Chemical Science

EDGE ARTICLE

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Cite this: *Chem. Sci.*, 2017, **8**, 6904

Stereochemistry and biological activity of chlorinated lipids: a study of danicalipin A and selected diastereomers†

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Received 17th July 2017
Accepted 7th August 2017

DOI: 10.1039/c7sc03124f

rsc.li/chemical-science

The syntheses of (+)-16-*epi*- and (+)-11,15-di-*epi*-danicalipin A (**2** and **3**) are reported. The conformations of the parent diols **5** and **6** as well as the corresponding disulfates **2** and **3** were determined on the basis of *J*-based configuration analysis and supported by calculations. The impact of configuration on membrane permeability in Gram-negative bacteria and mammalian cell lines was assessed as well as cytotoxicity. Although diastereomer **2** showed similar behavior to natural (+)-danicalipin A (**1**), strikingly, the more flexible C11,C15-epimer **3** had no effect on permeability and proved equally or more toxic towards multiple cell lines.

Introduction

(+)-Danicalipin A (**1**) is a prominent member of the chlorosulfolipid family of natural products isolated in the 1960's from microalgae, which were later anecdotally associated with seafood poisoning (Fig. 1A). More recently, it has captured the interest of research groups globally.[1,2] Hexachlorosulfolipid **1** is the main polar component in the flagellar membrane of the golden-brown alga *Ochromonas danica*. The presence of other unusual, rare lipids renders its membrane a fascinating system for study.[3] Coinciding with our interest in halogenated compounds of relevance to drug discovery and halosulfolipids, we set out to identify ways of investigating the chemical properties and biological activities of (+)-danicalipin A (**1**).[4,5] Halogenation is known to influence electronic properties, lipophilicity, and metabolic stability of bioactive molecules.[6,7] Additional subtle effects may be manifest in conformational preferences[8] especially in aliphatic systems as notably highlighted by Hoffmann,[8b] O'Hagan,[8c] and more recently by Gademann.[8d] We were interested in the question: Do configurational isomers **2** and **3** (Fig. 2B and C) exhibit differences, chemically or biologically, from the natural product? Herein we report the syntheses of diastereomers **2** and **3** and accompanying structure–activity studies. Strikingly, a significant deviation of toxicity and membrane permeability was revealed and correlated to flexibility.

Many biologically active natural products feature characteristic configurational patterns that enable seemingly flexible molecules to adopt defined shapes.[9] For example, the configuration of polyketides has been linked to their biological mode of action. Vicinal dichlorides and chlorohydrins are known to be conformationally biasing in simple systems when compared to the parent hydrocarbons.[10,11] In contrast to polyketides, complex polychlorinated natural products have not been the focus of stereochemical investigations to date. The enigmatic biological role of (+)-danicalipin A (**1**) and its structure, in particular its chlorination pattern, render it an ideal target to probe a link, if any, between configuration and bioactivity. Therefore, we set out to identify diastereomers whose conformations would differ from **1**.

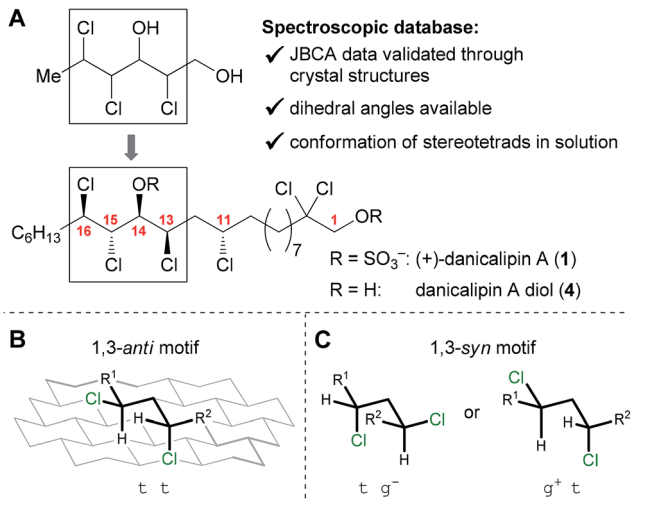

Fig. 1 Identification of interesting danicalipin A isomers. (A) Information of the stereotetrad set by a spectroscopic database. (B) Conformation of the 1,3-*anti* motif ($R^1 \neq R^2$). (C) Possible conformations of the 1,3-*syn* motif ($R^1 \neq R^2$).

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c7sc03124f

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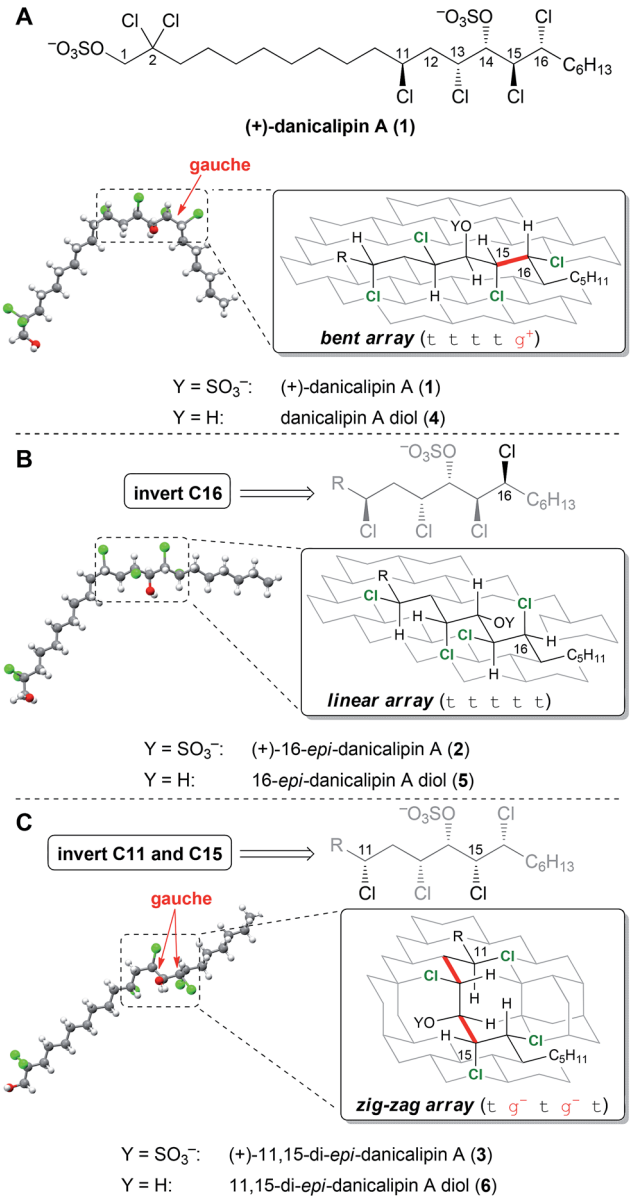


Fig. 2 (A) (+)-Danicalipin A (**1**) and danicalipin A diol (**4**), (B) (+)-16-*epi*-danicalipin A (**2**) and 16-*epi*-danicalipin A diol (**5**), (C) (+)-11,15-di-*epi*-danicalipin A (**3**) and 11,15-di-*epi*-danicalipin A diol (**6**); R = $(CH_2)_8(CCl_2)CH_2OY$. Lowest-energy structures (DFT) of diols **4**, **5**, and **6** shown in ball-and-stick models; 3D representation of the C11 to C16 array superimposed on a diamond lattice using JBCA and DFT (along the principle chain: $g^+ = +60°$, $g^- = -60°$, $t = 180°$).

Results and discussion

Target selection

Collectively, there are 34 992 possible staggered conformations (C10 to C17) for the 16 diastereomers of (+)-danicalipin A (**1**, 2187 conformers per diastereomer). Consideration of previous work suggests that various derivatives of **1** (Y = H, SO_3^-, Ac, TBS) have similar conformations.[12] Consequently, the parent diols were chosen as the focus of our efforts in this study. Utilization of a conformational database of stereodefined trichlorinated hexane-1,3-diols[10,13] (Fig. 1) narrowed the selection of low-energy conformers centered around the C13 to C16 stereotetrad to 18 structures (see ESI†).

According to models generated from the database, **5** and **6** were suggested to display defined structures different from danicalipin A diol (**4**, Fig. 2). Examination of models for **4**, **5**, and **6** revealed one, zero, and two *gauche* interactions in the C11 to C16 region, respectively. Conformational DFT analysis of **4** led to a structure that was in agreement with one produced from a solution-state NMR study (*ttttg*$^+$).[14–16] Calculations also supported the predicted all-*trans* arrangement in the chlorinated segment of 16-*epi*-danicalipin A diol (**5**, *ttttt*). *A priori*, substituents in a 1,3-*syn* relationship (*cf.* C11 to C13 in **6**) ought to give rise to two energetically similar conformers (tg^- or g^+t, Fig. 1).[9c,d,17] Yet, in 11,15-di-*epi*-danicalipin A diol (**6**), computations showed a preference for one (tg^-tg^-t). In order to gain insight into their solution-state conformations, diols **5** and **6** were prepared through *de novo* synthesis.

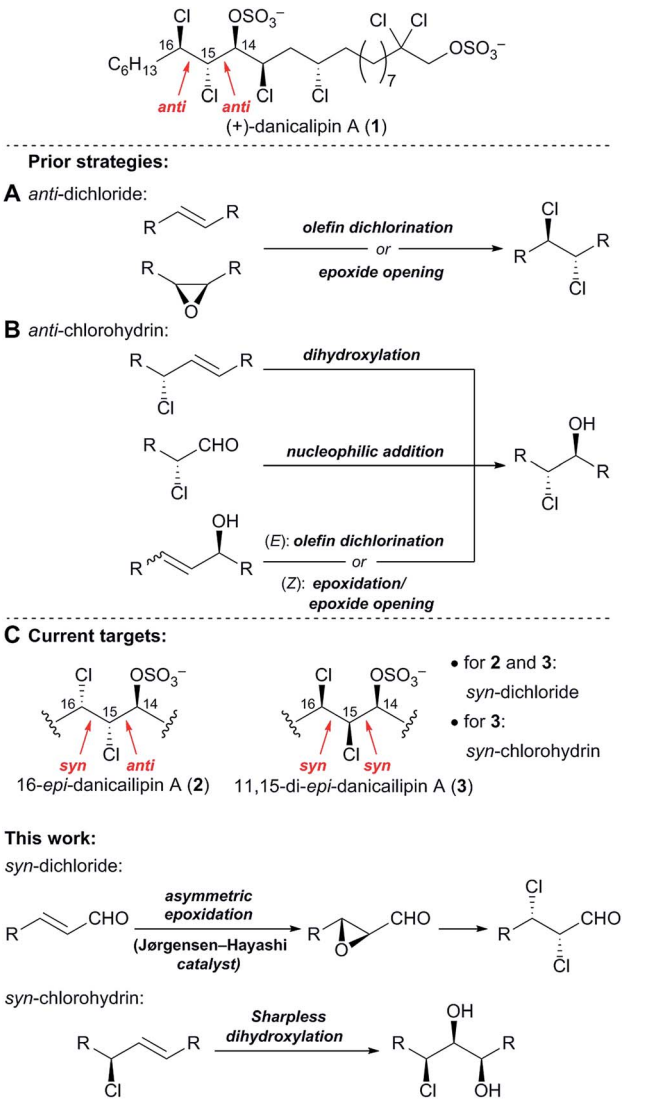


Fig. 3 (A) Prior strategies to access the *anti*-dichloride and (B) the *anti*-chlorohydrin in (+)-danicalipin A (**1**), (C) different configurations in diastereomers **2** and **3**.

Syntheses of diastereomers

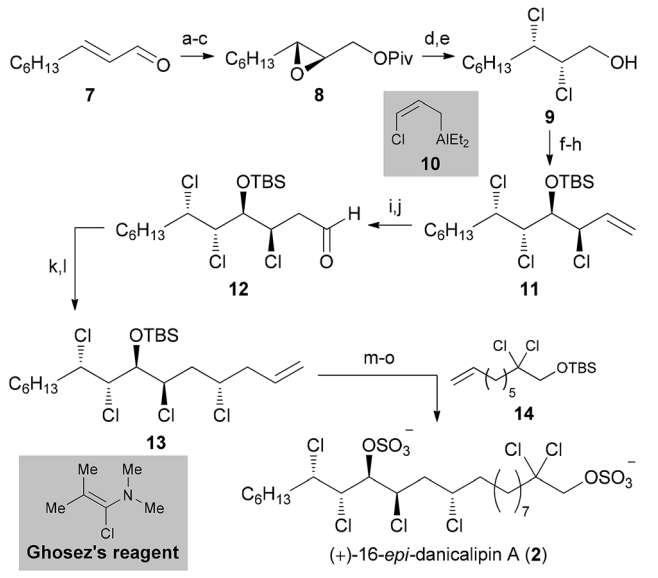
In prior syntheses of (+)-danicalipin A (**1**), the *anti*-relative configuration of the C15,C16-*vic*-dichloride employed *trans*-olefin dichlorination[18a,b,d,e] or *cis*-epoxide[2a,18c] opening (Fig. 3A). The *anti*-configuration of the C14,C15-chlorohydrin was set by substrate-controlled diastereoselective transformations (Fig. 3B).[2a,18] The relative configuration present in **2**/**5** and **3**/**6** precluded the implementation of these earlier strategies (Fig. 3C).

Synthesis of 16-*epi*-danicalipin A. The synthesis of (+)-16-*epi*-danicalipin A (**2**) (Scheme 1) commenced with the asymmetric organocatalytic epoxidation of (*E*)-non-2-enal (**7**) using the (*S*)-Jørgensen–Hayashi catalyst,[19] followed by reduction and protection of the hydroxy group as its pivaloate ester. The enantiomeric ratio was determined to be >20 : 1 by Mosher ester analysis of the free alcohol (not shown).

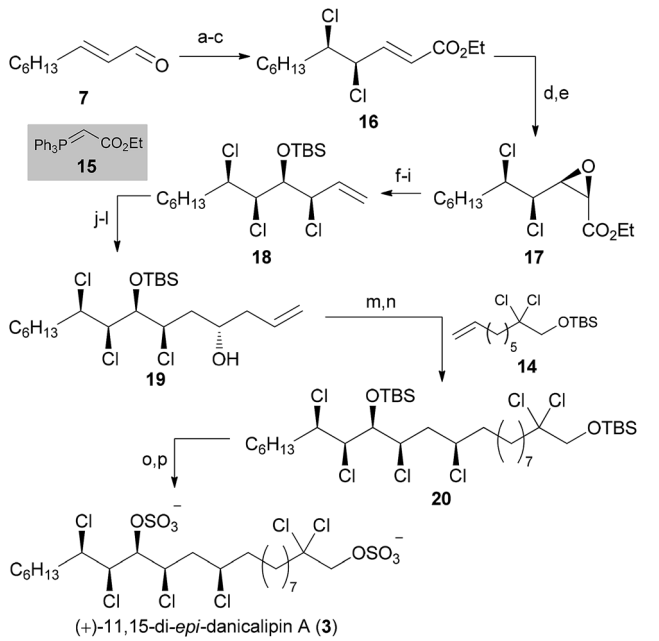
Opening of epoxide **8** with NCS and PPh_3 in toluene at 90 °C afforded a vicinal dichloride,[20a] which was deprotected using $(iBu)_2AlH$ to give **9**. Dess–Martin oxidation of alcohol **9** afforded an unstable and highly volatile α,β-dichloroaldehyde, which was directly subjected to chloroallylation conditions. For this purpose, γ-chloroallylaluminum reagent **10** was generated *in situ* from allyl chloride, LiTMP and Et_2AlCl.[21] As expected, the stereochemical outcome followed both the Felkin–Anh and Cornforth models with d.r. > 6 : 1. Protection of the secondary hydroxy group provided trichloride **11**. Hydroboration and subsequent oxidation furnished unstable aldehyde **12**. Brown allylation was performed using (+)-Ipc_2BCl and allylmagnesium bromide in THF at −100 °C.[22] The homoallylic alcohol was obtained in high yield (83%) and d.r. (>5 : 1).

Based on the knowledge gained in our research group,[2a] this allylic hydroxy group was converted to the corresponding chloride in high yield and with complete inversion utilizing Ghosez's reagent.[23] This highly reactive α-chloroenamine is proposed to be ionized *in situ* to a keteneiminium ion, which is readily attacked by nucleophiles (*e.g.* the free secondary hydroxy group at C11). Hence, fragment **13** was synthesized in 12 steps from commercially available material. One-pot cross metathesis/hydrogenation with known olefin **14** (ref. 2a) established the complete C_{22} chain.[24] The two TBS groups were removed with an excess of acetyl chloride in MeOH at 80 °C. Final sulfation with SO_3·pyridine gave (+)-16-*epi*-danicalipin A (**2**) in 87% yield.

Synthesis of 11,15-di-*epi*-danicalipin A. In a similar manner, the synthesis of (+)-11,15-di-*epi*-danicalipin A (**3**) (Scheme 2) commenced by epoxidizing (*E*)-non-2-enal (**7**) enantioselectively[19] and subjecting the formed epoxyaldehyde to stabilized Wittig reagent **15**. Subsequent epoxide opening under modified Yoshimitsu's conditions[20b] (PPh_2Cl/NCS in CH_2Cl_2)



Scheme 1 Reagents and conditions: (a) (*S*)-Jørgensen–Hayashi catalyst (10 mol%), H_2O_2, CH_2Cl_2, r.t., e.r. > 20 : 1; (b) $NaBH_4$, MeOH, 0 °C, 62% over 2 steps; (c) PivCl, pyridine, CH_2Cl_2, r.t., 82%; (d) PPh_3, NCS, toluene, 90 °C; (e) $(iBu)_2AlH$, CH_2Cl_2, −78 °C, 78% over 2 steps; (f) DMP, $NaHCO_3$, CH_2Cl_2, r.t.; (g) **10**, THF, −78 °C, d.r. > 6 : 1; (h) TBSOTf, Et_3N, CH_2Cl_2, r.t., 47% over 3 steps; (i) Cy_2BH, THF, 0 °C, then $NaBO_3$·$4H_2O$, r.t., 69%; (j) DMP, CH_2Cl_2, r.t.; (k) (+)-Ipc_2B(allyl), THF, −100 °C to r.t., 83% over 2 steps, d.r. > 5 : 1; (l) Ghosez's reagent, $CHCl_3$, then Et_3N, r.t., 86%; (m) **14** (3 equiv.), Grubbs II (10 mol%), CH_2Cl_2, 45 °C, then PtO_2 (10 mol%), H_2 (1 atm), r.t., 90%; (n) AcCl, MeOH, 80 °C, 84%; (o) SO_3·pyridine, THF, r.t., 87%.



Scheme 2 Reagents and conditions: (a) (*R*)-Jørgensen–Hayashi catalyst (10 mol%), H_2O_2, CH_2Cl_2, r.t., e.r. > 20 : 1; (b) **15**, CH_2Cl_2, 0 °C, 61% over 2 steps; (c) NCS, Ph_2PCl, CH_2Cl_2, r.t., 45%; (d) AD-mix β, $NaHCO_3$, $MeSO_2NH_2$, *t*BuOH/H_2O (1 : 1), 0 °C, 70%, d.r. = 6 : 1; (e) *p*-nitrobenzenesulfonyl chloride, Et_3N, CH_2Cl_2, 0 °C, 74%; (f) $(iBu)_2AlH$, toluene, −78 °C; (g) $MePPh_3Br$, KN(TMS)$_2$, THF −78 to 0 °C, 91% over 2 steps; (h) TMSCl, EtOAc; r.t.; (i) TBSOTf, Et_3N, CH_2Cl_2, r.t., 76%, d.r. = 8 : 1; (j) Cy_2BH, THF, 0 °C to r.t., then $NaBO_3$·$4H_2O$, 0 °C to r.t., 85%; (k) DMP, CH_2Cl_2, r.t.; (l) (−)-Ipc_2B(allyl), THF, −100 °C to r.t., then $NaBO_3$·$4H_2O$, 0 °C to r.t., 73% over 2 steps, d.r. > 10 : 1; (m) **14** (3 equiv.), Grubbs II (10 mol%), CH_2Cl_2, 45 °C, then PtO_2 (10 mol%), H_2 (1 atm), 66%; (n) Ghosez's reagent, $CHCl_3$, 0 °C to r.t., then Et_3N, 0 °C to r.t., 78%; (o) AcCl, MeOH, 80 °C, 93%; (p) SO_3·pyridine, THF, r.t., quant.

gave vicinal dichloride **16** in 45% yield, along with 28% of an elimination product (see ESI†). To overcome the inherent preference of the molecule for *anti*-functionalization,[18] α,β-unsaturated ester **16** was dihydroxylated under catalyst-controlled Sharpless' conditions[25] in high yield (70%) and d.r. (8 : 1) favoring the all-*syn* product. Prolonged reaction times furnished substantial amounts of elimination products, further demonstrating the instability of the all-*syn* motif. Surprisingly, after selective nosylation of the α-hydroxy group[26] *cis*-epoxide **17** directly formed *in situ*. Reduction of the ester with $(iBu)_2AlH$ was followed by Wittig reaction to give a terminal olefin. According to previous results by our group,[4a,27] epoxide opening with TMSCl in EtOAc occurred with inversion of configuration (d.r. = 8 : 1).

With a route to all-*syn* trichloride **18**, we pursued the same approach as for (+)-16-*epi*-danicalipin A (**2**). Thus, hydroboration/oxidation preceded Dess–Martin oxidation and Brown allylation.[22] When homoallylic alcohol **19** was subjected to Ghosez's reagent,[23] an inseparable 1 : 1 mixture of tetrachloride and conjugated diene was isolated (not shown).[28] Although this mixture could then be taken forward to **20**, a higher yielding procedure resulted from cross metathesis of homoallylic alcohol **19** with **14** using Grubbs 2nd generation catalyst, and direct reduction of the olefin.[24] The secondary alcohol could then be substituted using Ghosez's reagent, without concomitant elimination. Deprotection and sulfation of **20** was then achieved *via* the same procedure as for (+)-16-*epi*-danicalipin A (**2**).[29]

Biological investigations

The consequence of the configurational differences in disulfates **1**, **2**, and **3** on biological activity was then examined. We have previously reported that (+)-danicalipin A (**1**) affects the integrity of cell membranes in Gram-negative bacteria (*E. coli* DH5α) and mammalian cell lines (Hepa 1–6, HT-29).[2a]

In the assay, incubation of *E. coli* with **1** compromised bacterial membranes, an effect quantified by measuring an increase in fluorescence of a DNA stain.[30] At 125 μM concentrations of (+)-danicalipin A (**1**), incorporation of Hoechst 33342 into *E. coli* was increased 5-fold over the negative control (Fig. 4),[31] while incubation with (+)-16-*epi*- danicalipin A (**2**) showed a 3-fold increase. Remarkably, no permeability enhancement was observed with (+)-11,15-di-*epi*-danicalipin A (**3**) even at toxic concentrations ≥ 250 μM (see ESI†).

Additional experiments were conducted in Hepa 1–6 murine liver cells to assess permeability enhancement (Table 1). At 25 μM concentrations, both (+)-danicalipin A (**1**) and (+)-16-*epi*-danicalipin A (**2**) compromised cell membranes as shown with a Hoechst 33342/Sytox Green assay (*cf.* A/B with C/D),[2a,32] consistent with positive control (20% EtOH, see ESI†). By contrast, using the same assay, we observed that (+)-11,15-di-*epi*-danicalipin A (**3**) resulted in minimal Sytox Green staining (E/F, Table 1) similar to negative control (1% DMSO, see ESI†).

To enable direct comparison with other known halosulfolipids and analogs toxicity towards brine shrimp was examined.[2,15,18b,c] The observed LC_{50} values, 1 : 2.5 μM; 2 : 5.7 μM; 3 : 4.5 μM, indicate that configuration has no influence on brine shrimp toxicity. Interestingly, the fact that all diastereomers (**1–3**) are significantly more toxic than the parent non-chlorinated lipid (1,14-docosane disulfate)[2a] emphasizes the importance of chlorination on activity. In mammalian-cell toxicity assays **1–3** showed similar EC_{50} values against Hepa 1–6 and A549 cell lines (see ESI†). However, C11,C15-epimer **3** was noticeably more toxic towards HT-29 cells (3.7 ± 0.6 μM) than both C16-epimer **2** (10.9 ± 0.1 μM) and (+)-danicalipin A (**1**, 14.7 ± 0.4 μM).

Given the biological data, the answer to the question serving as the focus of this study is yes: configurational isomers **1–3** display differential biological profiles. We were then interested in understanding the nature of the relationship between observed bioactivity and configuration. The data resulting from investigation of disulfates **1–3** by NMR spectroscopy was insufficient to confidently determine their solution-state conformations. In order to elucidate the limited spectroscopic data, additional DFT analyses were undertaken.[34] The calculated conformations of the chlorinated segments in **1** and **2** were identical to those found in diols **4** and **5** (Fig. 2), respectively. These were also consistent with data obtained from NMR spectra. Interestingly, computational analysis of **3** revealed a small energy difference ($\Delta\Delta G$ = 0.1 kcal mol^{-1}) between two conformations along C11–C13.[35] The inconclusive spectroscopic data in conjunction with DFT analysis suggests **3** exists as a mixture of rapidly interconverting conformers (see ESI†). Thus, while the chlorinated segments of **1** and **2** have defined minima in solution, the *syn*-C11,C13-configuration promotes flexibility for **3**.

The biological observations indicate that the configuration of naturally-occurring (+)-danicalipin A (**1**) plays a pronounced role in structurally compromising phospholipid-based membranes, such as those in *E. coli*, murine, and human

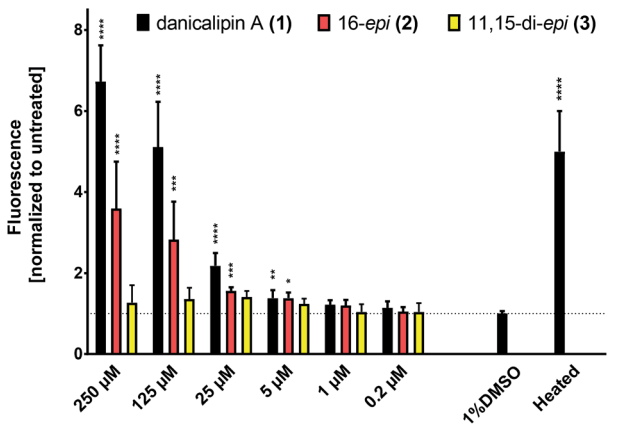
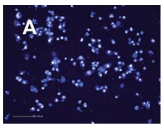
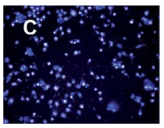
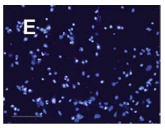
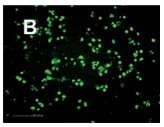
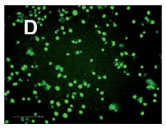
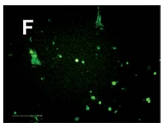


Fig. 4 Membrane permeability enhancement in bacteria: fluorescence response due to nuclear staining of *E. coli* DH5α by Hoechst 33342 as a function of the concentration of **1**, **2**, or **3** as well as positive and negative control experiments. Data are normalized to the untreated results. The significance of each result *vs.* DMSO is shown above the bar: $p < 0.033$ = *; $p < 0.01$ = **; $p < 0.001$ = ***; $p < 0.0002$ = ****.

Table 1 Membrane permeability enhancement in mammalian cells[a]

	(+)-Danicalipin A (**1**)	(+)-16-*epi*-Danicalipin A (**2**)	(+)-11,15-Di-*epi*-danicalipin A (**3**)
Hoechst 33342 (sampled cells)			
Sytox Green (cells with compromised membranes)			

[a] Fluorescent images of Hepa 1–6 cells after incubation with compounds **1**, **2**, and **3** at 25 μM concentration. Blue: sampled cells, visualized with Hoechst 33342. Green: cells with compromised cell membranes, visualized with Sytox Green.[33]

cell lines. This feature may be relevant to its unspecified role in the membrane of *O. danica*. In this respect, the membrane domains in spermatozoa from the fish *Sparus aurata* have been reported to consist of varying proportions of (poly) unsaturated fatty acids in accordance with their function. Specifically, a high content of such lipids in the flagellar membrane is correlated with improved sperm viability and motility, as well as increased membrane fluidity.[36] We speculate that the chlorinated array in **1** acts in analogy to *cis*-unsaturation in fatty acids by introducing a kink in the lipid chain.[37] Consequently, we hypothesize that **1** confers similar effects on motility in the flagellum of *O. danica*.[38] This suggests new directions to advance understanding of this unicellular organism.

Conclusions

In conclusion, we have synthesized two unnatural diastereomers of (+)-danicalipin A (**1**), namely, 16-*epi*- and 11,15-di-*epi*-danicalipin A (**2** and **3**). These were selected by a combined database/computational approach to examine, for the first time, the effect of complex chlorination patterns on chemical and biological properties. We observed a striking result in which one of the diastereomers displays a significantly different biological profile when compared to the natural product, which can be correlated to solution-state flexibility. Thus, **1** and **2** had an effect on permeability, while **3** displayed no such activity. Yet, **3** was more toxic against HT-29 cell lines. Noteworthy, comparison of the biological data for **1** and **3** further reveals that there is no obvious link between toxicity and permeability enhancement. Our work underscores that the configurational pattern of chlorinated lipids influences the conformational landscape and also impacts biological profiles. More broadly, stereodefined chlorinated arrays may find use as conformationally biasing elements with applications in materials science and medicinal chemistry.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We are grateful to Dr Stefan Ruider for assistance with DFT calculations, Dr M.-O. Ebert, R. Arnold, R. Frankenstein, P. Zumbrunnen and S. Burkhardt for NMR measurements, and to the European Research Council (320666-CHLIP) for financial support.

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3 The novelty of the membrane composition of *O. danica* has raised significant questions and stimulated debate regarding its ability to self-assemble.[37a] In addition to chlorosulfolipids, other major components include rare betaine lipids, such as 1(**3**),2-diacylglyceryl-(**3**)-*O*-4′-(*N*,*N*,*N*-trimethyl)homoserine, incorporating saturated as well as unsaturated C14–C20 fatty acids, and sterols, with poriferasterol as the most abundant. None are commercially available or are easily accessible. For the isolation of these membrane components, see: (*a*) A. E. Brown and J. Elovson, *Biochemistry*, 1974, **13**, 3476; (*b*) M. G. Gershengorn, A. R. H. Smith, G. Goulston, L. J. Goad, T. W. Goodwin and T. H. Haines, *Biochemistry*, 1968, **7**, 1698.

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