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Abstract

New synthetic pathways are proposed for the synthesis of a new set of bolaamphiphilic derivatives starting from microbiologically produced sophorolipids. A total set of 43 new derivatives was synthesized *via* reductive amination of a previously synthesized sophorolipid aldehyde with diamines and primary amines. The new derivatives were evaluated for their antimicrobial activity against Gram-negative and Gram-positive bacteria. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined for the active compounds. Transfection efficiencies were also evaluated for some of the deprotected derivatives *via* the assessment of their capacity to transfect three different eukaryotic cell lines *in vitro*. Finally, the self-assembly properties were evaluated for the deprotected derivatives. Antimicrobial activities were mostly observed for the peracetylated mono- or dicationic bolaamphiphiles and only the deprotected monocationic bolaamphiphile with an octadecyl chain on the nitrogen atom was eligible for the evaluation of its transfection properties. Evaluation of the self-assembly properties indicated that the presence of an octadecyl chain was necessary for micelle formation. Both micelle formation and the net charge of the compounds seem to have an influence on the antimicrobial activity and transfection efficiency. These results are promising for use of bolaamphiphilic sophorolipids in medical and self-assembly applications.

Keywords

Sophorolipids, chemical modification, bolaamphiphiles, antimicrobial activity, transfection efficiency, quaternary ammonium salt, self-assembly

Synopsis

Sophorolipids, renewable biosurfactants, are used for the synthesis of innovative bolaamphiphilic compounds for application in the medical sector.

Introduction

Bolaamphiphiles possess not one but two hydrophilic heads, coupled by a hydrophobic linker. Currently, there is a lot of interest in synthetic bolaamphiphiles because of the unusual architectures created by such molecules.[1] They form monolayer membranes which can organize into micelles, vesicles, nanotubuli, *etc*. These supramolecular features often arise spontaneously, they are so-called self-organizing structures. The best known natural examples of bolaamphiphiles are the tetraether lipid membranes of *Archaea* which are able to grow under very extreme temperatures and at very high salt concentrations. In this context bolaamphiphiles likely contribute to the stabilization of membranes.[1-2] As a result, they can for example be used in drug or nucleic acid delivery applications to form or stabilize vesicles.[3-4] After all, most liposomes currently used for this purpose face stability problems such as leakage of the content before reaching the target location.[5] Moreover, bolaamphiphiles can be used as membrane-spanning linkers in biosensors to detect proteins, antibodies, viruses, *etc*.[6] They can also serve as channels for ion transport.[1] Fluorescent bolaamphiphiles can be used as transmembrane probes for lipid imaging to visualize for example ether lipids in the brain.[7]

In view of the growing ecological awareness and the necessity to develop alternatives for fossil resources, the production of amphiphiles from renewable resources is becoming increasingly important. However, among the synthetic bolaamphiphiles which are described in literature, only a few are produced from renewable resources.[8] These renewable based bolaamphiphiles are mostly fatty acid - for example derived from vernonia and castor oil - and sugar based derivatives.[9-19] The use of glutamic acid, proline and vitamin C was also reported for the synthesis of bolaamphiphilic compounds.[20-22] Examples of naturally occurring bolaamphiphiles are the carotenoid glycoside crocin **1**, present in saffron up to 25%, and sophorolipid acid **2** (Figure 1).[23-25] Sophorolipids are a class of glycolipid biosurfactants which are produced by different yeast species, mainly *Starmerella bombicola*, from renewable resources. They consist of a hydrophilic sophorose head and a hydrophobic fatty acid tail. The main sophorolipid fermentation products are C18:1 sophorolipid acid **2** and diacetylated C18:1 sophorolipid lactone **3** which both include oleic acid in their structure (Figure 1). Natural sophorolipids feature interesting biological activities such as anti-cancer, antimicrobial, dermatological, immunoregulatory, spermicidal and antiviral activity.[26-27] Moreover, they possess self-assembly properties with a high variety in the type of nanostructures formed from different sophorolipid derivatives.[28-32] For example, C18:1 sophorolipid acid **2** forms micelles with the charge dependent on the pH, while its saturated derivative forms nanoscale ribbons with supramolecular chirality.[31] Recently, the fermentative production of sophorolipid based bolaamphiphiles with two sugar heads was also reported.[33-34]

In this work, the synthesis of a new class of bolaamphiphilic sophorolipids is described starting from the major microbial fermentation product, *i.e.* diacetylated sophorolipid lactone **3**. A modification pathway previously developed in our group is used for the synthesis of an intermediate sophorolipid aldehyde **6** (Scheme 1).[35] This aldehyde intermediate is then further modified towards different nitrogen containing bolaamphiphilic sophorolipids. With this procedure, the length of the linker can be customized and optimized, which is not the case for derivatives produced *via* fermentation. The new bolaamphiphilic derivatives were evaluated regarding their antimicrobial, transfection and self-assembly properties.

Previously synthesized quaternary ammonium sophorolipids proved to have good antimicrobial and transfection properties when an octadecyl chain was present on the nitrogen atom.[35-36] With the described reaction procedures, an overall yield of 23% was obtained for the quaternary ammonium sophorolipids

with the ozonolysis and reductive amination being the crucial steps.[37] If the yields of these reaction steps could be heightened towards 90% in an industrial setting by avoiding automated chromatography purification, the overall yield of the reaction pathway could be raised to 71%. The carbon efficiency and atom economy were respectively 69% and 62% for the sophorolipid aldehyde **3** and respectively 70% and 48% for the quaternary ammonium sophorolipids. The valorization of the methyl 9-oxononanoate byproduct resulting from the ozonolysis step is taken into account. For all reaction steps, the use of green solvents was aimed at, resulting in the replacement of dichloromethane with methanol for the ozonolysis step.[38]

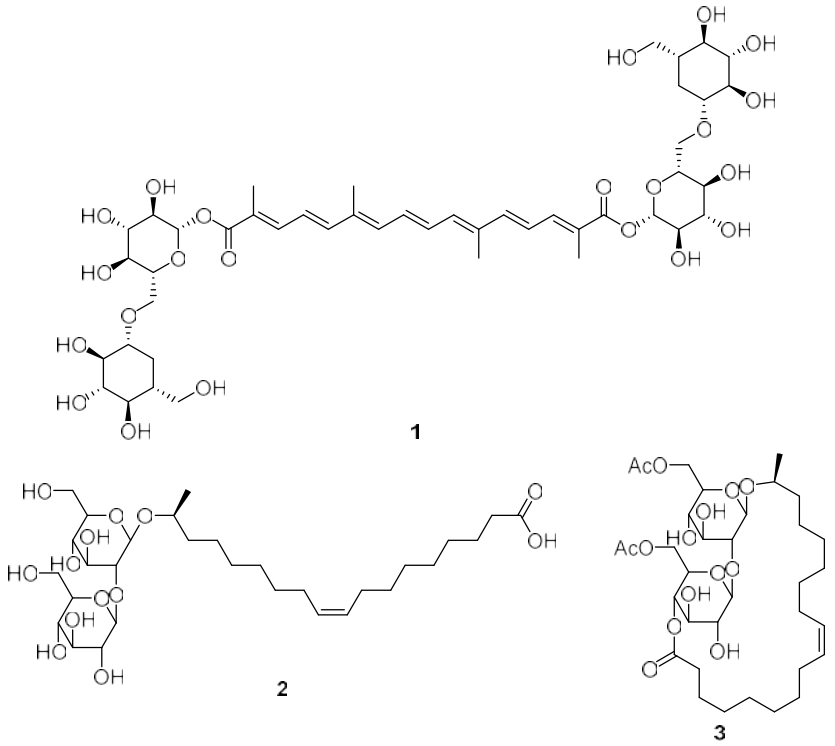
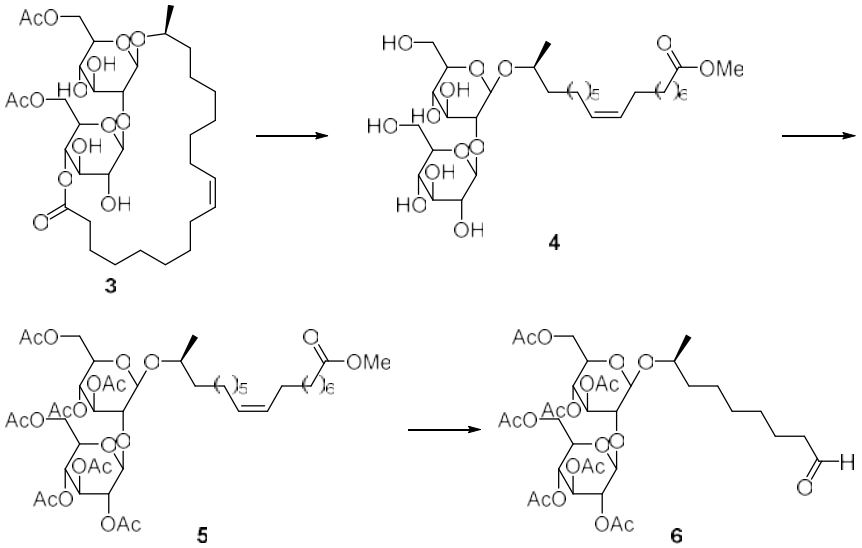


Figure 1. Crocin **1**, sophorolipid acid **2** and sophorolipid lactone **3**



Scheme 1. Modification pathway towards sophorolipid aldehyde **6**

Experimental section

Determination of the antimicrobial activity. Antimicrobial activity of all bolaamphiphilic sophorolipids against *E. coli* LMG 8063, *K. pneumoniae* LMG 2095, *P. aeruginosa* PAO1, *S. aureus* ATCC 6538 and *S. aureus* Mu50 was assessed by a broth microdilution method (CLSI, 2012).[39] Strains with LMG designation were obtained from the BCCM/LMG Bacteria Collection (Ghent, Belgium), while strain ATCC 6538 was obtained from the American Type Culture Collection (Manassas, VA). *S. aureus* strain Mu50 was a kind gift of P. Vandamme (Ghent, Belgium). All strains were grown aerobically at 37 °C on Mueller Hinton agar (LabM, Heywood, UK). The minimal inhibitory concentration (MIC) that inhibited growth completely compared to the untreated control and the minimal bactericidal concentration (MBC) at which no surviving organisms can be recovered were used as a measure of activity. MIC and MBC values were determined using flat-bottomed 96-well microtiter plates (TPP, Trasadingen, Switzerland). Concentrations of compounds tested ranged from 1.22 to 2500 µg/mL in Mueller Hinton Broth (LabM). The inoculum was standardized at approximately 5 x 10^4 colony forming units/mL. The plates were incubated at 37 °C for 24 h, and the optical density was determined at 590 nm using a multilabel microtiter plate reader (Envision Xcite, PerkinElmer LAS, Waltham, MA).

Liposome formulation. The liposomal solutions were prepared by the lipid film hydration method. A 1.5 mM solution (in 1 mL) of each compound was prepared in chloroform, formulated with or without DOPE (1:1 compound/DOPE) and evaporated under reduced pressure to produce a thin lipid film. Water (1 mL) was added to rehydrate this lipid film in a time period of 7 days at room temperature. The solution was vortexed (10 s) and sonicated (30 min at 50 °C) at 45 kHz using a VWR ultrasonic bath. The size and zeta potential were determined for each liposomal formulation.

DNA complexation. Lipoplexes were prepared by mixing pDNA (pEGFP-Luc, Clontech) with each liposomal solution in OptiMEM (Gibco). Addition of pDNA to the liposomal solutions was performed at concentrations corresponding to CR ranging from 1 to 4. The obtained mixtures were incubated at room temperature for 1 h before being subjected to electrophoresis in a 0.8% agarose gel at 100 V, 90 mA. The gel was stained with SYBRgold nucleic acid gel staining (Life Technologies) and visualized under UV light using a UV trans-illuminator (Fischer Bioblock).

Transfection efficiency. The *in vitro* reporter gene assay *via* luciferase measurement was carried out as reported previously.[40-41] Data were expressed as relative light units (RLU) per milligram of total proteins (means ± SD with n=3). Lipofectamine 3000 (Invitrogene) was used as standard.

Cell viability. The Vialight kit (Lonza) was used to estimate the viability of the cells following transfection. For this purpose, 48 h after exposition to the lipoplexes to evaluate, cells were lysed and their ATP content was determined, as recommended by the manufacturer. Non-transfected cells were used to express viability results in the form of percentages (% of the reference).

Small Angle X-ray Scattering (SAXS). SAXS experiments are performed at 25 °C immediately after sample preparation on the BioSAXS BM29 beamline at the ESRF synchrotron facility (Grenoble, France) using 12.5 keV energy and a sample-to-detector distance of 2.867 m, imposed by the beamline standard configuration. The energy is calibrated by measuring the L_I and L_{III} edges of platinum and the sample-to-detector distance is determined using silver behenate (d_{ref} = 58.38 Å). (http://www.esrf.eu/home/UsersAndScience/Experiments/MX/About_our_beamlines/bm29.html).[42] For this experiment, the automatic samples changer for liquids was employed using the 96-well plates and about 100 µL of each sample.[43] The liquid sample is automatically loaded into a 1.8 mm quartz glass capillary and ten acquisitions of 1 s each are taken as the sample passes the beam. Individual

frames are manually controlled for systematic changes and averaged for better statistics if none are found. Eventual changes can be either due to intrinsic sample heterogeneity or radiation damage. The signal of the Pilatus 1M 2D detector, used to record the data, is integrated azimuthally with PyFAI to obtain the *I(q) vs. q* spectrum ($q = \frac{4\pi}{\lambda} sin\theta$, , where 2θ is the scattering angle) after masking systematically wrong pixels and the beam stop shadow.[44] Absolute intensity units were determined by measuring the scattering signal of water (0.0163 cm^{-1}). Radii of gyration, R_g, have been calculated by the Guinier analysis of the SAXS data using the SasView software, available free of charge at the developer's website (http://www.sasview.org).

Results and discussion

Synthesis of the new derivatives

A first set of bolaamphiphilic sophorolipid derivatives was synthesized *via* reductive amination of sophorolipid aldehyde **6** with several diamines. A set of secondary diamines **7** with an ethylene-, hexamethylene, or *o*-phenylenelinker and methyl, butyl or octadecyl groups was selected for the synthesis of the desired bolaamphiphilic sophorolipids (Figure 2). With this set of amines, it was envisioned to assess the influence of the length of the alkyl linker, the presence of an aromatic group in the linker and the length of the substituents on the properties of the derivatives. Since only *N,N*'-dimethylethylenediamine **7a** and *N,N*'-dimethylhexamethylenediamine **7b** are commercially available, other diamines had to be synthesized.

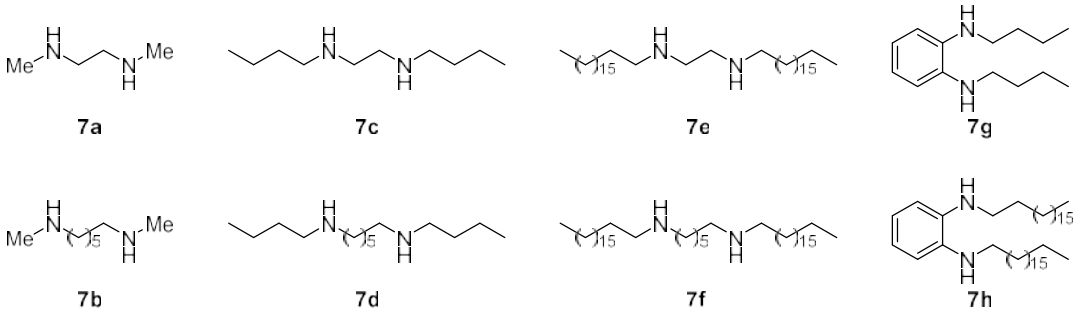
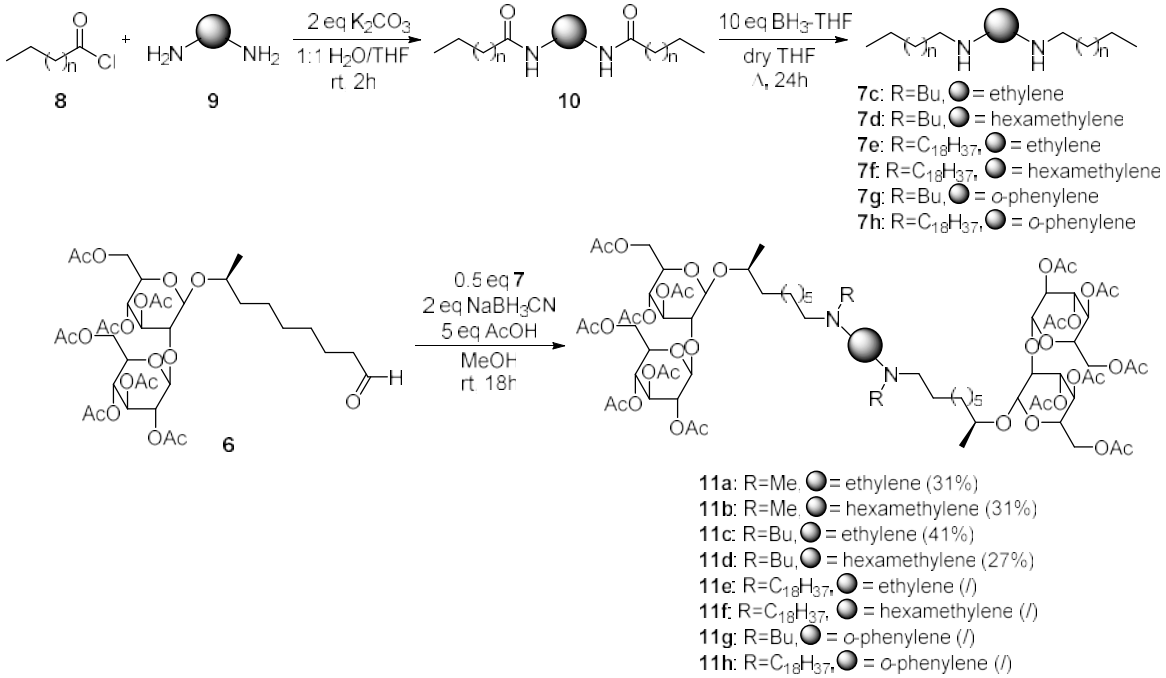


Figure 2. *N,N*'-dialkyldiamines **7** as substrates for the reductive amination towards bolaamphiphilic sophorolipids

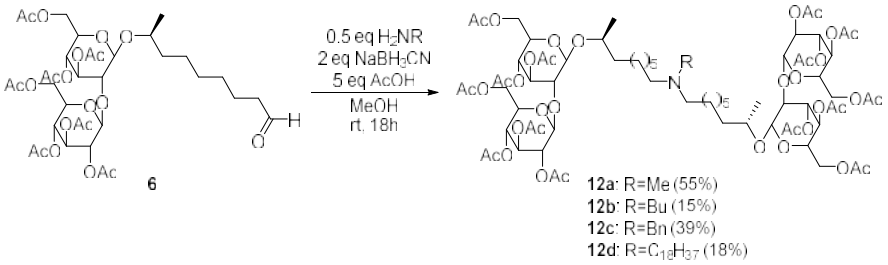
The intermediate diamides **10** were synthesized *via* a Schotten-Bauman reaction with acid chlorides **8** and diamines **9** (Scheme 2). Subsequently, these intermediate diamides were reduced with a borane tetrahydrofuran complex towards the desired *N,N*'-dialkyldiamines **7**. With this procedure, a total set of eight different *N,N*'-dialkyldiamines **7** was available, including the commercially available *N,N*'-dimethylethylenediamine **7a** and *N,N*'-dimethylhexamethylenediamine **7b**. These intermediate diamines were not fully characterized and therefore reaction yields were not determined.

The synthesis of the desired bolaamphiphilic sophorolipids **11** was performed according to the reaction conditions described for the previously synthesized sophorolipid amines (Scheme 2).[35] The reductive amination of sophorolipid aldehyde **6** with *N,N*'-dialkyldiamines **7a-d** resulted in the synthesis of the desired bolaamphiphilic sophorolipids **11a-d**. However, reductive amination with *N,N*'-dialkyldiamines **7e-h** was not successful. In the case of *N,N*'-dialkyldiamines **7e, 7f** and **7h**, poor solubility of the diamines in the reaction solvent prevented the synthesis of the desired bolaamphiphilic sophorolipids. When tetrahydrofuran was evaluated as solvent instead of methanol, no reductive amination occurred either. In the case of *N,N*'-dialkyldiamine**7g**, NMR-analysis indicated that the reductive amination did occur partially, but no complete conversion could be obtained due to the steric hindrance caused by the close proximity of the two butyl groups.



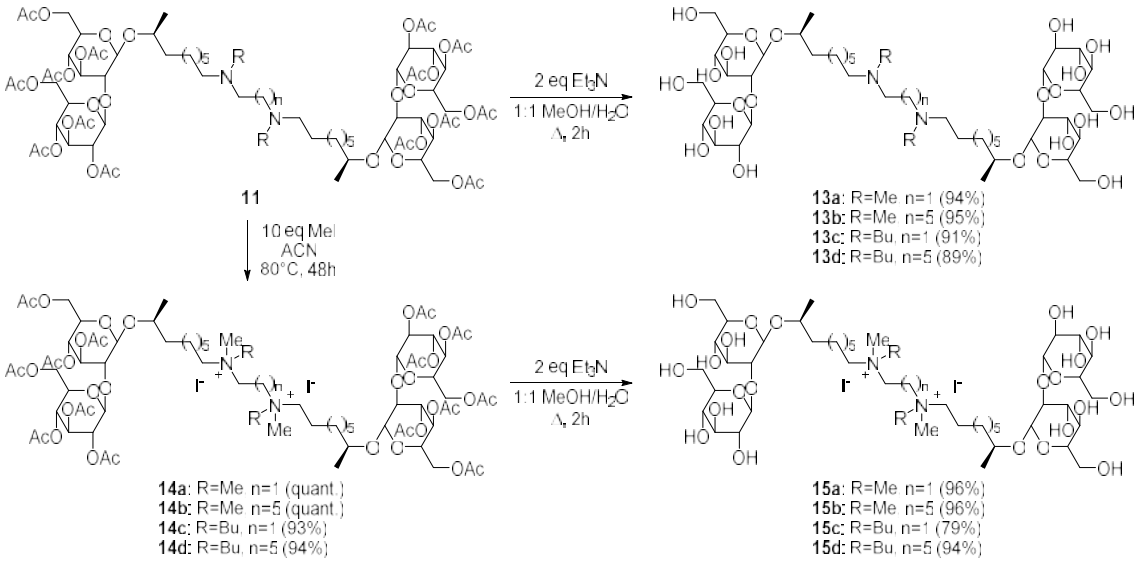
Scheme 2. Synthesis of *N,N*'-dialkyldiamines **7** and reductive amination towards bolaamphiphilic sophorolipids **11**

The formation of another class of bolaamphiphilic sophorolipids was evidenced in the course of the evaluation of the reductive amination of sophorolipid aldehyde **6** with primary amines. When the same reaction conditions were applied as for the reductive amination with secondary amines, a mixture of secondary sophorolipid amines and bolaamphiphilic sophorolipid amines was obtained. The selective formation of *N*-alkyl bolaamphiphilic sophorolipid amines **12** was accomplished by using only 0.5 equivalents of primary amine and applying the same reaction conditions as described for the *N,N*'-dialkyl bolaamphiphilic sophorolipids **11** (Scheme 3).



Scheme 3. Reductive amination with primary amines towards bolaamphiphilic sophorolipids **12**

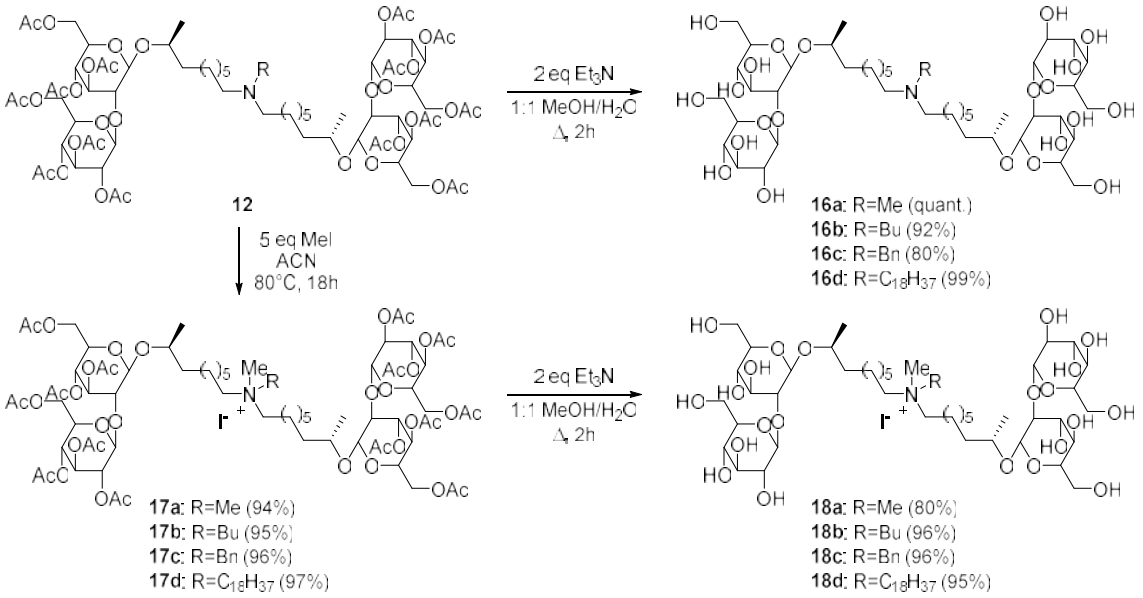
The set of four *N,N*'-dialkyl bolaamphiphilic sophorolipids **11** was transformed into the deprotected *N,N*'-dialkyl bolaamphiphilic sophorolipids **13**, peracetylated dicationic bolaamphiphilic sophorolipids **14** and deprotected dicationic bolaamphiphilic sophorolipids **15** (Scheme 4). The work of De Vos, De Coen *et al*. showed that the double quaternization of a bis(7-azabicyclo[2.2.1]heptane) derivative with an ethylene linker to a dicationic compound was unsuccessful, even upon reaction with 20 equivalents of methyl iodide at room temperature.[45-46] Therefore, it could be anticipated that the large charge repulsion on the small ethylene linker could also inhibit the formation of the dicationic bolaamphiphilic sophorolipids **14a** and **14c**. However, NMR-analysis clearly confirmed the successful synthesis of both compounds.



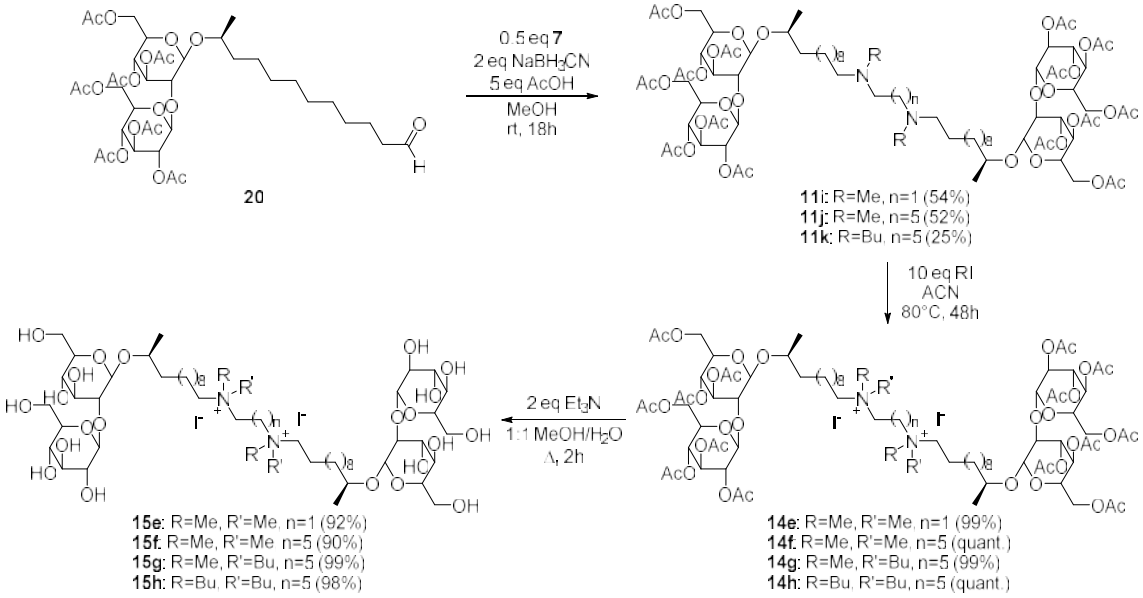
Scheme 4. Modification of *N,N*'-dialkyl bolaamphiphilic sophorolipids **11** *via* quaternization and deprotection

Alternative procedures were evaluated for the synthesis of *N,N*'-dialkyl bolaamphiphilic sophorolipids with octadecyl groups on the nitrogen atom. At first, the introduction of the octadecyl groups was attempted *via* quaternization of the *N,N*'-dimethyl bolaamphiphilic sophorolipids **11a** and **11b** with octadecyl iodide. However, no quaternization occurred in acetonitrile or toluene as solvent after 72 h. An alternative procedure was designed which comprised the formation of secondary bolaamphiphilic sophorolipid amines as intermediates. To prevent similar overalkylations which resulted in the formation of the *N*-alkyl bolaamphiphilic sophorolipid amines **12**, the mixture of sophorolipid aldehyde **6** and diamine was stirred for 1 h at room temperature prior to the addition of sodium cyanoborohydride and acetic acid. Attempts to alkylate these secondary bolaamphiphilic sophorolipid amines with octadecyl iodide were unsuccessful. Also reductive amination of these secondary bolaamphiphilic sophorolipid amines with octadecanal failed, although this dual reductive amination procedure proved to work for the synthesis of *N,N*'-dibutyl bolaamphiphilic sophorolipid **11c**. The set of four *N*-alkyl bolaamphiphilic sophorolipids **12** was also transformed into the deprotected *N*-alkyl bolaamphiphilic sophorolipids **16**, peracetylated monocationic bolaamphiphilic sophorolipids **17** and deprotected monocationic bolaamphiphilic sophorolipids **18** (Scheme 5).

To extend the set of *N,N*'-dialkyl bolaamphiphilic sophorolipids **11**, reductive amination of a C12 sophorolipid aldehyde **20** was performed with *N,N*'-alkyldiamines **7a, 7b** and **7d** (Scheme 6). This C12 sophorolipid aldehyde was obtained *via* incorporation of petroselinic acid into the sophorolipid structure, resulting in an aldehyde intermediate with a longer lipid chain after the ozonolysis reaction.[47] The resulting *N,N*'-dialkyl bolaamphiphilic sophorolipids **11i**, **11j** and **11k** were subsequently quaternized with methyl or butyl iodide towards the peracetylated dicationic bolaamphiphilic sophorolipids **14e-h**. In view of the antimicrobial activities for the C9 sophorolipid aldehyde derived bolaamphiphilic compounds (*vide infra*), only the peracetylated dicationic bolaamphiphilic sophorolipids **14e-h** were deprotected towards the dicationic bolaamphiphilic sophorolipids **15e-h**.



Scheme 5. Modification of *N*-alkyl bolaamphiphilic sophorolipids **12** *via* quaternization and deprotection



Scheme 6. Modification of C12 sophorolipid aldehyde **19** towards *N,N'*-dialkyl bolaamphiphilic sophorolipids

Different sustainable chemistry metrics were evaluated for the synthesis of the bolaamphiphilic sophorolipid derivatives (Table 1). Overall reaction yields ranging from 8 to 33% were obtained for the new derivatives, with the ozonolysis and reductive amination being the crucial steps.[37] If the yields of these reaction steps could be heightened towards 90% in an industrial setting by avoiding automated chromatography purification, the overall yield of the reaction pathways could be raised to 71%. For the calculation of the carbon efficiency (CE) and the atom economy (AE), the valorization of the methyl 9-oxononanoate byproduct resulting from the ozonolysis step is taken into account. The CE estimates the percentage of carbon from the reactants that is contained in the final product. This metric does not take into account the amount of solvent used and waste generated during the process. The AE estimates the total amount of reactants that are incorporated into the final product. Also here, only the reactants which are incorporated into the final product are taken into account. Solvent use and waste generation are not quantified. Metrics which do take the solvent use and waste generation into account are not qualified

here since these largely depend on the scale at which the reactions are performed and scale-up of the reaction procedures was out of the scope of this work. For all reaction steps, the use of green solvents was aimed at.

Table 1. Overview of the sustainable chemistry metrics for the production of the bolaamphiphilic sophorolipid derivatives. ε = reaction yield, CE = carbon efficiency, AE = atom economy.

	ε (%)	CE (%)	AE (%)		ε (%)	CE (%)	AE (%)
Peracetylated *N,N'*-dialkyl bolaamphiphilic SL				**Deprotected *N,N'*-dialkyl bolaamphiphilic SL**			
11a	19	69	60	**13a**	17	44	37
11b	19	70	61	**13b**	18	45	38
11c	24	70	61	**13c**	22	46	38
11d	16	71	62	**13d**	24	48	39
11i	32	69	60				
11j	31	70	61				
11k	15	71	62				
Peracetylated *N*-alkyl bolaamphiphilic SL				**Deprotected *N*-alkyl bolaamphiphilic SL**			
12a	33	69	60	**16a**	33	43	37
12b	9	70	61	**16b**	8	44	37
12c	23	70	61	**16c**	19	45	38
12d	11	72	63	**16d**	11	49	41
Peracetylated Dicationic bolaamphiphilic SL				**Deprotected Dicationic bolaamphiphilic SL**			
14a	18	70	63	**15a**	18	44	42
14b	18	70	64	**15b**	18	46	42
14c	23	71	64	**15c**	18	47	43
14d	15	72	64	**15d**	14	48	43
14e	32	70	63	**15e**	29	45	41
14f	31	70	64	**15f**	28	46	42
14g	15	72	64	**15g**	15	48	43
14h	15	71	65	**15h**	14	49	45
Peracetylated Monocationc bolaamphiphilic SL				**Deprotected Monocationc bolaamphiphilic SL**			
17a	31	69	62	**18a**	25	44	39
17b	9	70	63	**18b**	8	45	40
17c	22	71	63	**18c**	21	46	40
17d	10	73	65	**18d**	10	49	43

Evaluation of the antimicrobial activity

The antimicrobial activity of the peracetylated and deprotected *N,N'*-dialkyl bolaamphiphilic sophorolipids **11** and **13**, peracetylated and deprotected dicationic bolaamphiphilic sophorolipids **14** and **15**, peracetylated and deprotected *N*-alkyl bolaamphiphilic sophorolipids **12** and **16**, and peracetylated and deprotected monocationic bolaamphiphilic sophorolipids **17** and **18** was evaluated. As reference compounds, the previously synthesized quaternary ammonium sophorolipids **19a** and **19b** with an octadecyl chain on the nitrogen atom were taken into account (Figure 3).

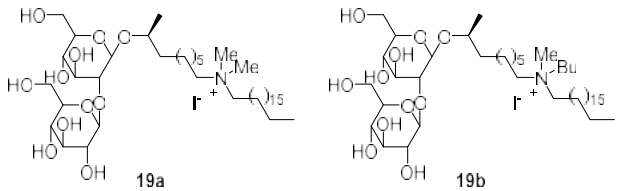


Figure 3. Quaternary ammonium sophorolipids **19a** and **19b** having demonstrated high transfection efficiencies[36]

The Gram-negative bacteria *Escherichia coli* LMG 8063, *Klebsiella pneumoniae* LMG 2095 and *Pseudomonas aeruginosa* PAO1, and the Gram-positive bacteria *Staphylococcus aureus* ATCC 6538 and *Staphylococcus aureus* Mu50 were chosen as test organisms because of their clinical relevance.

This set includes bacteria of high medical concern, some being part of the WHO priority pathogen list for the development of new antibiotic alternatives.[48] The bioassay was carried out in 96-well plates in a concentration series ranging from 2500 to 1.22 µg/mL of test compound and approximately 5 x 10^4 bacteria in a final volume of 200 µL. None of the evaluated compounds inhibited growth of any of the three Gram-negative bacteria tested. A set of nine bolaamphiphilic sophorolipids showed activity against one or both *S. aureus* strains. For all nine compounds, both the minimal inhibitory concentration (MIC, *i.e*. the lowest concentration that inhibits growth) and minimal bactericidal concentration (MBC, *i.e*. the lowest concentration that kills cells) values were determined against the two *S. aureus* strains (Table S1 ESI). All activities were determined in duplicate or triplicate. Results are considered reproducible when the repetitions don't differ more than one dilution. In case a higher difference is obtained, the highest value is reported.

Within this set of bolaamphiphilic sophorolipid derivatives, the peracetylated dicationic and monocationic congeners **14** and **17** proved to be the most active groups of derivatives. The highest activity was obtained for peracetylated *N,N*'-dibutyl-*N,N'*-dimethylethylene bolaamphiphilic sophorolipid diammonium di-iodide **14c** with MIC and MBC values against *S. aureus* ATCC 6538 of 39 and 312 µg/mL (=20 and 158 µM), respectively, and MIC and MBC values against *S. aureus* Mu50 of 156 and 312 µg/mL (=79 and 158 µM), respectively. For a better comparison of the active bolaamphiphilic derivatives with each other and with the previously synthesized quaternary ammonium sophorolipids, the MIC and MBC values were converted based on their molecular weight (Table 2). On this basis, *N,N*'-dibutyl-*N,N'*-dimethylethylene bolaamphiphilic sophorolipid diammonium diodide **14c** still has the highest activity among all bolaamphiphilic derivatives. However, these MIC values are still fourfold higher than the ones obtained for the deprotected quaternary ammonium sophorolipids **19** with an octadecyl chain on the nitrogen atom.

Table 2. MIC and MBC values (µM) for the active compounds against S. aureus ATCC 6538 and S. aureus Mu50. The best results for the new derivatives are highlighted in bold. For comparison, the results

(µM)		**11b**	**14a**	**14b**	**14c**	**14d**	**17a**	**17b**	**17c**	**18d**	**19a**	**19b**
S. aureus	**MIC**	375	165	80	20	38	369	360	353	116	2.18	2.09
ATCC 6538	**MBC**	>1501	330	321	158	153	1476	720	>1412	233	8.76	2.09
S. aureus	**MIC**	1501	660	321	79	153	185	360	176	233	4.37	4.18
Mu50	**MBC**	1501	1320	321	158	614	1476	1440	706	>1861	17.5	16.7

Antimicrobial activities were also evaluated for the petroselinic acid based peracetylated and deprotected dicationic bolaamphiphilic sophorolipids (Table 3, Table S2 ESI). Low activities were observed against the Gram-negative bacteria, but five of the derivatives showed moderate to high activity against the Gram-positive bacteria. The highest activity was obtained for the peracetylated *N,N'*-dibutyl,-*N,N'*-dimethylhexamethylene bolaamphiphilic sophorolipid diammonium di-iodide **14g** and *N,N,N',N'*-tetrabutylhexamethylene bolaamphiphilic sophorolipid diammonium di-iodide **14h**, which are almost in the same range as for the previously synthesized deprotected quaternary ammonium sophorolipids with an octadecyl chain on the nitrogen atom. The natural sophorolipid lactone and acid were also evaluated in the antimicrobial analysis. The activity for oleic acid based sophorolipid acid **2** and lactone **3** are displayed in Table 3. These results illustrate that the chemical modification enables an increase in the antimicrobial activity by a factor 100.

In the evaluation, an *S. aureus* Mu50 strain is included, which is a methicillin-resistant *Staphylococcus aureus* (MRSA) strain with vancomycin resistance. MIC values for activity of vancomycin and clindamycin against this *S. aureus* Mu50 were reported to be 8 µg/mL (=6 µM) and 512 µg/mL (=1205 µM), respectively.[49] Therefore, the activities obtained with compounds **14g** and **14h** against both *S. aureus* strains are reasonably good. However, it should be taken into account that these results are obtained with *in vitro* testing and that further *in vivo* testing is necessary to determine the actual antibiotic

potential of these compounds. In a next step, toxicity on eukaryotic cells should be evaluated to know whether the antimicrobial activity occurs at a lower concentration.

Table 3. MIC and MBC values (μM) for the active compounds against *E. coli* LMG 8063, *K. pneumoniae* LMG 2095, *S. aureus* ATCC 6538 and *S. aureus* Mu50. ND = not determined.

(μM)		2	3	14e	14f	14g	14h	15h	19a	19b
E. coli	**MIC**	>1607	>1453	>506	492	472	454	>620	>1122	>1071
LMG 8063	**MBC**	ND	ND	ND	ND	ND	ND	ND	ND	ND
K. pneumoniae	**MIC**	>1607	>1453	253	246	236	227	>620	>1122	>1017
LMG 2095	**MBC**	ND	ND	ND	ND	ND	ND	ND	ND	ND
S. aureus	**MIC**	>1607	182	63.2	15.3	3.69	3.55	620	2.18	2.09
ATCC 6538	**MBC**	ND	363	>506	30.7	7.37	3.55	ND	8.76	2.09
S. aureus	**MIC**	>1607	363	63.2	15.3	7.37	7.09	310	4.37	4.18
Mu50	**MBC**	ND	1453	>506	246	14.8	7.09	ND	17.5	16.7

Evaluation of the transfection efficiency

Transfection efficiencies for the bolaamphiphilic sophorolipids were evaluated in a similar way as for the previously synthesized quaternary ammonium sophorolipids.[36] High transfection results were obtained for the two derivatives having an octadecyl chain on the nitrogen atom (Figure 3). Lipofectamine 3000 (LFM), a commercial lipofection reagent, was used as a reference compound in the transfection experiments. The application of quaternary ammonium derivatives for the compaction and delivery of plasmid DNA (pDNA) and nucleic acids to different cell lines, both *in vitro* and *in vivo*, is already widely known.[50-54] Renewable based gene delivery vectors are of special interest since they are expected to have an increased biocompatibility.[40, 55-57]

From the set of thirty-two bolaamphiphilic sophorolipids, three cationic compounds (**15b**, **15d** and **18d**) were evaluated for their ability to form supramolecular aggregates in water solution by using the lipid film hydration method. For all liposomal solutions, the size of the particles and their surface charge were determined *via* Dynamic Light Scattering (DLS) and zeta measurements, respectively. The formation of a homogeneous formulation is a necessary prerequisite for the evaluation of the suitability of quaternary ammonium salts as vectors for gene delivery. The compounds were formulated with or without 1,2-dioleyl-*sn*-glycero-3-phosphoethanolamine (DOPE). Formulation with DOPE proved to be necessary to obtain homogenous formulations (Table 4), as was previously reported for similar compounds.[36] For the monocationic bolaamphiphilic sophorolipid **18d**, a positive zeta potential was obtained as is expected for liposomes generated from cationic lipid derivatives. However, for both dicationic bolaamphiphilic sophorolipids **15b** and **15d**, negative zeta potentials were obtained for formulations both with and without DOPE. Therefore, only compound **18d** was considered for further evaluation of the transfection efficiency.

Table 4. Size and zeta potential measurements of liposomal solutions prepared at 1.5 mM after 2 days of hydration without (left) or with (right) DOPE. ND = not determined.

	Size (nm)	Polydisp. index	Zeta (mV)	Size (nm)	Polydisp. index	Zeta (mV)
					+ DOPE	
15b	ND	ND	ND	432 ± 15	0.41	-20.1
15d	73 ± 11	1	-0.39	175 ± 0.1	0.13	-24.1
18d	ND	ND	ND	103 ± 1	0.28	44

The capacity to compact pDNA was evaluated for the liposomal formulation of compound **18d** with DOPE by pDNA retardation assays on agarose gel electrophoresis (Figure 4). This was performed at different charge ratios (CR) in a similar way as for the previously synthesized quaternary ammonium sophorolipids **19a-b**. CR is defined as the number of positive charges provided by the cationic lipid

derivative divided by the number of negative charges carried by the pDNA. The previously synthesized quaternary ammonium sophorolipids **19a-b** show a low capacity to complex pDNA at each CR. A similar behavior was observed for compound **18d**.

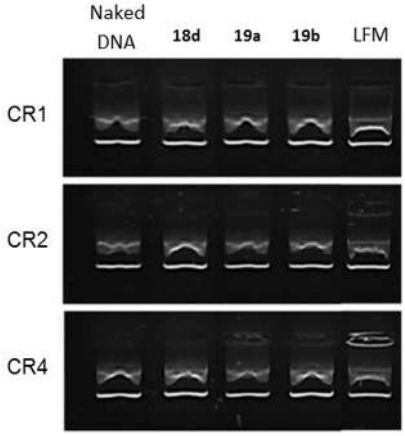


Figure 4. DNA complexation ability of compound **18d (**formulated with DOPE) as determined *via* the agarose gel retardation assay for different charge ratios. Previously synthesized quaternary ammonium sophorolipids **19a** and **19b**, naked DNA and LFM (Lipofectamine 3000) were used as controls.

In the transfection assay, compound **18d** was evaluated considering its ability to deliver DNA to three human-derived cell lines, namely melanoma cells (SKMEL28) and two airway epithelial cells *i.e.* (i) lung carcinoma (A549) and (ii) normal bronchial (16HBE) cells. A reporter (luciferase-encoding) pDNA was used that allowed the determination of the transfection efficiency *via* highly sensitive luminescence measurements. The formulation of compound **18d** with DOPE was evaluated at different CR. Compared with the previously synthesized quaternary ammonium sophorolipids **19a-b** and the control LFM, compound **18d** was almost ineffective for the transfection of the three cell lines (Figure 5). No real toxicity was observed towards any cell line (Figure 6).

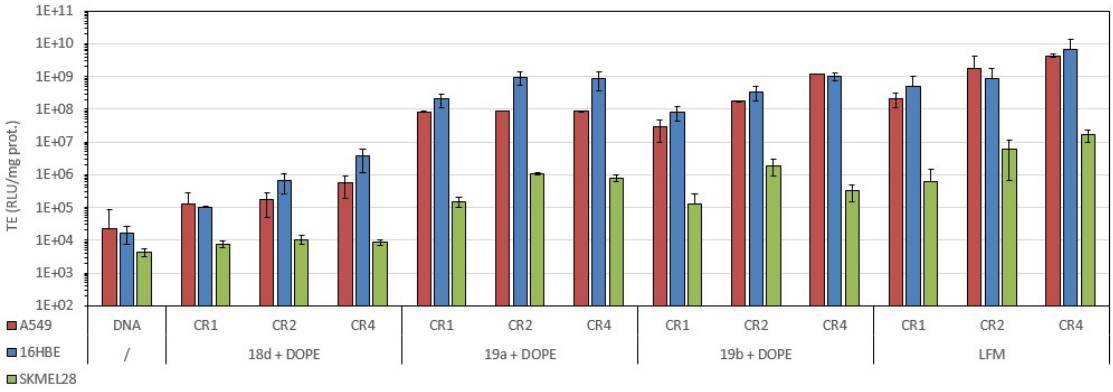


Figure 5. Transfection efficacies (TE) of compound **18d** formulated with DOPE on three cell lines (A549, 16HBE and SKMEL28) using luciferase-encoding pDNA (DNA). TE are expressed in RLU per mg of proteins (n = 3). Lipofectamine (LFM) and naked (uncomplexed) pDNA were used as positive and negative controls, respectively.

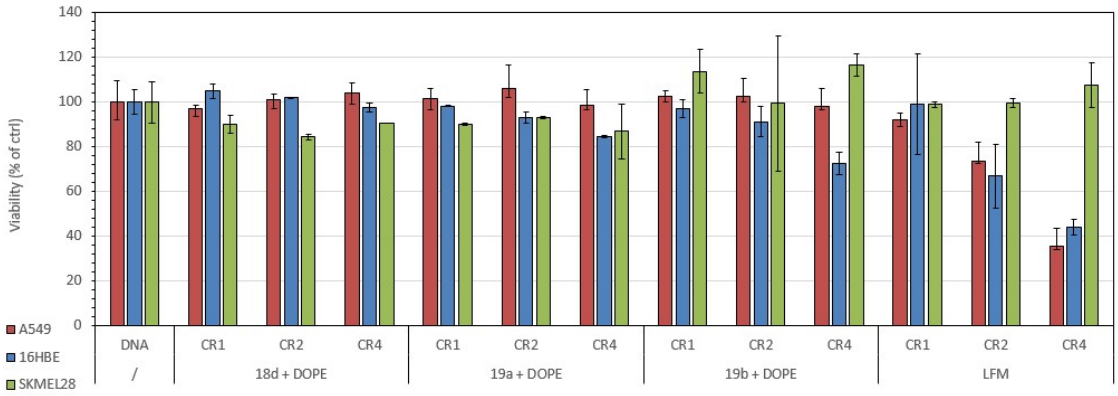


Figure 6. Cell viability determined 48 h after incubation of the cells with lipoplexes prepared with compound **18d** formulated with DOPE. Naked pDNA (DNA) was used as the negative control. Values are expressed as a percentage of the viability determined with untransfected cells.

Evaluation of the self-assembly properties

In an attempt to correlate the antimicrobial and transfection data, the self-assembly behavior in water of the bolaamphiphilic sophorolipids was evaluated *via* Small-Angle X-ray Scattering (SAXS) analysis. All samples were analyzed in milli-Q grade water within 24 h after sample preparation in a broad concentration range (0.78-100 mg/mL). In the following discussion, we only report the SAXS data at 6.25 mg/mL (=3.88-4.79 mM) for the dicationic bolaamphiphilic sophorolipids **15**, at 10 mg/mL (=8.32-10.38 mM) for the *N*-alkyl bolaamphiphilic sophorolipids **16** and at 12.5 mg/mL (=9.30-11.31 mM) for the monocationic bolaamphiphilic sophorolipids **18**. The choice to show this set of concentration values is a good compromise between a scattering signal with a satisfactory signal-to-noise ratio and the range of concentrations used in the previous experiments. Figures 7-9 show the SAXS data of only deprotected dicationic bolaamphiphilic sophorolipids **15a-15g**, *N*-alkyl bolaamphiphilic sophorolipids **16a-16d** and monocationic sophorolipids **18a-18d**, because the peracetylated derivatives are poorly soluble in water. According to the SAXS data, the self-assembly properties of the quaternary ammonium bolaamphihilic sophorolipids are neither uniform nor straightforward to interpret. The group of compounds **15** (**a, e-h**) shows a poor, although clearly present, scattering signal. A qualitative analysis of the data using a Guinier approach provides a set of radii of gyration (R_g) below 1 nm (Table 4).[58] By using the classical Tanford formula

$$l_c\ (Å) = 1.5 + 1.265n_c \quad (1)$$

(with l_c and n_c being the hydrocarbon chain length and the number of carbon atoms in the chain respectively) to estimate the length of the fully extended hydrocarbon chain, one can estimate the approximate overall size of any of these compounds to range between 5 and 6 nm.[59] Under these circumstances, the aggregates probed by SAXS must be composed of few, folded, bolaform compounds **15** (**a,e-h**). The set of compounds **15b-d**, of which the R_g is difficult to estimate, seems to be composed of larger self-assembled objects of undefined morphology. The value of the slope in the log(I)-log(q) profiles can help, in some cases, identifying the qualitative nature of the morphology, or the fractional dimension.[58] In the present case, compounds **15c** and **15d** have slopes close to -1 (Table 4), which could indicate the presence of elongated, cylindrical objects although compound **15d** has a more complex profile. The value of the slope found for compound **15b** (-1.79 ± 0.03) and its poor overall scattering signal do not allow a precise interpretation although values between -1 and -2 have been reported for flexible cylinders.

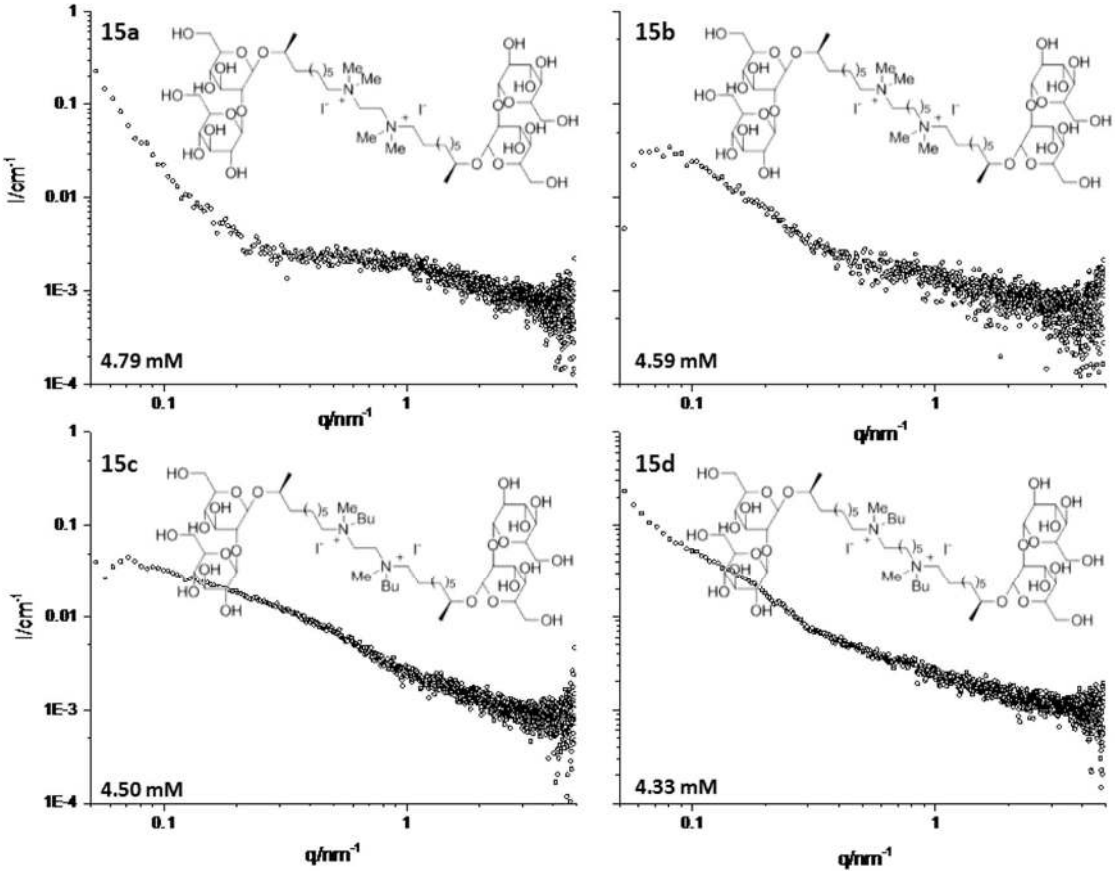


Figure 7. SAXS data of oleic acid based dicationic bolaamphiphilic sophorolipids **15a-15d** at a concentration of 6.25 mg/mL (=4.33-4.79 mM).

In the family of compounds **16** and **18**, one can find strong similarities in the self-assembly behavior. Compounds **16a**, **16b**, **18a** and **18b** are all characterized by a scattering profile providing an estimated $R_g < 1$ and, above all, an important low-q scattering signal indicating the presence of larger objects. For compounds **18a** and **18b**, the slopes can be evaluated in the vicinity of -1.6 (Table 5), which is a value observed for elongated wormlike micelles.[28, 60-62] Compounds **16c** and **18c** have typical scattering profiles of crystalline flat fibers, characterized by a -2 slope in the low-q range and a diffraction peak, here localized at $q = 2.28\ nm^{-1}$, and indicative of a repeating distance between the bolaform lipid planes of $2\pi/q = 2.75$ nm. Similar data have been reported for several sophorolipid-based systems forming twisted nanoscale fibers.[31, 63] Finally, compounds **16d** and **18d** have the typical profile of spheroidal micelles in the presence of repulsive interactions, which can be recognized by the flattened low-q signal for **16d** and the broad interaction peak for **18d**. Similar data have been reported for acidic sophorolipids and acidic sophorolipids mixed with sodium dodecyl sulfate.[30, 64]

As a general comment, irrespective of the mono- and diammonium linker in the bolaform sophorolipids, the nature of the substitute strongly impacts the self-assembly behavior of these molecules. In the case of methyl or butyl groups (compounds **15a-h**, **16a-b** and **18a-b**), the self-assembly properties are rather poor. In some cases, small molecular aggregates are formed in the presence of larger objects of which the morphology is rather unclear without further microscopy studies, which are out of the scope of this work. On the contrary, the octadecyl hydrocarbon group on samples **16d** and **18d** drives the self-assembly of the bolaform sophorolipids into micelles, most likely through the hydrophobic interaction which is a well-known fact in the theory of the self-assembly of amphiphiles.[59, 65-66] Similar results have been observed on several long-chained quaternary ammonium sophorolipids such as compounds **19a**

and **19b** (unpublished results). Intermicellar repulsive interactions characterize these systems and the strength of the interaction depends on the charge localized on the nitrogen. An ammonium group (**18d**) favors electrostatic interactions (well-defined, broad scattering peak) which were previously described also for sophorolipid systems, while an amino group favors less pronounced, excluded volume, interactions (flattened low-q signal).[30, 64] Finally, the presence of an aryl group favors π-π stacking which is known to drive low molecular weight molecules, including bolaform glycolipids, towards the formation of self-assembled fibers.[67]

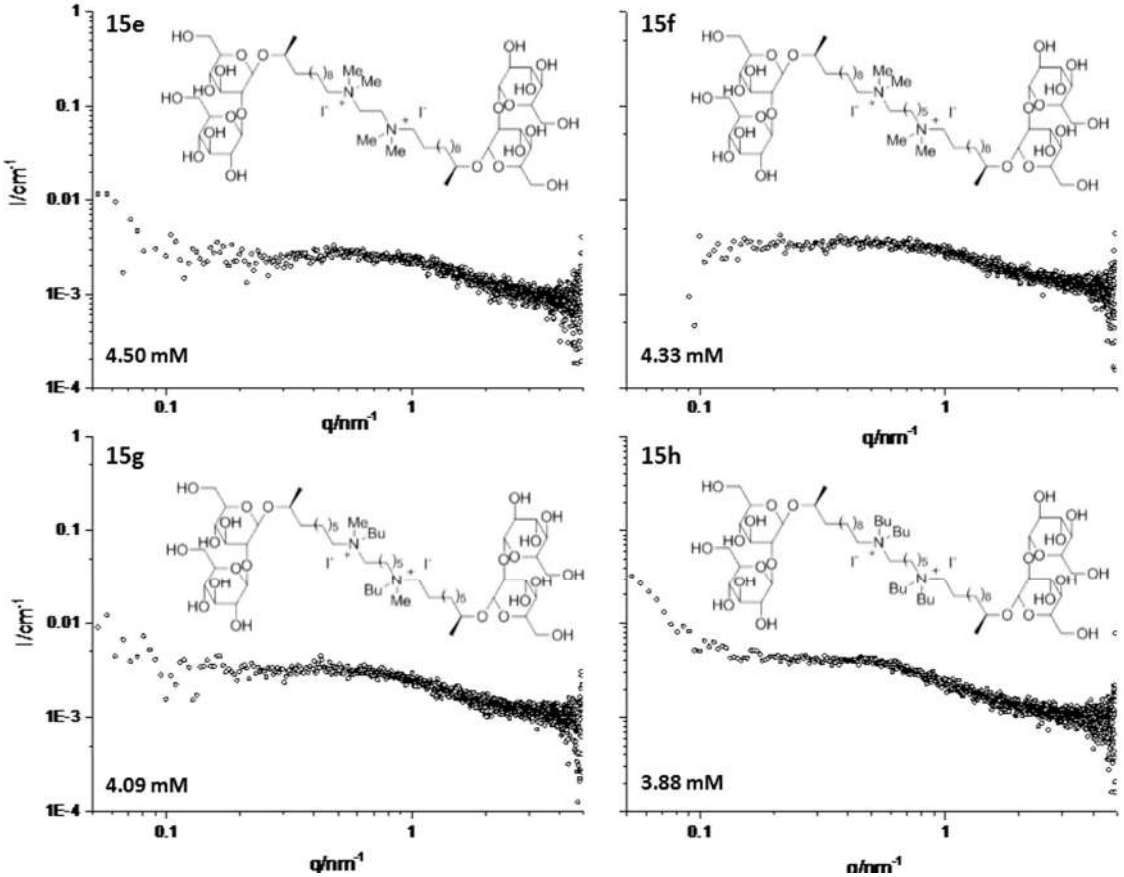


Figure 8. SAXS data of petroselinic acid based dicationic bolaamphiphilic sophorolipids **15e-15h** at a concentration of 6.25 mg/mL (=3.88-4.50 mM).

Among all non-acetylated molecules, monocationic bolaamphiphilic sophorolipid **18d**, having an octadecyl substituent, was the only compound which proved to be interesting for the evaluation of the transfection properties. However, this compound was much less efficient in comparison to the previously synthesized compounds **19a-b**, and the reference lipofection reagent Lipofectamine. Interestingly, **18d** was the only non-acetylated bolaamphiphilic compound for which antimicrobial activity could actually be observed. Also here, the activity was much lower compared to the previously synthesized compounds **19a-b**. Recent work on several long-chained quaternary ammonium sophorolipids, such as compounds **19a** and **19b**, demonstrates a strong correlation between the antimicrobial or transfection efficiencies and the self-assembly properties of the compounds (submitted results). The explanation is not straightforward for the relationship between self-assembly and transfection efficiency. Different experimental conditions were applied for the measurement of the transfection and self-assembly properties. Self-assembly properties were evaluated with compounds independently solubilized in water, whereas sophorolipids are associated with DOPE (1:1 ratio) to form liposomes that are subsequently mixed with pDNA for the transfection experiments. In consequence, the SAXS results are worth only for the sophorolipid derivatives placed in water but cannot be extended

to the liposomal solutions where DOPE likely plays an important role for the supramolecular structuration. Mixtures with DOPE are not included in the self-assembly analysis because of the overlap between the signal of micelles and liposomes in the 0.8 nm^{-1} < q < -3 nm^{-1} region. In the current case, self-assembly confirms that compound **18d** is better suited for transfection and antimicrobial action with respect to the other non-acetylated compounds. However, if micelle formations seems to be a necessary condition for one of the above-tested non-acetylated compounds to display antimicrobial and transfection properties, it is definitely not sufficient to guarantee a high performance, which can depend on the interaction between the compound and DOPE, for transfection, and between the molecule and the bacterial strain, for the antimicrobial activity.

Table 5. Radii of gyration, R_g, low-q slopes and position of diffraction peaks obtained from the SAXS data presented in Figure 7, Figure 8 and Figure 9. Values of R_g have been determined using the Guinier treatment.

	Rg (nm)	Slope	Peak (nm^{-1})
15a	0.67	-3.12 ± 0.09	
15b		-1.79 ± 0.03	
15c		-0.94 ± 0.01	
15d		-0.82 ± 0.03 (q> 0.3) -1.40 ± 0.01 (q< 0.15)	
15e	0.67		
15f	0.66		
15g	0.69		
15h	0.82		
16a	0.67		
16b	0.51		
16c		-1.90 ± 0.01	2.28
16d	3.80		
18a	0.74	-1.67 ± 0.01 (q< 0.20)	
18b	0.75	-1.72 ± 0.02 (q< 0.20)	
18c		-2.04 ± 0.02	2.28
18d	3.20		

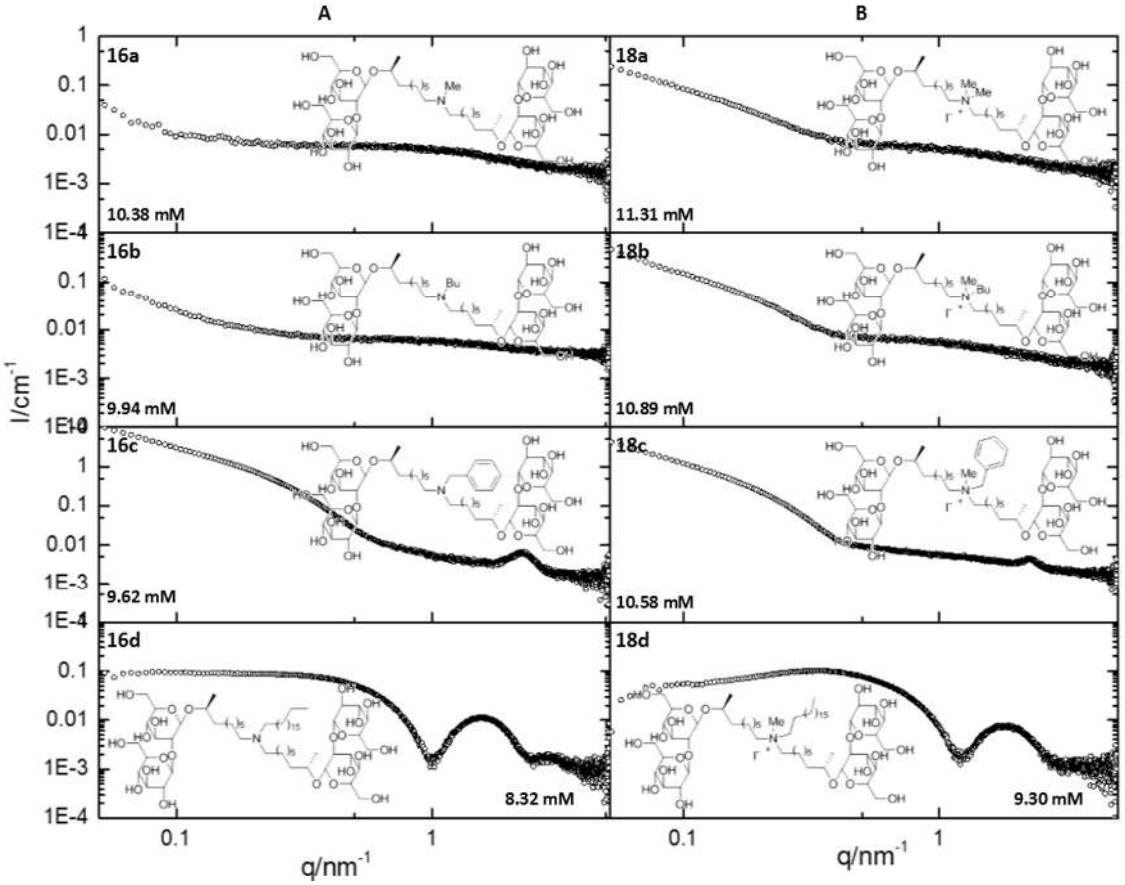


Figure 9. A) SAXS data of *N*-alkyl bolaamphiphilic sophorolipids **16a-16d** at a concentration of 10 mg/mL (=8.32-10.38 mM) and pH 7. **B)** SAXS data of monocationic bolaamphiphilic sophorolipids **18a-18d** at a concentration of 12.5 mg/mL (=9.30-11.31 mM).

Conclusions

A new set of sophorolipid derivatives, *i.e.* bolaamphiphilic sophorolipids, was synthesized starting from a sophorolipid aldehyde intermediate *via* reductive amination with diamines or primary amines. Attempts to introduce octadecyl substituents or an aromatic linker on the diamine bolaamphiphilic sophorolipids were unsuccessful, probably due to the poor solubility of the *N*,*N*'-octadecyldiamines and steric hindrance induced by the aromatic linker. The new bolaamphiphilic sophorolipids were evaluated for their antimicrobial, transfection and self-assembly properties. The antimicrobial activity was evaluated against the Gram-positive bacteria *S. aureus* ATCC 6538 and *S. aureus* Mu50, and against the Gram-negative bacteria *E. coli* LMG 8063, *K. pneumoniae* LMG 2095 and *P. aeruginosa* PAO1. Fourteen of the derivatives were active against the Gram-positive bacteria tested, while five of them also showed low activity against the Gram-negative bacteria. The peracetylated dicationic and monocationic congeners **14** and **17** proved to be the most active groups of derivatives. The best results were obtained for the peracetylated *N,N*'-dibutyl-*N,N'*-dimethylhexamethylene bolaamphiphilic sophorolipid diammonium di-iodide **14g** and *N,N,N',N*'-tetrabutylhexamethylene bolaamphiphilic sophorolipid diammonium di-iodide **14h**, with activities almost in the same range as for the previously synthesized deprotected quaternary ammonium sophorolipids with an octadecyl chain on the nitrogen atom. Transfection efficiencies were evaluated for three cationic bolaamphiphilic sophorolipids, namely **15b**, **15c** an **18d**. Formulation with DOPE was required to obtain homogeneous liposomal solutions and a positive zeta potential (as expected for liposomes with cationic lipid derivatives) was only obtained for the *N*-octadecyl bolaamphiphilic sophorolipid ammonium iodide **18d**. Then, the ability of the liposomal

formulation with compound **18d** to compact pDNA was assessed. A low compaction was observed for compound **18d**, as was also the case for the previously synthesized quaternary ammonium sophorolipids. The capacity of compound **18d** to achieve transfection of eukaryotic cells was assessed on three cell lines (A549, 16HBE and SKMEL28). Compared with the previously synthesized quaternary ammonium sophorolipids and the control LFM, compound **18d** was almost inefficient for the transfection of the three cell lines and no real toxicity was observed towards any of these cell lines. Evaluation of the self-assembly properties indicated that the presence of an octadecyl chain was necessary for the compounds to self-assemble as micelles. The molecular self-assembly and the net charge of the compounds seem to have an influence on the antimicrobial activity and transfection efficiency. These results are promising to further extend the existing set of bolaamphiphilic sophorolipids and their use for medical and self-assembly applications.

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Supporting information

An experimental section on the general instrumental methods and general synthesis procedures is included in the supporting information, together with two tables on the antimicrobial activities of the compounds (µg/mL), the characterization of the synthesized compounds and the accompanying ^{1}H- and ^{13}C-NMR spectra.

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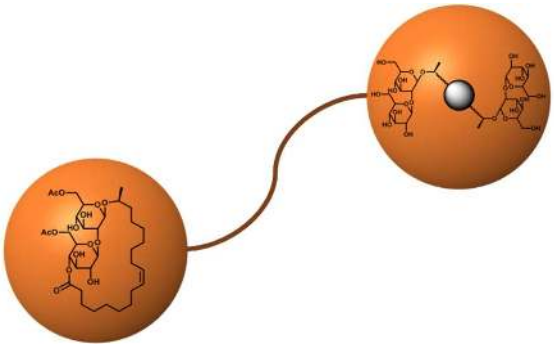

Sophorolipids, renewable biosurfactants, are used for the synthesis of innovative bolaamphiphilic compounds for application in the medical sector.