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Review

Supramolecular pore formation as an antimicrobial strategy

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ABSTRACT

Although synthetic amphiphiles that form ion-conducting pores have surged in interest within the chemical community, their potential as antimicrobials is little appreciated. The formation of pores in bacterial membranes has at least two obvious consequences. First, insertion of the amphiphile into the bacterial membrane results in membrane disruption or disorganization, enhancing membrane permeability. Second, to the extent that ion-conducting pores form within the membranes, ion homeostasis is likely to be affected. Changes in ion balance will have secondary effects on cytosolic proteins that may adversely affect bacterial survival.

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1. Introduction

The emergence of penicillin during the second world war was followed by a growing range of derivatives and other antimicrobial agents [1]. Medicinal chemists have prepared numerous derivatives of this remarkable β-lactam and many other antibiotic families have been discovered [2]. In many, if not most, cases antibiotics have been discovered from natural sources, such as the protective secretions of bacteria themselves. As a result of such discoveries, many new and potent antibiotic compounds appeared by the 1960s and these seemed to have conquered bacterial disease. Sadly, overuse of antibiotics, incomplete patient regimens, and bacterial genetics have led to resistance. A limited antibiotic pipeline is also a concern [3]. Currently, resistance to all of the clinically used antibiotics has been reported.

The antibiotic resistance crisis has been trumpeted by warnings issued by both the Centers for Disease Control and Prevention (CDC) [4] and the World Health Organization (WHO) [5]. The problem of resistance is paramount, but an exacerbating factor is a limited pipeline of new antimicrobials. New discovery techniques [6] have permitted culturing of several novel antibiotics and such

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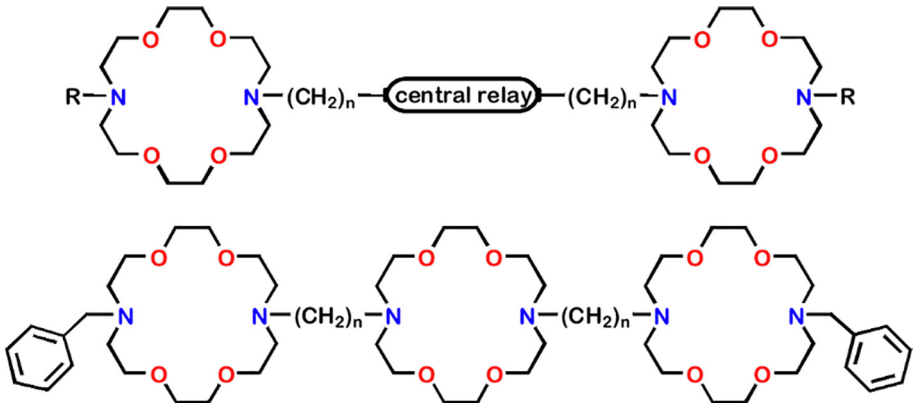

Fig. 1. Top: General hydraphile structure showing the terminal, spacer chain, and central relay modules. Bottom: The benzyl hydraphile family, n = 6–20.

developments are clearly of great value. However, gene transfer between bacteria is likely to lead to resistance, no matter the microbial source or the antibiotic type [4a].

The present article discusses an approach to antimicrobial efficacy different from conventional discovery efforts. The compounds presented here were not discovered, but were designed and prepared to penetrate membranes and to exhibit at least some of the properties known for protein ion channels [7]. The quest for compounds that could transport ions through membranes began with many examples of ion binding agents whose host guest complexes could diffuse through liquid or bilayer membranes [8]. Early examples of synthetic pore-formers reported transport of such metal cations as Co^{2+} [9]. By the very early 1990s, however, several examples of synthetic amphiphiles that formed pores to transport biologically important alkali metal cations were reported [10]. The design approaches to these molecules differed, but more detailed studies followed that confirmed their successful channel-like function. Subsequently, numerous imaginative designs have led to families of pore-formers that are highly varied in structure and function [11].

Our contribution to the alkali-metal ion transport effort resulted in the family of molecules we call hydraphiles [12]. These compounds have the overall structure shown at the top of Fig. 1. A number of macrocyclic rings and other residues have been incorporated into the module designated as "central relay." The spacer chain lengths ranged from n = 6 to n = 20. As discussed below, both chemical and biological function varied with chain length. The groups shown as "R" have varied from *n*-dodecyl to dansyl [13] to benzyl. Arguably the most studied hydraphiles are the compounds shown below in the general structure, which incorporates the latter as the terminal residues. The most active ion transporters in the dibenzyl, three diaza-18-crown-6 family that have been prepared thus far have n = 12 and n = 14.

Extensive biophysical studies have confirmed the following characteristics of such active compounds as the C_{12} benzyl hydraphile. (1) They insert into liposomal membranes and transport alkali metal cations [14]. (2) The transport is 4-fold selective for Na^+ over K^+.[12] (3) Transport is blocked by Ag^+.[12] (4) Transport efficacy correlates to bilayer membrane thickness: longer hydraphiles are more active in thicker membranes than are shorter hydraphiles, which may be inactive [15]. (5) The hydraphiles form unimolecular channels [16]. (6) Planar bilayer voltage clamp ("BLM") experiments show open-close behavior typical of some protein channels. The hydraphiles form robust channels that show open-close behavior for several hours in BLM experiments [13]. (7) The hydraphiles functioned as channels in vital cells (patch clamp experiment) [17]. (8) Photomicrographs of a fluorescent hydraphile showed localization of the channel within *E. coli*'s boundary layer [18]. (9) The disruption of ion homeostasis in bacteria resulted in antimicrobial activity of hydraphiles [19].

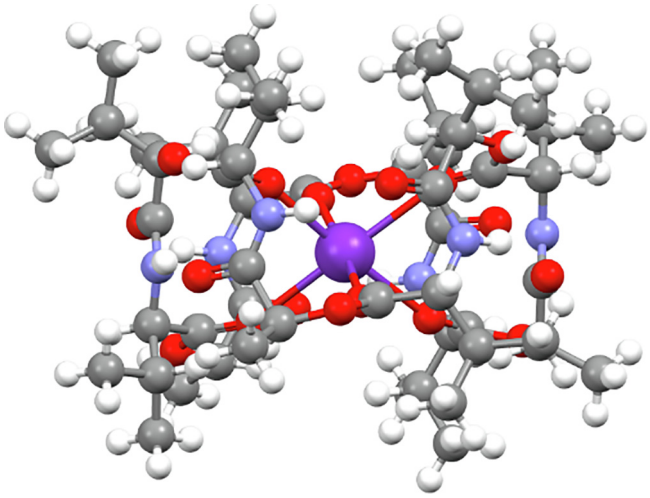

Fig. 2. Structure of the valinomycin•K^+ complex from the Cambridge Structural Database, code: VALINK.

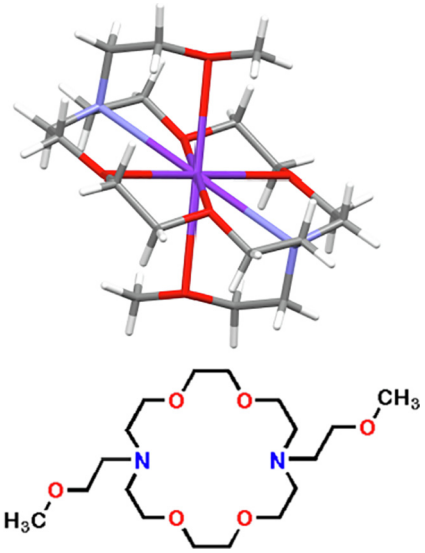

Fig. 3. Crystal structure (CSD:DUGHIW) of the potassium cation complex of the illustrated lariat ether.

Precursors to the hydraphile family of compounds were the lariat ethers [8], also developed in our laboratory. These compounds emerged from a desire to mimic the natural ion carrier known as valinomycin. This cyclododecadepsipeptide has the structure [(L-lactate)-(L-valine)-(D-hydroxyisovalerate)-(D-valine)]$_3$ [20]. The alternating D,D-L,L-stereochemistry allows the molecule to bind K^+ ion with high selectivity over Na^+ by forming what has been called a "tennis ball seam" complex about the cation [21]. This structure permits both enveloping complexation of the cation while retaining high binding dynamics. A structure rendered by Mercury software is shown in the ball and stick metaphor and was rendered from CSD VALINK (Fig. 2).

Crown ethers bind alkali metal cations in such polar solutions as water or methanol with high complexation and decomplexation rates [22]. The result is modest binding, but high dynamics. In contrast, cryptands bind cations with modest rates and release bound cation very slowly [23]. The result is high complexation strength, but poor dynamics. The concept in developing lariat ethers was to merge the high binding dynamics of crown ethers with one or more additional donor group-bearing side arms that could provide a flexible third dimension. Quite a range of compounds was prepared by us and by others [24]. The ion carrier ability of these compounds was extensively explored. The three-dimensional complexation of cations was confirmed by solid state structure analysis for both one- and two-armed structures (Fig. 3).

Fig. 3 shows *N,N*-di-2-methoxyethyl-4,13-diaza-18-crown-6 in a structural drawing and its corresponding KI complex (CSD: DUGHIW). Potassium resides snugly within the macrocycle and each of the 2-methoxyethyl sidearms provide apical oxygen donors to augment complexation. Like valinomycin, the potassium cation is enveloped by the ring and sidearms, but there is no covalent secondary bridge to hinder dynamics. It should be noted that the conformation shown in Fig. 3 is possible because the side arms are attached to invertable nitrogen. This means that they may interact from the same or opposite sides of the macrocycle. If the side arms were attached to macroring carbons, their stereochemistry would be fixed either on one side or on opposite sides of the cycle.

It was only recently discovered that *N,N*'-di-*n*-alkyldiaza-18-crown-6 compounds form ion conducting pores in bilayer membranes [25]. The earlier ion carrier studies were conducted on compounds in which the side arm or side arms contained donor atoms such as ether oxygens. A typical lariat ether having such donor-group-embedded side arms is shown in the lower panel of Fig. 3. The sidearms in the pore-forming lariat ethers lack side arm donor groups and exhibited distinctly different behavior in bilayer membranes. Rather than functioning as carriers within an asolectin membrane, which may also occur, channels exhibiting distinct open-close behavior were apparent. The solid state structure of a potassium iodide complex of *N,N*'-didodecyl-4,13-diaza-18-crown-6 (CSD: HUTGUY) is shown in Fig. 4. Note that iodide anion interacts with the ring-bound cation through a water molecule and the non-donor side arms align with each other. When represented in the space-filling metaphor, it is clear that the chains pack very closely, supporting the proposed aggregation mechanism for ion conduction.

The solid-state structures shown in Fig. 4 are for the didodecyl lariat ether, represented in shorthand as C_{12} < N18N > C_{12} or simply as C_{12}LE [26]. The planar bilayer conductance studies were performed with a close analog, C_{11} < N18N > C_{11}. The lariat ethers were added to planar asolectin membranes and currents were recorded for the transport of cations. Analysis of the planar bilayer traces showed that the pores formed by the dialkyl lariat ethers were trimeric, tetrameric, or pentameric. Pores formed from different numbers of lariat monomers formed aggregates having internal diameters of different sizes. By comparing molecular models and the conductances associated with trimer, tetramer, and pentamer structures, the sizes of the internal channels formed by the three different sizes of aggregates could be estimated. This was particularly interesting because the cations did not pass through the crown ethers, but rather the aggregate pore. This mechanism is more akin to the barrel stave model [27] than to the unimolecular hydraphile transporter. Perhaps this is not remarkable as even valinomycin has been reported to form channels in ultrathin membranes [28].

Fig. 5 shows planar bilayer voltage clamp traces for *N,N*'-*bis*(undecyl)-4,13-diaza-18-crown-6, a dialkyl lariat ether. The baseline indicates no channel activity, *i.e.*, the channels are closed. The flat-topped peaks indicate open channels and current flowing. In one case, more than one channel is open. An irregular peak indicates an unstable or dynamic pore. This occurs when pores form by different levels of monomer self-assembly. Details of such experiments are beyond the scope of this article, but may be found in Refs. [12] and [13].

The remainder of this article deals with the remarkable array of biological activity shown by lariat ethers and hydraphiles. These two families of compounds appear to share a similar ability to penetrate bilayer membranes and to alter both permeability and ion homeostasis. The result is that both families of compounds show a range of biological activities.

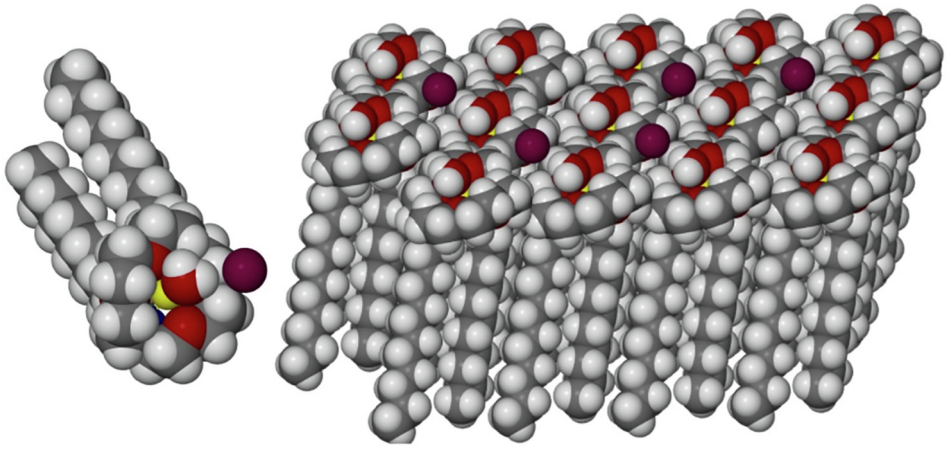

Fig. 4. The solid state structure of *N,N*'-didodecyl-4,13-diaza-18-crown-6•KI in the CPK metaphor (left) and packed in the solid matrix (right).

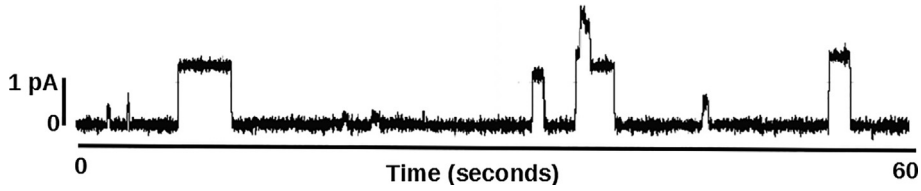

Fig. 5. Planar bilayer trace for *N,N'-bis*(undecyl)-4,13-diaza-18-crown-6 recorded in asolectin bilayer membranes.[26] The applied potential is 30 mV.

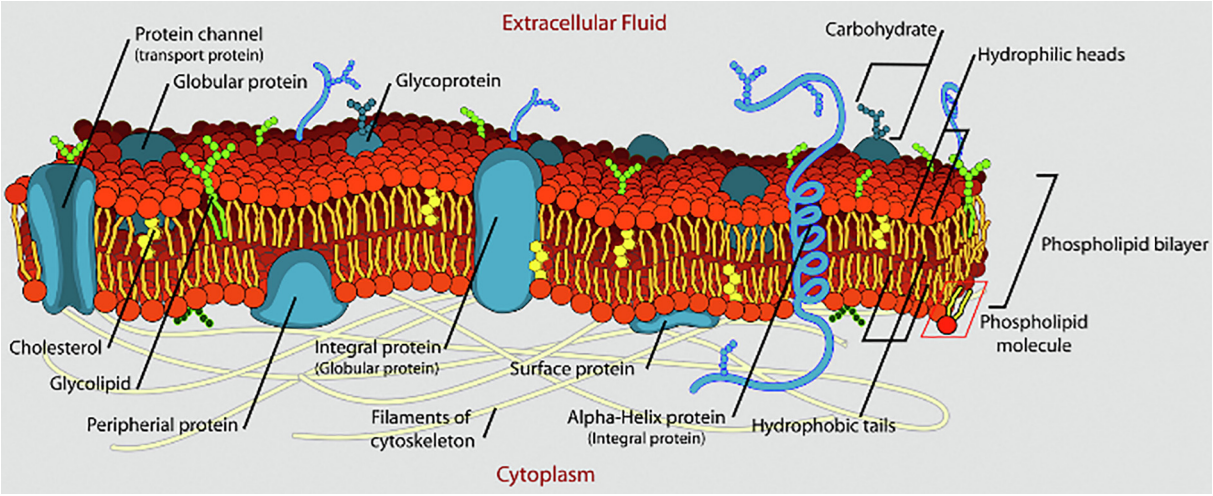

Fig. 6. Image of a cellular bilayer illustrating the numerous components present in addition to the phospholipids.[43]

2. Artificial and natural membranes

The bulk of the studies conducted to understand the ion conducting properties of lariat ethers and hydraphiles used artificial or reconstituted membranes. Two common membrane systems were phosphatidylcholine/phosphatidic acid and soybean asolectin. These have been used both in liposomal and planar bilayer forms. Ion release from liposomes may be conducted in a variety of ways. Various dyes that influx or efflux may be detected or the movement of a cation such as $^{7}Li^{+}$ of $^{23}Na^{+}$ may be observed directly by NMR [29]. The planar bilayer voltage clamp method directly observes the transport of charge through a membrane such as asolectin [30].

Mammalian membranes are far more complex than the artificial membranes often illustrated in introductory chemical textbooks. Monomers in these membranes are typically drawn as a circle with two lines pendant. Such illustrations are sometimes referred to the "lollipop model." A much more realistic image is shown in Fig. 6, which includes the many components that are present in addition to phospholipid monomers.

Bacterial membranes present an entirely different problem. For the present discussion, such membranes fall into three categories: Gram negative, Gram positive, and mycobacterial. The cytosol of Gram positive organisms is encompassed by a plasma membrane and a peptidoglycan cell wall. In addition, techoic acid chains extend from the plasma membrane through the peptidoglycan layers. Mycobacterial membranes are also Gram positive, but are more complex. In the membranes of such organisms as *Mycobacterium tuberculosis*, the cytosol is bounded by a mycolic acid layer. The long chains of mycolic acid fold into a third, and relatively impermeable layer.

The cytosol of Gram negative bacterial membranes is bounded by an inner lipid bilayer, a periplasmic space, and a peptidoglycan layer. The latter is thinner than in Gram positive bacteria. A second, outer bilayer membrane has lipopolysaccharides on its outer surface. Another difference between Gram positive and negative membranes is that the latter contains porins in the outer membrane layer. The porins are β-barrel proteins within the membrane that permit the passage of various molecules through it (Fig. 7).

The complexity of natural bacterial membranes compared to completely synthetic or even such membranes as soybean asolectin prevents a direct comparison between the two types. Moreover, mycobacterial membranes are significantly different from either Gram negative or positive membranes. Remarkably, however, planar bilayer model studies do give an indication of channel-like function for synthetic amphiphiles in bacteria. To the extent they are available for the amphiphiles discussed, they will be noted along with the biological activity.

3. Biological studies

We have been interested in two types of biological activity. First, we wished to evaluate the potency of various amphiphiles against a range of organisms. Second, we queried whether combinations of synthetic amphiphiles with FDA approved antimicrobials would be beneficial. In addition to our own lariat ether studies, biological activity of many novel structures has been evaluated. Comparisons among the various structural types is complicated by differences in organisms and the different types of test that have been reported. These have been extensively reviewed in Ref. [31]. In general, crown ether compounds that show activity are more potent against Gram positive than Gram negative organisms.

Potencies are typically expressed as minimum inhibitory concentrations or MICs [32]. They may be reported either in μM or in μg/mL. The latter is more often used in clinical reports and the former in the chemical/bioorganic literature. For comparison, a compound having a molecular weight of 400 Daltons and a MIC

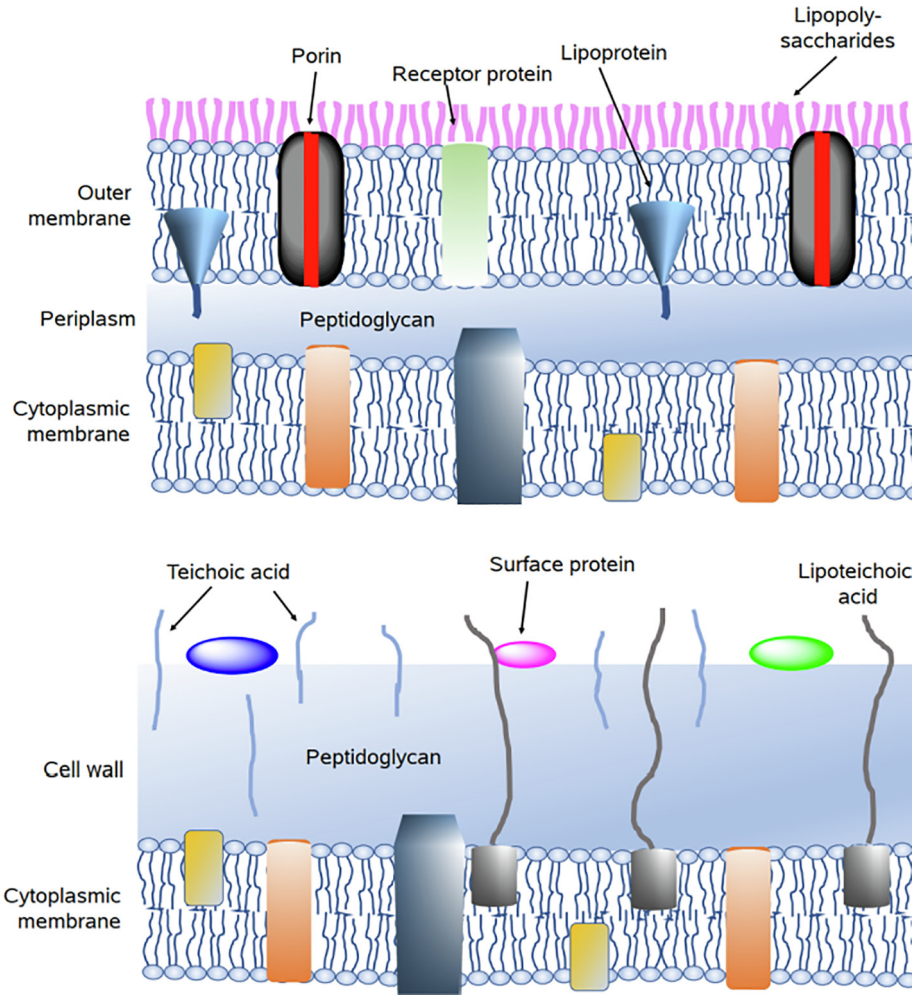

Fig. 7. Schematic representation of Gram negative (top) and Gram positive bacterial membranes. This diagram was adapted from https://laboratoryinfo.com/ gram-staining-principle-procedure-interpretation-and-animation/.

of 4 μg/mL, converts/translates to a MIC of 40 μM. The use of μg/mL helps to evaluate solubility. However, potencies that are expressed in μM units may be compared directly without considering molecular weight differences between or among compounds.

It should be noted that Okahara, Kato, and coworkers, who reported early examples of lariat ethers, investigated the biological potency of a range of compounds against several organisms [33]. In particular, they studied C-alkyl-substituted crown ethers and *N*-alkylazacrowns. For convenience, we may represent 15-crown-5 as <15>. *N,N*'-Didecyldiaza-18-crown-6 can be abbreviated as C_{10} < N18N > C_{10}. Okahara *et al.* reported MICs in the range of 2.5–5 μg/mL for *n*-dodecyl-15-crown-5 and *n*-decyl-18-crown-6 against *Bacillus subtilis*, *Bacillus cereus*, and *Staphylococcus aureus* (all Gram positive). The *N*-alkyl crowns that were studied, <15N> $C_{10,12}$ and <18N> $C_{10,12}$ showed lower potency.

4. Lariat ether potency

Our initial study of lariat ether bioactivity involved *N,N*'-di-*n*-alkyldiaza-18-crown-6 compounds. A series of compounds was prepared having the structure C*n* < N18N > C*n* where n was 8, 10, 12, 14, 16, and 18 [34]. We represent these compounds using the further shorthand C_nLE. Their potencies were evaluated against the bacteria *Escherichia coli* and *B. subtilis*. Only the *n*-octyl, *n*-decyl, and *n*-dodecyl compounds showed activity. Against Gram positive *B. subtilis*, the MICs were C_8LE = 28 μM, C_{10}LE = 2.8 μM, and C_{12}LE = 2.5 μM. Against Gram negative *E. coli*, the respective MICs were >200 μM, 11 μM, and >200 μM.

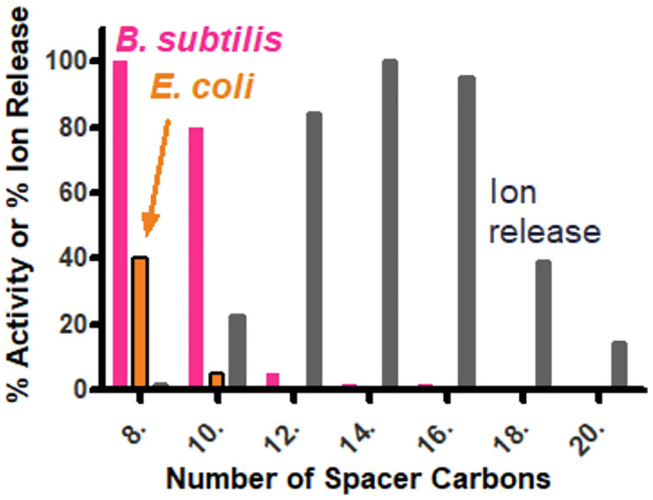

Fig. 8. Comparison of ion release by hydraphiles (dark bar) of varied chain lengths with their potency against Gram positive *B. subtilis* (red bar) and Gram negative *E. coli*. (orange bar). Each value represents a minimum of three separate experiments. Error bars are omitted for clarity. No biological data was recorded for n = 18–20.

A number of physicochemical variables were assessed in an effort to correlate structure and activity. Sodium release from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dierucoyl-*sn*-glycero-3-phosphocholine (DEPC) liposomes was assessed by using a sodium-selective electrode. Fractional Na^+ release from either DOPC or DEPC vesicles was ~0.6 for C_8LE, ~1 for C_{10}LE, and ~0.8 for C_{12}LE. No significant cation release was detected for lariat ethers having alkyl side arms ranging from 14 to 18 carbons [36]. A membrane depolarization study using C_{10}LE and the membrane dye 3,3′-dipropylthiadicarbocyanine [DiSC3 (5)] confirmed similar effects in ion balance for both *B. subtilis* and *E. coli*.

5. Hydraphile potency

The hydraphiles also showed biological activity. They were studied to determine their activity against *B. subtilis* and *E. coli*. The biological activity was measured by using the standard method to determine MICs [34]. The cutoff for biological activity was arbitrarily set at 100 μM. Sodium cation release from liposomes was measured by using an ion selective electrode or by the ^{23}Na NMR method of Riddell and coworkers [30]. Ion transport was measured for compounds having spacer chains of octylene to eicosylene, but no biodata were obtained for the two longest chain hydraphiles.

The graph of Fig. 8 shows MIC data compared to the percentage of ion release. It was anticipated that the greater the ion release in liposomes, the greater would be the membrane disruption in bacterial cells. This projected a direct relationship between transport efficacy and antibacterial potency. In graphical form, it would appear as an inverse relationship because high ion release percentages should lead to lower MICs that reflect higher potency.

As noted above, bacterial membranes differ substantially from liposomal bilayers formed from simple phospholipids. It was therefore useful to confirm the ion release capability of hydraphiles in a bacterial system. The experiment that was undertaken used *E. coli*. A K^+-sensitive ion selective electrode was used to monitor K^+ efflux over time. Four benzyl hydraphiles having C_8, C_{10}, C_{12}, and C_{14} spacer chains were used in the study. These compounds are abbreviated on the graph of Fig. 9 as BC_8H to $BC_{14}H$. Each hydraphile was used at a concentration of 4 μM. Negative controls were *E. coli* alone and 0.5% DMSO, the commonly used solubilizing solvent [35]. Gramicidin-D was used as a positive control at 30 μM. As in the case of liposomes, $BC_{14}H$ and $BC_{12}H$ were active and the shorter chained hydraphiles were less effective in transporting K^+ through the bacterial boundary membranes.

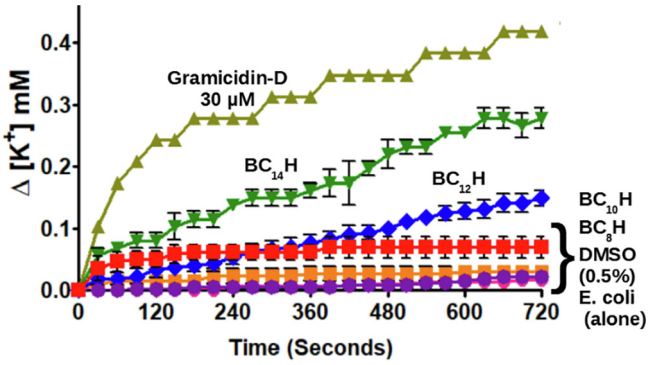

Fig. 9. Potassium cation release from *E. coli* in the presence of Gramicidin-D and hydraphiles. Release was monitored by K^+-sensitive ISE. Each hydraphile was present at a concentration of 4 μM.

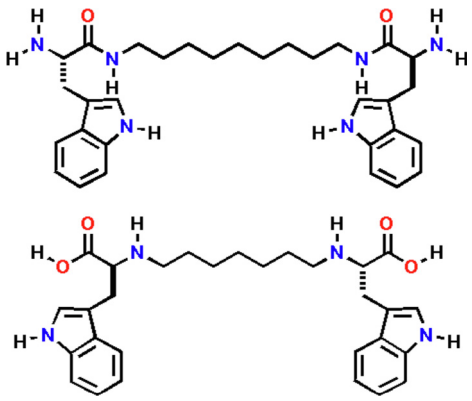

Fig. 10. Two hypothetical *bis*(tryptophan)s linked as *bis*(amides) (top) and as diamines (bottom).

6. *Bis*(tryptophan)s — novel amino acid-based amphiphiles

We have developed a family of tryptophan compounds that were designed to take advantage of this amino acid's special anchoring properties. It is known, for example, that the bacterial dimer channel gramicidin has tryptophans at the opposite ends of the channel that serve as membrane anchors [36]. The KcsA voltage gated channel is a complex protein with multiple alpha helices. The only tryptophans present in its structure are at the proximal and distal boundaries of the channel [37]. In very early work, we discovered that simple *n*-alkyl derivatives of indole, the side chain heterocycle of tryptophan, could form stable vesicles [38]. The vesicles formed whether the single alkyl chain was linked to the *N*- or the 3-position of indole.

Our hypothesis was that compounds of the type Trp-linker-Trp would comprise bolaamphiphiles, many of which are known to insert in membranes. These bis(tryptophan)s or BTs were not expected to function as ion channels, but rather to insert into membranes, disrupt the intrinsic order, and enhance permeability.

6.1. Synthetic Access to bis(Tryptophan)s

Two different strategies seemed obvious. Tryptophan could be coupled to *bis*(amines) or to dicarboxylic acids. The two types of structures would share similar features, but there would be critical differences. Fig. 10 shows the two obvious alternatives using aliphatic linkers of arbitrary length for illustrative purposes.

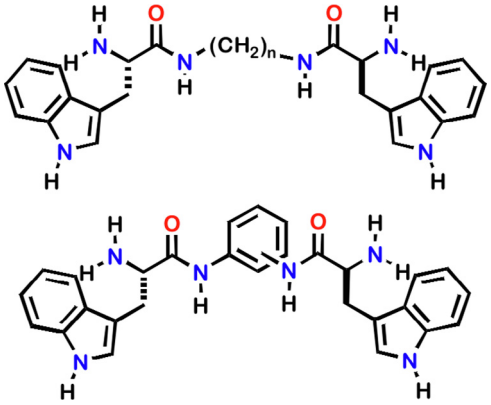

Fig. 11. Trp-linker-Trp amphiphiles. In the upper figure, n was 1, 2, 3, 6, and 12. In the lower structure, *ortho*, *meta*- and *para*-phenylenediamines were studied. In addition, the *meta*-compound was prepared with both D,D- and L,L-tryptophan stereochemistries.

Table 1
MICs (μM) of synthetic ionophores against bacteria.

Bacteria	[$BC_{14}H$] μM	[$C_{10}LE$] μM	[$C_{12}BT$] μM
Gram-Negative Bacteria			
E. coli (R:tet)[a]	2	8	10
P. aeruginosa (R: Mero)[b]	47	>30	>18
K. pneumoniae (ESBL, HSV-2)[c]	12	>30	>18
A. baumannii (WT)[d]	6	ND	N.D.
Gram-positive Bacteria			
S. aureus 1199B	1.5	8	4
Methicillin Resistant *S. aureus* (MRSA)	1.5	30	9
Vancomycin resistant *E. faecalis* (VRE)	1.5	30	9
S. pneumoniae (R: Levo)[e]	3	13	9

[a] Tetracycline resistant.
[b] Meropenem resistant.
[c] Extended spectrum beta lactamase resistance.
[d] Wild type.
[e] Levofloxacin resistant.

The top structure shown in Fig. 10 was more appealing than the lower one for two reasons. First, the linkage of tryptophan with any diamine could be accomplished by standard coupling peptide methods. Numerous diamines are available and the coupling procedure is standard chemistry. This contrasts with the lower structure which would be more complicated to prepare. One approach would be to treat *C*-protected tryptophan with a dihalide in the hope that only monoalkylation would occur. Even if a single connection resulted at each end of the alkyl chain, deprotection of the terminal acids would still be required. The alternative of using a diacid and forming a *bis*(amide) from the C-protected tryptophan would require the selective reduction of the internal and not the distal amides.

The second consideration concerned the boundary layers of bacteria. Bacterial surfaces are typically negative and would show affinity for a diamine protonated at physiologic pH (*i.e.* an ammonium cation). In the alternative structure, the diacid structure would be ionized, but the negative carboxylate anion would show no affinity for a negative cellular surface.

Several BTs were prepared on the diamide model (Fig. 11). The precursor diamines are shown in Fig. 11. In addition to the BTs prepared from these, all of which can be represented as Trp-diamine-Trp, both the L,L- and D,D- forms of 1,3-diaminobenzene were used to form BTs. This diamine as also coupled to *bis*(homotryptophan). In addition, a number of control compounds, such as Gly-*m*-C_6H_4-Gly, were prepared. The range of BT compounds studied was admittedly limited, but the results were gratifying.

7. Synthetic amphiphile potency

Table 1 shows the potencies of lariat ethers, hydraphiles, and bis-tryptophans against tetracycline-resistant *E. coli* (Tet^R), *Pseudomonas aeruginosa* (resistant to meropenem), *Klebsiella pneumoniae* (ESBL, HSV-12), *Acinetobacter baumanii* (wild type), *Staphylococcus aureus* 1199B, methicillin resistant *Staphylococcus aureus*, vancomycin resistant *Enterococcus faecalis*, and laevofloxacin resistant *Streptococcus pneumoniae*. All are dangerous bacterial strains. Methicillin resistant *Staphylococcus aureus*, also called MRSA, and vancomycin resistant *Enterococcus faecalis* are no longer susceptible to the drugs used to control them and are particularly dangerous. *K. pneumoniae* is resistant to extended spectrum *beta*-lactamase drugs. Wherever resistance has developed, infections can be severe and difficult or impossible to treat effectively [39].

Table 1 shows the innate potency of a single representative of each family discussed herein against the range of organisms described above. These are *N,N*′-di-*n*-decyl-4,13-diaza-18-crown-6 ($C_{10}LE$), dibenzyl-C_{14}-hydraphile ($BC_{14}H$) (Fig. 12), and Trp-NH-$(CH_2)_{12}$-NH-Trp ($C_{12}BT$) (Fig. 10). The first row of Table 1 shows activity to the tetracycline resistant R:tet (or Tet^R) strain of *E. coli* that we prepared. Its MIC against tetracycline is ~900–1000 μM. Each of the synthetic amphiphiles shows *in vitro* potency of less than 10 μM. Among the Gram negative bacteria, the potencies of $BC_{14}H$ are shown against wild type *A. baumanii* (6 μM) and ESBL *K. pneumoniae* (12 μM).

It is an empirical observation that many antimicrobials, synthetic or otherwise, are more potent against Gram positive organisms. This is likely due to the presence of a less complex cellular boundary membrane in Gram positive bacteria than in Gram negative organisms (*see* Fig. 7). The results shown in Table 1 illustrate that $BC_{14}H$ is potent against *S. aureus* 1199B, methicillin resistant *S. aureus* (MRSA), vancomycin resistant *E. faecalis* (VRE), and *S. pneumoniae* (R: Levo) at $\leq$ 3 μM *in vitro*. $C_{12}BT$ does reasonably well against these same four bacteria at $\leq$9 μM *in vitro*.

8. Preparation of a tetracycline-resistant *E. coli*

As part of the studies described here, we required a model organism to evaluate efficacy and to assess potential synergy. Tetracycline is one of the most common antibiotics and *Escherichia coli* is an extremely common pathogen. We therefore prepared a strain of *E. coli* resistant to tetracycline that we call Tet^R. Tet^R *E. coli* was made by transforming JM109 competent *E. coli* (Promega) with pBR322 plasmid (Carolina Biological) using the manufacturer's protocol. The minimum inhibitory concentration (MIC)

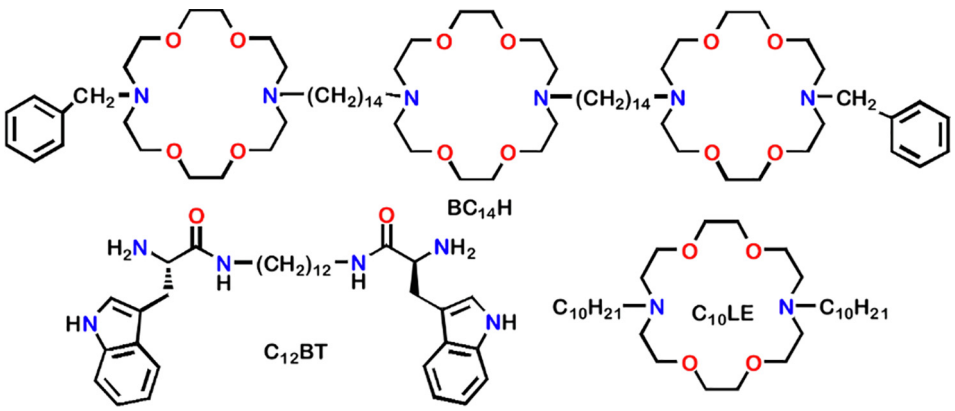

Fig. 12. Structures of three bioactive synthetic amphiphiles: hydraphile (top), *bis*(tryptophan) (bottom left) and lariat ether (*see* Table 1).

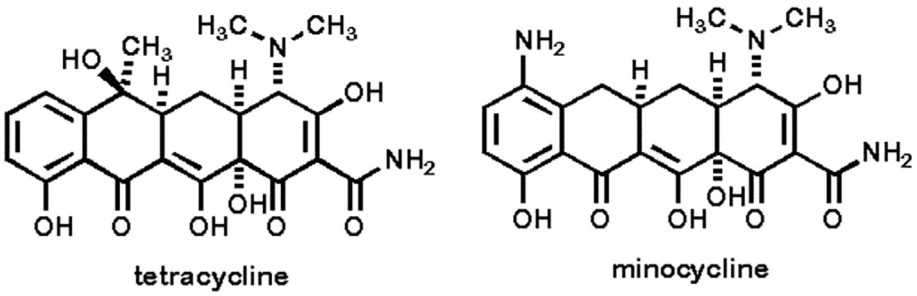

Fig. 13. The structures of tetracycline and minocycline.

[40] for Tet^R *E. coli* was determined as noted above.3[4] MICs are determined by allowing bacteria to grow in the presence of an antibiotic. The concentration of antimicrobial is serially diluted by half in a series of experimental samples. The MIC is assigned at the boundary between samples at which growth and no growth is visually apparent. The MIC for Tet^R *E. coli* against tetracycline reflects the resistance: it was found to be between 900 μM and 1 mM. This contrasts with the normal MIC against *E. coli* of 12 μM for tetracycline and 18 μM for structurally related minocycline (Fig. 13). The potency difference between non-resistant and Tet^R is a difference of ~75-fold.

8.1. Combination therapy

Our hypothesis that hydraphile penetration of bilayer membranes could translate to enhanced permeability led us to combine hydraphiles with several different FDA approved antibiotics. In these initial studies, we used the DH5α strain of *E. coli* as the model organism. In later studies, the more robust, but non-pathogenic, K12 strain was used with similar results. The approach was to determine the MIC for each of the hydraphiles against the microbe and use half the MIC in additive experiments. When *E. coli* was grown in the absence or presence of ½ MIC of any of the hydraphiles tested, no effect was observed during ~35 generations of reproduction. The antibiotics chosen for this study were erythromycin, kanamycin, rifampicin, and tetracycline. The structures of these four compounds differ significantly and their modes of action are well established and different. The MICs of each antibiotic against *E. coli* were determined in the absence of any additive. For the combination experiment, ½ MIC of the hydraphile was added to a suspension of *E. coli* and the new MIC for each antibiotic was determined.

These experiments differ somewhat from typical combination experiments. In all cases, the amphiphile is used at a concentration at which it is ineffective as an antibiotic. Indeed, at the concentrations used, no effect is apparent on the growth of the bacterium over an extended period. However, the ability of each of these compounds to enhance membrane permeability and perhaps exhibit other effects, made them promising adjuvants.

We conducted combination studies using the checkerboard method. The data of Table 2 include only ½ and ¼ [MIC] of the amphiphiles rather than the results of the entire checkerboard. Table 2 columns 3 and 4 indicate the concentrations of antibiotics required in the presence of our amphiphile to inhibit bacterial growth and the fractional inhibitory concentration.

Three drug resistant bacteria were screened. The ability of the amphiphiles to recover antibiotic potency against resistant bacteria was determined. For example, in the first row under tet^R *E. coli*, the activity of tetracycline was recovered from 900 μM to 360 μM at ¼ [MIC] of $BC_{14}H$. This results in an FIC of 0.64, which is the total of fractional MICs of the two compounds used. Any FIC number greater than 1 is considered antagonist, 0.5 to 1 is additive and lower than 0.5 is synergistic. In this particular case, the combination was additive. Recovery of tetracycline activity was also observed against *K. pneumoniae*, a serious bacterium that may cause pneumonia. We also observed synergistic interactions of lariat ethers against Gram-positive *S. aureus*.

Table 2
Recovery of antibiotic activity against antibiotic-resistant bacteria.

Amphiphile Used	[Amphiphile] μM	[Tetracycline] μM	FIC index
Tet^R *E. coli*[a]			
None	0	900	n/a
$BC_{14}H$	0.5 ($^1/_4$ MIC)	360	0.65
$BC_{14}H$	1 (½ MIC)	220	0.74
$C_{10}LE$	6	225	0.5
$C_{10}LE$	9	56	0.56
$C_{12}BT$	5	225	0.75
$C_{12}BT$	2.5	450	0.75
K. pneumoniae (ATCC BAA 2146)[a]			
None	0	1,000	n/a
$BC_{14}H$	5	62.5	0.56
S. aureus 1199B[b]			
Amphiphile Used	[Amphiphile] μM	[Norfloxacin] μM	FIC index
None	0	64	n/a
$BC_{14}H$	0.25	16	0.5
$C_{10}LE$	2	0.5	0.27

[a] Tetracycline resistant.
[b] Norfloxacin resistant.

In all the cases represented above, it is crucial to note that (1) potency of antibiotic that was ineffective against a resistant bacterium was recovered and (2) this also potentially reduces the spread of antibiotic resistance by use of combination antibiotics.

9. Ion balance and efflux pump inhibition

Different types of cells have different concentrations of a relatively small number of cations. In both bacterial and mammalian cells, the internal concentration of K^+ is higher than Na^+, with the ratio of K^+/Na^+ higher in bacteria. Divalent magnesium and calcium are present as well, with [Mg^{2+}] being higher in bacteria and [Ca^{2+}] being higher in mammalian cells. Our initial postulate was that the hydraphiles disrupted membrane organization, enhancing permeability. To the extent that hydraphiles are present in the membrane(s), ion regulation will be disrupted as well.

The hydraphiles form ion-conducting channels, but they are symmetrical and non-rectifying. As a result, the regulated flow of cations across the boundary membranes is disrupted as is ion homeostasis. The loss of ion balance affects proteins that are dependent upon appropriate ion gradients for function. Efflux pumps [41] are such proteins. When ion balance is lost, the ability of these proteins to expel such xenobiotics as antimicrobials is reduced [42].

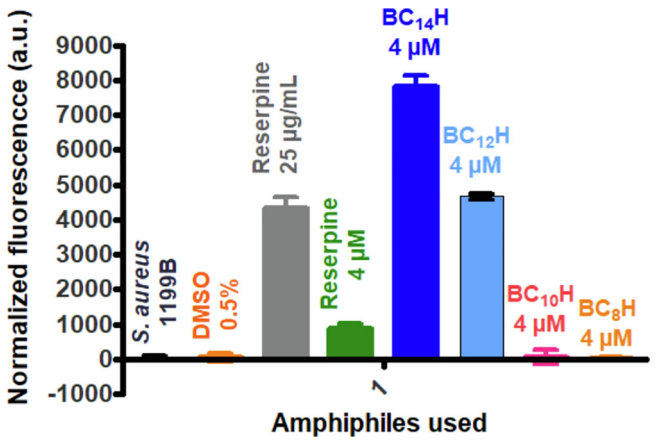

Fig. 14. Graph showing the accumulation of ethidium bromide in S. aureus in the absence and presence of various efflux pump inhibitors. Each bar represents a minimum of three separate experiments. Error bars show mean ± SD.

Fig. 14 shows the result of exposing *S. aureus* to various efflux pump inhibitors in the presence of ethidium bromide. Ethidium bromide does not normally penetrate cellular membranes. When it does, it interacts with DNA producing enhanced fluorescence. In the experiments reflected in the graph of Fig. 14, various efflux pump inhibitors were added. To the extent efflux pump function is inhibited, ethidium bromide that does penetrate the membrane will not be ejected, and fluorescence will increase. Of course, ethidium bromide cellular penetration will also be assisted by permeability enhancement.

In the study graphed in Fig. 14, no fluorescence enhancement was observed in the absence of any additive (*S. aureus* alone) or when 0.5% DMSO, added for solubility, was present. Reserpine was added as a positive control because it is a well-known efflux pump inhibitor. It is typically administered at 25 μg/mL, which corresponds to 41 μM. Surprisingly, both $BC_{12}H$ and $BC_{14}H$ at 4 μM exceeded its effect as an efflux pump inhibitor at 4 μM or at ten times that concentration. In contrast to $BC_{12}H$ and $BC_{14}H$, $BC_{10}H$ and BC_8H, showed little inhibition.

10. Toxicity

The question of toxicity of any potential drug is a complex problem. It is obvious that whatever biological interaction is expected for a drug to be administered to humans, it cannot be more toxic. Of course, toxicity is relative in the sense that many anticancer drugs are generally toxic to the most rapidly growing cells. This toxicity is acceptable when the disease is life-threatening. Likewise, an antibacterial agent can exhibit some toxicity if an infection is dire. Although not discussed herein, toxicity data for the compounds discussed show good survival of the mammalian cell types human embryonic kidney (HEK-293) and monkey kidney (Cos-7).[41]

11. Conclusions

Our hypothesis that synthetic amphiphiles can enhance membrane permeability seems viable, but incomplete. Increased penetration of antimicrobials does permit greater concentrations of exogenous substances into the bacterial cytosol. This is clear from the ethidium bromide experiments. However, the enhancements of potency also result from the fact that certain of these amphiphiles are efflux pump inhibitors. We speculate that these structurally different molecules do not target a particular enzymatic site. Instead the known ability of these compounds to form ion conducting channels, likely leads to disruption of ion homeostasis. This, in turn, impedes that ion-regulated function of efflux pumps. The combination of enhanced permeability leading to greater penetration and flaccid efflux pump ejection increases potency.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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