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A new class of antimicrobial biosurfactants: quaternary ammonium sophorolipids†

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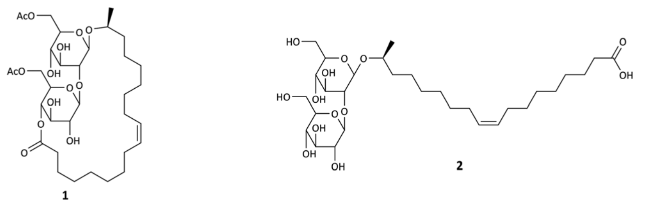
New synthetic pathways are proposed for the production of a broad range of innovative sophorolipid amines and sophorolipid quaternary ammonium salts starting from microbially produced sophorolipids. The selective formation of an intermediate sophorolipid aldehyde proved to be a key synthetic step of the new derivatives. The sophorolipid quaternary ammonium salts were evaluated for their antimicrobial activity against Gram-negative and Gram-positive test strains. Minimum inhibitory concentration (MIC) values were determined for the active compounds. Derivatives with an octadecyl group on the nitrogen atom proved to be more active than the antibiotic gentamicin sulfate against all tested Gram-positive strains. The results show great promise for modified sophorolipids in the medical sector.

Introduction

In our modern society, the concept of sustainability gains more interest from day to day.[1] Indeed, over the last decades, the transition towards a bio-based economy has initiated. The significance of renewable resources for the chemical industry is gradually increasing because they are considered good alternatives for fossil resources, whose supplies are limited and which have a major environmental impact.[2] At present already more than 8% of all the chemicals produced in Europe are based on renewable resources.[3] Most of these renewable resources are directly used as biomaterials such as paper, plastics, textile and fibres or as biochemicals in paint, detergents, and cosmetics. However, the incorporation of renewable resources in high value added products is mostly based on the same concept as the one adopted for fossil resources, *i.e.* starting from small hydrocarbon building blocks. Traditionally, fossil and renewable resources are broken down into base chemicals which are then used for the synthesis of the desired products. Using this methodology, the cost of renewable based pharmaceuticals outweighs the cost of their fossil counterparts, giving renewable resources a competitive disadvantage for the synthesis of these high value products. Obviously the business case would be far more interesting if renewable resources with a complex structure would be directly used as building blocks in a synthetic pathway. This will reduce the number of required steps to obtain the desired compounds and will additionally contribute to the sustainability of both process and end product. Target compounds for this 'green synthesis' approach should preferably possess the desired properties or biological activities which the traditionally obtained competing molecules lack.

In this light, sophorolipids are very useful building blocks for chemical derivatization. They occur in a lactonic form or an open acid form (Scheme 1). These glycolipids are produced by micro-organisms from renewable resources through fermentation.[4] The yeast *Starmerella bombicola* is the preferred producer, with output of around 400 g L^{-1} and a production price of 2 to 5€ kg^{-1}.[4a] Due to the presence of a hydrophilic carbohydrate head and a hydrophobic lipid tail, sophorolipids possess surface-active properties classifying them as biosurfactants. They are low foaming surfactants which can be used for hard surface cleaning and automatic dishwashing rinse aid applications.[5] Nevertheless, application of sophorolipids in the detergent sector is limited because they have a competitive disadvantage compared to synthetic surfactants in terms of production cost. Therefore, it is desirable to look for



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Scheme 1 Microbial produced sophorolipid lactone **1** and sophorolipid acid **2**.

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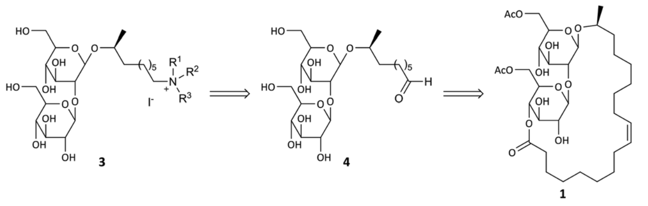


Scheme 2 Retrosynthetic scheme for sophorolipid quaternary ammonium salts **3**.

other application areas which are economically more attractive, *e.g.* in the medical sector. Sophorolipids feature beneficial biological activities such as anticancer, anti-HIV, sperm-immobilizing and antibacterial activity.[6] Recently, the self-assembly properties of sophorolipids have been described, demonstrating the formation of nanostructures with supramolecular chirality.[7] Until now, sophorolipid production is restricted to only a few derivatives. Consequently, the variation in their surface-active properties is limited and their biological activities have not yet been optimized. Chemical modification offers the opportunity to extend the limited set of microbial derivatives. In this paper, chemical modification pathways are evaluated which increase the applicability of sophorolipids for medical applications.

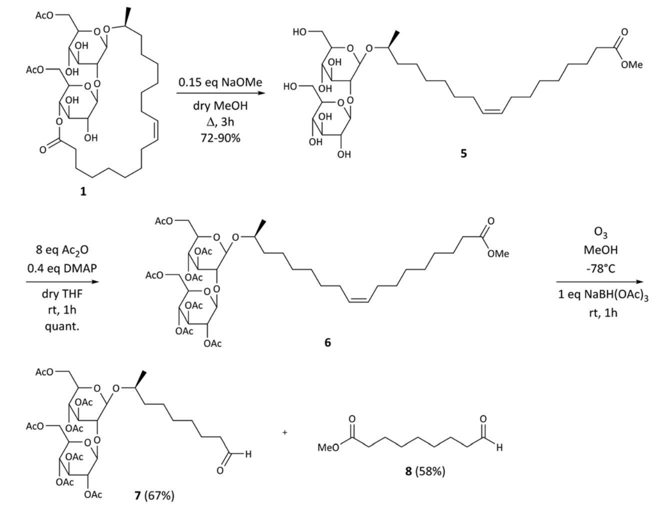
At present, chemical and enzymatic modification of sophorolipids have been mostly limited to the sugar head or the lipid tail.[8] Cleavage of the double bond, however, has only been described for the synthesis of short-chained sophorolipid acids or in ring-opening cross-metathesis reactions.[9] It was never included in a synthetic pathway for the production of a functionalized building block towards modified derivatives. This is surprising because such modification would result in the formation of sophorolipid analogues with shorter chain lengths, which would be better soluble in water.

Therefore in this work, a series of chemical modification pathways is described for sophorolipids towards quaternary ammonium salt derivatives (Scheme 2). First, an ozonolysis reaction is performed on sophorolipids in order to transform the double bond into a reactive site. In a second key step, a nitrogen functionality is incorporated. Further modification leads to a library of sophorolipid quaternary ammonium salts, which are evaluated as antimicrobial compounds.

Results and discussion

Synthesis of the sophorolipid aldehyde intermediate

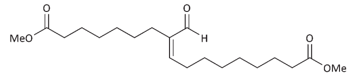
A chemical modification pathway for sophorolipids was developed starting from the major microbial product, *i.e.* the diacetylated sophorolipid lactone **1**.[10] This lactone was transformed into sophorolipid methyl ester **5** and, subsequently, into peracetylated analogue **6** according to literature procedures.[8c,d] Cleavage of the double bond through ozonolysis, offering an aldehyde functionality, would create opportunities for the incorporation of nitrogen. These nitrogen containing derivatives can then easily be transformed into cationic surfactants.



Scheme 3 Chemical modification towards sophorolipid aldehyde **5**.

The double bond was cleaved through ozonolysis in MeOH, and reductive work-up with $NaBH(OAc)_3$ furnished the peracetylated sophorolipid aldehyde **7** and methyl 9-oxononanoate side product **8** (Scheme 3).[11] At first, ozonolysis was attempted in CH_2Cl_2, followed by reductive work-up with dimethyl sulfide. Analysis of the reaction mixture demonstrated the presence of the desired peracetylated sophorolipid aldehyde **7**, but methyl 9-oxononanoate **8** could not be detected. Instead, only the presence of a stable 1,2,4-trioxolane (ozonide) intermediate was observed. The presence of high concentrations of residual ozonides after work-up with dimethyl sulfide has already been described by Dussault *et al.*[12] Therefore, reaction conditions were changed towards the use of the more environmental friendly $NaBH(OAc)_3$. Besides, isolation of methyl 9-oxononanoate **8** is most desirable for a green synthetic pathway. This side product could be used as a valuable building block for other applications. Direct ozonolysis of the diacetylated sophorolipid lactone **1** was also evaluated. An intermediate dialdehyde was obtained after ozonolysis, but transesterification to the desired sophorolipid aldehyde was not possible. Analysis of the reaction mixture demonstrated the presence of aldol condensation product **9** formed from methyl 9-oxononanoate indicating the non-compatibility of the aldehyde function with alkaline reaction conditions (Scheme 4).

Care should be taken during the ozonolysis reaction due to the formation of unstable ozonides and peroxides, and the use of methanol as a solvent. The reaction is performed in a taped washing flask on a small scale of 10–15 g peracetylated



Scheme 4 Observed aldol condensation product **9**.

sophorolipid methyl ester **6** at −78 °C. Ozone is generated from dry air and is purged through the reaction mixture at a flow of 2.15 L min^{-1} and a concentration of 6.2 mg L^{-1}. For large scale reactions, use of microreactor equipment should be considered.[13]

The purification of the sophorolipid aldehyde **7** proved to be a challenge due to the presence of saturated sophorolipids from the fermentation process and degradation products from the ozonolysis reaction. Multiple column chromatography purifications were evaluated, giving the highest yield and purity for automated flash chromatography with a gradient elution of ethyl acetate and hexane. A sodium bisulfite addition reaction was performed after the chromatography purification to improve the purity but resulted in a very low yield.

Synthesis of a quaternary ammonium salt library

Next, a nitrogen functionality was introduced through reductive amination of the aldehyde function of **7** with a variety of secondary amines (Table 1). Complete conversion to the resulting sophorolipid tertiary amines **10** was obtained, encompassing methyl, butyl, benzyl and octadecyl groups. Purification of sophorolipid tertiary amines **10** was necessary to avoid purification of sophorolipid quaternary ammonium salts **11** in the next step, but resulted in low yields.

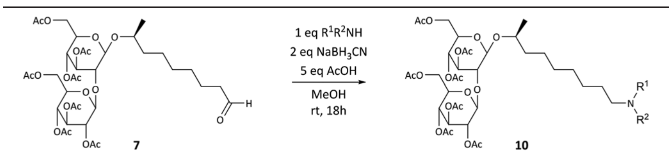
Subsequently, the sophorolipid tertiary amines **10** were quaternized with alkyl iodides in pressure vials to obtain the desired peracetylated sophorolipid quaternary ammonium salts **11** (Table 2). In a final step, the sugar head group of the quaternary ammonium salts **11** was deprotected to obtain the water soluble sophorolipid quaternary ammonium salts **3** (Table 3).

Evaluation of the antimicrobial activity

The antimicrobial activity of peracetylated sophorolipid aldehyde **7**, peracetylated sophorolipid amines **10a**, **10b**, **10c**, **10d**, **10f** and **10g**, peracetylated sophorolipid quaternary ammonium salts **11** and deprotected sophorolipid quaternary ammonium salts **3** was evaluated. The Gram-negative strains *Escherichia coli* LMG 8063 and *Klebsiella pneumoniae* LMG 2095, and the Gram-positive strains *Staphylococcus aureus* LMG 8064 and *Bacillus subtilis* LMG 13579 were chosen as test strains. The bioassay was carried out in 96-well microtiter plates at a concentration of approximately 0.5 mg mL^{-1} of the test compound and 10^4 CFU mL^{-1} test bacteria. None of the test compounds showed significant growth inhibition of *Escherichia coli* LMG 8063 and *Klebsiella pneumoniae* LMG 2095. Compounds **11b**, **11c**, **11d**, **11e**, **11f**, **11g**, **11h**, **11i**, **3b**, **3h** and **3i** showed significant growth inhibition of *Staphylococcus aureus* LMG 8064 and *Bacillus subtilis* LMG 13579.

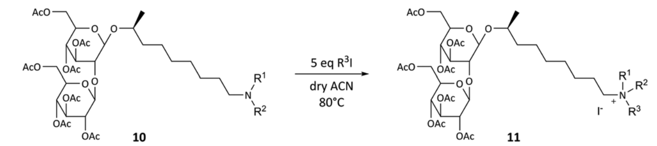
For the active compounds, the minimum inhibitory concentration (MIC) was determined against a test panel of four Gram-positive bacterial strains, namely *Staphylococcus aureus* LMG 8064, *Enterococcus faecium* LMG 11397, *Bacillus subtilis* LMG 13579 and *Streptococcus pneumoniae* LMG 16738. The MIC value is considered as the lowest concentration of the test compound for which a lack of visible bacterial growth is observed. This bioassay was carried out in 96-well microtiter plates at a concentration range between 100 and 2.5 μg mL^{-1} or 1000 and

Table 1 Reductive amination towards sophorolipid amines **10**



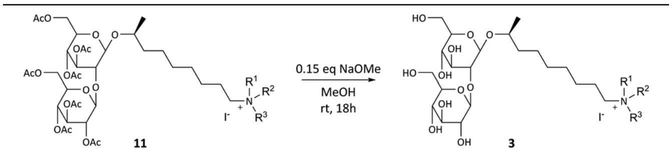
Entry	R^1R^2NH	Yield (%)	
1	Dimethylamine	**10a**	38
2	*N*-Methylbutylamine	**10b**	52
3	Dibutylamine	**10c**	53
4	*N*-Methylbenzylamine	**10d**	40
5	*N*-Butylbenzylamine	**10e**	49
6	Dibenzylamine	**10f**	48
7	*N*-Methyloctadecylamine	**10g**	39

Table 2 Quaternization towards peracetylated sophorolipid quaternary ammonium salts **11**



Entry	10	R^3I	Time (h)	Yield (%)	
1	**10a**	Methyl iodide	18	**11a**	91
2	**10b**	Methyl iodide	18	**11b**	89
3	**10c**	Methyl iodide	18	**11c**	96
4	**10c**	Butyl iodide	48	**11d**	94
5	**10d**	Methyl iodide	18	**11e**	Quant.
6	**10e**	Methyl iodide	18	**11f**	89
7	**10f**	Methyl iodide	18	**11g**	Quant.
8	**10g**	Methyl iodide	18	**11h**	98
9	**10g**	Butyl iodide	48	**11i**	Quant.

Table 3 Transesterification towards sophorolipid quaternary ammonium salts **10**



Entry	11	Yield (%)	
1	**11a**	**3a**	Quant.
2	**11b**	**3b**	88
3	**11c**	**3c**	Quant.
4	**11d**	**3d**	Quant.
5	**11e**	**3e**	Quant.
6	**11f**	**3f**	Quant.
7	**11g**	**3g**	99
8	**11h**	**3h**	97
9	**11i**	**3i**	66

Table 4 MIC values (μg mL^{-1}) for the active compounds and the antibiotic gentamicin sulfate

	11b	11c	11d	11e	11f	11g	11h	11i	3b	3h	3i	Gentamicin sulfate
S. aureus	>100	>100	>100	500	>100	50	10	10	>100	5	5	5
E. faecium	>100	>100	>100	>1000	>100	>100	10	10	>100	5	5	10
B. subtilis	>100	25	>100	1000	>100	50	10	10	>100	5	5	5
S. pneumoniae	>100	100	>100	1000	>100	100	10	10	>100	5	5	25

Table 5 MIC values (μM) for the active compounds and the antibiotic gentamicin sulfate

	11b	11c	11d	11e	11f	11g	11h	11i	3b	3h	3i	Gentamicin sulfate
S. aureus	>101	>97	>93	489	>94	45	8	8	>144	6	5	10
E. faecium	>101	>97	>93	977	>94	>91	8	8	>144	6	5	21
B. subtilis	>101	24	>93	977	>94	45	8	8	>144	6	5	10
S. pneumoniae	>101	97	>93	>977	>94	91	8	8	>144	6	5	52

5 μg mL^{-1} of the test compounds for respectively strong or weak inhibitors and 10^4 CFU mL^{-1} test bacteria. MIC values for the active compounds are given in Table 4 together with MIC values for the antibiotic gentamicin sulfate. Microscopic analysis in addition to the determination of the MIC values revealed that lysis of the cells occurred at the active concentrations.

The lowest MIC value of 5 μg mL^{-1} was obtained with compounds **3h** and **3i** against all four Gram-positive test strains. Low MIC values of 10 μg mL^{-1} were obtained with compounds **11h** and **11i**, also against all four Gram-positive test strains. Interestingly, these activities lie in the same concentration range as that of the antibiotic gentamicin sulfate. All four compounds perform as good or better as gentamicin sulfate against *E. faecium* and *S. pneumoniae*. Compounds **3h** and **3i** even perform as good as gentamicin sulfate against *S. aureus* and *B. subtilis*. For better comparison, the MIC values were converted on the basis of their molecular weight (Table 5). On this basis, we can conclude that compounds **3h**, **3i**, **11h** and **11i** are more active against all four Gram-positive test strains than the antibiotic gentamicin sulfate. These results show great promise for further evaluation of the biological activities of the active derivatives such as synergistic effects of multiple compounds against specific test strains or activities of specific compounds against multiple test strains. The active derivatives also offer opportunities for their use in medical applications such as the inhibition of biofilm formation. These sophorolipid quaternary ammonium salts represent a new class of antimicrobial surfactants.

Conclusions

Microbial produced sophorolipids have been chemically modified into a series of novel sophorolipid amines and sophorolipid quaternary ammonium salts *via* an intermediate sophorolipid aldehyde. The sophorolipid amines and sophorolipid quaternary ammonium salts have been evaluated for their antimicrobial activity against the Gram-negative strains *Escherichia coli* LMG 8063 and *Klebsiella pneumoniae* LMG 2095, and the Gram-positive strains *Staphylococcus aureus* LMG 8064 and *Bacillus subtilis* LMG 13579. Eight of the peracetylated sophorolipid quaternary ammonium salts and three of the deprotected sophorolipid quaternary ammonium salts showed inhibition against the Gram-positive strains, but not against the Gram-negative strains. For the active compounds, their minimum inhibitory concentration (MIC) has been determined against the four Gram-positive strains *Staphylococcus aureus* LMG 8064, *Enterococcus faecium* LMG 11397, *Bacillus subtilis* LMG 13579 and *Streptococcus pneumoniae* LMG 16738. The best results are obtained with *N*,*N*-dimethyl,*N*-octadecyl-(8-L-[(2″,3′,3″,4′,4″,6′,6″-heptaacetoxy-2′-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)-oxy])nonan-1-ammonium iodide **11h**, *N*-benzyl,*N*-methyl,*N*-octadecyl-(8-L-[(2″,3′,3″,4′,4″,6′,6″-heptaacetoxy-2′-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)-oxy])nonan-1-ammonium iodide **11i**, *N*,*N*-dimethyl,*N*-octadecyl-(8-L-[(2β-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)-oxy])nonan-1-ammonium iodide **3h** and with *N*-benzyl,*N*-methyl,*N*-octadecyl-(8-L-[(2β-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)-oxy])nonan-1-ammonium iodide **3i**. These four compounds are more active than the antibiotic gentamicin sulfate against all four Gram-positive test strains. These results show great promise for further evaluation of the biological activities of sophorolipid quaternary ammonium salts with an octadecyl group on the nitrogen atom and their use for medical applications. These sophorolipid quaternary ammonium salts represent a new class of antimicrobial surfactants.

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