Design and Synthesis of Potent Sensitizers of Gram-Negative Bacteria Based on a Cholic Acid Scaffolding

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Received November 12, 1997

The outer membrane of Gram-negative bacteria provides a protective barrier against proteases, lysozymes, and many types of antibiotics.[1] Consequently, numerous antibiotics that are active against Gram-positive bacteria are much less active against Gram-negative strains. Lipid A is a primary component of the outer membrane of Gram-negative bacteria and plays an essential role in cell wall integrity. Compounds known to associate strongly with lipid A disrupt the organization of the outer cell wall and thereby sensitize Gram-negative bacteria to antibiotics.[2] These compounds have generally been derivatives of polymyxin B (**1**, Figure 1), the most common member of a family of related peptide antibiotics. Derivatives of polymyxin B such as polymyxin B nonapeptide (**2**, Figure 1), and polymyxin B heptapeptide (**3**, Figure 1) sensitize bacteria to antibiotics without causing toxic effects that limit the use of polymyxin B.[3,4] However, compounds such as **2** and **3** are difficult to prepare and purify.[2c,i] We have prepared simple, nonpeptide mimics of polymyxin B that act as potent sensitizers of Gram-negative bacteria to antibiotics.

Design of the new sensitizers was based upon modeling of the lipid A binding domain of polymyxin B and consideration of the conserved residues found in polymyxin B and related antibiotics. Through the pioneering work of Vaara and co-workers,[2c] the lipid A binding domain of polymyxin B has been identified as the macrocyclic portion of the molecule (**3**). Molecular modeling[5] of **3** with constraints derived from reported NOESY experiments[6] and predicted peptide turn formation[7] provided a low-energy structure in which the three amine groups derived from diaminobutyric acid residues are oriented on one face of the molecule (Figure 2). Since these diaminobutyric acid groups are conserved among the related antibiotics polymyxins A, B_1, B_2, D_1, E_1, and E_2, circulin A, and octapeptins A_1, A_2, A_3, B_1, B_2, B_3, and C_1, we included three primary amines in the design of our sensitizers. The fact that simple polyamines and linear versions of **3** do not sensitize Gram-negative bacteria to antibiotics[8] suggests that a specific arrangement of amine groups is required for sensitization activity. Our modeling demonstrated that appropriate functionalization of cholic acid results in orientation of three amine groups in a conformation comparable to that predicted for **3** (Figure 2). Our hypothesis was that if association of **3** with lipid A were mediated by the diaminobutyric acid side chains, then the cholic acid derivatives would bind to lipid A and sensitize bacteria to antibiotics.

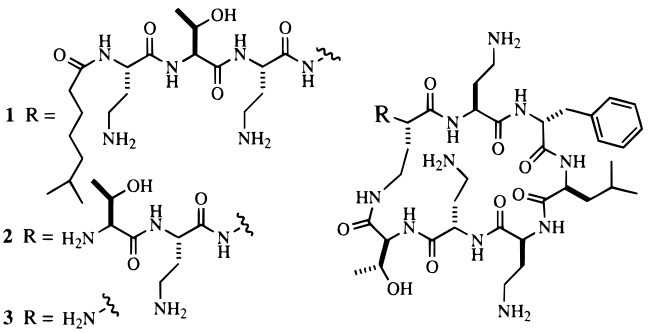

Figure 1. Polymyxin B_2 (**1**), polymyxin B nonapeptide (**2**), and polymyxin B heptapeptide (**3**).

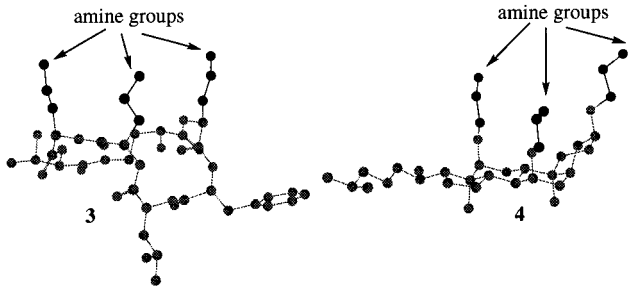

Figure 2. Conformations of **3** and **4** predicted using molecular mechanics (MM3 parameters using the program Spartan). Primary amine groups are indicated. Hydrogens and the phenyl group of **4** have been omitted for clarity.

Preparation of the cholic acid derivatives is shown in Scheme 1. Tethers of either two or three carbons were used between the steroid and the amine groups. Also, because guanidine groups were expected to interact strongly with phosphates on lipid A,[9] **4** and **5** were converted to the tris(guanidine) analogues **6** and **7**.[10]

The ability of **4**−**7** to sensitize bacteria to antibiotics and to inhibit the growth of Gram-negative bacteria was assayed by measuring minimum inhibition concentration (MIC) values.[11] To distinguish between bacteristatic and bactericidal activity, we also measured minimum bactericidal concentration (MBC) values.[12]

Prior to measuring sensitizing activity of **4**−**7**, we determined the effects of these compounds alone on bacterial growth. Unexpectedly, **4**−**7** exhibited bacteristatic and bactericidal activity. We first measured MIC values of **4**−**7** with *Escherichia coli* strain ATCC 10798, and the results are shown in Figure 3. The

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(5) Using MM3 parameters with the program Spartan.

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(11) An MIC measurement consists of incubating a known concentration of bacteria for 24 h in a nutrient broth containing incrementally varied concentrations of the compound of interest followed by determination of bacterial growth via cell counting and turbidity measurements. The MIC value is the concentration of the studied compound at which the number of bacteria remains constant or decreases during incubation. (For example, see: Pitt, W. G.; McBride, M. O.; Lunceford, J. K.; Roper, R. J.; Sagers, R. D. *Antimicrob. Agent Chemother.* **1994**, *38*, 2577.) In our experiments, each MIC determination was repeated three times with MIC values varying by less than 10%.

(12) An MBC value is the concentration at which fewer than 0.1% of bacteria survive incubation with the compound of interest.

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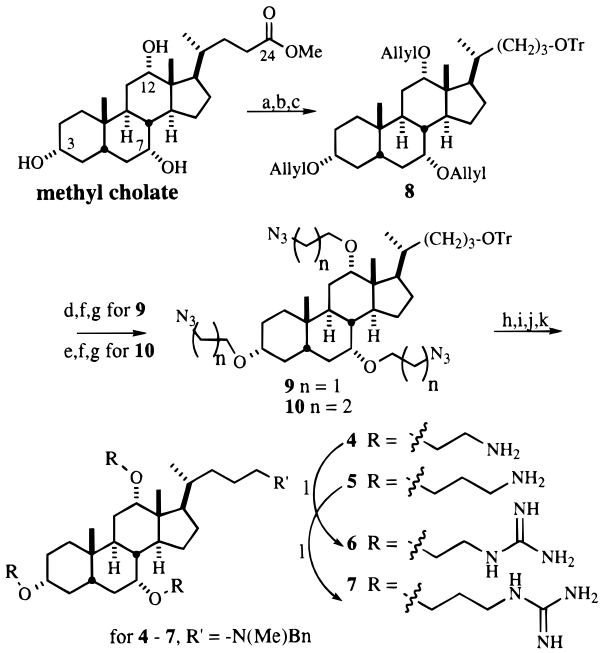
Published on Web 03/12/1998

Scheme 1



Reagents (reaction yields in parentheses): a) $LiAlH_4$, THF (98%). b) tritylchloride, Et_3N, DMF (70%). c) allylbromide, NaH, THF (96%). d) O_3, CH_2Cl_2, MeOH; Me_2S; $NaBH_4$ (95%). e) 9-BBN, THF; H_2O_2, NaOH (80%). f) MsCl, CH_2Cl_2, Et_3N (78%, 99%). g) NaN_3, DMSO (66%, 95%). h) TsOH, MeOH (94%, 95%), i) MsCl, CH_2Cl_2, Et_3N (99%, 97%). j) N-benzylmethylamine (95%, 96%). k) $LiAlH_4$, THF (95%, 99%). l) $NH_2C(NH)SO_3H$, MeOH (91%, 89%).

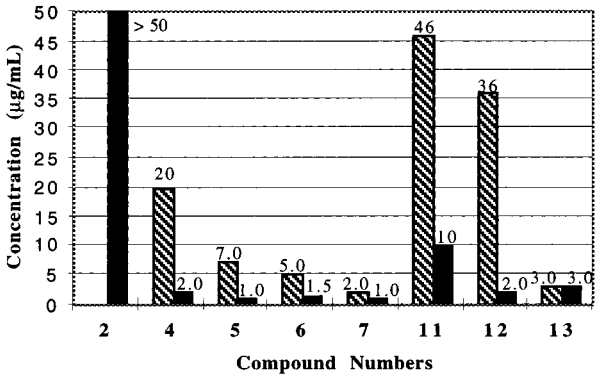


Figure 3. Hatched bars: MIC values for compounds **4**−**7** and **11**−**13** with *E. coli* (ATCC 10798). Solid bars: concentrations of compounds **2**, **4**−**7**, and **11**−**13** required to lower the MIC of erythromycin from 70 to 1 μg/mL with *E. coli* (ATCC 10798).

data suggest that longer tethers between the steroid backbone and the primary amines increase activity as well as replacement of amines with guanidine groups. MBC values were also measured for **4**−**7** and were found to be approximately 1.5 times as high as the MIC values.

We used erythromycin to measure the ability of **4**−**7** to sensitize bacteria to other antibiotics. Erythromycin is not as active against Gram-negative bacteria as it is against Gram-positive strains due to the inability of the large, partially hydrophobic antibiotic to traverse the outer membrane of the former.[1] The MIC of erythromycin with *E. coli* (ATCC 10798) is 70 μg/mL, and our bench mark was to lower the MIC of erythromycin to 1 μg/mL.[13] Cultures containing 1 μg/mL of erythromycin were incubated with incrementally varied concentrations of **4**−**7**. The results are shown in Figure 3. All four compounds displayed a synergistic inhibition of bacterial growth with erythromycin. With **4**, the MIC of erythromycin was lowered by a factor of 70 and the MIC of **4** by a factor of 10. To compare **4**−**7** with known bacterial sensitizers, we used the same conditions to measure synergism with **2**, a compound described as a potent sensitizer of Gram-negative bacteria.[2] The amount required to lower the MIC of erythromycin to 1 μg/mL was over 50 μg/mL.

To demonstrate that the activity of the cholic acid derivatives was not antibiotic dependent, we repeated the sensitization experiments with novobiocin, an antibiotic unrelated to erythromycin. The MIC of novobiocin with *E. coli* (ATCC 10798) is >500 μg/mL. Concentrations of 4 μg/mL of **4** or 2 μg/mL of **5** lowered the MIC of novobiocin to 1 μg/mL.

To verify that the effects of the cholic acid derivatives were not strain or species dependent, we measured MIC and sensitization data with an additional strain of *E. coli* and with a strain of *Pseudomonas aeruginosa*. The MIC values and sensitization properties of **4**−**7** with *E. coli* (ATCC 25922) proved to be very similar to those measured with *E. coli* (ATCC 10798). *P. aeruginosa* strains are typically more resistant to antibiotics than *E. coli*,[4] and the MIC values of erythromycin, **4**, and **5** with *P. aeruginosa* (ATCC 27853) were 240, 45, and 25 μg/mL, respectively. The sensitization properties of our cholic acid derivatives also decreased, although the trends remained the same (e.g., 12 μg/mL of **4** or 7 μg/mL of **5** lowered the MIC of erythromycin to 5 μg/mL). These results demonstrate that the cholic acid derivatives are capable of sensitizing multiple strains and species of Gram-negative bacteria to antibiotics.

To probe the requirements for a spatial arrangement of amine groups in bacterial sensitization, we prepared the C-3-epimer of **4** by inverting[14] the alcohol group at C-3 on cholic acid and following the procedures outlined in Scheme 1 to give **11**. With **11**, we found a significant rise in the MIC (as compared to **4**) and a decrease in sensitization properties of this compound (Figure 3). These results suggest the importance of a specific arrangement of amines in the cholic acid derivatives.

Bactericidal and bacteristatic activity of polymyxin B (**1**) can be eliminated while preserving sensitization properties by removal of the fatty acid and any number of the exocyclic amino acids from the antibiotic giving compounds such as **2** and **3**.[2] We hypothesized that there might be an equivalent effect from similar variations of the group extending from C-24 in the cholic acid derivatives. To address this issue, we prepared **12** (compound **5** with R′ = OH) and **13** (compound **5** with R′ = -O$(CH_2)_7CH_3$). We expected that if the group at C-24 were involved in bacteristatic activity, the MIC of **12** would increase compared to that of **5** and that the MIC of **13** would decrease. Indeed, these effects were observed (Figure 3). However, even more striking is that the abilities of the two compounds to sensitize bacteria were nearly indistinguishable (Figure 3). These data suggest that sensitization properties can be separated from bacteristatic activity in a fashion similar to that observed with polymyxin B and its derivatives.

We have demonstrated that appropriate arrangements of primary amines or guanidine groups on a steroid scaffolding can produce potent sensitizers of Gram-negative bacteria. These sensitizers may allow use of antibiotics against Gram-negative bacteria that have traditionally been used primarily against Gram-positive bacterial infections.

Acknowledgment. We thank Brigham Young University for financial support, Cynthia Burrows and Morris Robins for helpful suggestions, and William Pitt for help initiating MIC measurements.

Supporting Information Available: Experimental data including synthetic details for compounds **4**−**7** and **11**−**13** and representative procedures for MIC and MBC measurements (30 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(13) Many clinically useful antibiotics against Gram-negative bacteria have MIC values of ca. 1 μg/mL (see ref 4).

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