Chlorosulfolipids

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Biological Investigations of (+)-Danicalipin A Enabled Through Synthesis

*Adrian M. Bailey, Susanne Wolfrum, and Erick M. Carreira**

Abstract: *A total synthesis of the chlorosulfolipid (+)-danicalipin A has been accomplished in 12 steps and 4.4% overall yield. The efficient and scalable synthesis enabled in-depth investigations of the lipid's biological properties, in particular cytotoxicity towards various mammalian cell lines. Furthermore, the ability of (+)-danicalipin A to increase the uptake of fluorophores into bacteria and mammalian cells was demonstrated, indicating it may enhance membrane permeability. By comparing (+)-danicalipin A with racemic 1,14-docosane disulfate, and the diol precursor of (+)-danicalipin A, we have shown that both chlorine and sulfate functionalities are necessary for biological activity.*

Chlorosulfolipids are a unique family of natural lipids, the first of which were isolated in the 1960's independently by the groups of Haines and Elovson from the fresh water alga *Ochromonas danica*.[1] Since then, several additional chlorosulfolipids have been isolated and characterized, namely malhamensilipin A from *Poterioochromonas malhamensis*[2] and mytilipin A–C (Figure 1a) from unspecified, harmful microalgae, which accumulate in Mediterranean sea mussels *Mytilus galloprovincialis* rendering them toxic.[3] These chiral lipids are characterized by polychlorinated, sulfated aliphatic chains, structural features uncommon in nature.[4] Despite a number of total syntheses of these structurally complex chlorosulfolipids,[5] little is known about their biological properties. Herein, we disclose a scalable total synthesis of (+)-danicalipin A (**1**) and evidence that this lipid has a significant effect on the membranes of mammalian cells and the walls of Gram-negative bacteria.

Chlorosulfolipids are most abundant in *O. danica*, contributing to 14.4% of the total lipid weight, whereas they account for only 2.3% of the total lipid weight of *P. malhamensis*.[6] Furthermore, in the case of *O. danica*, these lipids comprise more than 80 mol% of the total polar lipid content of the entire cell.[7] This observation has led to the speculation that the larger contribution of the chlorinated lipids is related to an unspecified important role in membrane structure and function in *O. danica*.[1f]

Most membrane lipids have well defined domains including hydrophilic heads and hydrophobic tails (Figure 1b); these structural attributes enable formation of lipid bilayers through self-assembly.[8] A comparison of the conventional phospholipid structure to that of **1** reveals a number of differences. Firstly, in contrast to typical phospholipids, **1** is water soluble and highly polar.[1f,9] This is presumably a consequence of the pair of anionic *O*-sulfates. Secondly, the charged domains of phospholipids are localized at one extremity of the lipid chain, which facilitates interaction between the extra- and/or intracellular space of the cell with the interior of the membrane.[8] Chlorosulfolipid **1** has two charged *O*-sulfates separated by an expanse of fourteen methylenes. It has been proposed that the *O*-sulfate positioned towards the center of the lipid chain, would find itself inside the hydrophobic region of a biomembrane, and presumably lead to its destabilization.[1f] Lastly, the fatty acid components of phospholipids incorporate long carbon chains, with palmitic (C_{16}) and oleic (C_{18}) acids being most abundant in mammalian cells.[10] By contrast, the hydrophobic domains in **1** are much shorter. This presents a problem in understanding the structural integrity of membranes largely comprised of **1**.[11] Collectively, these structural features of this chlorosulfolipid are at odds with the generally accepted characteristics and associated functions of lipids in biomembranes.

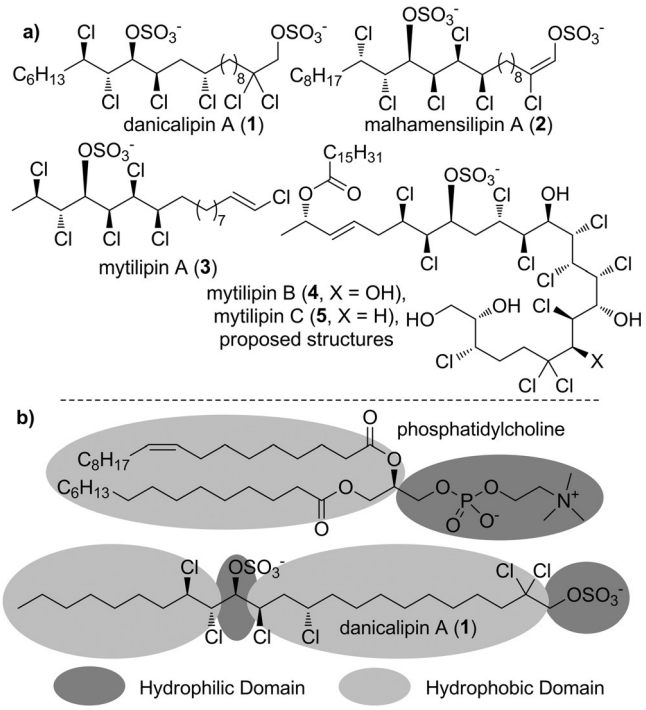

Figure 1. a) The structures of the most complex chlorosulfolipids, and b) the polar and apolar regions of **1** and phosphatidylcholine.

[*] A. M. Bailey, Dr. S. Wolfrum, Prof. Dr. E. M. Carreira
Laboratorium für Organische Chemie, Eidgenössische Technische Hochschule Zürich, HCI H335
Vladimir-Prelog-Weg 3, 8093 Zürich (Switzerland)
E-mail: carreira@org.chem.ethz.ch

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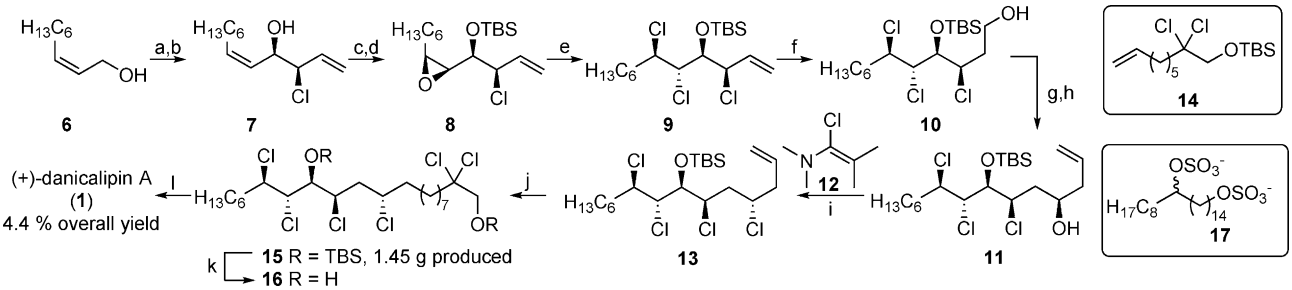
The biological data for **1** is limited. It is associated with other chlorosulfolipids (**3**–**5**) for which there are anecdotal reports of their causing seafood poisoning,[5e,12] its toxicity towards an unspecified genus of brine shrimp,[5b,13] and that it is one of the dominant polar components of the membrane of *O. danica*.[7b,14] The lack of any kind of understanding of their role and function stimulated us to investigate these rare lipids. Specifically for **1**, we sought to gain insight into its effect on cell membranes, to generate an array of biological assays, and to compare biological data with related non-chlorinated lipids. To achieve these goals, we required the development of a route towards appreciable quantities of this synthetically challenging, intriguing target.

To date, **1** has been synthesized four times[5a–c,h] with the most elegant approach in 14 total steps and 4.6% overall yield.[5h] Despite its conciseness, midway through the route is a step that requires the use of a catalyst that is not commercially available and must be prepared in 3 steps to effect a kinetic resolution reaction. This is followed by a low yielding cross metathesis reaction (29% yield) with 30 mol% of a catalyst (single turnover) that has only recently become commercially available.[15] Consequently, we looked to formulate a synthesis utilizing robust, scalable chemistry to facilitate the production of large quantities of material (>1 g).

Synthesis of **1** commenced with MnO_2-mediated oxidation of commercially available (*Z*)-non-2-en-1-ol (**6**) to the corresponding (*Z*)-enal (*Z*/*E* = 24:1) isolated without purification in 97% yield (Scheme 1). Oehlschlager–Brown haloallylation[16] selectively yielded *syn*-halohydrin **7** ($\alpha/\gamma = 5{:}1$; *syn*/*anti* > 20:1) utilizing (−)-Ipc$_2$BOMe, which was prepared following the procedure of Lautens.[17] Difficulties were encountered in attempts to effect separation of the desired product from the co-product, isopinocampheol. However, subjecting the unpurified mixture to directed epoxidation (*m*CPBA, d.r. = 11:1 *syn*/*anti* as determined by NMR)[18] followed by TBS protection afforded the all *syn* protected halohydrin **8** in 30% yield over 3 steps. Subsequent application of Yoshimitsu's conditions for dichlorination of epoxides[19] to **8** provided trichloride **9** as a single diastereomer. Hydroboration/oxidation of this electron deficient olefin proved problematic and intriguing. Surprisingly, the use of BH_3·THF furnished dehalogenated material in addition to small quantities (15–30%) of desired product.[20] A screening of various hydroboration reagents and conditions led to the use of dicyclohexylborane followed by work up with sodium perborate[21] to provide primary alcohol **10** in 75% yield. After benzoylation, the enantiomeric excess of **10** was determined to be 91% by chiral HPLC analysis. Despite the undesired propensity for elimination of β-chloroaldehydes to α,β-enals, oxidation with Dess–Martin periodinane[22] to the unpurified β-chloroaldehyde proceeded smoothly with no elimination evident as determined by ^{1}H NMR analysis. Subsequent Brown allylation[23] of the crude β-chloroaldehyde yielded homoallylic alcohol **11** in 8.3:1 d.r. and 71% yield over two steps. Conversion of **11** to the desired secondary chloride with complete inversion was achieved in 93% yield, utilizing Ghosez's reagent (**12**).[24] One-pot cross-metathesis of **13** with **14** and subsequent hydrogenation[25] afforded **15** in 77% yield. To complete the synthesis, **15** was deprotected with hot methanolic HCl, and the free diol was sulfated with $ClSO_3H$ in CH_2Cl_2 yielding **1** in 82% yield over 2 steps. For the longest linear sequence, the route required 12 steps and afforded 1.45 g of **15**, with an overall yield of 4.4%.

With sufficient quantities of **1** in hand, it was then examined in the sole biological assay reported in which its toxicity towards brine shrimp (unspecified genera) was investigated.[5b,13] *Artemia salina* brine shrimp were hatched and allowed to grow for 24–96 h. Following distribution into tissue culture plates (well volume 2 mL), yeast extract (aq.) along with **1**, **16**, or **17** were added. After 24 h, live-dead shrimps were counted, and the results were plotted to provide LC_{50} data (see Supporting Information, SI). The value measured for the toxicity of **1** ($LC_{50} = 5.3\ \mu M = 3.8\ \mu g\,mL^{-1}$; Table 1) was in agreement to that previously reported ($LC_{50} = 3.3\ \mu g\,mL^{-1}$).[5b,13] Unfortunately, in the previous studies, non-chlorinated sulfolipids were not examined. Interestingly, docosane disulfate (**17**)[26,27] has been proposed to be a biosynthetic precursor to **1**; consequently, we decided to investigate it and assess the effect of chloride substitution. For



Scheme 1. Reagents and conditions: a) MnO_2 (25 equiv), CH_2Cl_2, RT, *Z*:*E* = 24:1; b) (−)-Ipc$_2$BOMe (1.0 equiv), allyl chloride (1.3 equiv), $(C_6H_{11})_2NLi$ (1.6 equiv), BF_3·OEt_2 (2.6 equiv), Et_2O:THF (1.8:1), −78 °C to RT, then ethanolamine (1.0 equiv), Et_2O; c) *m*CPBA (1.1 equiv), CH_2Cl_2, 0 °C to RT, d.r. = 11:1; d) TBSCl (1.3 equiv), imidazole (2.0 equiv), DMAP (0.1 equiv), CH_2Cl_2, 0 °C to 40 °C, 30% (3 steps); e) NCS (3.6 equiv), $ClPPh_2$ (3.0 equiv), CH_2Cl_2, 0 °C to RT, 44%; f) $(C_6H_{11})_2BH$ (2.4 equiv), THF, 0 °C, then $NaBO_3$ (30 equiv), THF:H_2O (1.4:1), RT, 75%; g) DMP (1.2 equiv), CH_2Cl_2, 0 °C to RT; h) (+)-Ipc$_2$BCl (2.0 equiv), allylMgBr (1.0 M in Et_2O, 1.5 equiv), THF, −78 °C to −100 °C to RT, d.r. = 8.3:1, 71% (2 steps); i) **12** (3.0 equiv), $CHCl_3$, 0 °C to RT, 93%; j) **14** (3.0 equiv), Grubbs II (10 mol%), PtO_2 (10 mol%), H_2, 40 °C to RT, 77%; k) AcCl (50 equiv), MeOH, 0 °C to 80 °C, 89%; l) $ClSO_3H$ (30 equiv), CH_2Cl_2, 0 °C to RT, 92%. Ipc = isopinocampheyl, *m*CPBA = *m*-$ClC_6H_4CO_3H$, TBS = *t*$BuMe_2Si$, DMAP = *p*-$(Me_2N)C_5H_5N$, NCS = *N*-chlorosuccinimide, DMP = Dess–Martin periodinane, Ac = acyl.

Table 1: Toxicity of **1**, **16**, and **17** towards brine shrimp and various cell lines.

Target[a]	Compound toxicity		
	1	**16**	**17**
Brine shrimp LC_{50}	5.3	>141[b]	63.8
A549 cells EC_{50}	26.5 ± 0.9	41.4 ± 0.9	69.3 ± 1.2
HT-29 cells EC_{50}	15.5 ± 1.2	>166	84.4 ± 0.7
Hepa 1-6 cells EC_{50}	14.3 ± 0.7	17.3 ± 0.1	39.1 ± 0.2

[a] LC_{50} and EC_{50} data are reported in units of μM. [b] The exact LC_{50} of this compound could not be determined due to its limited water solubility above 100 $\mu g\,mL^{-1}$.

comparison purposes, we included the non-sulfated chlorolipid **16**,[13] which was the penultimate intermediate in our synthesis. In the assay, danicalipin A (**1**) is an order of magnitude more toxic than **17** and at least 2 orders of magnitude more toxic than **16**.

We next decided to expand the panoply of assays to include exploration of cytotoxicity towards various cell lines: A549 and HT-29 human adenocarcinomic cell lines, as well as Hepa 1–6 murine liver cell line (Table 1). Our aim was to establish robust cell culture conditions for more than one cell line that could ultimately be used to study the effect of **1**, **16**, **17**, and any future derivatives on membrane permeability. The cell lines were chosen based on high and consistent growth capacity as well as batch to batch homogeneity to assure high reproducibility vis-à-vis cell numbers and minimal phenotypic variation. Similar criteria were applied in the selection of Gram-negative bacteria (see below). Before beginning experiments on cell cultures, toxicity thresholds were determined towards the three cell lines (Table 1; see SI for details). The EC_{50} was determined for each compound, wherein **1** was shown to be most toxic. With this information, we began to investigate the effect of these lipids on cells at or below this threshold value.

A staining method was developed to distinguish cells with compromised membranes from healthy cells. Two DNA staining dyes were selected: 1) Hoechst 33342 stain, capable of crossing healthy membranes and subsequently staining the DNA of the cell,[28] and 2) Sytox Green, known to only penetrate cells with compromised plasma membranes.[29] Upon exposure of HT-29 cells to **1** (Figure 2, entries 1–3) a vast majority of the cells stained positive for both dyes, a result that is particularly evident at 10 μM (Figure 2, entry 2). A qualitative positive correlation between the concentration of **1** and the number of cells stained with Sytox Green was observed. In contrast, exposure of HT-29 cells, under equivalent conditions, to **16** or **17** resulted in minimal DNA staining by Sytox Green at concentrations below their EC_{50} values (Figure 2, entries 4–9), consistent with results from negative control experiments (1% DMSO). Alternatively, positive control experiments (20% EtOH) furnished results in alignment with those produced by **1** (see SI). The data strongly implicate, for the first time, that the natural product danicalipin A affects the integrity of the cellular membrane. Similar observations were made with Hepa 1–6 cells (see SI), demonstrating that the effect of **1** is not cell line specific.

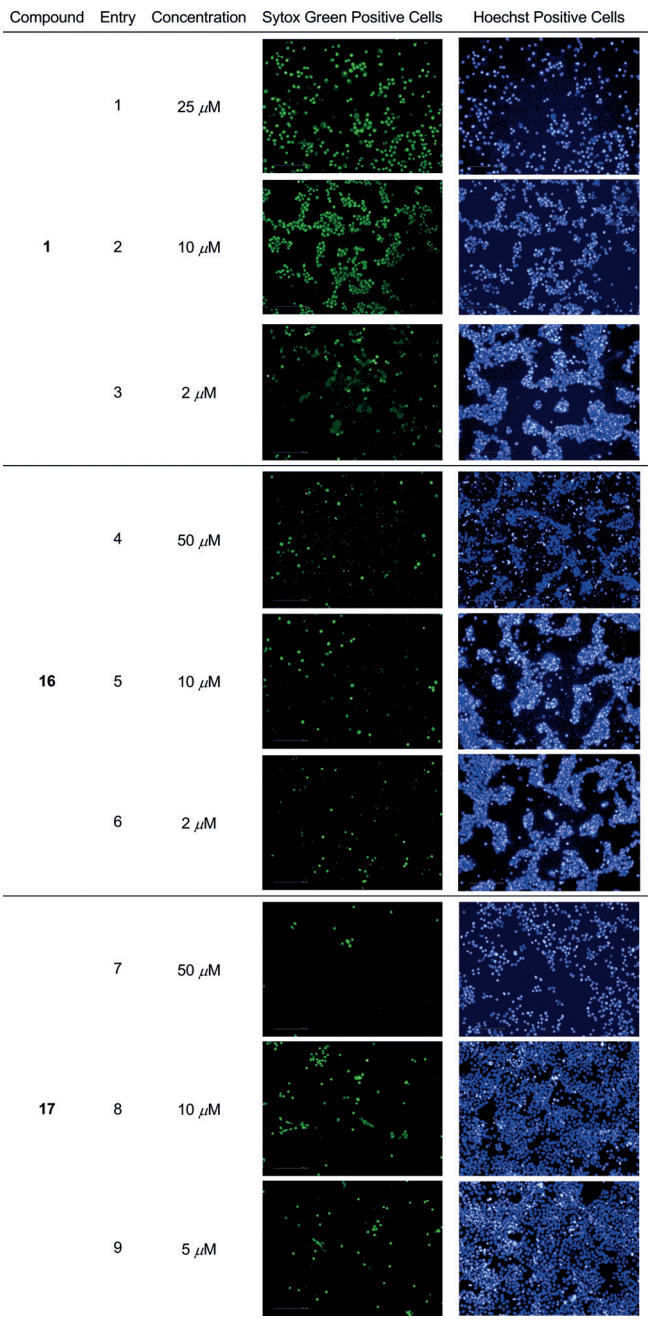


Figure 2. Fluorescent images of HT-29 cells following exposures to **1**, **16**, or **17** at various concentrations.

To further probe the role of these lipids, we targeted a structurally distinct organism, namely, *E. coli* DH5α, a strain of Gram-negative bacteria. These bacteria were chosen as they have two protective layers, namely an outer membrane comprised largely of lipopolysaccharides and proteins and a peptidoglycan cell wall.[30] Utilization of an MTT assay[31] demonstrated that viability of bacteria was reduced (70–90%) compared to untreated cells only upon exposure to **1** at ≥250 μM. Non-chlorinated lipid **17** was shown to be slightly antibacterial in a dose independent manner at all concentrations ≤250 μM. Bacterial viability was not affected upon treatment with **16**, even at 250 μM (see SI). Thus, the presence

of chlorines in danicalipin A renders it somewhat less toxic towards bacteria (>90% viability) when compared to the dechlorinated alkyl disulfate **17** (70–90% viability).

We then moved on to examine whether **1** would have similar effects on membrane integrity, in the more challenging bacterial setting, as was observed with mammalian cell lines. Hoechst 33342 staining of *E. coli* DH5α bacterial cultures in the presence of 1% DMSO was taken as baseline, while dye uptake following heat inactivation of the bacteria served as a positive control.[32]

Incubation of bacteria in the presence of Hoechst 33342 and **1** elicited a dramatic dose-dependent positive correlation between the fluorescence measured and the concentration of **1** (Figure 3). Compounds **16** and **17** had minimal effect on the

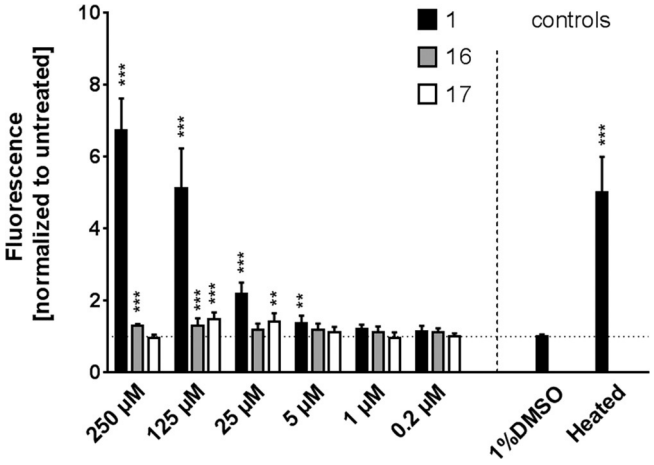


Figure 3. The fluorescence response due to nuclear staining of bacteria *E. coli* DH5α by Hoechst 33342 as a function of the concentration of **1**, **16**, or **17** as well as positive and negative control experiments. The data is normalized to the untreated results. The significance of each result vs. DMSO is shown above the bar: $p<0.01$ = **; $p<0.001$ = ***. For a color image, see SI.

amount of dye entering the cell, even at 250 μM, and produced results similar to the negative control. Notably, at the 125 μM concentration of **1**, a 5-times greater fluorescence response was observed and bacterial viability remained >90%. In analogy to the mammalian cell experiments, the bacterial analysis shows that the chlorides and sulfates of danicalipin A are necessary for effective uptake of Hoechst 33342 into *E. coli* DH5α cells.

It is generally accepted that sterols, particularly cholesterol and ergosterol, govern the fluidity and mechanical strength of membranes.[33,34] The effect of sterols on the structure of biomembranes is conserved among a large variety of living organisms including vertebrates, fungi, protozoa, higher plants, and algae. The optimal membrane composition defined at one end by high permeability and at the other by intransigency, has come about through evolutionary pressures and is unique to each organism's particular set of membrane sterols. The remarkable ability of **1** to increase the amount of dye to pass through biological membranes without leading to cell death is rare for a lipid outside of sterols.[35] At present there is much uncertainty about the mechanism of action of **1** but this study has shown that this unique property is dependent on the chlorines and the sulfates, indicating that both are essential. These structural features in particular, given the effect on membrane fluidity, may be pertinent to the presence of danicalipin A in the flagella of *O. danica*.[7]

In conclusion, we have accomplished a concise and scalable synthesis of (+)-danicalipin A (**1**), utilizing haloallylation, chlorination, and one-pot metathesis/hydrogenation reactions providing significant quantities of material. Its purported casual association with seafood poisoning renders its study essential. We have also documented a comparative study of **1** with its non-chlorinated biosynthetic precursor **17**. Through the application of DNA staining experiments, we have shown that both the chlorines and the sulfates are necessary to compromise the cellular membranes of mammalian (e.g. human colorectal) cells as well as Gram-negative bacteria. In this respect, the study described with danicalipin A provides a crucial benchmark for any future investigations. Further efforts toward establishing the biophysical role of **1** in the membrane of *O. danica* and a more detailed analysis of the biological properties of **1**, its stereoisomers, and its congeners are currently ongoing and will be reported in due course.

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