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Synthesis and Characterization of Peptide–Cationic Steroid Antibiotic Conjugates

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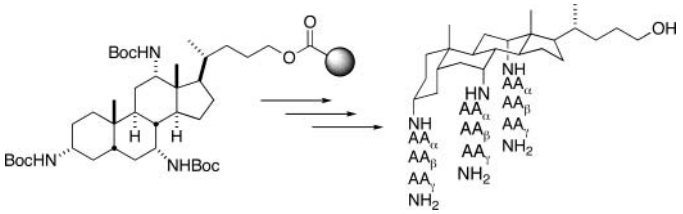
ABSTRACT



New cationic steroid antibiotics have been prepared by conjugating tripeptides to a triamino analogue of cholic acid. These compounds were synthesized on a solid phase in an indexed library that was screened for antibacterial activity against Gram-negative and Gram-positive bacteria. Selected compounds were synthesized on a larger scale, and minimum inhibition concentrations were measured. These results correlated very well with the screening of the indexed library of antibiotics. The most active antibiotics demonstrate a marked improvement over a related and well characterized cationic steroid antibiotic.

Cationic steroid antibiotics (CSAs) were developed to mimic the antibacterial behavior of endogenous peptide antibiotics.[1] This behavior includes selective association of the antibiotics with and disruption of bacterial membranes.[2,3] This activity results in rapid bactericidal activity with a minimal potential for causing the emergence of resistance. These antibiotics, in general, adopt cationic, facially amphiphilic[4] conformations (e.g., see **1** in Figure 1), which appears to be the key requirement for antibacterial activity, and membrane selectivity is primarily derived from ionic recognition of negatively charged bacterial membranes. However, bacterial membrane components present more that anionic groups, and it is anticipated that additional, associative noncovalent interactions would increase affinity for membrane-active antibiotics for bacterial membranes and increase antibacterial activity. Consequently, we have explored means of increasing the functionality presented by CSAs. Due to the relatively rigid and preorganized forms of CSAs, it is clear that

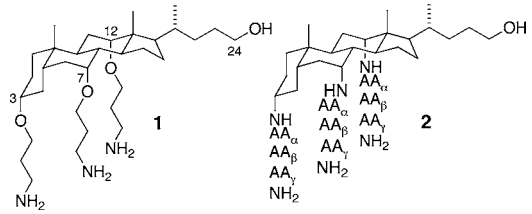


Figure 1. Structure of CSA **1** and a schematic representation of a CSA−peptide conjugate (**2**).

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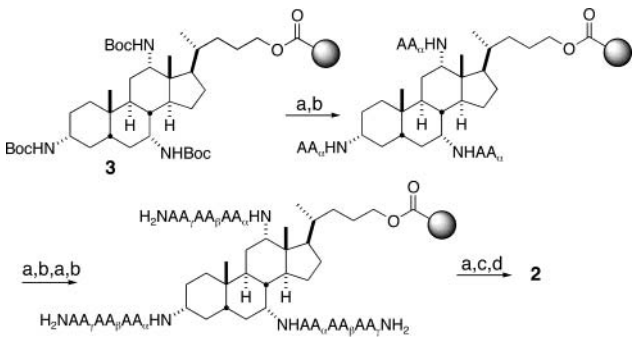
attachment of functionality designed to interact with bacterial membrane components should be on the polar face of the molecules. Derivatization of cholic acid, and analogues, with diverse functional groups has been very effective in the development of receptors for a variety of small molecules.[5]

We have developed an effective means of preparing combinatorial libraries of CSAs derived from a steroid scaffolding and amino acids.[6] We now report extension of this methodology to generation of libraries of peptide−CSA conjugates. Libraries of peptide-steroid conjugates have been reported with enzyme-like activity and as receptors for other peptides.[7] These libraries have incorporated only two peptide chains on the steroid nucleus and were not screened for antibacterial activity. Using a triamino analogue reported earlier[8] and in a parallel synthesis format, we have prepared an indexed library of tripeptide-containing CSAs, using six different amino acids, yielding 216 different compounds (**2** in Figure 1). The six amino acids used, histidine, lysine, methionine, phenylalanine, tryptophan, and valine, were selected for an initial library because this group contains basic amino acids that will contribute to the facial amphiphilicity of the CSAs and hydrophobic and aromatic amino acids that may interact with the nonpolar components of bacterial membranes. Following preparation of the library, we screened the compounds for activity against Gram-negative and Gram-positive bacteria. Tripeptides were used in these compounds because modeling of peptide-appended cholic acid derivatives suggested that peptide chains of up to three amino acids in length were sufficiently short to maintain the facially amphiphilic morphology necessary for antibacterial activity. Following the screening, we prepared and purified relatively large amounts of selected compounds characterized in the screening process, and we have verified that the screening process yields meaningful information about antibacterial activity. From this effort we have found sequences of tripeptides that yield CSA−peptide conjugates with good antibacterial properties.

Generation of the tripeptide-containing library began with **3** immobilized on carboxylic acid-modified polystyrene beads (Scheme 1). The Boc groups were removed, and the beads were partitioned into six groups. Each group was reacted with a different activated Boc-protected amino acid (δ-Fmoc lysine) to give amino acids directly attached to the amine groups on the steroid (AA_α).

Scheme 1. Preparation of Indexed Library **2**



This procedure was repeated two additional times adding AA_β and AA_γ, giving tripeptide-containing CSAs on approximately 5 mg of beads in each group. The remaining Boc groups were removed, followed by cleavage of the Fmoc groups on lysine. The beads were then treated with a 0.1 M solution of sodium methoxide in methanol to release the CSAs from the beads. The beads were removed; the solutions were neutralized with dilute hydrochloric acid in methanol, and the solvent was removed. The resulting materials were dissolved in 1 mL of water and serially diluted for use in antibacterial screening assays. In larger scale synthesis of compounds represented by **2**, we found that we could isolate approximately 200 mg of material from 1 g of beads. Consequently, the concentrations of the libraries of CSAs were expected to be 1 mg/mL before the dilutions described below.

The indexed libraries were screened for antibacterial activity against Gram-negative (*Escherichia coli* (ATCC 25922)) and Gram-positive bacteria (*Staphylococcus aureus* (ATCC 25923) using a micro-broth dilution method.[9] The solutions of CSAs were introduced to bacterial suspensions at fractions of the original concentrations (1/4, 1/16, 1/64, 1/256), and the lowest concentration of each CSA that inhibited bacterial growth (24 h incubation) was used to classify the antibiotic as weakly, moderately, or strongly active. A number of the antibiotics were active against *S. aureus* at a dilution of 1/64 (Figure 2); CSAs with AAα of

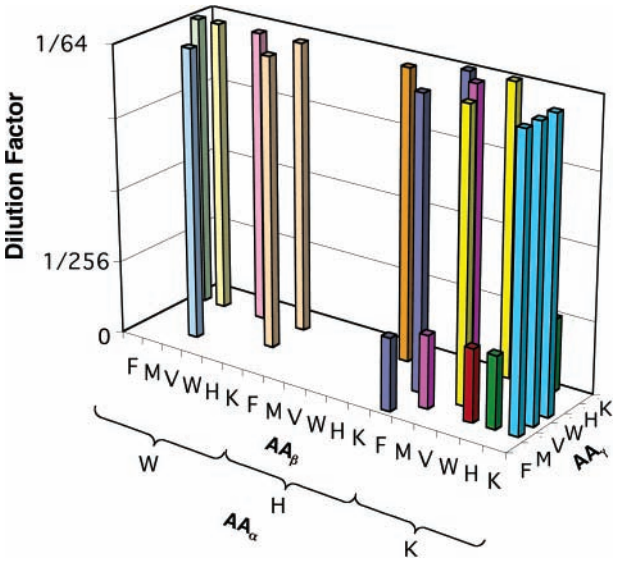


Figure 2. Dilutions at which indexed CSA libraries inhibited the growth of *S. aureus*.

F, M, or V did not show activity at or below dilution of 1/64 and are not described in Figure 2. The most active compounds inhibited growth at a dilution of 1/256. These antibiotics, containing the tripeptides FFK, MMK, MWK, KHK, and MHK, as well as a majority of the CSAs active at a dilution of 1/64, contain lysine attached directly to the steroid. The requirement for lysine at the AAα position may

be due to selection of compounds that are strongly facially amphiphilic, whereas compounds with amine groups found only at the N-termini of the peptide chains may not retain the necessary amphiphilic morphology.

The antibiotics that were active against *E. coli* included compounds that were active against *S. aureus*, but there were some differences upon screening with Gram-positive vs Gram-negative bacteria. As with *S. aureus*, compounds with AAα of F, M, or V were not active at dilutions below 1/16, and only compounds active at dilutions of 1/64 and 1/256 are described in Figure 3. The antibiotics active at a dilution

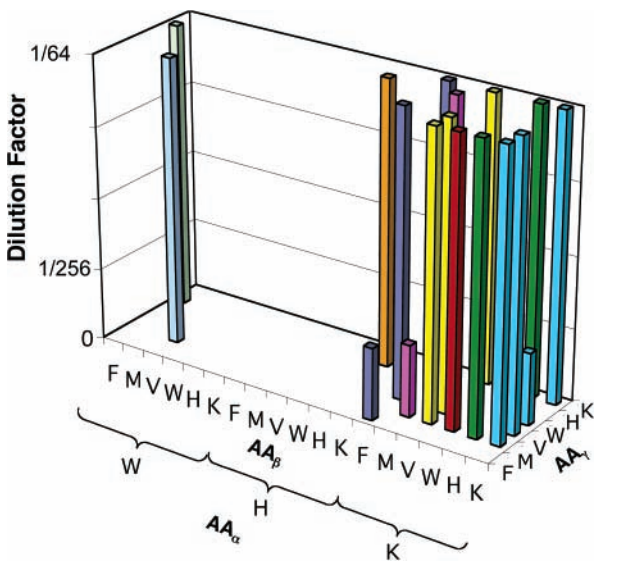


Figure 3. Dilutions at which indexed CSA libraries inhibited the growth of *E. coli*.

of 1/256 were FFK, MMK, and WKK. In this case an even stronger preference for lysine at the AAα position was observed, a feature of active antibiotics possibly necessary for effective interaction with the outer membranes of Gram-negative bacteria.

To verify that the solid-phase synthesis and screening procedures allowed identification of novel active antibiotics, we selected three compounds with varied activities to prepare on a larger scale for comparison of minimum inhibition concentrations (MICs). As the least active compound, we prepared the CSA containing VKF; as a compound with intermediate activity, we prepared the CSA containing VKW; and as the most active compound, we prepared the CSA containing FFK. In the screening experiments, these three compounds inhibited growth of *S. aureus* at dilutions of 1/16, 1/64, and 1/256, respectively.

Efficient syntheses of the three targeted CSAs in the relatively large scale necessary for characterization and MIC measurements required modification of the synthetic procedures used in preparing the indexed libraries. To maximize the convergent nature of the synthesis, tripeptides were prepared and purified before attachment to the steroid scaffolding. The tripeptides were assembled using standard peptide coupling procedures (Boc-protected amino acids, diisopropylcarbodiimide, *N*-hydroxysuccinimide) beginning with the methyl ester of the C-terminal amino acid. For lysine, a δ-Cbz protecting group was used, and after generation of the tripeptide, this group was replaced by a Boc protecting group to allow deprotection of all amine groups in one step. The methyl ester was hydrolyzed with lithium hydroxide in methanol, and no epimerization was observed in the corresponding ^{1}H NMR spectrum of each tripeptide. The resulting acid was coupled to the required triamino analogue of cholic acid using diisopropylcarbodiimide and *N*-hydroxysuccinimide. Use of 4-(dimethylamino)-pyridine as a catalyst in this step resulted in significant amounts of epimerization as observed by ^{13}C NMR, and while the reaction was relatively slow without this catalyst, epimerization was not observed via NMR. Removal of the six Boc groups on each compound generated compounds **4**−**6** (Figure 4).

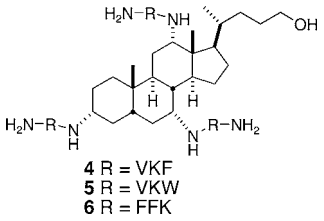


Figure 4. Structures of tripeptide-containing CSAs prepared for comparison of MIC values.

MIC values for compounds **4**−**6** with *S. aureus* were measured using macro-broth dilution methods.[9] These values are >100, 40, and 8 μg/mL, respectively. As we had anticipated, the trend of increasing antibacterial activity (**4** to **5** to **6**) is consistent with the screening results (Table 1). In the screening assay, **4** and **5** inhibited growth of *E. coli* at dilutions of 1/16, while **6** inhibited growth at a dilution of 1/256. The respective MIC values of these compounds with *E. coli* are >100, >100, and 8 μg/mL, again consistent with the outcome of the screening experiments. Taken together, these results suggest that solid-phase synthesis and

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(9) National Committee for Clinical Laboratory Standards. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard M7-A4*, 4th ed.; NCCLS: Villanova, PA, 1997.

Table 1. Comparison of the Dilutions at Which Compounds **4**−**6** from Solid-Phase Synthesis Inhibited Bacterial Growth and the MIC Values of These Compounds Measured Independently

	compounds		
	4	**5**	**6**
S. aureus			
active dilution[a]	1/16	1/64	1/256
MIC (μg/mL)	>100	40	8
E. coli			
active dilution[a]	1/16	1/16	1/256
MIC (μg/mL)	>100	>100	8

[a] Dilution of the initial solutions from the library (see text) that inhibit bacterial growth.

micro-broth dilution screening for antibacterial activity yield meaningful information about the types of amino acids that can be incorporated into active CSAs.

We have demonstrated that the nature of the group at the C24 position of CSAs (see Figure 1 for steroid numbering) strongly influences their antibacterial activities.[10] For example, **1** has an MIC of 36 μg/mL against *E. coli*, and if the hydroxyl group at C24 is replaced by an octylamine group, the MIC of the resulting CSA is 3 μg/mL. As compared to **1**, CSA **6** has a relatively low MIC value. It is anticipated that incorporation of an octylamine or polyamine chain at C24 will similarly result in a dramatic decrease in MIC values. Before pursuing this line of research, however, it would be advantageous to expand the screening of peptide-containing CSAs. While the libraries reported contain 216 compounds, use of 20 common amino acids will yield 8000. Using micro-broth dilution screening, it will be possible to screen this size of library in a reasonable amount of time. In these libraries, the peptides at C3, C7, and C12 are identical, and because we have developed means of *sequentially* adding different amino acids at each of these positions on the steroid scaffolding, there is enormous potential for generation of chemical diversity (use of 20 amino acids would yield over 500 billion combinations).

We have demonstrated that peptide-containing CSAs can be effectively prepared in indexed libraries and rapidly screened for antibacterial activities. MIC values measured with CSAs characterized in the screening process correlate very well with screening results. From this work, we have identified key requirements for peptide-containing CSAs and compounds that appear to offer an improvement in antibacterial activity over simpler CSAs. While a limited number of amino acids were used in preparing the libraries, wide variation in antibacterial activities was observed. It is anticipated that expansion of the libraries and screening will result in discovery of compounds with improved antibacterial activities against both Gram-negative and Gram-positive bacteria.

Acknowledgment. Generous support from the National Institutes of Health (NIGMS) is gratefully acknowledged.

Supporting Information Available: Detailed description of the micro-broth antibacterial screening method and experimental procedures for the preparation of **4**−**6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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