Micelle Formation and Antimicrobial Activity of Cholic Acid Derivatives with Three Permanent Ionic Head Groups**

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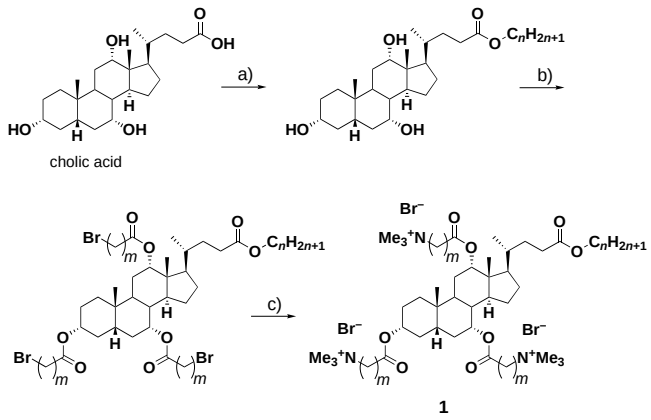
Cholic acid, a main bile acid, is a biosurfactant involved in the digestion of dietary lipids. With the carboxylate group as the ionic head group it resembles a "classical" surfactant. However, the steroid part with three hydroxy groups on one side is often considered to be a facial amphiphile. These hydroxy groups can have specific interactions, such as those found in inclusion compounds,[1,2] organogelators,[3–6] and receptors[7,8] based on cholic acid. Furthermore, the hydroxy groups, each with a different reactivity,[9] make cholic acid an ideal starting point for a synthetic procedure. The polarity of the hydroxy groups can be increased to emphasize the facial amphiphilicity of the steroid unit. The resulting facial amphiphiles have many applications in ion transport,[10,11] combinatorial chemistry,[12] vesicle fusion,[13] and improvement of membrane permeability.[14] Even facial amphiphiles with three ionic groups are reported.[15,16] However, the properties of these cationic (NH_3^+) or anionic (COO^-) facial amphiphiles are dependent on the pH of the solution. Herein, a series of new facial amphiphiles with a permanent ionic character is presented. Three cationic trimethylammonium groups were attached to cholic acid and the carboxylate group was esterified, to yield a new class of three-headed surfactants. We know of only one other example of a surfactant with three permanent ionic head groups and only one hydrophobic tail.[17] We report on the aggregation of this new type of surfactant into spherical micelles and on its antimicrobial activity.

Synthesis of this series of ionic facial amphiphiles was completed in three steps from cholic acid (Scheme 1). First, the carboxylate group was esterified with a long alkyl group.



Scheme 1. a) $C_nH_{2n+1}OH$, *p*-toluenesulfonic acid, benzene, 90 °C, 24 h, 75 %; b) Br-$(CH_2)_m$-COCl, DMAP, benzene, pyridine, RT, 24 h, 65 %; c) NMe_3, EtOH, 100 °C, 24 h, 90 %. Prepared compounds: **1a** ($n=8$, $m=3$), **1b** ($n=10$, $m=3$), **1c** ($n=12$, $m=3$), **1d** ($n=12$, $m=4$), **1e** ($n=12$, $m=5$), **1f** ($n=12$, $m=7$), and **1g** ($n=16$, $m=3$). DMAP = *N*,*N*-dimethylamino pyridine.

Then, a spacer was attached to all three hydroxy groups using an ω-bromoalkanoic chloride. Finally, the bromine atoms were substituted by trimethylamine to give trimethylammonium groups on **1**. Variation was made in the length of the alkyl tail ($n=8$, 10, 12, or 16) and in the length of the spacer ($m=3$, 4, 5, or 7).

These new compounds are very hygroscopic. Solutions with accurately defined concentration were therefore prepared from lyophilized solutions. Because of the long aliphatic tail, aggregation occurs at rather low concentrations, about 1 mM, depending on the length of both the alkyl tail and the spacer.

Evidence for micelle formation was obtained from cryo-TEM measurements. For several compounds, cryo-TEM images were recorded of aqueous solutions (1 wt %, about 8 mM). Two examples are shown in Figure 1. The images show

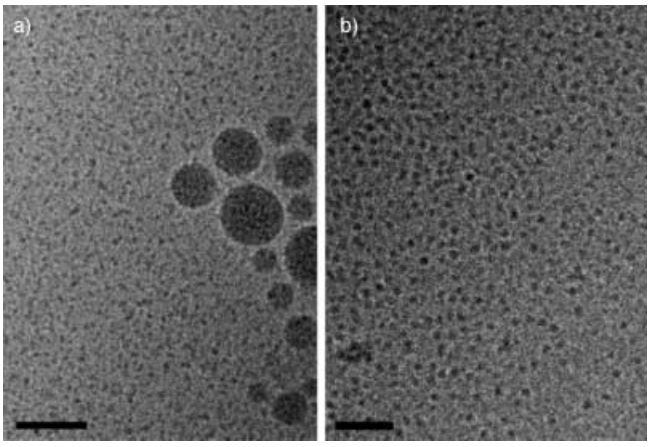


Figure 1. Cryo-TEM images of 1 wt % aqueous solutions of **1f** (a), and **1g** (b); scale bar indicates 50 nm.

small aggregates with an average diameter of 3–6 nm. The aggregates formed from **1a** ($n=8$, $m=3$) are smaller than from **1g** ($n=16$, $m=3$; 3–4 nm and 5–6 nm respectively), which demonstrates the influence of the alkyl tail length on micellar size. No elliptical or bar-shaped aggregates are

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visible, which indicates that the aggregates are spherical micelles. To verify this, a density histogram was created for a single aggregate; this indeed shows a Gaussian distribution. Occasionally, larger aggregates with a diameter of 15–50 nm can be seen. These aggregates have the same density variation as the small ones, so they are not vesical structures. We assume the occurrence of secondary micelles, formed by clustering of the small, primary micelles, similar to the two-step aggregation behavior of cholic acid itself.[18]

Dynamic light scattering (DLS) measurements on aqueous solutions of compounds **1b** ($n = 10$, $m = 3$) and **1g** ($n = 16$, $m = 3$) roughly show the presence of many aggregates with a diameter of about 5 nm and a few larger aggregates with a diameter of several tens of nanometers, assuming a spherical shape of the aggregates. These values correspond with those obtained from cryo-TEM images.

Several cholic acid-derived facial amphiphiles have been reported that improve the permeability of membranes such as bacterial cell walls.[14] These compounds, which make bacteria vulnerable to other antibiotics, have nitrogen-containing groups such as amine or guanidine groups, which are coupled to the hydroxy groups. The positive charges associate with the negatively charged membrane. Attaching a hydrophobic chain of about eight carbon atoms to these molecules facilitates "self-promoted" transport across the bacterial outer membrane and therefore results in strong antimicrobial activity.[19,20] Our surfactants contain the same structural elements (three nitrogen-containing, positively charged groups and a hydrophobic alkyl tail), therefore some tests were performed to investigate their antimicrobial activity. The minimum inhibition concentration (MIC) was measured using two bacterial cultures: *Escherichia coli* and *Enterococcus faecalis*. Compounds **1a** ($n = 8$, $m = 3$) and **1b** ($n = 10$, $m = 3$) inhibited growth of both bacterial cultures. The MIC was established to be 25 and 12.5 $\mu g\,mL^{-1}$ for **1a** and **1b**, respectively. This is far below the critical micelle concentration (CMC), so the inhibition mechanism does not involve aggregate structures but single molecules. The activity of **1b** is almost equal to that of the well-known antibiotic chloramphenicol. It is noteworthy that these compounds act as competent bacterial growth inhibitors against both gram-positive and gram-negative bacterial cultures. The other compounds with longer alkyl tails showed no inhibitory effect, not even in concentrations up to 200 $\mu g\,mL^{-1}$. The hydrophobic tail of 12 or 16 carbon atoms may be too long to facilitate transport through the bacterial membrane because of stronger hydrophobic interaction with the membrane.

In conclusion, we report the synthesis of a series of new facial amphiphiles, based on cholic acid, with a permanent ionic character. These compounds act as three-headed surfactants, forming small primary and larger secondary spherical micelles. Micellar size depends on the lengths of the alkyl tail. Two compounds display antimicrobial activity against gram-positive as well as gram-negative bacteria. Further research to the micelle-forming behavior of this new class of three-headed ionic surfactants as a function of their molecular structure is ongoing.

Experimental Section

General procedure for the synthesis of **1**: Alkyl cholate (3 mmol)[4] was dissolved in benzene (10 mL) and pyridine (2 mL). A catalytic amount of DMAP was added and the mixture was stirred for 45 min at room temperature. A solution of ω-bromoalkanoic acid chloride (15 mmol) in benzene (10 mL) was added and the mixture stirred overnight at room temperature. The reaction mixture was filtered, diluted with CH_2Cl_2, and washed with 3 M HCl solution. The organic phase was dried and the solvent was evaporated under vacuum. The residue was purified by column chromatography on silica, using CH_2Cl_2:MeOH (99.5:0.5) as eluent, to give a yellow oil. 1 mmol of this 24-(alkyloxy)-24-oxo-3,7,12-tris{[ω-bromoalkanoyl]oxy}cholane was dissolved in a solution of trimethylamine in ethanol (20 wt %, 15 mL) and the solution stirred for 24 h in a closed reaction vessel at 100 °C. After cooling and concentrating the mixture, chloroform was added. The precipitate was filtered and evaporation of the filtrate under vacuum gave a white solid. The overall yield of this route is about 45 %. Spectroscopic data for all compounds are listed in the Supporting Information.

Cryo-TEM: a drop of sample suspension was placed on a glow-discharged, perforated carbon film. Excess liquid was blotted away with filter paper and the grid was subsequently vitrified in liquid ethane and cooled with liquid nitrogen. The frozen, hydrated grid was mounted in a Gatan cryostage (Gatan model 626) and observed in a Philips CM120 cryotransmission electron microscope operating at 120 kV. Images were recorded on a slow-scan charge-coupled device (CCD) camera under low-dose conditions.

DLS measurements: 1 wt % aqueous solutions were placed in a Lexel 85 Argon Ion Laser beam with $\lambda = 514.5$ nm and power = 100–300 mW; scattering was recorded at an angle of 90°, using an ALV-5000 digital correlator, ALV-125 goniometer, ALV-800 transputerboard from ALV-GmbH (Langen, Germany). 30 measurements of 20 s were accumulated and analyzed with Contin-fit.

Antimicrobial tests: cultures of gram-negative *Escherichia coli* (JM109, Promega) and gram-positive *Enterococcus faecalis* (93103, kind gift from VTT Biotechnology, Finland) were grown in LB broth or M17 broth with 1 % of glucose, respectively. A fresh overnight culture of *E. coli* (16 h at 37 °C) was inoculated into LB broth containing 100 $\mu g\,mL^{-1}$ of a compound and incubated for 24 h at 37 °C. On the compounds which showed no turbidity after incubation, a more extensive test was performed.[21] The MIC (the lowest concentration of surfactant that showed no turbidity) was measured using a series of glass test-tubes containing the compound in concentrations of 200, 100, 50, 25, and 12.5 $\mu g\,mL^{-1}$. Each tube was inoculated with exponential-growth-phase organisms (*E. coli* or *E. faecalis*) to a concentration of 1 % and incubated at 37 °C for 20 h. The optical density at 660 nm was measured before and after incubation. Broth containing bacteria alone was used as a positive control and, for comparison, bacteria were also tested in broth supplemented with 15 μg of chloramphenicol mL^{-1}. Tests were performed in duplicate.

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Polymorphism in the Crystal Structure of the Cellulose Fragment Analogue Methyl 4-*O*-Methyl-β-D-Glucopyranosyl-(1-4)-β-D-Glucopyranoside**

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Cellulose, the most abundant polymer on earth, usually consists of a mixture of highly crystalline and amorphous regions. The crystalline parts display one chain conformation, made up of a twofold ribbon, but many possible packing arrangements, with either parallel chains (cellulose I, III_I, and IV_I) or antiparallel chains (cellulose II, III_{II}, and IV_{II}) (for a review see ref. [1]). The three-dimensional structure of native cellulose is highly complex and not yet completely resolved because two distinct crystalline forms, cellulose Iα and Iβ[2] showing triclinic and monoclinic symmetry, respectively, coexist. The crystalline phases Iα and Iβ occur in variable proportions according to the source of the cellulose; that of primitive organisms (bacteria, alga, etc.) is enriched in the Iα phase whereas that of higher plants (woody tissues, cotton, ramie, etc.) consists mainly of the Iβ phase,[3] and cellulose of the outer membrane of marine animals is uniquely composed of the Iβ phase.[4] Modeling studies have established that the two crystalline arrangements correspond to two low-energy structures arising from parallel associations of cellulose chains.[5]

Cellobiose has been crystallized in its native form and in the form of derivatives and salts.[6] In all crystals, the packing arrangement corresponds to a low-energy arrangement of small molecules and does not provide any clues to the polymorphism of the polysaccharide. Only in crystals of methyl β-cellotrioside[7] and β-cellotetraose[8,9] chain-like arrangements were found. In these two cases, the molecules are arranged in an antiparallel way, which represents the packing of lowest energy, and the resulting structures are similar to that of cellulose II. We describe here the crystal structure analysis of a cellulose fragment that displays a parallel arrangement of molecules and that can therefore be directly compared with the two polymorphs of natural cellulose I.

Crystals of methyl 4-*O*-methyl-β-D-glucopyranosyl-(1-4)-β-D-glucopyranoside were obtained in the triclinic space group *P*1 (form **I**) using conditions which decreased the crystallization rate as compared to the crystallization of the monoclinic polymorph (group $P2_1$, form **II**).[10] The crystal structure of form **II** has been described recently from data collected at low temperature (220 K).[11] We redetermined the structure at room temperature[12] and found the differences in cell dimensions, cell volumes, and geometrical details to be less than 0.8%. Therefore, the structure is not shown here, but its data will be used in the tables to allow direct comparison between form **I** and form **II** geometries.

The molecular structure of form **I** is shown in Figure 1. Geometry calculations and ORTEP illustrations of the crystal structure were obtained with PLATON.[13] Both glucose rings have the usual $^4C_1$ shape. Intra-ring bond lengths and valence angles are in the range of standard values (Table 1). The short C1′–O4 bond at the glycosidic linkage is in agreement with the anomeric effect.[14] The C5–C6 and C6–O6 linkages of the

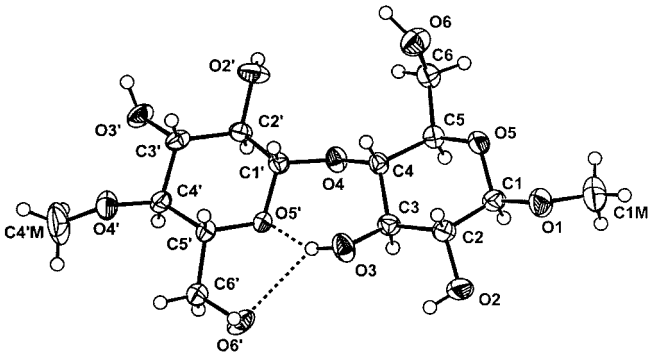

Figure 1. ORTEP representation of the molecular structure of form **I**. Thermal ellipsoids are drawn at the 50% probability level. Intramolecular hydrogen bonds are represented as dashed lines.

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