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ARTICLE TYPE

Biologically active binaphthol-scaffolded imidazolium salts

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This work describes the antimicrobial activity and selectivity for gram-positive bacteria of imidazolium-functionnalized binols, as a result of their insertion into the lipid membrane and alteration of its permeability. The most potent compound displayed micromolar minimal inhibitory concentrations against Gram-positive *Bacillus thuringiensis* and *Listeria seeligeri*. At these concentrations, the rate of survival after 24 hours of mammalian cells were superior to 90% of that measured in the absence of these compounds and its hemolysis percentage was only about 10%.

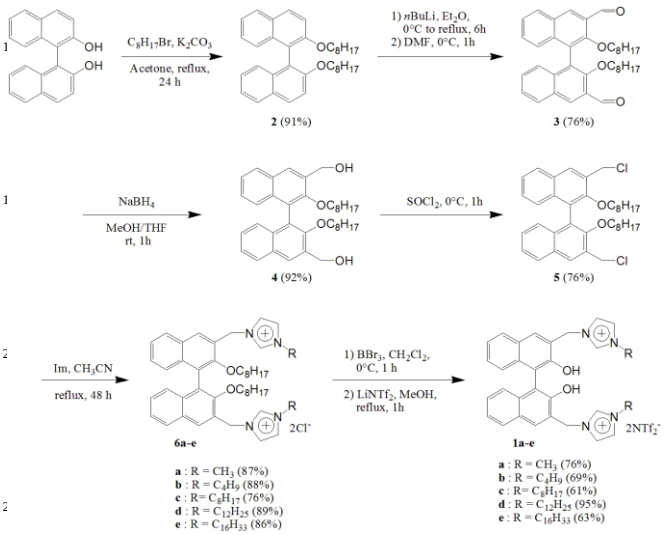
The rise in antibiotic resistance[1] among pathogenic bacteria and the declining rate of novel drug discovery are common concerns in medicine,[2] driving research into new antibacterial classes and novel drugs in order to maintain the existing ability to treat infectious diseases, especially those caused by multidrug-resistant organisms.[3] Membrane targeting offers advantages over standard methods of drug design and antibiotic activity due to the wide variety of active structures and a reduced development of resistance mechanisms.[4] The development of antimicrobial peptides (AMPs) as pharmaceutical agents showed great promise this last decade, with a variety of natural and synthetic compounds currently in development.[1] Most AMPs are cationic (polar) molecules with spatially separated hydrophobic and charged regions. The shortest cationic lipopeptides resemble antimicrobial surfactants[5] and are effective against fungi and Gram-positive and Gram-negative bacteria, with moderate hemolytic activity in the more active variants.[6] AMPs generally adopt highly amphiphilic conformations that appeared to be important for insertion into cytoplasmic membrane[6] and it was further demonstrated that an appropriate hydrophilic/hydrophobic balance were generally important for obtaining active compounds.[7] In efforts to minimize the size of cyclic synthetic AMPs, a binaphthyl scaffold was recently introduced in dicationic peptoids in order to enable hydrophobic interactions with the cell membrane. Dicationic peptoids resulted from these studies as promising new antibacterial subclass of cationic peptides with antibacterial potency against a range of Gram-positive pathogens including strains of *S. aureus* resistant to vancomycin, methicillin and linezolid.[8] On the other hand, great effort has been made toward the development of imidazolium salts as antimicrobial compounds and different series of imidazolium salts have been reported having significant antibacterial and antifungal activities.[9] The general trend retained from all the previously published studies is that short-chain 1-alkyl-3-methyl imidazolium salts with chloride anions demonstrated the weakest antimicrobial activity. Exchange of the halide by other anions generlly results in an increase of antibacterial and antifungal activities, those with the bis(trifluoromethylsulfonyl)imide (NTf_2) anion being the most active, but their minimal inhibitory concentrations (MIC) are generally in the mM range, their antimicrobial action being associated to their interaction with cytoplasmic membrane of bacteria and subsequent change of permeability properties on the membrane.[10]

Herein we report the synthesis and antibacterial activities for derivatives that possess a 2,2'-binaphthol (binol) hydrophobic scaffold and have incorporated charged alkyl-imidazolium moieties attached in positions 3 and 3'. We demonstrate that the antimicrobial activity against Gram-positive of the most potent octyl-derivative is a combination of interaction and insertion into the cell membrane of bacteria, without being significantly toxic to mammalian cells.

Synthesis. The synthesis of the binol-functionalized imidazolium salts started with the protection of the alcohol groups of racemic 2,2'-binaphtol (binol). The protection of the alcohols with an *n*-octyl chain instead of commonly used methyl or ethyl groups afforded an increase in the solubility of the compounds during imidazolium functionalization and increased the yield of the diimidazolium salt formation. The protected binol was formylated in positions 3 and 3' and followed by common reduction and chlorination steps. Diimidazolium salts were obtained by the nucleophilic substitution of chlorides with different substituted imidazoles in acetonitrile. Compounds **1a-e** were obtained after the deprotection of the alcohol groups and an anion exchange step with NTf_2 anion (Scheme 1).

Transmembrane ion transport in liposomes. The ion balance is crucial for cell survival. External modification of the ion permeability of membranes, upsetting the normal ion balance can be the mechanism for cell death (either apoptosis or cell lysis). We previously demonstrated that transport properties of benzimidazolium salts in liposomes were a direct evidence of their activity in living bacteria.[11] In order to assess the capacity of compounds **1a-e** to interact with phospholipid membranes, we investigated their ability to transport of anions across liposomes bilayers.[11] The chloride efflux in the presence of compounds **1a-e** was studied using egg yolk phosphatidylcholine large unilamelar vesicles (EYPC LUVs) loaded with the fluorescent dye lucigenin.

Using this method, the translocation of chloride anions outside the vesicles can be monitored as an increase of lucigenin's fluorescence. In general, compounds able to alter the membrane's integrity show high lucigenin fluorescence plateaus, achieved at faster rates.[11] When compounds **1a-e** were studied at 6.25 mol% (relative to EYPC concentration), **1c** was able to efficiently transport chloride anions outside the EYPC LUVs, reaching a 68% chloride efflux in only a few seconds (Figure 1 and Table 1).



Scheme 1. Synthesis of imidazolium-functionalized binols **1a-e**.

Compounds **1b** and **1d** were both able to transport chloride, but were less efficient than **1c**. Compound **1c** may possess the appropriate hydrophilic/hydrophobic balance in order to be able to insert into the phospholipid bilayer and transport chloride anions across membranes. In order to corroborate the results obtained in the Cl^-/NO_3^- antiport with those for biological relevant anions, the most active compound **1c** was studied in three series of measurements performed using anions with different permeabilities in the extravesicular medium. The nitrate (ΔG_{hyd} = -300 kJ/mol) is a more hydrophobic ion than carbonate (ΔG_{hyd} = -335 kJ/mol) and sulfate (ΔG_{hyd} = -1080 kJ/mol).[12] The results presented in Figure 2 show a higher selectivity for the HCO_3^- anion and support a Cl^-/HCO_3^- antiport mechanism. The effectiveness (*i.e.* their EC_{50} values, Table 1) of compounds **1a-e** for chloride transport across the EYPC bilayer were also determined from these studies. The EC_{50} value of compound **1c** in a Cl^-/NO_3^- system was 4.62% (relative to the EYPC concentration). This result is comparable to the EC_{50} obtained for the other imidazolium or benzimidazolium anion transporters reported that previously showed biological activities.[11]

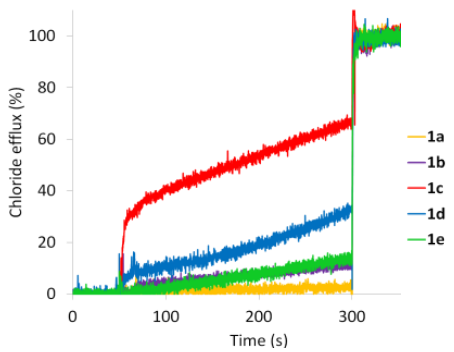


Figure 1. Relative chloride transport activity of **1a-e** at 6.25% mol relative to EYPC. Intravesicular: 100 mM NaCl, 10 mM phosphate buffer, lucigenin 1 mM. Extravesicular: 100 mM $NaNO_3$, 10 mM phosphate buffer (pH 6.4).

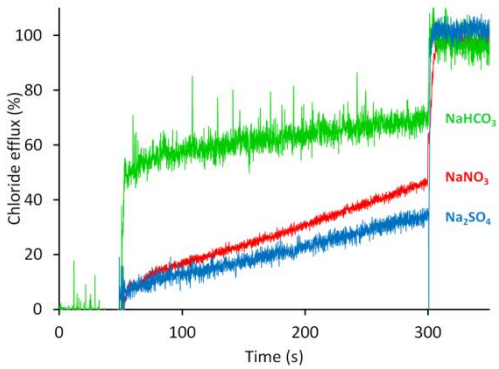


Figure 2. Lucigenin-based transport assay. Intravesicular: 100 mM NaCl, 10 mM phosphate buffer (pH = 6,4). Extravesicular: 100 mM Na^+ A^- (A^- = NO_3^-, HCO_3^-) or 100 mM Na_2SO_4, 10 mM phosphate buffer (pH = 6,4). Compound **1c** was injected at t = 50 sec, ratio **1c**/EYPC = 0.05. Aqueous 10% Triton-X was injected at t = 300 sec. Temperature was set to 37°C.

Antimicrobial Properties. The antimicrobial activities of compounds **1a-e** were tested against a range of pathogenic and non-pathogenic microorganisms, including Gram-negative bacteria (*Escherichia coli*, *Alcaligenes faecalis*) and Gram-positive bacteria (*Bacillus thuringiensis*, *Listeria seeligeri*). Broth dilutions were compared in determining the MICs of **1a-e**. The results (Table 1 and ESI) showed that most MIC values for a given organism for compound **1c** were significantly lower than the MICs for the other compounds. Compound **1c** had also a larger spectrum of activity against the Gram-positive bacteria. The MIC values for **1c** against the Gram-positive bacteria tested were 10^3 times lower that the corresponding 1-alkyl-1-methyl imidazolium salts and very close to those of biologically active synthetic hydraphiles or AMPs.[13] The antimicrobial activities of these salts are directly related to the structure and the length of the alkyl groups attached to the imidazolium moiety, playing an important role in the mechanism of action.

Mechanistically, there are two major steps that may contribute to the antimicrobial activity of these imidazolium salts. First the cationic head group may approach the negatively charged cell membrane of microbes because of the electrostatic interactions. These electrostatic interactions are also entropically favored due to the release of a large number of counterions. Next, the hydrophobic tail of the molecule could undergo self-promoted transport across the cell membrane resulting in disruption in the bilayer and the release of the intracellular constituents leading to cell death. In this step, an optimum hydrophobicity of the amphiphile possibly allows **1c** to penetrate more easily and diffuse in the non-polar environment created by the lipid bilayers of the cell membrane. The lipophilicity (expressed as logP in Table 1) of the compounds is crucial for the interaction and insertion into the bacterial membrane. Compound **1b** is probably too hydrophilic to penetrate in the hydrophobic part of the

membrane, while **1d** is probably too hydrophobic to cross the hydrophilic surface of the bacterial membrane.

The composition of the cell membrane of microbes plays a crucial role in determining the efficiency and specificity of these antimicrobial agents. The cell membrane of Gram- positive bacteria is composed of a peptidoglycan layer, a class of polysaccharides, whereas Gram-negative bacteria contain an outer layer consisting of lipopolysaccharide and phospholipids in addition to the peptidoglycan layer. This additional protection in Gram-negative bacteria was presumably the main reason behind the generally higher MIC values obtained (Table 1) for these organisms. The better antibacterial efficiency of compounds **1b, 1c** and **1d** against Gram-positive bacteria can be attributed to the presence of high amounts of negatively charged lipids in their membranes[14] resulting in stronger electrostatic interactions. However, these electrostatic interactions are not the only parameter responsible for their antibacterial activity as compounds **1 b** and **1d** showed MICs values 8-10 times higher than compound **1c**. Small-molecules antibiotics kill bacteria over the course of several hours, whereas active amphiphilic antimicrobials kill bacteria faster, in less than two hours.[15]

Table 1.

		MIC (μM) Gram-negative		MIC (μM) Gram-positive		EC_{50} (% mol) [b]	IC_{50} (μM) [c]
	logP[a]	E. *coli DH5α*	A. *faeca-lis*	B. *thurin-giensis*	L. *seeli-geri*		
1a	5.9	> 100	> 100	> 100	> 100	-	-
1b	8.4	> 100	> 100	> 100	30	-	23.8 ± 2
1c	11.6	> 100	> 100	4	3	4.62	11.7 ± 1.8
1d	14.7	> 100	> 100	100	25	9.3	9.2 ± 2.3
1e	17.8	> 100	> 100	> 100	> 100	18.1	-

[a] calculated using Hyperchem 8.0. [b] % mol relative to EYPC necessary to obtain 50% of chloride transport, calculated at 250 s from dose-response analysis. For compounds **1a** and **1b**, no plateau was reached even at 50% mol. [c] determined by the MTT assay.

The observed resistance of Gram-negative bacteria to compounds **1b-d** can occur by a variety of mechanisms, including failure of the compound to cross the cell membrane, modification or degradation of the drug, creation of permeability barriers, or active export of the drug. It is increasingly recognized that active efflux plays a major role in the resistance of many organisms to a plethora of agents.[16] A wide variety of antibiotics are exported from *E. coli* by one of several active efflux systems.[17] At least two of these systems, the AcrAB and EmrAB efflux pumps, have been shown to depend on the outer membrane protein TolC.[18] To determine whether the Gram-negative resistance was due to an efflux pump, we examined the antiobiotic activity of compounds **1b** and **1c** in mutant *E. coli* strain (SK037) in which *tolC* had been inactivated by the Tn*10* insertion. As shown in Figure 3, SK037 E. coli was sensitive to 50 and 10 μM **1c**, while the reference wild-type *E. coli* DH5α strain remained resistant to **1b** and **1c** at these concentrations. These results suggest that the observed resistance in Gram-negative bacteria is mediated by a TolC- containing multidrug resistance efflux pump. Compound **1c** showed fast growth inhibition of bacterial strain SK037 within the first 2 h, at concentrations 10 and 5 times lower than their MICs (Figure 2). The rate of bacterial growth inhibition in SK037 strain was dose-dependent, faster and greater in magnitude for **1c** than **1b**. These results suggested that the higher antibacterial activity of compound **1c** may be attributed to its greater capacity of insertion in the outer membrane of Gram-negative bacteria, and the resistance of wild-type *E.coli* to this compound is the result of their pump export.

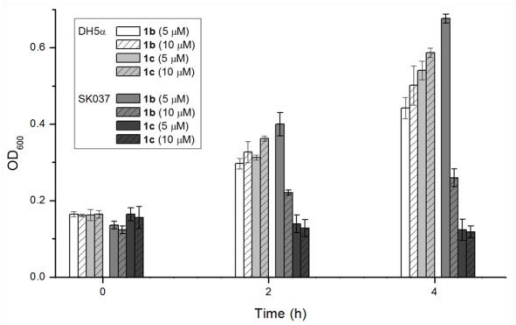


Figure 3. Relative antibacterial activities of compounds **1b** and **1c** in gram-negative SK037 and DH5α *E. coli*. *E. coli* were grown at 37°C over a 4h time period in presence of **1b** and **1c** at different concentrations, lower than their MICs.

Cytotoxicity. The cytotoxicity of compounds **1b-d** was determined by assessing viability of human embryonic kidney (HEK 293T) cells using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide] (MTT) assay (Table 1 and Figure 4).[19] From Figure 3, it can be observed that 60 % of the initial cells survived in the presence of compound **1b** even at 50 and 100 μM. For compounds **1c**, it can be observed that at 3.1 μM (which represent the MIC for Gram-positive bacteria) more than 90% of the cell survived after 24 hours. However, 25 μM of compounds **1b** and **1c** which corresponds to the MIC for the L. seeligeri, was enough to kill 100% HEK293T cells. The IC_{50} was not determined for compound **1e** as it did not show any selectivity.

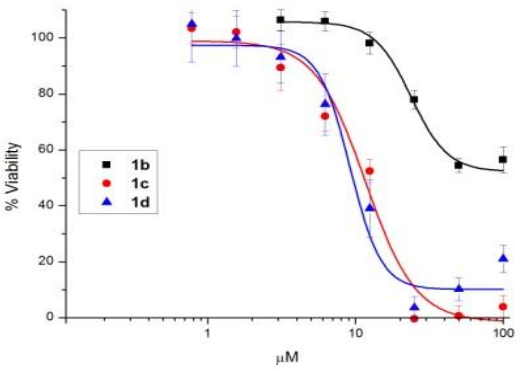


Figure 4. HEK 293 cells viability after 24 hours of incubation with compounds **1b-d**. The points represent the average of three independent measurements.

Haemolysis. To evaluate the potential use of compounds **1b-d** as therapeutic candidates, their hemolytic activities[20] were examined after 1 and 24 hours (Figure 5). It was notable that haemolytic activities against human red blood cells after 1 hour is the same for compounds **1b-d**, all of them being inferior to 10%. Based on the % haemolysis values after 24 hours, where compound **1c** displayed an haemolytic activity inferior to 15% in his MIC ranges (3-4 μM), this compound is a promising template for the development of effective therapeutics, through further

optimization of its structure. However, at higher concentrations compound **1c** showed important haemolytic activity.

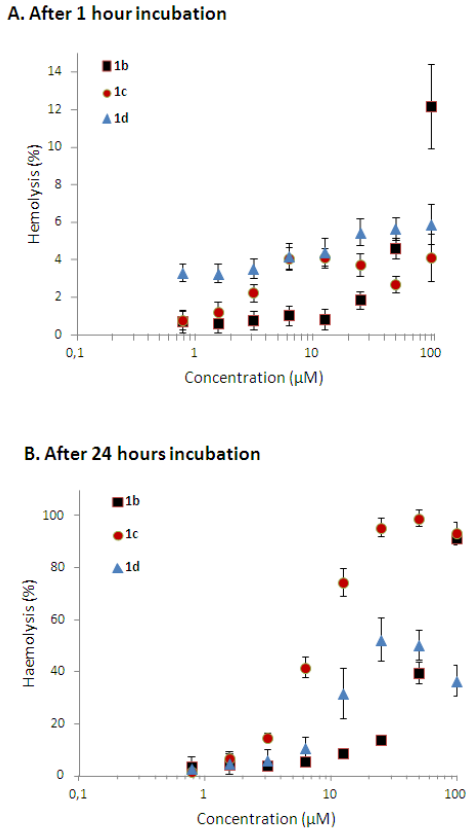


Figure 5. Haemolytic activities of **1b-d**. The points represent the average of three independent measurements.

Conclusions

Taken together, our findings lead us to propose that the antimicrobial effect of imidazolium-functionalized binaphthols may be the result, at least partially, from the insertion of the compounds into the lipid membrane, causing alterations of membrane permeability and allowing ions to cross the cell membrane. Besides being related to their physicochemical characteristics (such as lipophilicity and water solubility), this effect seems to be dependent on lipid composition and net surface charge of microbial membranes. The interaction with bacterial membranes appears to be responsible for the higher selectivity observed for **1c**. Compound **1c** exhibits a high activity against Gram-positive bacteria, with MIC of 3 and 4 µM for *L. seeligeri* and *B. thuringiensis*, concentrations allowing about 90% of mammalian cells survival compared to untreated cells. The appropriate hydrophobicity of compound **1c** can explain its ability to penetrate into the hydrophobic-water interface of the bacterial membrane. Current studies are underway to optimize the structure of these imidazolium salts and their potential use mainly as disinfectant, detergents, or topical antimicrobial agents.

Notes and references

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† Electronic Supplementary Information (ESI) available: Synthesis, chloride transport assays, dose-response curves, MICs curves and NMR spectra.

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1 B. Findlay, G. G. Zhanel, F. Schweizer, *Antimicrob. Agents Chemother.* 2010, **54**, 4049.
2 J. Song, *Int. J. Antimicrob. Agents* 2008, **32,** S207.
3 M. R. Mulvey, A. E. Simor, *CMAJ* 2009, **180,** 408; J. A. Otter, G. L. French, *Lancet Infect. Dis.* 2006, ***6***, 753.
4 M. Zasloff, *N. Engl. J. Med.* 2002, **347**, 1199.
5 R. N. Mitra, A. Shome, P. Paul, P. K. Das, *Org. Biomol. Chem.* 2009, **7**, 94.
6 M. Zasloff, *Nature* 2002, **415**, 389.
7 M. A. Schmitt, B. Weisblum, S. H. Gellman, *J. Am. Chem. Soc.* 2007, **129**, 417-428.
8 J. B. Bremner, P.A. Keller, S.G. Pyne, T.P. Boyle, Z. Brkic, D.M. David, A. Garas, J. Morgan, M. Robertson, K. Somphol, M.H. Miller, A.S. Howe, P. Ambrose, S. Bhavnani, T.R. Fritsche, D.J. Biedenbach, R.N. Jones, R.W. Buckheit Jr., K.M Watson, K. M. Baylis, J.A. Coates, J. Deadman, D. Jeevarajah, A. McCracken, D.I. Rhodes, *Angew. Chem. Int. Ed. Engl.* 2010, **49**, 537; M. Robertson, J. B. Bremner, J. Coates, J. Deadman, P. A. Keller, S. G. Pyne, K. Somphol, D. I. Rhodes, *Eur. J. Med. Chem.* 2011, **46**, 4201.
9 J. Ranke, A. Skrzypczak, G. Lota, E. Frackowiak, *Chem. Eur. J.* 2007, **13**, 3106; J. Cybulski, A. Wiśniewska, A. Kulig-Adamiak, L. lewicka, A. Cieniecka-Rostonkiewicz, K. Kita, A. Fojutowski, J. Nawrot, K. Materna, J. Pernak, *Chem. Eur. J.* 2008, **14**, 9305; A. Busetti, D. E. Crawford, M. J. Earle, M. A. Gilea, B. F. Gilmore, S. P. Gorman, G. Laverty, M. McLaughlin, K. R. Seddon, *Green Chem.* 2010, **12**, 420; L. Carson, P. K. W. Chau, M. J. Earle, M. A. Gilea, B. F. Gilmore, S. P. Gorman, M. T. McCann, K. R. Seddon, *Green Chem.* 2009, **11**, 492; J. Luczak, C. Jungnickel, S. Stolte, J. Hupka, *Green Chem.* 2010, **12**, 593; M. Bahnous, A. Bouraiou, M. Chelgroum, S. Bouacida, T. Roisnel, F. Smati, C. Bentchouala, P. C. Gros, A. Belfaitah, *Bioorg. Med. Chem. Lett.* 2013, **23**, 1274.
10 D. Demberelnyamba, K.-S. Kim, S. Choi, S.-Y. Park, H. Lee, C.-J. Kim, I.-D. Yoo, *Bioorg. Med. Chem.* 2004, **12**, 853.
11 C.-R. Elie, N. Noujeim, C. Pardin, A. R. Schmitzer, *Chem. Commun.* 2011, **47**, 1788; C.-R. Elie, A. Hébert, M. Charbonneau, A. Haiun, A. R. Schmitzer, *Org. Biomol. Chem.* 2013, **11**, 923; C.-R. Elie, M. Charbonneau, A. R. Schmitzer, *Med. Chem. Commun.* 2012, **3**, 123.
12 N. Busschaert, P.A. Gale, C.J. Haynes, M.E. Light, S.J. Moore, C.C. Tong, *Chem. Comm.* 2010, **46**, 6252.
13 a) J. Ranke, A. Skrzypczak, G. Lota, E. Frackowiak, *Chem. Eur. J.* 2007, **13**, 3106; b) J. Pernak, P. Chwala, *Eur. J. Med. Chem.* 2003, **38**, 1035.; c) J. Pernak, M. Smiglak, S. T. Griffin, W. L. Hough, T. B. Wilson, A. Pernak, J. Zabielska-Matejuk, A. Fojutowski, K. Kita, R. D. Rogers, *Green Chem.* 2006, **8**, 798; d) J. Cybulski, A. Wiśniewska, A. Kulig-Adamiak, L. lewicka, A. Cieniecka-Rostonkiewicz, K. Kita, A. Fojutowski, J. Nawrot, K. Materna, J. Pernka, *Chem. Eur. J.* 2008, **14**, 9305; e) A. Busetti, D. E. Crawford, M. J. Earle, M. A. Gilea, B. F. Gilmore, S. P. Gorman, G. Laverty, M. McLaughlin, K. R. Seddon, *Green Chem.* 2010, **12**, 420; f) J. Pernak, K. Sobaszkiewicz, J. Foksowicz-Flaczyk, *Chem. Eur. J.* 2004, **10**, 3479; g) J. Pernak, K. Sobaszkiewicz I. Mirska, *Green Chem.* 2003, **5**, 52; i) K. M. Docherty, C. F. Kulpa, *Green Chem.* 2005, **7**, 185; h) J. Pernak, I. Goc, I. Mirska, *Green Chem.* 2004, **6,** 323; i) L. Carson, P. K. W. Chau, M. J. Earle, M. A. Gilea, B. F. Gilmore, S. P. Gorman, M. T. McCann, K. R. Seddon, *Green Chem.* 2009, **11**, 492; j) S. Kanjilal, S. Sunitha, P. S. Reddy, K. P. Kumar, U. S. N. Murty, R. B. N. Prasad, *Eur. J. Lipid Sci. Technol.* 2009, **111**, 941; k) J. Luczak, C. Jungnickel, S. Stolte, J. Hupka, *Green Chem.* 2010, **12**, 593; n) M. Bahnous, A. Bouraiou, M. Chelgroum, S.

Bouacida, T. Roisnel, F. Smati, C. Bentchouala, P. C. Gros, A. Belfaitah, *Bioorg. Med. Chem. Lett.* 2013, **23**, 1274.
14 J.-F. Frantz, B. Desbat, E.J. Dufourc, *FASEB J.* 2009, **23**, 3692.
15 S.K.Vooturi, C.M. Cheung, M.J. Rybak, S.M. Firestine, *J. Med. Chem.* 2009, **52**, 5020.
16 S.B. Levy, *Antimicrob. Agents Chemother.* 1992, **36**, 695; H. Nikaido, *Science* 1994, **264,** 382.
17 H. Nikaido, *J Bacteriol* 1996, **178,** 5853.
18 R. Aono, N. Tsukagoshi, M. Yamamoto, *J Bacteriol. 1998,* **180,** 938.
19 T. Mosmann, *J. of Immun. Meth.* 1983, **65**, 55.
20 L. Liu, Y. Huang, S. N. Riduan, S. Gao, Y.Yang, W. Fan, Y. Zhang, *Biomaterials* 2012, **33**, 8625.