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Preparation of Amino Acid-Appended Cholic Acid Derivatives as Sensitizers of Gram-Negative Bacteria

Atiq-ur-Rehman, Chunhong Li, Loren P. Budge, Sarah E. Street and Paul B. Savage*

Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah 84602

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Abstract. Attachment of amino acids to cholic acid derivatives yielded compounds that sensitize Gram-negative bacteria to hydrophobic antibiotics. Incorporation of three amino acid onto a cholic acid derivative via ester bonds was only achieved with non-α-branched amino acids. Three non-α-branched or three α-branched amino acids were coupled to the steroid backbone via amide bonds.

The outer membranes of Gram-negative bacteria provide a formidable permeability barrier to many types of hydrophobic antibiotics.[1] Consequently, Gram-negative bacteria are resistant to many antibiotics that are active against Gram-positive organisms.[2] A number of peptides have been identified that increase the permeability of the outer membranes of Gram-negative bacteria and sensitize these organisms to hydrophobic antibiotics that ineffectively traverse the outer membranes.[3] The best studied of these peptides are the polymyxin B derivatives.[3a,b] We have modeled polymyxin B derivatives to determine potential active conformations and determined functionality conserved among antibiotics related to polymyxin B. This conserved functionality was incorporated onto a steroid scaffolding yielding compounds that sensitize Gram-negative bacteria to hydrophobic antibiotics.[4] Compounds we have previously reported are comprised of amine-bearing groups linked to a cholic acid scaffolding via ether linkages.

We now report preparation of cholic acid derivatives in which amine-bearing groups are attached to the steroid via ester or amide bonds. Preparation of these compounds results from our effort to increase the affinity of the cholic acid derivatives for components of the outer membranes of Gram-negative bacteria by taking advantage of associative interactions that might be offered by the side chains of amino acids coupled to a cholic acid scaffolding. As part of this effort, we have developed efficient means of appending cholic acid derivatives with three amino acids. A number of the resulting compounds effectively sensitize Gram-negative bacteria to erythromycin and novobiocin.

We first attempted to attach three amino acids to a cholic acid scaffolding using ester bonds. Amino acids attached to steroids have been reported;[5] however, we are unaware of any report of three amino acids linked to the 3, 7 and 12 positions of cholic acid. We reacted BOC-glycine with DCC, DMAP and cholic acid derivative **1**[4b] (Scheme 1) to give triester **2a** in good yield. A similar reaction incorporating BOC-β-alanine was also successful giving **2b**. However, attempts to form triesters with α-branched amino acids failed; only diesters were isolated. Use of excess reagents, heating and long reaction times did not yield triester products. Reactions using CBZ-protected amino acids also failed. Molecular modeling of a triester of BOC-alanine suggested that unfavorable steric interactions may have prevented triester formation. Alternatively, steric interactions may have made the triester unstable and caused it to be readily hydrolyzed.

Deprotection of **2a** and **2b** with HCl in dioxane and purification gave triesters **3a** and **3b** in good yield. However, **3a** and **3b** proved to be marginally unstable under the acidic conditions used in the deprotection. At least one of the ester bonds (presumably the least sterically hindered at C3) was susceptible to hydrolysis.

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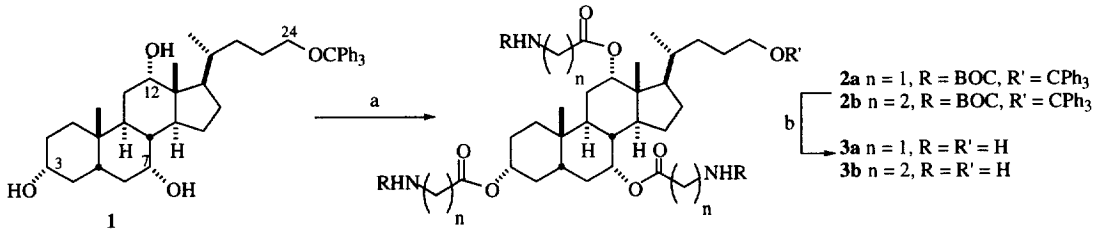
Carefully controlled reaction times allowed deprotection of **2a** and **2b** with minimal ester hydrolysis. Purification of triesters **3a** and **3b** was straightforward using SiO_2 chromatography with a CH_2Cl_2/MeOH/NH_4OH eluent. Surprisingly, the triesters were stable in the presence of ammonium hydroxide. Apparently, the esters were sufficiently sterically hindered that nucleophilic attack was inhibited.

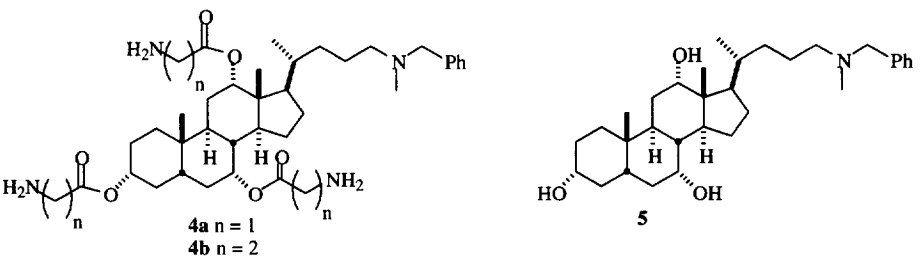
Scheme 1.



Reagents (reaction yields in parenthesis): a) BOC-glycine or BOC-alanine, DCC, DMAP, CH_2Cl_2 (60%, 94%). b) 4 *M* HCl in dioxane (74%, 71%).

In our previous work with cholic acid derivatives,[4] we found that the nature of the group at C24 influenced the activity of these compounds. Consequently, we prepared triesters **4a** and **4b**, both with a benzylmethyl amine group at C24 (Figure 1). These triesters were synthesized in a manner similar to **3a** and **3b** starting from amine **5**.

Figure 1. Structures of **4a**, **4b** and **5**.

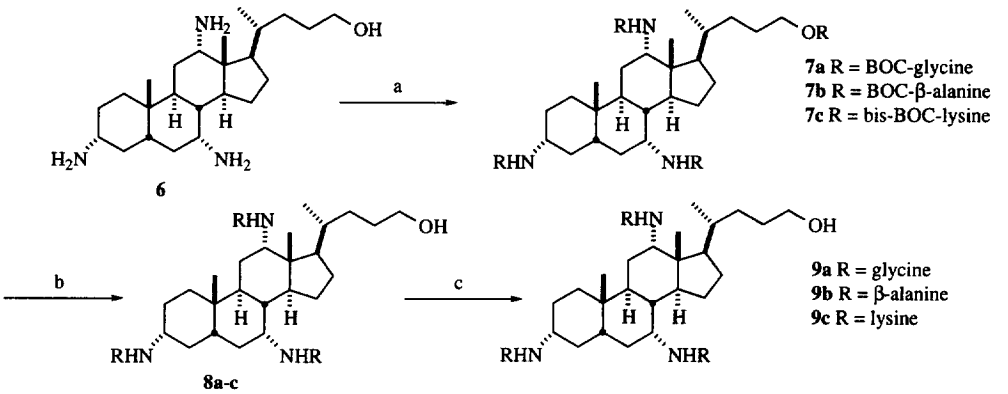


In an effort to prepare amino acid-functionalized cholic acid derivatives that would have greater stability, we investigated formation of triamides derived from a triamine analog of cholic acid (**6**).[6] It was also anticipated that we could form triamides with α-branched amino acids linked to **6**. We attempted amide formation using an N-protected amino acid, DCC and N-hydroxysuccinimide. Under these conditions we expected to avoid ester formation at C24. However, even after prolonged periods at reflux in dichloromethane, only trace amount of the desired triamide formed. Use of DMAP as a catalyst allowed formation of the triamide, but the C24 ester was also produced (Scheme 2). Triamides of glycine and β-alanine (**7a** and **7b**, respectively) were formed in this manner. We found that we could also form triamides with α-branched amino acids. Under the conditions described, we formed a triamide with bis-BOC-lysine (**7c**). The C24 esters of **7a-c** were hydrolyzed with LiOH in THF and methanol to give alcohols **8a-c**. Deprotection using HCl in dioxane (**8a-c**) gave triamides **9a-c** in good yield. In addition, alcohols **8a** and **8b** were mesylated and reacted with benzylmethyl amine. Deprotection of the resulting compounds with HCl in dioxane gave triamides **10a** and **10b** (Figure 2).

With the triesters **3a,b** and **4a,b** and triamides **9a-c** and **10a,b** in hand, we were prepared to determine the abilities of these compounds to sensitize Gram-negative bacteria to hydrophobic antibiotics. Against *Escherichia coli* (ATCC 10798), erythromycin and novobiocin have minimum inhibition concentrations (MICs) of 70 and > 500 μg/mL, respectively. These MIC values are much higher than those observed with Gram-positive organisms (generally ~ 1 μg/mL for both antibiotics).[2] Erythromycin and novobiocin are

unrelated in structure and mechanism of action. Consequently, sensitization of Gram-negative bacteria to these antibiotics would most likely result from increased permeability of the outer membranes of the bacteria caused by the cholic acid derivatives.

Scheme 2.

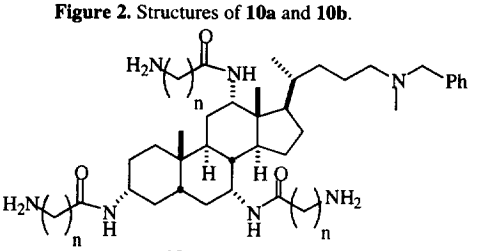


Reagents (reaction yields in parenthesis): a) BOC-glycine, BOC-alanine or bis-BOC-lysine, DCC, DMAP, CH_2Cl_2. b) LiOH, THF, MeOH (71-85% for two steps). c) 4 *M* HCl in dioxane (~100%).

We used *E. coli* (ATCC 10798) previously to characterize the bacterial sensitizing properties of cholic acid derivatives and found that results with this strain were representative of behavior with other Gram-negative organisms.[4] To characterize synergism of the cholic acid derivatives with erythromycin and novobiocin, we determined the concentrations of the cholic acid derivatives necessary to lower the MIC values of these antibiotics to 1 μg/mL (a concentration at which many clinically useful antibiotics are active).[2] This measurement entailed incubating a known population of bacteria for 24 h in a nutrient broth with erythromycin or novobiocin and incrementally varied concentrations of the cholic acid derivatives. MIC values were determined by plating out diluted samples on a nutrient agar and counting colonies after an additional 24 h incubation. The minimum concentrations of the cholic acid derivatives that caused the populations of bacteria to decrease are shown in Table 1. Also in Table 1 are the MIC values of the cholic acid derivatives alone.

In contrast to other cholic acid derivatives we have reported, the compounds listed in Table 1 alone are not potent antibacterial agents. However, **3b**, **4b** and **10a** display potent synergism with

Figure 2. Structures of **10a** and **10b**.



10a n = 1
10b n = 2

Table 1. Antibacterial properties of **3a,b**, **4a,b**, **9a-d** and **10a,b**.

Compound	MIC (μg/mL)	a (μg/mL)	b (μg/mL)
3a	85	18	55
3b	80	4	10
4a	85	15	40
4b	70	3	13
9a	>100	25	75
9b	>100	40	75
9c	85	45	60
10a	80	6	18
10b	100	15	40

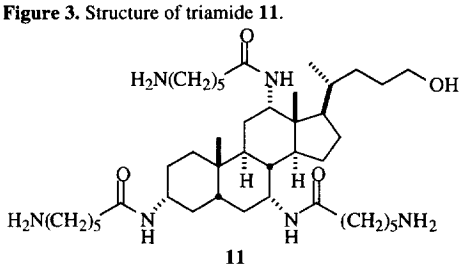
a: concentration of the cholic acid derivatives required to lower the MIC of erythromycin to 1 μg/mL.

b: concentration of the cholic acid derivatives required to lower the MIC of novobiocin to 1 μg/mL.

erythromycin and novobiocin. In the triester series (**3a,b** and **4a,b**), the β-alanine derived compounds are more active than those from glycine. Also of note is that substitution at C24 had minimal effect on the activity of these compounds (compare **3b** and **4b**). Triamides **9a-c** were less active than the esters possibly due to conformational constraints imposed by the amide bonds. With the triamides, substitution at C24 had significant effects on the activity of the compounds (compare **9a** and **10a**). In this series, the glycine derivative was more active than the corresponding β-alanine derivative.

The relative lack of synergism displayed by the lysine derivative may be attributable to the length of the side chain. As a control, we prepared **11** (Figure 3), a derivative of **9c** lacking the α-amine groups, and found it to be less active than **9c**. Compound **6** also proved to be ineffective as a sensitizer of Gram-negative bacteria. These results suggest that the optimal length for the tether between the steroid and the amine functionality is between zero and six atoms. As we have shown, compounds with tethers of two and three atoms can act as effective sensitizers.[4]

Figure 3. Structure of triamide **11**.



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In summary, we have prepared series of cholic acid derivatives with three amino acids incorporated via amide and ester bonds that can act as potent sensitizers of Gram-negative bacteria. The synergism of the most active compounds (**3b**, **4b** and **10a**) with certain hydrophobic antibiotics greatly improves the activity of these antibiotics against Gram-negative strains, thus potentially making available a larger arsenal of antibiotics for use against Gram-negative organisms. Because the cholic acid derivatives act synergistically with unrelated hydrophobic antibiotics, compounds **3b**, **4b** and **10a** most likely act as outer membrane permeabilizers. In addition, by demonstrating that triester and triamide derivatives of cholic acid can be readily prepared, we hope to have facilitated expansion of the types of combinatorial chemistry that can be performed with cholic acid scaffoldings.

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6. See the preceding Letter.