid film to form an emulsion that was sonicated for approximately 2 minutes to encapsulate the CF. LUVs were made by extrusion of the lipid emulsion through two stacked polycarbonate filters with 100 nm pore size (Nuclepore, Maidstone, UK), using a mini-extruder (Avanti Polar Lipids, Alabama, US). External CF

was removed by gel-filtration on a Sephadex G-25 mini-column (GE Healthcare) with Tris-HCl 10 mM and NaCl 300 mM as eluent. The lipid content of the LUV preparations was quantified by measuring inorganic phosphate, following the procedure of Rouser *et al.* [60].

Carboxifluorescein leakage by violacein

LUV permeabilization was investigated as described by Mojsoska et al. (2017) [59]. Due to the high concentration used in this experiment, CF is self-quenched in the internal aqueous compartment of the LUVs. When an added external compound damages the LUVs bilayer, the CF is released to the external aqueous compartment and its fluorescence increases. Violacein was serially diluted in 96-well- microdilution plates at a range of 256 to 0.125 μM in 10 mM Tris-HCl, pH 7.4, NaCl 300 mM and the LUV suspension was added to each well to a final concentration of 20 μM of lipids in 150 μL. Final concentration of DMSO (from violacein stock) in samples was no greater than 0.6%, a concentration that does not interfere on CF leakage experiments. Wells containing only LUVs and 10 mM Tris-HCl, pH 7.4, NaCl 300 mM were used as negative control. The fluorescence was determined using a multi-mode microplate reader (BioTek Synergy™ H1 Hybrid Reader) immediately after LUV addition, with excitation and emission wavelengths of 490 and 520 nm, respectively, every one minute, for 1 hour at 37°C. At the end of the time course, 2 μL of Triton X-100 (10%) was added to all wells to obtain the maximum CF release. CF leakage was calculated using the following equation:

$$R\% = 100 \times [(F_v - F_o) / (F_{Max} - F_o)] \quad \text{Equation 1}$$

where F_o and F_v represent the initial and final values of fluorescence before and after violacein addition, respectively. F_{Max} is the fluorescence value after the addition of Triton X-100, representing maximal LUV damage. The experiment was performed in triplicate and repeated three times.

Binding of violacein to LUVs

Fluorescence spectra of violacein with different LUVs were collected in a Hitachi F-7000 spectrofluorimeter (Hitachi, Japan), exciting the samples at 350 nm and recording the emission from 680 to 720 nm. Emission scanning speed was 240 nm/min, with the photomultiplier at 700 V and using excitation and emission slits of 2.5 nm, at room temperature. Violacein concentration was 10 μM in 10 mM Tris-HCl buffer, pH 7.4, NaCl 300 mM, and lipid concentration was varied from 0.16 to 22.2 mM. Lipids were re-suspended in 10 mM Tris-HCl buffer, pH 7.4, NaCl 300 mM, and extruded as described above to obtain 100 nm LUVs. Spectra of water and the vesicles without violacein taken under the same conditions were subtracted from violacein's spectra for correction.

Violacein' affinity for the different LUVs was calculated considering a two-state model of the violacein (free and bound forms). The following Hill equation was fitted to the binding isotherms to determine the lipid concentration where half of total amount of violacein is bound and the apparent binding constant (K_B, M^{-1}). Experiment was repeated three times.

$$\text{Fraction bound} = [\text{Lipid}]^n / (1/K)^n + [\text{Lipid}]^n \qquad \text{Equation 2}$$

Molecular dynamics simulations

The force field used for violacein molecule was GAFF [61], and Slipids [62,63] for the membranes. We performed 200 ns simulations with violacein in three conditions: in the presence of POPC, POPG and POPC:POPG (50 : 50) bilayers. In all simulations, one violacein molecule was positioned on one side of the bilayer, approximately 2 nm above the phosphorus atoms. Membranes composed of 64 lipids per monolayer were hydrated in 55 water molecules per lipid ratio with the TIP3P water model. POPG lipids had their negative charges counter-balanced with Na^+ ions**.** Prior to simulation, the bilayers were thermalized for 500 ns. The temperature was maintained at 37°C using the V-rescale thermostat (with frequency of 1 ps) and semi-isotropic pressure coupling at 1 bar with the Parinello-Rahman barostat, in a frequency of 10 ps. The simulations were carried out using the GROMACS 5.0.2 [64,65] package and visual inspections and renderings of images by VMD 1.9.2 [66].

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: XXXXXXXXXX.

Growth curve of violacein effect at high cell density; fluorescence microscopy of violacein at 5 x MIC; ATP release experiment controls; CF release experiment controls; Violacein binding affinity raw data and calculated constants.

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References

(1) Cox, G., and Wright, G. D. (2013) Intrinsic antibiotic resistance: Mechanisms, origins, challenges and solutions. *Int. J. Med. Microbiol. 303*, 287–292. DOI:10.1016/j.ijmm.2013.02.009.

(2) Dever, L. A., and Dermody, T. S. (1991) Mechanisms of bacterial resistance to antibiotics. *Arch. Intern. Med. 151*, 886–95. DOI: 10.1001/archinte.1991.00400050040010.

(3) Ventola, C. L. (2015) The antibiotic resistance crisis: part 2: management strategies and new agents. *P T 40*, 344–52.

(4) Walsh, C. T., and Wencewicz, T. A. (2014) Prospects for new antibiotics: A molecule-

centered perspective. *J. Antibiot. (Tokyo). 67*, 7–22. DOI: 10.1038/ja.2013.49.

(5) Durán, N., Justo, G. Z., Durán, M., Brocchi, M., Cordi, L., Tasic, L., Castro, G. R., and Nakazato, G. (2016) Advances in Chromobacterium violaceum and properties of violacein-Its main secondary metabolite: A review. *Biotechnol. Adv. 34*, 1030–1045. DOI:10.1016/j.biotechadv.2016.06.003.

(6) Hakvåg, S., Fjærvik, E., Klinkenberg, G., Borgos, S. E. F., Josefsen, K. D., Ellingsen, T. E., and Zotchev, S. B. (2009) Violacein-producing Collimonas sp. from the sea surface microlayer of costal waters in Trøndelag, Norway. *Mar. Drugs 7*, 576–588. DOI: 10.3390/md7040576.

(7) Matz, C., Webb, J. S., Schupp, P. J., Phang, S. Y., Penesyan, A., Egan, S., Steinberg, P., and Kjelleberg, S. (2008) Marine biofilm bacteria evade eukaryotic predation by targeted chemical defense. *PLoS One 3*, 1–7. DOI: 10.1371/journal.pone.0002744.

(8) McCarthy, S. A., Johnson, R. M., Kakimoto, D., and Sakata, T. (1985) Effects of various agents on the pigment (violacein) and antibiotic production of *Alteromonas luteoviolacea*. *Bull. Japanese Soc. Sci. Fish. 51*, 1115–1121. DOI: 10.2331/suisan.51.1115.

(9) Wang, H., Jiang, P., Lu, Y., Ruan, Z., Jiang, R., Xing, X. H., Lou, K., and Wei, D. (2009) Optimization of culture conditions for violacein production by a new strain of Duganella sp. B2. *Biochem. Eng. J. 44*, 119–124. DOI: 10.1016/j.bej.2008.11.008.

(10) Pantanella, F., Berlutti, F., Passariello, C., Sarli, S., Morea, C., and Schippa, S. (2007) Violacein and biofilm production in Janthinobacterium lividum. *J. Appl. Microbiol. 102*, 992–999. DOI: 10.1111/j.1365-2672.2006.03155.x.

(11) Boisbaudran, P.-É. L. de. (1882) Matière colorante se formant dans la colle de farine. *C. R. Hebd. Seances Acad. Sci. 94*, 562–563.

(12) Vasconcelos, A. T. R. de, Almeida, D. F. de, Hungria, M., Guimarães, C. T., Antônio, R. V., Almeida, F. C., Almeida, L. G. P. de, Almeida, R. de, Alves-Gomes, J. A., Andrade, E. M., Araripe, J., Araújo, M. F. F. de, Astolfi-Filho, S., Azevedo, V., Baptista, A. J., Bataus, L. A. M., Batista, J. da S., Beló, A., Berg, C. van den, Bogo, M., Bonatto, S., Bordignon, J., Brigido, M. M., Brito, C. A., Brocchi, M., Burity, H. A., Camargo, A. A., Cardoso, D. das D. de P., Carneiro, N. P., Carraro, D. M., Carvalho, C. M. B., Cascardo, J. C. de M., Cavada, B. S., Chueire, L. M. O., Creczynski-Pasa, T. B., Cunha-Junior, N. C. da, Fagundes, N., Falcão, C. L., Fantinatti, F., Farias, I. P., Felipe, M. S. S., Ferrari, L. P., Ferro, J. A., Ferro, M. I. T., Franco, G. R., Freitas, N. S. A. de, Furlan, L. R., Gazzinelli, R. T., Gomes, E. A., Gonçalves, P. R., Grangeiro, T. B., Grattapaglia, D., Grisard, E. C., Hanna, E. S., Jardim, S. N., Laurino, J., Leoi, L. C. T., Lima, L. F. A., Loureiro, M. de F., Lyra, M. do C. C. P. de, Madeira, H. M. F., Manfio, G. P., Maranhão, A. Q., Martins, W. S., Mauro, S. M. Z. di, Medeiros, S. R. B. de, Meissner, R. de V., Moreira, M. A. M., Nascimento, F. F. do, Nicolás, M. F., Oliveira, J. G., Oliveira, S. C., Paixão, R. F. C., Parente, J. A., Pedrosa, F. de O., Pena, S. D. J., Pereira, J. O., Pereira, M., Pinto, L. S. R. C., Pinto, L. da S., Porto, J. I. R., Potrich, D. P., Ramalho-Neto, C. E., Reis, A. M. M., Rigo, L. U., Rondinelli, E., Santos, E. B. P. do, Santos, F. R., Schneider, M. P. C., Seuanez, H. N., Silva, A. M. R., Silva, A. L. da C. da, Silva, D. W., Silva, R., Simões, I. de C., Simon, D., Soares, C. M. de A., Soares, R. de B. A., Souza, E. M., Souza, K. R. L. de, Souza, R. C., Steffens, M. B. R., Steindel, M., Teixeira, S. R., Urmenyi, T., Vettore, A., Wassem, R., Simpson, A. Z., and Andrew John George. (2003) The Complete Genome

Sequence of Chromobacterium violaceum Reveals Remarkable and Exploitable Bacterial Adaptability. *Proc. Natl. Acad. Sci. U. S. A. 100*, 11660–11665. DOI: 10.1073/pnas.1832124100.

(13) Durán, N., and Menck, C. F. M. (2001) Chromobacterium violaceum: A review of pharmacological and industiral perspectives. *Crit. Rev. Microbiol. 27*, 201–222. DOI: 10.1080/20014091096747.

(14) Dantas, C., Volpe, P. L. O., Durán, N., and Ferreira, M. M. C. (2012) The violacein biosynthesis monitored by multi-wavelength fluorescence spectroscopy and by the PARAFAC method. *J. Braz. Chem. Soc. 23*, 2054–2064. DOI: 10.1590/S0103-50532012005000083.

(15) Lichstein, H. C., and Van de Sand, V. F. (1945) Violacein, an antibiotic pigment produced by Chromobacterium violaceum. *J. Infect. Dis. 76*, 47–51. DOI: 10.1093/infdis/76.1.47.

(16) Brucker, R. M., Harris, R. N., Schwantes, C. R., Gallaher, T. N., Flaherty, D. C., Lam, B. A., and Minbiole, K. P. C. (2008) Amphibian chemical defense: Antifungal metabolites of the microsymbiont Janthinobacterium lividum on the salamander Plethodon cinereus. *J. Chem. Ecol. 34*, 1422–1429. DOI: 10.1007/s10886-008-9555-7.

(17) Lopes, S. C. P., Blanco, Y. C., Justo, G. Z., Nogueira, P. A., Rodrigues, F. L. S., Goelnitz, U., Wunderlich, G., Facchini, G., Brocchi, M., Duran, N., and Costa, F. T. M. (2009) Violacein extracted from Chromobacterium violaceum inhibits Plasmodium growth in vitro and in vivo. *Antimicrob. Agents Chemother. 53*, 2149–2152. DOI: 10.1128/AAC.00693-08.

(18) Andrighetti-Fröhner, C. R., Antonio, R. V., Creczynski-Pasa, T. B., Barardi, C. R. M., and Simões, C. M. O. (2003) Cytotoxicity and Potential Antiviral Evaluation of Violacein Produced by Chromobacterium violaceum. *Mem. Inst. Oswaldo Cruz 98*, 843–848. DOI: 10.1590/S0074-02762003000600023.

(19) De Azevedo, M. B. M., Alderete, J., Rodriguez, J. A., Souza, A. O., Rettori, D., Torsoni, M. A., Faljoni-Alario, A., Haun, M., and Durán, N. (2000) Biological Activities of Violacein, a New Antitumoral Indole Derivative, in an Inclusion Complex with β-Cyclodextrin. *J. Incl. Phenom. Macrocycl. Chem. 37*, 93–101. DOI: 10.1023/A:1008138807481.

(20) Konzen, M., De Marco, D., Cordova, C. A. S., Vieira, T. O., Antônio, R. V., and Creczynski-Pasa, T. B. (2006) Antioxidant properties of violacein: Possible relation on its biological function. *Bioorganic Med. Chem. 14*, 8307–8313. DOI: 10.1016/j.bmc.2006.09.013.

(21) Cazoto, L. L., Martins, D., Ribeiro, M. G., Durán, N., and Nakazato, G. (2011) Antibacterial activity of violacein against Staphylococcus aureus isolated from Bovine Mastitis. *J. Antibiot. (Tokyo). 64*, 395–397. DOI: 10.1038/ja.2011.13.

(22) Wang, H., Wang, F., Zhu, X., Yan, Y., Yu, X., Jiang, P., and Xing, X. H. (2012) Biosynthesis and characterization of violacein, deoxyviolacein and oxyviolacein in heterologous host, and their antimicrobial activities. *Biochem. Eng. J. 67*, 148–155. DOI: 10.1016/j.bej.2012.06.005.

(23) Mojib, N., Philpott, R., Huang, J. P., Niederweis, M., and Bej, A. K. (2010) Antimycobacterial activity in vitro of pigments isolated from Antarctic bacteria. *Antonie*

van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 98, 531–540. DOI: 10.1007/s10482-010-9470-0.

(24) Choi, S. Y., Yoon, K. H., Lee, J. Il, and Mitchell, R. J. (2015) Violacein: Properties and production of a versatile bacterial pigment. *Biomed Res. Int. 2015*. DOI: 10.1155/2015/465056.

(25) Aruldass, C. A., Masalamany, S. R. L., Venil, C. K., and Ahmad, W. A. (2017) Antibacterial mode of action of violacein from Chromobacterium violaceum UTM5 against Staphylococcus aureus and methicillin-resistant Staphylococcus aureus (MRSA). *Environ. Sci. Pollut. Res. 25*, 5164–5180. DOI: 10.1007/s11356-017-8855-2.

(26) Aruldass, C. A., Rubiyatno, Venil, C. K., and Ahmad, W. A. (2015) Violet pigment production from liquid pineapple waste by Chromobacterium violaceum UTM5 and evaluation of its bioactivity. *RSC Adv. 5*, 51524–51536. DOI: 10.1039/c5ra05765e.

(27) Subramaniam, S., Ravi, V., and Sivasubramanian, A. (2014) Synergistic antimicrobial profiling of violacein with commercial antibiotics against pathogenic micro-organisms. *Pharm. Biol. 52*, 86–90. DOI: 10.3109/13880209.2013.815634.

(28) Asencio, G., Lavin, P., Alegría, K., Domínguez, M., Bello, H., González-Rocha, G., and González-Aravena, M. (2014) Antibacterial activity of the Antarctic bacterium Janthinobacteriumsp. SMN 33.6 against 2 multi-resistant Gram-negative bacteria. *Electron. J. Biotechnol. 17*, 1–5. DOI: 10.1016/j.ejbt.2013.12.001.

(29) Nonejuie, P., Burkart, M., Pogliano, K., and Pogliano, J. (2013) Bacterial cytological profiling rapidly identifies the cellular pathways targeted by antibacterial molecules. *Proc.*

Natl. Acad. Sci. 110, 16169–16174. DOI: 10.1073/pnas.1311066110.

(30) Hurdle, J. G., O´Neill, A. J., Chopra, I., and Lee, R. E. (2011) Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. *Nat. Rev. Microbiol. 9*, 62–75. DOI: 10.1038/nrmicro2474.

(31) Matsuzaki, K., Harada, M., Handa, T., Funakoshi, S., Fujii, N., Yajima, H., and Miyajima, K. (1989) Magainin 1-induced leakage of entrapped calcein out of negatively-charged lipid vesicles. *BBA - Biomembr. 981*, 130–134. DOI: 10.1016/0005-2736(89)90090-4.

(32) Matsuzaki, K., Harada, M., Funakoshi, S., Fujii, N., and Miyajima, K. (1991) Physicochemical determinants for the interactions of magainins 1 and 2 with acidic lipid bilayers. *BBA - Biomembr. 1063*, 162–170. DOI: 10.1016/0005-2736(91)90366-G.

(33) Eun, Y.-J., Foss, M. H., Kiekebusch, D., Pauw, D. A., Westler, W. M., Thanbichler, M., and Weibel, D. B. (2012) DCAP: A broad-spectrum antibiotic that targets the cytoplasmic membrane of bacteria. *J. Am. Chem. Soc. 134*, 11322–11325. DOI: 10.1021/ja302542j.

(34) Ruhr, E., and Sahl, H. G. (1985) Mode of action of the peptide antibiotic nisin and influence on the membrane potential of whole cells and on cytoplasmic and artificial membrane vesicles. *Antimicrob. Agents Chemother. 27*, 841–845. DOI: 10.1128/AAC.27.5.841.

(35) Silverman, J. a, Perlmutter, N. G., Howard, M., and Shapiro, H. M. (2003) Correlation of daptomycin bactericidal activity and membrane depolarization in Staphylococcus aureus.

Antimicrob. Agents Chemother. 47, 2538–2544. DOI: 10.1128/AAC.47.8.2538.

(36) Higgins, D. L., Chang, R., Debabov, D. V, Leung, J., Wu, T., Krause, K. M., Sandvik, E., Hubbard, J. M., Schmidt, D. E., Gao, Q., Cass, R. T., Karr, D. E., Benton, B. M., and Humphrey, P. P. (2005) Telavancin , a Multifunctional Lipoglycopeptide , Disrupts both Cell Wall Synthesis and Cell Membrane Integrity in Methicillin-Resistant Staphylococcus aureus. *Antimi 49*, 1127–1134. DOI: 10.1128/AAC.49.3.1127.

(37) Belley, A., McKay, G. A., Arhin, F. F., Sarmiento, I., Beaulieu, S., Fadhil, I., Parr, T. R., and Moeck, G. (2010) Oritavancin disrupts membrane integrity of Staphylococcus aureus and vancomycin-resistant enterococci to effect rapid bacterial killing. *Antimicrob. Agents Chemother. 54*, 5369–5371. DOI: 10.1128/AAC.00760-10.

(38) El-Jastimi, R., and Lafleur, M. (1997) Structural characterization of free and membrane-bound nisin by infrared spectroscopy. *Biochim. Biophys. Acta - Biomembr. 1324*, 151–158. DOI: 10.1016/S0005-2736(96)00221-0.

(39) Mozsolits, H., Wirth, H. J., Werkmeister, J., and Aguilar, M. I. (2001) Analysis of antimicrobial peptide interactions with hybrid bilayer membrane systems using surface plasmon resonance. *Biochim. Biophys. Acta 1512*, 64–76. DOI: 10.1016/S0005-2736(01)00303-0.

(40) Breukink, E., Wiedemann, I., Van Kraaij, C., Kuipers, O. P., Sahl, H. G., and De Kruijff, B. (1999) Use of the cell wail precursor lipid II by a pore-forming peptide antibiotic. *Science (80-.). 286*, 2361–2364. DOI: 10.1126/science.286.5448.2361.

(41) Münch, D., Engels, I., Müller, A., Reder-Christ, K., Falkenstein-Paul, H., Bierbaum,

G., Grein, F., Bendas, G., Sahl, H. G., and Schneider, T. (2015) Structural variations of the cell wall precursor lipid II and their influence on binding and activity of the lipoglycopeptide antibiotic oritavancin. *Antimicrob. Agents Chemother. 59*, 772–781. DOI: 10.1128/AAC.02663-14.

(42) Yau, W. M., Wimley, W. C., Gawrisch, K., and White, S. H. (1998) The preference of tryptophan for membrane interfaces. *Biochemistry 37*, 14713–14718. DOI: 10.1021/bi980809c.

(43) Sasidharan, A., Sasidharan, N. K., Amma, D. B. N. S., Vasu, R. K., Nataraja, A. V., and Bhaskaran, K. (2015) Antifungal activity of violacein purified from a novel strain of Chromobacterium sp. NIIST (MTCC 5522). *J. Microbiol. 53*, 694–701. DOI: 10.1007/s12275-015-5173-6.

(44) Bilsland, E., Tavella, T. A., Krogh, R., Stokes, J. E., Roberts, A., Ajioka, J., Spring, D. R., Andricopulo, A. D., Costa, F. T. M., and Oliver, S. G. (2018) Antiplasmodial and trypanocidal activity of violacein and deoxyviolacein produced from synthetic operons. *BMC Biotechnol. 18*, 1–8. DOI: 10.1186/s12896-018-0428-z.

(45) Scheidt, H. A., Pampel, A., Nissler, L., Gebhardt, R., and Huster, D. (2004) Investigation of the membrane localization and distribution of flavonoids by high-resolution magic angle spinning NMR spectroscopy. *Biochim. Biophys. Acta - Biomembr. 1663*, 97–107. DOI: 10.1016/j.bbamem.2004.02.004.

(46) Ingólfsson, H. I., Thakur, P., Herold, K. F., Hobart, E. A., Ramsey, N. B., Periole, X., De Jong, D. H., Zwama, M., Yilmaz, D., Hall, K., Maretzky, T., Hemmings, H. C., Blobel, C., Marrink, S. J., Koçer, A., Sack, J. T., and Andersen, O. S. (2014) Phytochemicals

perturb membranes and promiscuously alter protein function. *ACS Chem. Biol. 9*, 1788–1798. DOI: 10.1021/cb500086e.

(47) Boggara, M. B., Mihailescu, M., and Krishnamoorti, R. (2012) Structural association of nonsteroidal anti-inflammatory drugs with lipid membranes. *J. Am. Chem. Soc. 134*, 19669–19676. DOI: 10.1021/ja3064342.

(48) Środa, K., Michalak, K., Maniewska, J., Grynkiewicz, G., Szeja, W., Zawisza, J., and Hendrich, A. B. (2008) Genistein derivatives decrease liposome membrane integrity - Calcein release and molecular modeling study. *Biophys. Chem. 138*, 78–82. DOI: 10.1016/j.bpc.2008.09.010.

(49) Sun, S., Sendecki, A. M., Pullanchery, S., Huang, D., Yang, T., and Cremer, P. S. (2018) Multistep Interactions between Ibuprofen and Lipid Membranes. *Langmuir 34*, 10782–10792. DOI:10.1021/acs.langmuir.8b01878.

(50) de Souza, K. D., Perez, K. R., Durán, N., Justo, G. Z., and Caseli, L. (2017) Interaction of violacein in models for cellular membranes: Regulation of the interaction by the lipid composition at the air-water interface. *Colloids Surfaces B Biointerfaces 160*, 247–253. DOI: 10.1016/j.colsurfb.2017.09.027.

(51) Epand, R. M., Walker, C., Epand, R. F., and Magarvey, N. A. (2016) Molecular mechanisms of membrane targeting antibiotics. *Biochim. Biophys. Acta - Biomembr. 1858*, 980–987. DOI: 10.1016/j.bbamem.2015.10.018.

(52) Matsuzaki, K. (2009) Control of cell selectivity of antimicrobial peptides. *Biochim. Biophys. Acta - Biomembr. 1788*, 1687–1692. DOI: 10.1016/j.bbamem.2008.09.013.

(53) Schroeder, J., and Simmons, L. (2013) Complete genome sequence of Bacillus subtilis strain PY79. *Genome Announc. 1*, 2164. DOI: 10.1128/genomeA.01085-13.

(54) Pulschen, A. A., Sastre, D. E., Machinandiarena, F., Crotta Asis, A., Albanesi, D., de Mendoza, D., and Gueiros-Filho, F. J. (2017) The stringent response plays a key role in Bacillus subtilis survival of fatty acid starvation. *Mol. Microbiol. 103*, 698–712. DOI: 10.1111/mmi.13582.

(55) CLSI. (2015) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. Approved Standard - Tenth Edition. CLSI Document M07-A10. Wayne, PA.

(56) Mempin, R., Tran, H., Chen, C., Gong, H., Kim Ho, K., and Lu, S. (2013) Release of extracellular ATP by bacteria during growth. *BMC Microbiol. 13*, 1–13. DOI: 10.1186/1471-2180-13-301.

(57) Folch, J., Less, M., and Sloane Stanley, G. H. (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem. 226*, 497–509.

(58) Bligh, E. G., and Dyer, W. J. (1959) A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. *Can. J. Biochem. Physiol. 37*, 911–917. DOI: 10.1139/o59-099.

(59) Mojsoska, B., Carretero, G., Larsen, S., Mateiu, R. V., and Jenssen, H. (2017) Peptoids successfully inhibit the growth of gram negative E. coli causing substantial membrane damage. *Sci. Rep. 7*, 1–12. DOI: 10.1038/srep42332.

(60) Rouser, G., Fleischer, S., and Yamamoto, A. (1970) Two Dimensional Thin Layer

Chromatographic Separation of Polar Lipids and Determination of Phospholipids by Phosphorus Analysis of Spots. *Lipids 5*, 494–496. DOI: 10.1007/BF02531316.

(61) Wang, J., Wolf, R. M., Caldwell, J. W., Kollman, P. A., and Case, D. A. (2004) Development and Testing of a General Amber Force Field. *J Comput Chem 25*, 1157–1174. DOI:10.1002/jcc.20035.

(62) Jä Mbeck, J. P. M., and Lyubartsev, A. P. (2012) An Extension and Further Validation of an All-Atomistic Force Field for Biological Membranes. *J. Chem. Theory Comput. 8*, 2938–2948. DOI: 10.1021/ct300342n.

(63) Jä Mbeck, J. P. M., and Lyubartsev, A. P. (2013) Another Piece of the Membrane Puzzle: Extending Slipids Further. *J. Chem. Theory Comput. 9*, 774–784. DOI: 10.1021/ct300777p.

(64) Van Der Spoel, D., Lindahl, E., Hess, B., Groenhof, G., Mark, A. E., and Berendsen, H. J. C. (2005) GROMACS: Fast, flexible, and free. *J. Comput. Chem.* DOI: 10.1002/jcc.20291.

(65) Abraham, M. J., Murtola, T., Schulz, R., Páll, S., Smith, J. C., Hess, B., and Lindahl, E. (2015) GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX 12*, 19–25. DOI: 10.1016/j.softx.2015.06.001.

(66) Humphrey, W., Dalke, A., and Schulten, K. (1996) VMD: Visual molecular dynamics. *J. Mol. Graph. 14*, 33–38. DOI: 10.1016/0263-7855(96)00018-5.

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