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Preparation and Characterization of Cholic Acid-Derived Antimicrobial Agents with Controlled Stabilities

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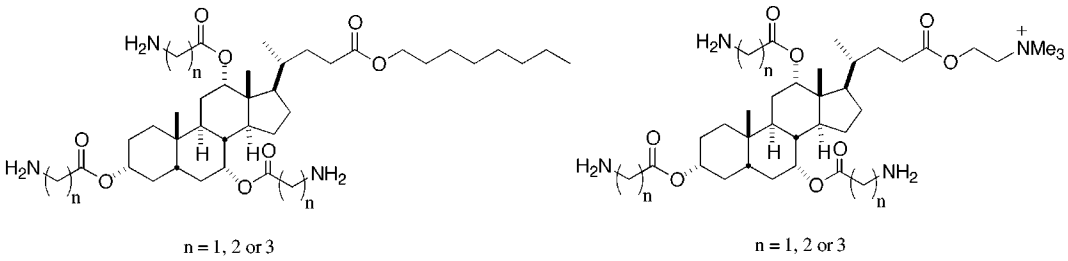
ABSTRACT



Novel cholic acid-derived antimicrobial agents that decompose under mildly basic conditions have been prepared. These compounds range in biological properties from potent antibacterial activity to effective permeabilization of the outer membranes of Gram-negative bacteria.

With the emergence of numerous strains of pathogenic multidrug-resistant bacteria comes the need for new antimicrobials to which bacteria are unlikely to develop resistance. A group of antimicrobial agents that has received considerable attention recently is membrane active cationic peptide antibiotics.[1] These compounds disrupt or permeabilize prokaryotic membranes.[2] Endogenous examples have been identified in many diverse organisms including animals and plants. Bacteria rarely develop resistance to membrane-active compounds because resistance requires alterations to the bulk membrane structure of the organisms. For example, few strains have been isolated that are resistant to polymyxin B, a commonly used cationic peptide antibiotic. Those that have been isolated exhibit membrane structural changes that influence the permeability of their membranes.[3]

We recently reported several series of cholic acid-derived compounds that, although not composed of amino acids, display a facial amphiphilicity similar to that of many cationic peptide antibiotics and mimic aspects of their behavior including membrane activity.[4] The antimicrobial activities of these compounds range from potent antibacterial activity to permeabilization of the outer membranes of Gram-negative bacteria and sensitization of these organisms to hydrophobic antibiotics.[4,5]

As an extension of our earlier work, we have become interested in antimicrobial agents that decompose into endogenous and nontoxic compounds under mild conditions including those encountered in the human gastrointestinal tract. Found within the gastrointestinal tract are large numbers

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(1) For a recent review, see: Epand, R. M.; Vogel, H. J. *Biochim. Biophys. Acta* **1999**, *1462*, 11.
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(4) (a) Li, C.; Budge, L. P.; Driscoll, C. D.; Willardson, B. M.; Allman, G. W.; Savage, P. B.*J. Am. Chem. Soc.* **1999**, *121*, 931. (b) Rehman, A.; Li, C.; Budge, L. P.; Street, S. E.; Savage, P. B. *Tetrahedron Lett.* **1999**, *40*, 1865. (c) Li, C.; Peters, A. S.; Meredith, E. L.; Allman, G. H.; Savage, P. B. *J. Am. Chem. Soc.* **1998**, *120*, 2961.
(5) Li, C.; Lewis, M. R.; Gilbert, A. B.; Noel, M. D.; Scoville, D. H.; Allman, G. W.; Savage, P. B. *Antimicrob. Agents Chemother.* **1999**, *43*, 1347.

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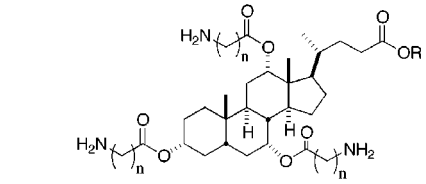
of bacteria[6] which play an important role in digestive processes. Elimination or reduction of the populations of bacteria in the gastrointestinal tract can influence digestion and facilitate opportunistic infections by pathogenic bacteria.[7] Oral ingestion of antimicrobial agents that are stable in the gastrointestinal tract can cause significant perturbations in bacterial populations.[8]

We have developed and now report a series of compounds, based on a cholic acid scaffolding, that are effective antibiotics yet decompose to endogenous and nontoxic compounds under mild conditions. An attractive potential use for compounds of this type is in controlling bacterial growth in food. Because these compounds decompose under mild conditions, they are expected to become inactive upon ingestion and therefore presumably not adversely affect the natural flora of the digestive system.

Nisin, a peptide antibiotic, is currently the only antimicrobial agent approved in many countries for use in food.[9] Nisin is active against Gram-positive strains but is nearly inactive against Gram-negative organisms such as *Escherichia coli* and strains of *Salmonella*.[10] Selected compounds in the series of new antimicrobials that we have developed are very active against these Gram-negative bacteria as well as against Gram-positive organisms.

To provide compounds capable of decomposition under mild conditions, we esterified the hydroxyl groups at C-3, C-7, and C-12 of cholic acid with amino acids including glycine, β-alanine, and γ-aminobutyric acid. The resulting compounds contained both ester and amine functionality, and this combination was expected to cause decomposition of the compounds under mildly basic conditions. Because of the possibility of pyrrolidone formation, we expected the cholic acid derivatives containing γ-aminobutyric acid to decompose especially rapidly.

In addition to consideration of functionalization of the hydroxyl groups at C-3, C-7, and C-12 of cholic acid, we took into account the influence of groups attached at C-24. Previous work[4a] established that the nature of the groups included at C-24 greatly influences the biological activity of the compounds: hydrophobic groups made the compounds active against Gram-negative strains, while shorter chains resulted in compounds capable of permeabilizing the outer membranes of Gram-negative bacteria and killing Gram-positive organisms. In previously reported cholic acid-derived antimicrobial agents, the C-24 position of the steroid was reduced.[4] To ensure that unmodified cholic acid would be released upon decomposition of the new compounds, the C-24 position of the steroid was not reduced. To observe the properties of compounds with hydrophobic and polar functionality at C-24, octyl esters and choline esters of cholic acid derivatives were prepared, giving **1**−**6** (Figure 1).



1 n = 1, R = -$(CH_2)_7CH_3$
2 n = 2, R = -$(CH_2)_7CH_3$
3 n = 3, R = -$(CH_2)_7CH_3$

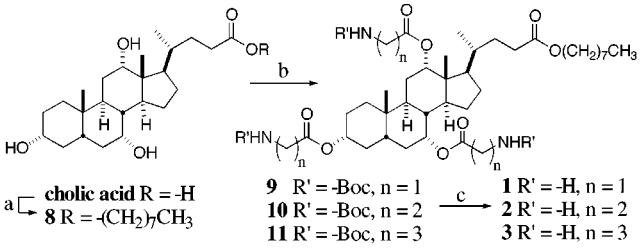
4 n = 1, R = -$CH_2CH_2N^+(CH_3)_3$
5 n = 2, R = -$CH_2CH_2N^+(CH_3)_3$
6 n = 3, R = -$CH_2CH_2N^+(CH_3)_3$
7 n = 1, R = -H

Figure 1. Structures of cholic acid derivatives **1**−**7**.

Compound **7** was also prepared to determine the effects of a carboxylate group at C-24 on antimicrobial activity.

Compounds **1**−**3** were prepared as described in Scheme 1. The carboxylic acid at C-24 of cholic acid was esterified

Scheme 1.[a] Preparation of **1**−**3**



a: **cholic acid** R = -H; **8** R = -$(CH_2)_7CH_3$
b
9 R' = -Boc, n = 1
10 R' = -Boc, n = 2
11 R' = -Boc, n = 3
c
1 R' = -H, n = 1
2 R' = -H, n = 2
3 R' = -H, n = 3

[a] (a) Octanol, TsOH (73%); (b) Bocglycine, Boc-β-alanine or Boc-γ-aminobutyric acid, DCC, DMAP, CH_2Cl_2 (91−95%); (c) HCl, dioxane (84−99%).

with octanol followed by incorporation of the Boc-protected amino acids, giving **9**−**11**. Cleavage of the Boc groups with HCl in dioxane yielded **1**−**3**. Preparation of **4**−**6** (Scheme 2) required protection of the carboxylic acid of cholic acid as the benzyl ester. Incorporation of the Boc-protected amino acids was followed by deprotection of the carboxylic acid, yielding **16**−**18**. Attempts to esterify the acid with choline failed using a variety of standard acid-activating reagents. The choline group was successfully incorporated via a two-step procedure: esterification with *N*,*N*-dimethylethanolamine using DCC and DMAP followed by treatment with methyl iodide. Removal of the Boc groups yielded **4**−**6**. Preparation of **7** only required removal of the Boc protecting groups of **16**. Because **7** proved to be inactive (vide infra), a complete series of acids, derived from deprotection of **17** and **18**, was not prepared.

(6) Bacterial populations have been estimated to reach 10^{14} cells at all sites of the human body (the human body contains ca. 10^{13} cells). Tannock, G. W. In *Normal Microfluora. An Introduction to Microbes Inhabiting the Human Body*; Tannock, G. W., Ed.; Chapman and Hall: London, 1994; pp 1−36.

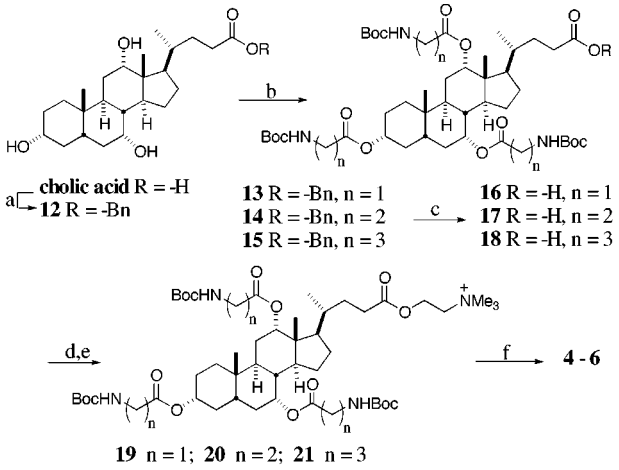
(7) For example see: (a) Vollaard, E. J.; Clasener, H. A. L. *Antimicrob. Agents Chemother.* **1994**, *38*, 409. (b) Hentges, D. J. In *Human Intestinal Microflora in Health and Disease*; Hentges, D. J., Ed.; Academic Press: New York, 1983; pp 311−328.

(8) For example, see: Finegold, S. M.; Mathisen, G. E.; George, W. L. In *Human Intestinal Microflora in Health and Disease*; Hentges, D. J., Ed.; Academic Press: New York, 1983; pp 356−438.

(9) Delves-Broughton, J.; Blackburn, P.; Evans, R. J.; Hugenholtz, J. *Antonie van Leeuwenhoek* **1996**, *69*, 193.

(10) De Vuyst, L.; Vandamme, E. J. In *Bacteriocins of Lactic Acid Bacteria*; De Vuyst, L.; Vandamme, E. J., Ed.; Blackie Academic and Professional: Glasgow, 1994; pp 151−221.

Scheme 2.[a] Preparation of **4**−**6**



[a] (a) Benzyl alcohol, TsOH (81%); (b) Bocglycine, Boc-β-alanine or Boc-γ-aminobutyric acid, DCC, DMAP, CH_2Cl_2 (68−78%); (c) H_2, Pd/C (97−99%); (d) $(CH_3)_2N(CH_2)_2OH$, DCC, DMAP, CH_2Cl_2 or THF (62−82%); (e) MeI, CH_2Cl_2. f) HCl, dioxane (83−90% for two steps).

The antimicrobial activities of the compound **1**−**7** were tested with standard strains of Gram-negative and Gram-postive bacteria, *E. coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923), respectively (Table 1). Minimum inhibition concentrations (MICs) were measured as described previously.[5] As anticipated, the compounds containing the hydrophobic chain at C-24 (**1**−**3**) were the most active against the Gram-negative strain. The compounds with choline at C-24 (**4**−**6**) were much less active alone against the Gram-negative strain yet retained the ability to inhibit the growth of the Gram-positive strain. This difference was likely due to the inability of compounds **4**−**6** to traverse the outer membrane of the Gram-negative strain. With a negatively charged group at C-24, **7** was inactive against either strain. Prokaryotes generally exhibit a net negative charge on their membranes.[11] Consequently, the negative charge of **7** likely inhibits interaction with the membranes of the bacteria.

Table 1. Antimicrobial Activities of Compounds **1**−**7**

compd	MIC[a] (μg/mL)	MIC[b] (μg/mL)	*c* (μg/mL)	MHC[d] (mg/mL)
1	1.8	1.0	0.7	4.0
2	4.0	7.0	3.0	2.0
3	1.2	3.5	3.5	<10
4	15	60	10	>200
5	11	30	2.0	>200
6	14	23	2.0	>200
7	>100	>100	>100	>100

[a] Mimum inhibition concentration (MIC) against *S. aureus* (ATCC 25923). [b] MIC against *E. coli* (ATCC 25922). [c] Concentration of the compounds required to lower the MIC of erythromycin from 30 to 1 μg/mL with *E. coli* (ATCC 25922). [d] Minimum hemolytic concentration.

The outer membranes of Gram-negative bacteria provide a permeability barrier to many hydrophobic molecules. Consequently, many hydrophobic antibiotics, such as erythromycin, are only weakly active against Gram-negative strains. Because cholic acid derivatives were expected to permeabilize the outer membranes of Gram-negative bacteria, measurements were made of concentrations of selected compounds that were necessary to lower the MIC of erythromycin from 30 μg/mL to a clinically useful level of 1 μg/mL (Table 1). Because **1**−**3** were active alone against *E. coli*, the concentrations at which they caused permeabilization were very near their MICs. Compounds **4**−**6** were effective permeabilizers of the outer membrane, while **7** was inactive.

A primary issue that may determine the utility of membrane-active antimicrobials in systemic applications is their selectivity for prokaryotic over eukaryotic membranes. Because prokaryotic membranes typically carry a net negative charge and their eukaryotic counterparts are generally composed of zwitterionic phospholipids,[11] it would be expected that compounds bearing positive charges would display selectivity for prokaryotic membranes. Minimum hemolytic concentrations (MHCs) can be compared to concentrations of compounds required for antibacterial activity as a measure of membrane selectivity. The MHC values of compounds **1**−**7** were measured as previously reported[5] and are included in Table 1. Compounds **1**−**3** exhibit very low MHCs; however, compounds **4**−**6** appear to be nonhemolytic presumably due to the additional positive charge at C-24. Nevertheless, if compounds **1**−**6** find use in preventing microbial growth in food, it is unlikely that their hemolytic behavior would be an issue because the compounds would likely be degraded to inactive components before absorption occurs.

To test the stabilities of the ester groups at C-3, C-7, and C-12, compounds **22**−**24**[4b] (Figure 2) were used because they contained a chromophore that facilitated monitoring of HPLC experiments. Stabilities of the compounds were tested at pH 2.0, 7.0, and 12.0 in a phosphate buffer at room temperature. Decomposition through ester hydrolysis yielded compounds

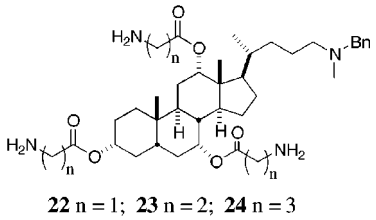


Figure 2. Structures of cholic acid derivatives **22**−**24**.

(11) Matsuzaki, K.; Sugishita, K.; Fugii, N.; Miyajima, K. *Biochemistry* **1995**, *34*, 3423.

that were less polar and easily separable from the starting compounds. Initially, only one benzene-containing decomposition product was observed; at longer reaction times two other decomposition products were observed which presumably corresponded to sequential ester hydrolysis. The half-lives of compounds **22**−**24** at the three pH values are shown in Table 2. At low pH the amine groups were protonated

Table 2. Stabilities (half-lives) of Compounds **22**−**24** in Phosphate Buffer

compd	pH 2.0	pH 7.0	pH 12.0
22	>37 days	28 days	<30 min
23	>37 days	37 days	<30 min
24	33 days	12 days	<30 min

and the compounds were relatively stable. At higher pH the amines were less strongly protonated and became involved in ester hydrolysis. As expected the γ-aminobutyric acid derived compound was especially susceptible to hydrolysis, presumably yielding pyrrolidone.

As we have shown,[4c] partial loss of facial amphiphilicity through inversion at C-3 in other cholic acid-derived antimicrobial agents results in dramatic increases in MIC values. Likewise, loss of amino acids resulted in a significant loss of antibacterial activity in compounds **1**−**6**; these gave relatively high MIC values after storage in solution for extended periods (>2 weeks).

Because compounds **1**−**6** are active antimicrobial agents and because their stability can be manipulated by controlling pH, these compounds may find use in situations in which antimicrobial compounds are required but long-term exposure is undesirable. As mentioned, these compounds may be ideal for use in controlling bacterial growth in food. The compounds could be applied to food, and after ingestion, the compounds would break down to endogenous and/or nontoxic components. Investigation of the ability of the compounds to inhibit microbial growth in food is ongoing and will be reported in due course.

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Supporting Information Available: Experimental details for the preparation of **1**−**7** and stability experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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