Review Article

Tryptophan in Membrane-active Synthetic Antimicrobials

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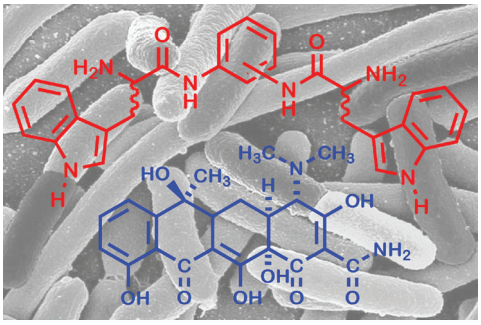

Abstract

Herein, we summarize the background data that has led to a new class of antimicrobial amphiphiles and present recent results showing reversal of resistance to antibiotics in an *Escherichia coli* strain incorporating a tetracycline-selective efflux pump. In addition, two types of amphiphiles that show antibiotic potency enhancement or resistance reversal are discussed, along with our knowledge on the evolution that led to the active structures. One family is based on macrocyclic crown polyethers, which are known to insert into both liposomal and bacterial bilayers; these compounds are termed as hydraphiles and consist of three diazacrown rings linked by alkyl spacers and terminated by benzyl groups. In contrast, the second type of amphiphiles use tryptophans as the terminal, membrane anchoring residues; in this group of amphiphiles, two tryptophans are connected by alkyl chains or aryl groups. In both cases, however, the antibacterial activity of certain members of each family was apparent. Further, the amphiphiles have been shown to act as adjuvants that increase the potency of such antimicrobials as tetracycline and even to have re-sensitized a tetracycline-resistant *E. coli* strain to the antibiotic.

Introduction

Among the 20 genetically-encoded amino acids, the occurrence of tryptophan (Trp, W) in proteins is the least common.[1] It is present at a frequency of about 1.3% in all known proteins. This compares with 3.9% for phenylalanine and 3.3% for tyrosine, the other electron-rich aromatic amino acids. Tryptophan typically occurs in peptides and proteins at the membrane interfaces, suggesting an amphiphilic role.[2] Examples are the bacterial peptide that forms ion channels, gramicidin, and the voltage-gated protein KcsA K^+ channel.[3–6] The structures of tryptophan and indole are shown in Scheme 1.

Gramicidin forms a channel by dimerizing and exposing its Leu-Trp repeats to the polar (external) surfaces of membranes. The dimeric nature and the organization within the bilayer have been extensively studied,[7] albeit not without the results representing some controversy.[7–9] A synthetic channel in which two gramicidin peptides were linked by a tartaric acid residue showed function similar to the individual peptides.[10] Other semi-synthetic

Keywords: Amphiphile; Antibiotic; Antibiotic Resistance; *Bis*(tryptophan); Cation-pi; *Escherichia coli*; Hydraphile; Indole; Re-sensitization; Resistant bacteria; Synthetic anion transporter; Tryptophan.

Abbreviations: Tet^R, tetracycline-resistant E. *coli*; THF, tetrahydrofuran; ^{23}Na-NMR, sodium-nuclear magnetic resonance spectroscopy; FDA, fluorescein diacetate.

Received: September 28, 2016; Revised: January 04, 2017; Accepted: January 05, 2017

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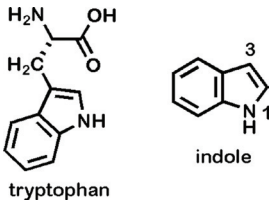
How to cite this article: Meisel JW, Patel MB, Negin S, Gokel MR, Cantwell R, Gokel GW. Tryptophan in Membrane-active Synthetic Antimicrobials. *J Explor Res Pharmacol* 2017;2(1):7–15. doi: 10.14218/JERP.2016.00025



Scheme 1. The chemical structures of tryptophan and indole. The 1- and 3- positions of indole are numbered.

gramicidin mimics have been prepared as well.[11,12] Figure 1 shows schematic illustrations of gramicidin (variant C), the KcsA K^+-conducting channel and the ClC Cl^--conducting channel.[13]

Our initial interest in tryptophan's indole was piqued by at least three factors. The first was its ubiquity at membrane boundaries, as noted above. The second was the observation of a high proportion of tryptophan in the periplasmic loops of the photosynthetic reaction center.[16] These loops connect the transmembrane segments, and a "needle and thread" mechanism was proposed, whereby tryptophans in periplasmic loops guide protein segments through bilayers during the process of translocation; furthermore, tryptophan is suggested to serve as an eventual protein anchor in these situations.[17]

The third stimulus of our interest was related to an interesting indole compound reported by Schore and Turro.[18] Those researchers prepared an indole derivative, which they referred to as "6-In-11" and in which the 3-carbon of indole was substituted by an *n*-hexyl group and the nitrogen was substituted by $-(CH_2)_{11}N^+(CH_3)_3$ Br^-. The compounds disclosed in the publication were designed as fluorescent probes of micellar systems; however, the compound 6-In-11 did not show the expected amphiphilic behavior. We speculated that both indole and the quaternary ammonium residue were competing as head groups, altering the anticipated extended conformation of the indole-containing chain.

Taken together, the remarkable properties of tryptophan and its aromatic residue, indole, led us to explore its potential as an amphiphile head group, as a pi-donor for alkali metal cations, and as a membrane-active synergist for antimicrobials against resistant bacteria. The results of those studies are recounted below.

Indole as an amphiphilic head group.

The formation of liposomes by twin-tailed amphiphiles is common.[19] Single-chained amphiphiles typically form micelles, although vesicle formation has long been known.[20] Our initial studies sought to determine if the indole residue was sufficiently polar to serve as a head group. The second question we investigated was whether an alkylindole having a sufficiently long chain would form micelles or liposomes. In either case, a third question was how, if at all, the formation of aggregates was altered by alkylation on nitrogen (1-position) or at the 3-position. Indole is linked at the 3-position in tryptophan.

Indole can be deprotonated at nitrogen to form an ambident anion.[21] We found that treatment with NaH in THF followed by reaction with an alkyl halide afforded the *N*-alkylated indoles in yields of about 60%.[22] The by-product was likely the 3-isomer, but this was not isolated. A similarly conducted reaction using LiH instead of NaH gave the corresponding 3-alkyl derivatives. The latter reaction conditions produced the 3-alkylindoles in only 20–40% yields after separation of the isomeric *N*-alkylated product.[23,24]

Aggregate formation was attempted by using either the reversed-phase or the lipid hydration method.[25,26] The methyl or *n*-hexyl substituted indoles designated **1**, **2**, **6**, and **7** failed to form any aggregate that could be detected either by light scattering or transmission electron microscopy (TEM). The other compounds did form aggregates, which fell within the size range of 1500 Å to 4000 Å (150–400 nm). Where possible, electron micrographs were obtained, and the sizes determined by the two methods were in reasonable agreement. The formation of vesicles from these single-tail amphiphiles was confirmed by TEM and by freeze-fracture and dye entrapment methods.[24]

A surprising finding of this study concerned aggregate stability. We anticipated that amphisomes which formed from 3-alkylindoles would be more stable than the isomeric *N*-alkylindoles. This was because the indole in tryptophan itself is substituted in the 3-position and has a free >N–H. Although similar isomer stabilities were observed in some cases, the 3-*n*-decylindole (**8**) aggregates proved to be frail and the aggregate size increased over time for 3-*n*-octadecylindole (**9**), but the aggregates formed from isomeric **4** were stable for more than a month.

Notwithstanding the surprising stability profiles, it was clear that indole itself could function effectively as an amphiphilic head group. Moreover, the evidence of formation of spherical aggregates that included a fluorescent dye confirmed the formation of vesicular, rather than micellar, assemblies.

Indole as a hydraphile head group

In a separate effort, we developed a family of cation-conducting pore-formers that we termed as 'hydraphiles'.[27] These molecules typically consist of three crown ether rings, connected by alkyl chains and terminated by alkyl, aryl, aralkyl or heteroaryl residues. A generalized structure is illustrated in Scheme 3.

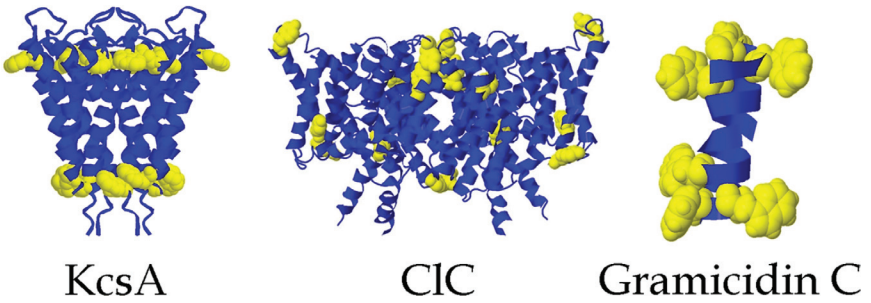
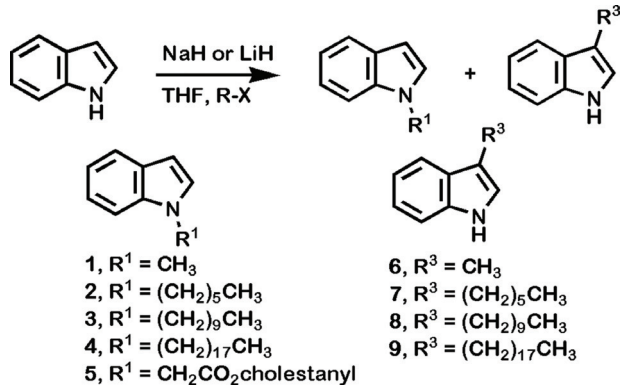


Fig. 1. Schematic representations (from solid state structures) of the KcsA K^+-conducting channel (PDB: 1BL8), the ClC Cl^--conducting channel (PDB: 1KPK), and the gramicidin variant known as "C" (PDB: 1JO4).[3,6,14,15]



Scheme 2. Synthesis of *N*(1)- or 3-substituted indoles by alkylation of the ambident anion.

The hydraphiles are synthetic amphiphiles that insert into liposomal bilayers and transport Na^+ cations by a pore or channel mechanism. Transport was confirmed by the ^{23}Na-NMR method and by planar bilayer conductance studies.[28,29] The channels were also shown to function in human embryonic kidney cells.[30] Moreover, they conduct ions as monomers, are selective for Na^+ over K^+ by *ca*. 4:1, and are blocked by Ag^+.[27] A fluorescent channel (R = dansyl in Scheme 3) was shown to localize in the boundary layer of *Escherichia coli*.[31] The hydraphiles show length-dependent transport function and toxicity to *E. coli* and *Bacillus subtilis*.[32,33] Toxicity to the primary eukaryote *Saccharomyces cerevisiae* was also observed, but correlated less well with changes in the spacer chain length. Of course, *E. coli* and *B. subtilis* are bacteria and *S. cerevisiae* is a primary eukaryote, so the cellular structures are significantly different.

There is considerable evidence that the hydraphiles adopt an extended conformation within phospholipid bilayers. This may be inferred from two lines of evidence in particular. First, cation release from phospholipid vesicles is length-dependent.[34] Thus, maximum ion release was observed when the spacer chains were dodecylene (12 methylenes), tetradecylene (14 methylenes), and hexadecylene (16 methylenes). The overall lengths of these three channels in an extended conformation correspond to the width of phospholipid bilayers. No ion transport was observed when the spacer chains were octylene (8 methylenes); however, when the chains were 18 or 20 methylenes, transport was observed but the rates were low. This was interpreted to mean that because of the excessive length, the overall conformation comprised a poorly organized conductance state. Second, in addition to our own experimental studies, computational studies verified the extended conformation and ion transport.[35] This work suggested that the extended conformation was maintained during ion transport. Additional support for the extended conformation was gained in recently reported quantum mechanical studies.[36,37]

When R in the structure of Scheme 3 is 3-indolylmethyl, the channel fails to transport cations.[38] It seems unlikely that protonation of the indole nitrogen is an issue, as its pK_A is about 2.3.[39] Further, the six aliphatic nitrogen atoms are more basic and will certainly protonate first. Evidence suggests that at least one indolyl N–H is hydrogen-bonding across the face of a distal macrocycle, preventing ions from passing through. Two pieces of evidence support this hypothesis. First, the infrared spectrum showed an intramolecular hydrogen bond that did not change even with 10^4-fold dilution. Second, replacing the proton on the indole nitrogen with a methyl group, which eliminates the possibility of H-bond formation, rescued the transport function.[34]

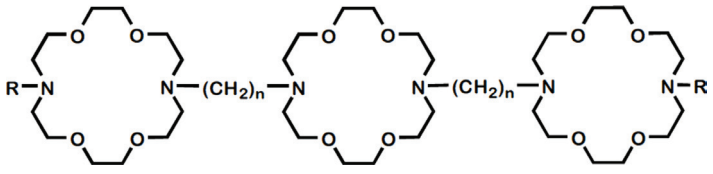
The original hydraphile design envisioned the two distal macrocycles as both head groups and entry and exit portals for transient cations.[40] The fact that indole's H-bond interactions prevented transport strongly supported this expectation. A hypothesis that tryptophan might also provide head group function engendered the synthesis of a central macrocycle having chains terminated by this amino acid. The anchoring potential of the tryptophan head groups suggested that their presence would augment pore stability, especially by protonation of the free amines.

The molecule was prepared from the macrocycle, having two side chains emanating from the nitrogen atoms. If we represent the parent macrocycle as H<N18N>H, then the intermediate would be $H_2N(CH_2)_{12}$<N18N>$(CH_2)_{12}NH_2$. Tryptophan was then coupled to each of the primary amines using standard protection-coupling methodology. However, when the product was evaluated for Na^+ transport it failed to show function. It was concluded that the distal macrocycles were required for effective pore formation, notwithstanding the potential anchoring effect of tryptophan.[41]

Indole as a pi-donor

Alkali metal and ammonium ion cation-pi interactions were demonstrated in the 1980s by mass spectrometry.[42,43] Meot-Ner and Deakyne demonstrated ammonium-pi interactions by mass spectrometry.[44] In the early 1990s, cation-pi interactions were suggested as a means to control ion selectivity in the voltage-gated potassium channel. Computational methods were used to evaluate the donor abilities of benzene (phenylalanine), phenol (tyrosine) and the indole of tryptophan—representing the three common arenes in amino acids that possess electron-rich aromatic side chains.[45] Site-directed mutagenesis experiments showed that this was not the explanation, but the potential for such interactions in supramolecular chemistry enhanced interest in the field.[46]

Our interest in tryptophan as a potential donor led us to use our lariat ether model system as a means to assess Na^+- and K^+-pi interactions.[47] Unfortunately, attempts to do so in the 1980s failed, owing to a poorly designed model system.[48] A slight modification of the lariat ether, from Ar–CH_2<N18N>CH_2–Ar to Ar–CH_2CH_2<N18N>CH_2CH_2–Ar, bore the hoped-for fruit.[49] Ultimately, cation-pi complexation of Na^+ and K^+ was demonstrated for phenyl, phenol and indole.[50,51] More recently, Petersen and coworkers have suggested that a cation-pi interaction involving tryptophan may play a role in interfacial stabilization.[52]



Scheme 3. Generalized structure of hydraphiles. R may be alkyl, aralkyl, aryl or heteroaryl, and *n* = 6–20.

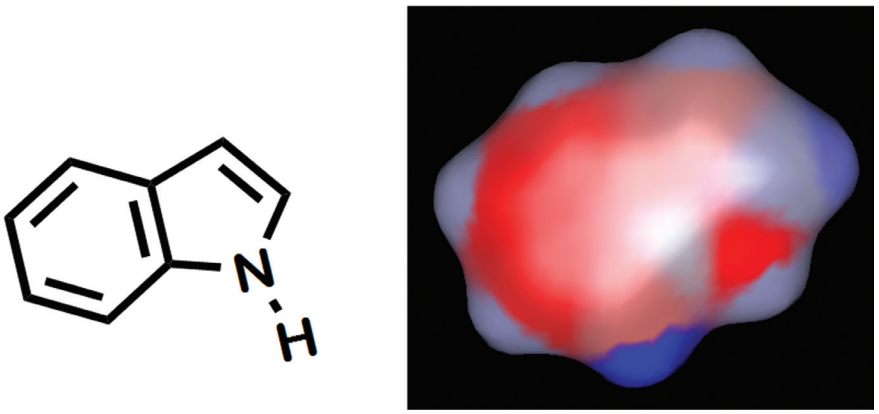


Fig. 2. Structure of indole represented in a line angle drawing and in a surface charge metaphor.

Two interesting facts emerged from these experimental studies. First, calculations of electron density were reported for benzene, phenol and indole. As expected, the donicity order was indole > phenol > benzene. Of the three, only indole has two rings that could potentially serve as the donor site. Calculations showed that the benzene ring of indole would be favored as a donor. This is apparent in the structures shown in Figure 2.

The panel on the right in Figure 2 shows the greatest electron density (in red) on the benzene ring, rather than the pyrrole. The prediction for cation-pi interactions is that complexation will favor an alignment of the cation with the benzene. When the structure of the lariat ether diindole complex of K^+ was obtained, the pyrrole end of the molecule was closest to the cation. This is apparent in the representation of the solid state structure shown in Figure 3. Two diindole-K^+ complexes are pictured with an iodide ion between. It is clear that both pyrrole residues in each complex are aligned with the cation.[53] Solution studies conducted by NMR methods showed that the alignment was similar in acetone-d_6 solution. When the lariat ether spacer chain was attached at position 5 (on the benzene ring), rather than 3, the 6-membered ring was the primary donor.

The second interesting observation to emerge from these studies concerned the orientation and spacing of the arenes from Na^+. Using the range of solid state structures that was obtained, we were able to determine average arene-cation contact distances and angles. We compared these criteria to data from protein structures in which the water-arene contacts were inferred. Since both water and Na^+ have similar electron densities and scatter similarly, it is hard to distinguish them on a density map. Knowing preferred distances and angles permitted us to show that at least in some cases, a sodium cation was more likely to be in a position assigned to a water molecule.[54]

Influence of tryptophan in synthetic anion transporters (SATs)

The successful development of cation-transporting hydraphiles inspired our effort to develop an anion-selective synthetic pore-former. We succeeded in this by designing a family of amphiphilic peptides, the generic structure for which is shown in Figure 4.[55] The key element of the design was the heptapeptide sequence. The G–X–X–P sequence in the presumed selectivity filter of the ClC channel fostered the selection of the G–G–G–P sequence that appears in most of the compounds prepared.[56] For simplicity and symmetry, the overall peptide sequence G_3PG_3 was adopted. These compounds proved generally to be active as Cl^- transporters when

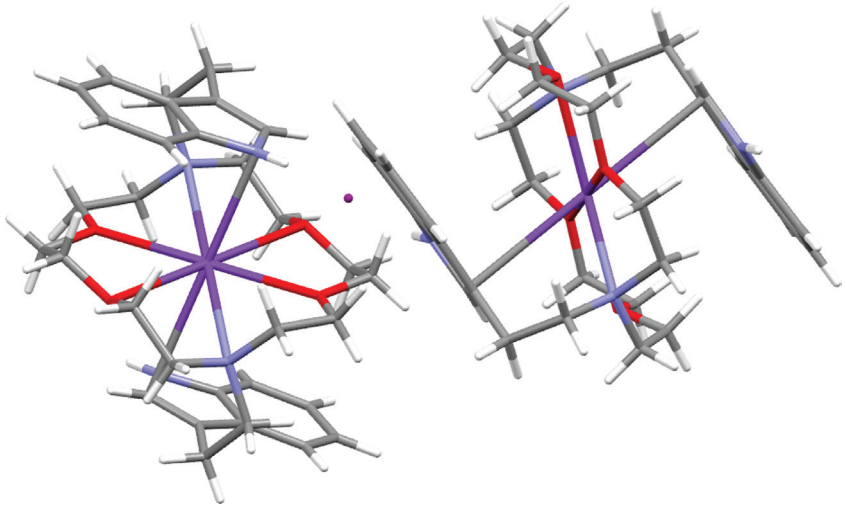


Fig. 3. Diindole lariat ether complex of KI showing the alignment of K^+ and the pyrrole ring, rather than the benzene ring, of indole.

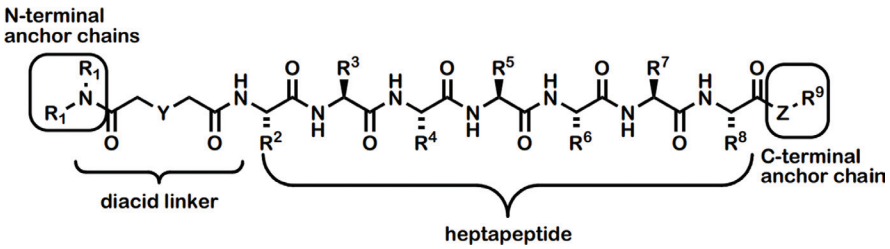


Fig. 4. Synthetic anion transporters (SATs). The four basic modules are illustrated and numerous structures were prepared that incorporate variations in each module.

the alkyl chain membrane anchors were ≥10 carbons, when the diacid linker was diglycolic acid (Y=O), and when the C-terminus (Z–R[9]) was benzyl or *n*-heptyl.[57–60]

It was found by NMR analysis that the principal H-bond donor interactions of the peptide chain occurred between Cl^- and ^{5}Gly and ^{7}Gly.[61] Attention was, therefore, focused on the C-terminal side of proline. Several SATs were prepared in which positions 5, 6 and/or 7 were occupied by tryptophan residues.[62] Bilayer conductance experiments showed clear and reproducible open-close behavior.[63] All of the compounds successfully released Cl^- from liposomes as shown in Table 1.

The chloride ion release experiments were conducted on DOPC:DOPA (7:3 w/w) liposomes that were filtered to an approximate size of 2000 Å (200 nm). Chloride release was monitored with a Cl^--selective electrode and the amount observed at 1800 s was compared to the total Cl^- obtained upon vesicular lysis. The factors that influence the 6-fold difference in Cl^- release were assessed in detail, but a correlation with the ability of the monomers to aggregate in aqueous solution was prominent.[58]

Hydraphiles that increase antibiotic potency

The fact that various hydraphiles insert into bilayers, conduct cations and alter ion homeostasis caused us to consider their potential as combination therapeutics. It was surmised that penetration of the bilayer might cause a membrane disruption that would enhance the entry of other substances. This potentially naïve notion was tested by treating the DH5α strain of *E. coli* with sub-minimal inhibitory concentrations (MICs) of hydraphiles and the antibiotics erythromycin, kanamycin, rifampicin and tetracycline.[64] The hydraphiles shown in Scheme 3 were studied, and the benzyl C_{14} and C_{16} hydraphiles proved to be most effective. The studies were conducted by first determining the MIC of the hydraphile against the bacterium.[65] The MIC was then determined for the antibiotic against the bacterium. In the co-administration experiment, bacteria were treated with a hydraphile at a concentration ½ to ¼ of its MIC and the MIC of the antibiotic determined for the combination. Enhancements in potency up to 30-fold were observed with certain combinations of bacteria, hydraphiles and antibiotics.[66]

Table 1. Percent chloride ion release from DOPC/DOPA (7:3) liposomes at 1800 s

Peptide sequencea	% Cl^- released
GGGPGGG	35
GGGPWGG	10
GGGPwGG	21
GGGPGWG	40
GGGPGGW	25
GGGPGGW(N–CHO)	60
GGGPGGW(N–CH_3)	30

a. All isomers are l except "w" which indicates D-Trp.

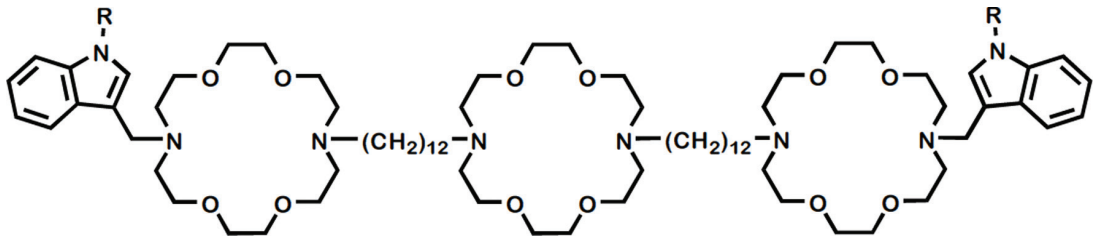
Tryptophan-based amphiphiles that reverse antibiotic resistance

Based on the numerous successful studies involving both indoles or tryptophans and amphiphiles, we prepared a family of *bis*(tryptophan)s (BTs).[67] Two groups of structures were prepared. In the first family, tryptophans were linked to 1,2-, 1,3-, or 1,4-diaminobenzene, affording different geometries and spacings of the tryptophans. In addition, the stereochemistry was varied to assess whether the presence of d-Trp in either relative position would alter the biological activity. In the second family, two tryptophans were linked to the α,ω-positions of linear diamines; these were clearly more flexible compounds that span a greater range of distances than the diaminobenzene derivatives. The compounds prepared are illustrated in Scheme 6.

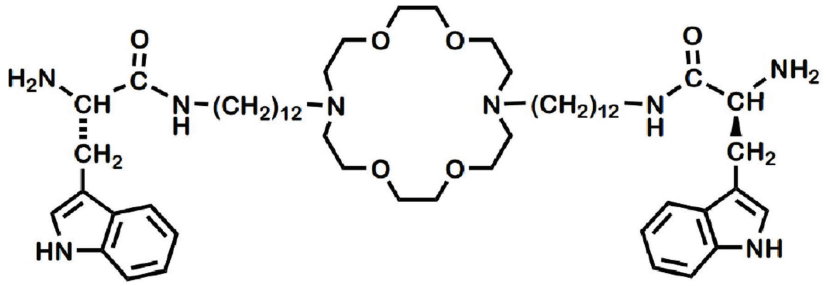
As described above, the goal was to determine if there was any enhancement in antibiotic potency in the presence of sub-MIC concentrations of any of these BTs. This study involved three different bacteria, two of which were obtained from the ATCC: *E. coli* K12 and *Staphylococcus aureus*. The third was a strain of competent *E. coli* that was transformed in our lab with a plasmid containing the tetA efflux pump gene. We refer to this tetracycline-resistant strain as Tet^R. The MIC of tetracycline against Tet^R was *ca*. 900 μM. The MIC of minocycline, which is a close structural relative of tetracycline, against Tet^R was 18 μM.

The MICs were determined for diaminobenzene BTs having side arms in the *ortho*-, *meta*- or *para*-positions against *E. coli* K12, *E. coli* Tet^R, and *S. aureus*. The MICs for the *ortho*- and *meta*-isomers ranged from 32 μM to 64 μM for all three bacteria. The *para*-isomer was less toxic than either of the others, but the MICs were ≥128 μM against all three bacteria. Similarly high MICs were observed for the alkyl BTs when $n = 3$, 4, or 6. In contrast, the MICs for Trp–$(CH_2)_{12}$–Trp against the three bacteria ranged from 4 μM to 10 μM.

We conducted the co-administration experiments described above using the BTs and Tet^R *E. coli.* In the absence of any adjuvant, the MIC for Tet^R *E. coli* was 900 μM (as noted above). The BT compounds were added to the growing bacteria at concentrations of either ½ or ¼ MIC and the MIC of tetracycline against the bacterium was determined. To the extent that any enhancement in antibacterial potency was observed, it is expressed as fold-recovery of resistance. Table 2 presents a few of the data obtained in an extensive set of experiments.



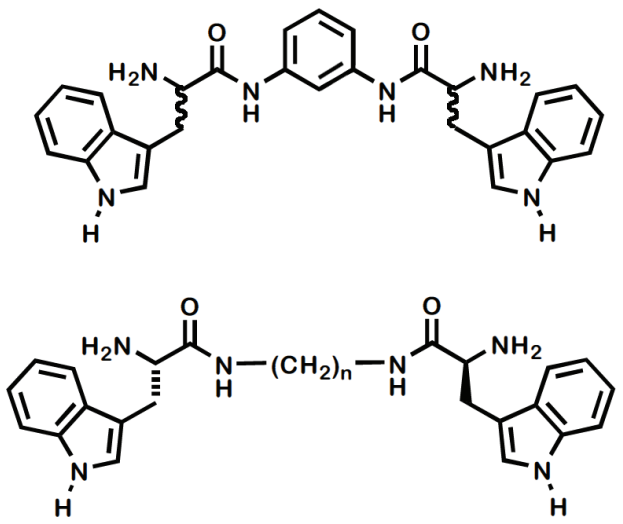
Scheme 4. Structures of 3-indolylmethyl hydraphiles. R may be H or CH_3.



Scheme 5. A hydraphile-inspired amphiphile having tryptophan termini.

The data shown in Table 2 were all obtained against Tet^R *E. coli*. Thus, any enhancement in tetracycline potency against this strain is not only an enhancement of efficacy, it is a reversal of resistance. The *meta*-phenylene compound was studied at both ½ and ¼ MIC (*i.e.* 24 μM or 12 μM BT was used in the experiment). Resistance reversal for this compound was 8-fold to 16-fold, depending on the adjuvant concentration. Similarly promising results were obtained for the BT in which the linker is propylene. A surprising result was that the BT having a C_{12} connector, representing the most toxic to Tet^R *E. coli* on its own, showed marginal activity as an adjuvant. At ½ MIC, a 4-fold resistance reversal was observed, so the 2-fold value is likely a real one.

One of the important questions regarding these potential adjuvants was if, and to what extent, they are toxic to mammalian cells. Thus, the three compounds shown in Table 2 were tested for toxicity against human embryonic kidney (*i.e.* HEK-293) cells and monkey kidney (*i.e.* Cos-7) cells. The *meta*-phenylene BT and the BT having a C_3 linker showed 100% survival (within experimental error) with both cell types. Cells administered with the BT having a C_{12} linker showed 100% survival for HEK-293 but only about 70% survival for Cos-7.



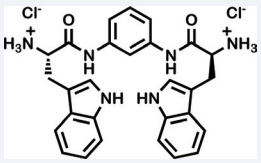
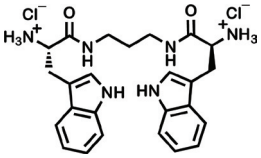
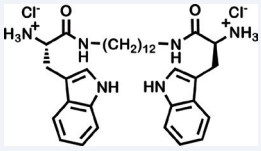
Scheme 6. Two families of *bis*(tryptophan)s. Top structure: *ortho-*, *meta-* and *para*-isomers and both d- and l-tryptophan stereochemistry. Bottom structure: *n* = 3, 4, 6, or 12.

It was important to obtain indications about the ability of the BTs to penetrate and be sustained in bacterial cells. This obviously needed to be contrasted with the undesired ability to penetrate mammalian cells. Experiments were conducted to determine the growth and microscopic characteristics of Tet^R *E. coli* in the absence of outside influences. A corresponding experiment was conducted in which 0.5% (v/v) DMSO was added, because this quantity of solvent was sometimes required to solubilize the amphiphilic adjuvants. The fluorescent dye propidium iodide (PI) was used as a reporter for cellular penetration; while it normally does not pass cellular boundary membranes, when it does, it interacts with intracellular DNA to afford a fluorescent signal.

The effects on Tet^R *E. coli* mediated by the *meta*-phenylene BT, the C_{12}-linked BT and the well-known detergent Triton X-100 were analyzed by confocal microscopy in the presence of PI. Internal fluorescein diacetate (FDA) was used as a reporter of cellular vitality, as the dye is hydrolyzed *in situ* and the diol is highly fluorescent; its green signal is apparent when no cellular damage has been done. The characteristic green fluorescence was observed for Tet^R *E. coli* alone and in the presence of 0.5% DMSO as well as both the *meta*-phenylene and C_{12}-linked BTs. No green fluorescence was observed when 0.1% of Triton X-100 was added.

No PI fluorescence was detected in the groups of bacteria alone or bacteria + DMSO controls. An intense red signal was apparent in the Triton X-100 case, which presumably resulted from an interaction between the DNA that is detritus subsequent to cellular rupture. PI penetration of the *meta*-phenylene and C_{12}-linked BTs was apparent. This, coupled with the significant FDA signal, confirmed successful penetration of the dye mediated by the BTs. A similar study was undertaken with HEK-293 cells, and in that case, the FDA signal was strong and the PI signal was weak for the control

Table 2. Reversal of Tet^R,s tetracycline resistance by co-administration of bis(tryptophans)

Adjuvant	Amount used	Observed MIC (μM)	Resistance reversal
None	0	900	n/a
	½ MIC ¼ MIC	56 112	16-fold 8-fold
	¼ MIC	112	8-fold
	¼ MIC	450	2-fold

cells. As above, Triton X-100 ruptured the cells. In the case of both the *meta*-phenylene and C_{12}-linked BTs, PI penetration was obvious and the FDA signal reported good vitality.[67] Additional and intense study of the BT class of compounds is currently underway in our laboratory.

Conclusions

In the past, we have reported the success of several projects involving either indole or tryptophan as a key element. The insights gained from these efforts have led us to a new class of BT amphiphiles that enhance antibiotic potency in sensitive bacteria, but also re-sensitize antibiotic-resistant bacteria. A factor in the success of this approach is certainly enhanced membrane penetration, but we cannot rule out other effects of these novel structures.

Acknowledgments

We thank the PRF, National Institutes of Health and National Science Foundation (most recent: CHE 1307324) for support of the work described herein that was conducted over many years.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Bis(tryptophan) synthesis and studies (JWM), antimicrobial studies (MBP), collaborating on studies (SN), preparing the draft for manuscript (SN), revising the manuscript critically for important intellectual content (SN), preparing the draft for manuscript (RC), collaborating on studies (MRG), preparing the draft for manuscript (MRG), drafting the manuscript (GWG).

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