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Membrane-Targeting DCAP Analogues with Broad-Spectrum Antibiotic Activity against Pathogenic Bacteria

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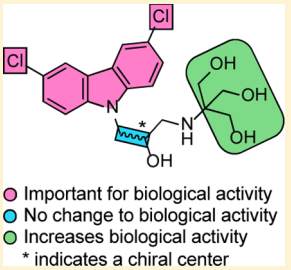
Supporting Information

ABSTRACT: We performed a structure−activity relationship study of 2-((3-(3,6-dichloro-9*H*-carbazol-9-yl)-2-hydroxypropyl)amino)-2-(hydroxymethyl)propane-1,3-diol (DCAP), which is an antibacterial agent that disrupts the membrane potential and permeability of bacteria. The stereochemistry of DCAP had no effect on the biological activity of DCAP. The aromaticity and electronegativity of the chlorine-substituted carbazole was required for activity, suggesting that its planar and dipolar characteristics orient DCAP in membranes. Increasing the hydrophobicity of the tail region of DCAP enhanced its antibiotic activity. Two DCAP analogues displayed promising antibacterial activity against the BSL-3 pathogens *Bacillus anthracis* and *Francisella tularensis*. Codosing DCAP analogues with ampicillin or kanamycin increased their potency. These studies demonstrate that DCAP and its analogues may be a promising scaffold for developing chemotherapeutic agents that bind to bacterial membranes and kill strains of slow-growing or dormant bacteria that cause persistent infections.



KEYWORDS: *Antibiotic, chemotherapeutic, antimicrobial, bacterial membrane, broad-spectrum activity*

An amalgamation of antibiotic resistance and drug discovery challenges has the potential of transforming bacterial infections into an emerging global threat. Exploiting new bacterial targets may close the gap between the disappointingly slow discovery of new antibiotics and the rate at which they lose potency against clinical pathogens, particularly in hospitals.[1−3] Dormant or slow-growing bacteria that are not susceptible to many classes of clinical antibiotics play a role in the loss of drug potency and the prevalence of antibiotic resistance. Inhibiting the replication of slow growing strains of bacteria using antibiotics can be particularly challenging because many drug targets (and mechanisms) are coupled to cell growth. These families of antibiotics may have limited impact on bacterial strains that grow slowly in response to the availability of nutrients and growth factors, physicochemical constraints and physiological changes associated with multicellular structures, and the environment within hosts.[4]

One emerging approach for inhibiting bacteria that grow slowly, are approaching stationary phase, or have reached the stationary phase is to target their membranes and alter the properties of this liquid crystalline material. Decreasing the transmembrane potential and increasing the permeability of phospholipid bilayers leads to cytotoxicity[5] and has been recently exploited to increase drug uptake.[6,7] Several families of antibiotics that fall into this functional class of small molecules have been described, including: (1) lipopeptides (e.g., daptomycin); (2) polypeptides (e.g., colistin and polymyxin B); and (3) natural and synthetic antimicrobial peptides.[5] Daptomycin is a clinical antibiotic that disrupts the membrane potential by inserting into the membrane, creating Ca^{2+}-dependent pores, and releasing intracellular ions.[8] Disrupting the transmembrane potential using daptomycin may also mislocalize membrane-associated proteins that are involved in essential cellular processes, ultimately leading to membrane permeability and cell death.[9]

We recently discovered 2-((3-(3,6-dichloro-9*H*-carbazol-9-yl)-2-hydroxypropyl)amino)-2-(hydroxymethyl) propane-1,3-diol (DCAP, **1**, Figure 1) in a high-throughput screen designed

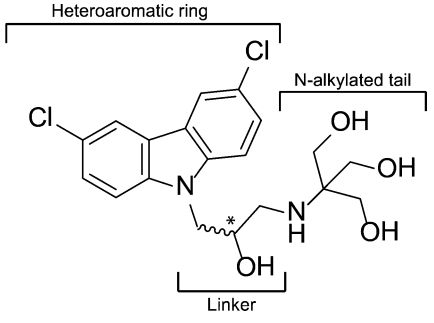


Figure 1. Chemical structure of **1** with the three distinct regions labeled: (1) heteroaromatic ring, (2) linker, and (3) *N*-alkylated tail. * indicates the chiral center.

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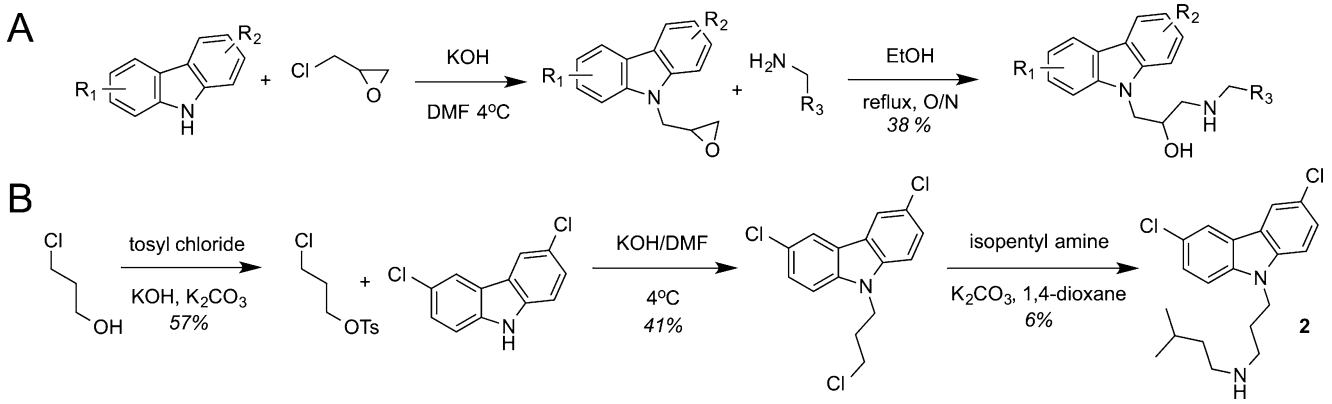
Scheme 1[a]



[a](A) The general synthetic strategy for the synthesis of **1** and its analogues. Reagents and conditions: (1) epichlorohydrin, KOH, DMF, 4 °C, overnight; (2) primary amine, EtOH, 78 °C, overnight. The yield included in this scheme is from the synthesis of **1** over the two steps. (B) The synthetic scheme for the synthesis of **2**. Reagents and conditions: (1) 4-toluenesulfonyl chloride, KOH, K_2CO_3, solvent-free; (2) KOH, DMF, 4 °C, overnight; (3) isopentyl amine, K_2CO_3, 1,4-dioxane, 100 °C, overnight. Yields listed in the scheme are for each individual synthetic step.

to identify small molecule inhibitors of MipZ, an ATPase that regulates the location of the division site in *Caulobacter crescentus*. Compound **1** was a weak inhibitor of the ATPase activity of *C. crescentus* MipZ in vitro; however, cells treated with the compound, including genera of bacteria that do not contain MipZ or homologous proteins, lysed rapidly. Our preliminary data demonstrated that **1** is a membrane-targeting small molecule in Gram-negative and Gram-positive bacteria and causes the mislocalization of membrane proteins that are sensitive to changes in membrane potential.[10]

The structural scaffold present in **1** occurs in a wide-range of biologically active compounds. Molecules effective against targets connected to several neurodegenerative diseases, such as Alzheimer's disease[11,12] and prion diseases,[13] contain the dichlorinated carbazolyl group and linker in **1**. This pharmacophore is also present in compounds with antiviral activity,[14] suppressors of mitochondrial apoptosis,[15] and cryptochrome activators.[16]

To improve the activity of DCAP, assess its toxicological properties, and study its biological function, we performed a preliminary structure−activity relationship (SAR) study. We tested the biological activity of these analogues against a broad panel of Biosafety Level 2 (BSL-2) and Biosafety level 3 (BSL-3) bacterial pathogens, including multidrug resistant (MDR) strains, and identified two analogues with promising activity against *Bacillus anthracis* and *Francisella tularensis*. This letter describes the results of these studies and provides a guide for developing the broad-spectrum activity of DCAP analogues as chemotherapeutic agents.

To perform a SAR study of **1**, we developed a linear, three-component synthesis. The synthetic strategy was designed to streamline the modification of the heteroaromatic ring and the *N*-alkylated tail group (Figure 1). This synthetic route involved alkylation of the carbazolyl amine with epichlorohydrin followed by opening the epoxide with a primary amine.[12] To synthesize **1** we reacted 3,6-dichlorocarbazole, racemic epichlorohydrin, and potassium hydroxide in dimethylformamide (DMF). The unpurified product was precipitated and reacted with tris base to provide **1** in 38% overall yield (two steps) (Scheme 1A). Creating a DCAP analogue devoid of the hydroxyl group in the linker required us to develop a synthetic strategy (Scheme 1B) using a different linker. To synthesize **2** we converted 3-chloropropanol to tosyl-protected 3-chloropropanol using tosyl chloride, potassium hydroxide, and potassium carbonate in solvent-free conditions (57% yield).[17] We reacted tosyl-protected 3-chloropropanol, 3,6-dichlorocarbazole, and potassium hydroxide in DMF to form the *N*-alkylated carbazole product in a yield of 41%.[12] The *N*-alkylated carbazole was added to a solution of potassium carbonate and isopentyl amine in 1,4-dioxane and refluxed for 24 h to form **2** in a yield of 6%.[18]

We tested the minimum inhibitory concentration (MIC) of DCAP analogues against *Escherichia coli* BW25113 (wildtype) and *E. coli* BW25113 Δ*tolC*; the latter strain lacks a primary component of drug efflux pumps and increases the intracellular concentration of drugs.[19] MIC measurements of analogues against a wildtype *E. coli* strain and *E. coli* BW25113 Δ*tolC* enabled us to gauge the efflux of these compounds (by comparing the two MIC values) and assess the biological activity of DCAP analogues. Although the membrane composition and properties of cells of *E. coli* K12 strains differ from many pathogenic bacteria, this model Gram-negative bacterium provides a convenient starting point for correlating changes in the structure of DCAP analogues and bioactivity. Comparing the MIC values of analogues of **1** against *E. coli* BW25113 made it possible for us to determine which portions of the molecule are important for its biological activity and which regions can be altered to increase potency. We were able to draw several correlations between structural components of **1** and antibacterial activity.

We investigated whether the stereochemistry of the secondary hydroxyl group in the linker of **1** is essential for its activity by synthesizing the S-enantiomer (**3**) and R-enantiomer (**4**) of DCAP (Figure 2A). The MIC values of **3** and **4** against *E. coli* BW25113 are both 80 μM, and the MIC values of both compounds against *E. coli* BW25113 Δ*tolC* are 20 μM, which is identical to the MIC of racemic **1** against these strains (Table 1). We also eliminated the stereocenter by synthesizing **2**. The MIC value of **2** against *E. coli* BW25113 and *E. coli* BW25113 Δ*tolC* is 80 and 5 μM, respectively, which is similar to the MIC of **10** against these strains. When combined with previous measurements of the influence of **1** on the transmembrane potential of bacteria,[10] these results support the hypothesis that

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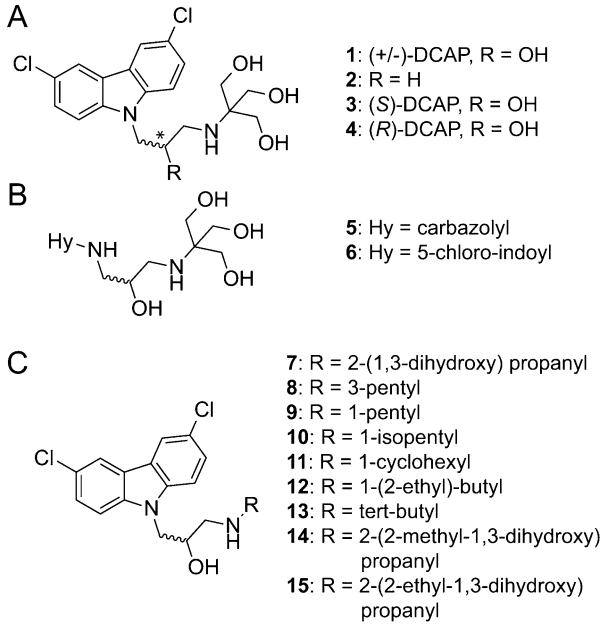

Figure 2. Chemical structures of DCAP analogues synthesized and tested are divided into three categories based on the region of the molecule manipulated: (A) the chiral center and hydroxyl group of the linker, (B) the heteroaromatic ring, and (C) *N*-alkylated tail.

Table 1. Measured MIC Values (μM) of Compounds 1−15 against *E. coli* BW25113 and *E. coli* BW25113 Δ*tolC* and Predicted cLogP Values

compd	*E. coli* BW25113	*E. coli* BW25113 Δ*tolC*	cLogP
1	80	20	1.46
2	80	5	6.05
3	80	20	1.46
4	80	20	1.46
5	>320	>80	0.24
6	>320	320	−0.62
7	40	20	2.26
8	80	5	5.10
9	80	5	5.16
10	40	2.5	5.04
11	40	2.5	4.92
12	80	2.5	5.50
13	160	40	4.32
14	80	40	2.42
15	160	20	2.88

the activity of **1** does not arise from binding a protein. We hypothesize that **1** binds to bacterial membranes directly.

By varying the chemistry of the heteroaromatic ring and measuring MICs, we established that the dichlorinated carbazolyl group is essential for the activity of **1**. Eliminating the electron-withdrawing chlorine atoms from the carbazolyl moiety (**5**) (Figure 2B) increased the MIC to >80 μM against *E. coli* BW25113 Δ*tolC*. Replacing the carbazolyl ring with a chlorinated indole ring (**6**) (Figure 2B) significantly increased the MIC (to 320 μM) against *E. coli* BW25113 Δ*tolC* (Table 1).

Manipulating the *N*-alkylated tail of **1** alters its activity (Figure 2C). Removing one of the hydroxyl groups from the tail provided 1,3-dihydroxy-propanyl analogue (**7**), which displayed a small decrease in MIC against *E. coli* BW25113. This result led us to increase the hydrophobicity of the tail region and eliminate the hydroxyl groups completely. As the hydrophobicity of the tail increased, the activity of analogues against *E. coli* BW25113 Δ*tolC* also increased. MIC values of analogues containing the linear pentyl group (**8**) and the branched pentyl group (**9**) were both 5 μM against *E. coli* BW25113 Δ*tolC*. MIC values of DCAP analogues containing isopentyl (**10**), cyclohexyl (**11**), and (2-ethyl)-butyl (**12**) groups against *E. coli* BW25113 Δ*tolC* were 2.5 μM (Table 1). These MIC values were 8-fold lower than the MIC of **1** (20 μM). Anticipating that replacement of the tris-hydroxyl moiety in **1** with an alkyl chain alters the physicochemical properties of the analogues, we calculated cLogP values to provide an estimate of hydrophobicity. Predicted cLogP values for **8**, **9**, **10**, **11**, and **12** were 5.10, 5.16, 5.04, 4.92, and 5.50, respectively; as a point of reference, the cLogP of **1** was 1.46 (Table 1). The general trend that we observed was that increasing the hydrophobicity of *N*-alkylated tails, and thus increasing cLogP values, improved the activity of analogues. Because of the increased potency of **10**, **11**, and **12** against *E. coli* BW25113 Δ*tolC* (compared to **1**), we decided to pursue these compounds further against a broad panel of BSL-2 and BSL-3 pathogens.

We tested the antibacterial activity of **10**, **11**, and **12** against a panel of clinically isolated BSL-2 pathogenic strains (Table 2).

Table 2. MIC Values (μM) of 1 and 2 against a Panel of BSL-2 Bacterial Strains

BSL2 strains	**1**	**10**	**11**	**12**
V. cholerae	50	6.25	6.25	6.25
S. typhimurium	100	50	100	200
S. boydii	100	25	50	25
E. tarda	100	25	50	12.5
K. pneumoniae	200	50	>200	>200
E. aerogenes	200	50	>200	>200
A. baumannii	200	12.5	25	25
M. morganii	200	50	>200	>200
S. aureus	40	5	5	5

BSL-2 pathogens used in this study, included *Vibrio cholerae*, *Salmonella enterica* Typhimurium (*Salmonella typhimurium*), *Shigella boydii*, *Edwardsiella tarda*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Acinetobacter baumanii*, *Morganella morganii*, and *Staphylococcus aureus*. As a comparison study we also determined MIC values of **1** against these organisms. Of the three analogues studied, **10** was more potent than **1** against BSL-2 pathogenic strains and was the most potent analogue against all the BSL-2 strains tested with the exception of *E. tarda* (which was most susceptible to **12**). Compounds **11** and **12** were more active than **1** against *V. cholerae*, *S. boydii*, *E. tarda*, *A. baumannii*, and *S. aureus*. The MIC of **12** against *S. boydii* and *E. tarda* was 25 and 12.5 μM, respectively, and the MIC of **11** and **12** against *A. baumannii* was 25 μM, and 5 μM against *S. aureus*. Compounds **11** and **12** did not display improved antibacterial activity against *S. typhimurium*, *K. pneumoniae*, *E. aerogenes*, and *M. morganii* compared to **1**. These results demonstrate that **10** was the most active analogue against these pathogenic strains.

We expanded our MIC study of the activity of **10** and **11** to include BSL-3 pathogens and multidrug resistant (MDR) strains. We tested **3** (as a reference compound), **10**, and **11** against a routinely used library of BSL-3 clinical isolates, including strains of *Francisella tularensis* (*FT*), *Bacillus anthracis*

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(*BA*), *Burkholderia mallei* (*BM*), *Yersinia pestis* (YP), and *Burkholderia pseudomallei* (*BP*) (Table 3). We measured MIC_{50}

Table 3. MIC_{50} and MIC_{90} (μM) Values of 3, 10, and 11 against a Panel of BSL-3 Bacterial Strains Measured at USAMIIRD

	3		10		11	
BSL-3 strains	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
FT	85	170	5.0	10	2.5	10
BA	42	42	5.0	5.0	5.0	10
BM	≥170	≥170	21	42	21	82
YP	≥170	≥170	21	42	41	82
BP	≥170	≥170	170	≥170	164	≥164

and MIC_{90} values against 30 isolates of each of these bacterial strains, which represent the concentration of compound that inhibited growth of 50% or 90% of the bacterial isolates, respectively (Appendices A and B in Supporting Information). MIC_{50} and MIC_{90} values for **10** and **11** were lower than **3** against all of the strains tested, except for *B. pseudomallei*. MIC_{90} of **10** and **11** against *B. mallei* and *Y. pestis* were 42 and 82 μM, respectively; MIC_{50} and MIC_{90} values for **3** were >170 μM. The MIC_{90} of **10** was 10 μM against *F. tularensis* and 5 μM against *B. anthracis*, and the MIC_{90} value of **11** was 10 μM against *F. tularensis* and *B. anthracis*. Compounds **10** and **11** were most efficacious against *F. tularensis* and *B. anthracis*. In an effort to increase the potency of **10**, we codosed **10** with clinical antibiotics that may hit targets that could complement the antibacterial activity of **10** and lead to synergistic effects.

Bacterial cells dosed with **10** and sublethal concentrations of clinical antibiotics (i.e., below the MIC) may have a synergistic effect, reduce the effective concentration of antibiotics, minimize the frequency at which antibiotic-resistant mutants emerge, and effectively extend the shelf life of therapeutic compounds.[20,21] To explore this concept, we varied the concentrations of **10** with ampicillin against *E. coli* MG1655 (Gram-negative bacteria) and *Staphylococcus aureus* (Gram-positive bacteria), measured MIC values, and visualized the data in a checkerboard format. Our rationale was that the combination of altered properties of membranes (**10**) and the peptidoglycan (ampicillin) might be particularly lethal as it would destabilize the cell envelope. We found synergy between **10** and ampicillin (Figure 3A). The MIC of ampicillin against *E. coli* MG1655 and *S. aureus* is 10 and 1 μM, respectively (Table S1); both checkerboard assays were performed with concentrations of ampicillin below these MIC values. The combination of >4 μM of ampicillin with any concentration of **10** inhibited cell growth in *E. coli* MG1655. Codosing cells with >200 nM of ampicillin with any concentration of **10** inhibited cell growth in *S. aureus*. This growth inhibition demonstrates a synergy between a peptidoglycan biosynthesis inhibitor and a compound that increases the membrane permeability of the membrane.

We also tested whether inhibiting the production of proteins could enhance the activity of **10**. Although our preliminary evidence indirectly suggested that **1** interacted with membranes,[10] we reasoned that if cell death ultimately occurred through mechanisms that were protein dependent, reducing the concentration of proteins may alter the susceptibility of cells to **10**. To test this hypothesis, we dosed *E. coli* MG1655 and *S. aureus* cells with varying concentrations of **10** and kanamycin (binds to the 30S subunit of the ribosome), determined MIC

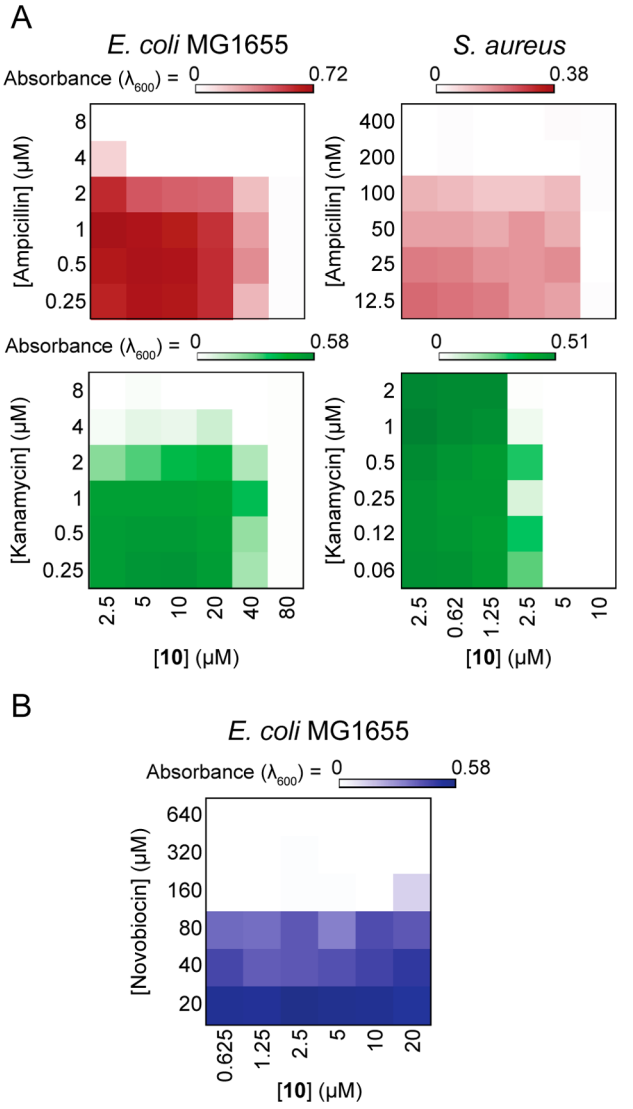

Figure 3. Synergy and additivity studies with **10** and ampicillin, kanamycin, and novobiocin. (A) Checkerboard assays with **10** and sub-MIC concentrations of ampicillin (red) and kanamycin (green) against *E. coli* MG1655 and *S. aureus*. (B) Checkerboard assay with **10** and novobiocin (blue) against *E. coli* MG1655.

values, and visualized the data in a checkerboard plot. We only found synergy between **10** and kanamycin against *E. coli* MG1655 (Figure 3A). The MIC of kanamycin against *E. coli* MG1655 and *S. aureus* is 10 and 2.5 μM, respectively (Table S1). Codosing *E. coli* MG1655 with **10** and sublethal concentration of kanamycin resulted in inhibited growth with >4 μM of kanamycin at any concentration of **10**. The combination of sublethal concentrations of kanamycin and any concentration of **10** did not inhibit the growth of *S. aureus*. These results suggest that **10** facilitates the transport of kanamycin across the cell wall of *E. coli*, but not *S. aureus*.

Finally, we tested the hypothesis that **10** may alter properties of cell membranes and increase the transport of clinical antibiotics that are excellent substrates of drug efflux pumps. Novobiocin is an aminocoumarin-based, clinically approved small molecule antibiotic that binds DNA gyrase, inhibits its ATPase activity, and suppresses negative supercoiling of the chromosome.[22,23] One of the challenges of using novobiocin as a chemotherapeutic agent is that it is rapidly pumped out of

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some bacteria and does not reach an intracellular concentration that creates a bacteriostatic effect.[24,25] This effect is clear in our MIC measurements of novobiocin against *E. coli* cells with and without the essential drug efflux pump component, TolC. The MIC of novobiocin against wildtype *E. coli* MG1655 cells was 320 μM, while the MIC against *E. coli* BW25113 Δ*tolC* was 1.2 μM (Table S1). To test this hypothesis, we codosed *E. coli* MG1655 cells with varying concentrations of novobiocin and **10**. A checkerboard plot demonstrates no significant change in the antibacterial activity of novobiocin when codosed with different concentrations of **10** (Figure 3B), suggesting that **10** does not alter membrane properties in a manner that influences the transport or passive diffusion of novobiocin into cells.

In conclusion, our retrosynthetic analysis led us to disconnect **1** into three structural components: (1) heteroaromatic ring, (2) linker region containing the secondary hydroxyl group, and (3) amine-linked tail region (Figure 1). We found that the stereochemistry of the linker had no influence on the biological activity of 1, signifying it does not target a specific protein but directly interacts with the cellular membrane. Through these studies we established that the carbazole moiety with chlorine substitutions is an important element for biological activity suggesting that its planar, hydrophobic, and dipolar characteristics are important for orienting the molecule in the membrane. Manipulation of the tail group affects the potency of the compound, and increasing its hydrophobicity improves the activity of the molecule, presumably through interacting with membranes through the lipophilic tail of the phospholipids. We discovered that the antibacterial activity of **10** can be enhanced by codosing with other antibiotics. Mouse infection model studies of **10** and **11** against *F. tularensis* and *B. anthracis* are currently underway with USAMRIID and will provide insight into the clinical capabilities of this class of antibiotics.

■ ASSOCIATED CONTENT

Supporting Information

All synthetic procedures, compound characterization data, and biological methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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