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Antibacterial activities of fluorescent nano assembled triphenylamine phosphonium ionic liquids

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ABSTRACT

Staphylococcus aureus, a Gram positive coccal bacterium is a major cause of nosocomial infection. We report the synthesis of new triphenylamine phosphonium ionic liquids which are able to self-assemble into multiwall nanoassemblies and to reveal a strong bactericidal activity (MIC = 0.5 mg/L) for Gram positive bacteria (including resistant strains) comparable to that of standard antibiotics. Time kill, metabolism and fluorescence confocal microscopy studies show a quasi-instantaneously penetration of the nanoassemblies inside the bacteria resulting of a rapid blocking (30 min) of their proliferation. As confirmed by rezasurin reduction monitoring, these compounds strongly affect the bacterial metabolism and a Gram positive versus Gram negative selectivity is clearly observed. These fluorescent phosphonium ionic liquid might constitute a useful tool for both translocation studies and to tackle infectious diseases related to the field of implantology.

Staphylococcus aureus is a threatening pathogen and is a major cause of human infections, while 30% of the human population is commensally colonized with the bacterium. Infections caused by this pathogen including bacteremia and infective endocarditis (IE) as well as osteoarticular, skin and soft tissue, pleuropulmonary, and device-related infections.[1] *S. aureus*, in addition to be responsible for many hospital's acquired infections,[2] belongs to the ESKAPE group that includes major human pathogens with multi-drug resistance.[3] Despite recent findings,[4] there is still an urgent need to discover new molecules to fight against these particularly adaptive pathogens which are continuously developing mechanisms of resistance.[5] In this context, the aim of our studies is the design of new ionic liquid and to evaluate the antibacterial potential of new phosphonium ionic liquids (PILs) derived from triphenylamine (TPA). Phosphonium ionic liquids are by far the less studied ILs in comparison to nitrogen ionic liquids such as alkylammonium or imidazolium salts. As a result, few examples of antibacterial activity have been reported in the literature.[6–8] Kanazawa et al.[8] have shown that phosphonium salts provide an advantage over the corresponding ammonium salts in bactericidal activity and killing rate particularly against *S. aureus*. However, the mechanism of action of phosphonium derivatives remained unclear and the conjugation of a fluorescent probe like TPA with PILs could provide a useful tool for bacteria localization of these new TPA phosphonium conjugated salts. Indeed, TPA group has been already used as fluorescent sensors or dyes[9,10] but to our knowledge only one example concerning a TPA coumarin derivative reports moderate activity against *S. aureus*.[11] TPA/PIL's derivatives described in this Letter are amphiphilic molecules susceptible to self-assembled into nanoassemblies and consequently they might represent a valuable advantage for the crossing of outer and/or inner bacterial membranes. In this Letter, we demonstrate the remarkable antibacterial activity of TPA/PIL's over a wide range of bacteria strains particularly against ESKAPE's Gram positive bacteria *S. aureus* and *Enterococcus faecium* as well as *S. aureus* resistant strains with a MIC comparable to that of the best known antibiotics.[12] Those TPA/PILs also display improved MIC compared to benzalkonium chlorides (BAC),[13] cationic surfactants which are the standard of comparison for antimicrobial ILs. Fluorescent microscopy of the nanoassemblies formed provides evidence of a

Abbreviations: ESKAPE, *Enterococcus faecium*, *Staphylococcus aureus*, Klebsiella species, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Enterobacter species; IL's, ionic liquids; MIC, minimal inhibitory concentration; TBAF, tetrabutylamonium fluoride; TEM, transmission electronic microscopy; TPA, triphenylamine; PIL's, phosphonium ionic liquids.

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quasi-instantaneously insertion onto the bacteria resulting of a time kill inferior to that of 1 h.

The target molecule **9**, **10** and **11** can be easily synthesized via a 5 to 6 steps sequence (Scheme 1). Firstly, a Vilsmeier–Hack formylation of commercial TPA affords the aldehyde **1** in good yield (77%, see SI).[14] Compound **1** was then successfully reduced with $NaBH_4$ in alcohol **2** in quantitative yield.[15] The conversion of **2** in corresponding sodium alkoxide using NaH allowed the Williamson etherification by reaction with previously prepared silylated bromohexanol **3** to give **4** (see SI). Protecting group was subsequently removed with TBAF and the corresponding alcohol **5** was obtained in quantitative yield. The following bromination reaction of **5** was the tricky step of the synthesis. Among the several attempts performed including Appel reactions, NBS/PPh_3 in DCM appeared to be the most reactive bromination agent affording **6** in 61% yield. Lastly, in order to optimize yield and purity of final ionic liquid **9**, we focused on a fast, clean and safe solvent free microwave activation (200 W). Under these conditions, final compound **9** was obtained in excellent yield (93%) within two hours. Compound **10** was obtained in 95% yield from **5** by treatment under Appel conditions to give the corresponding chlorinated compound **7** in 65% yield followed by a nucleophilic substitution under microwave**s**. The last iodide salt **11** was obtained in 95% yield by chloride-iodide exchange on **8** followed by substitution under microwaves.

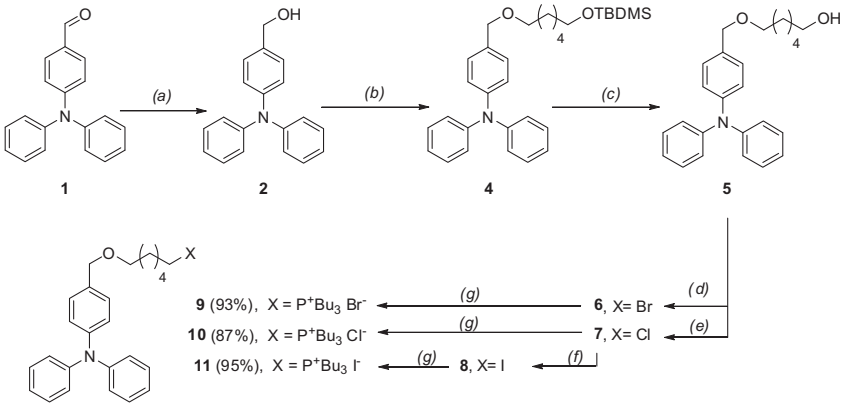
In order to complete our studies, the control's phosphonium salts described in Figure 1 were prepared through trivial syntheses detailed in the experimental part. Note that compounds **12** and **13** have been synthesized according to previous literature reported procedures.[16]

In order to prepare NAs from **9** a nano-precipitation method was used.[17] Compound **9** was solubilized into THF and the solution was added to pure water under stirring. The nano-structures were formed spontaneously. Following the removal of THF, a well-dispersed colloid of **9** NAs was thus obtained. The sample was characterized by TEM according to a known procedure[18] and nanoparticles inferior to that of 100 nm were observed (Fig. 1a), Figure 2b showing a single multiwall nanoassembly.

The Minimal Inhibitory Concentration (MIC) of each compound was determined against a series of strains representative of the ESKAPE pathogens[3] and are summarized in Table 1. While TPA and compounds **12**–**15** did not show antimicrobial activity in a range of concentration up to 64 mg/L, three compounds, **9**, **10** and **11** exhibited a significant activity against the Gram positive *Staphylococcus aureus* (MIC = 0.5–1 mg/L), and the difficult-to-treat Enterococci (MIC = 1–2 mg/L). It is noteworthy that the tetraalkyl-PIL's **12**–**14** as well as TPA alone didn't show any antimicrobial activity no matter the counterion nature. Moreover, compound **15** possessing an ether function similar to that of the active compounds **9**–**11** was found inactive.

This suggest that the unique combination of both TPA and tetraalkyl phosphonium part is determinant for the anti-bacterial activity. In addition, we also tested the activity of compounds on a strain of *S. aureus* overproducing the NorA efflux pump responsible for a multi-drug resistant phenotype.[19] The same efficacy was observed on the NorA overproducer SA1199B and its isogenic parent SA1199, suggesting that the compounds were not efflux-pump substrates. No activity however, was found on Gram negatives bacteria. The discrimination of activity between Gram positive and Gram negative bacteria strains could be explained by considering the structural diversity of the bacterial cell envelope. Whereas Gram positive bacteria have a cytoplasmic membrane recovered by a thick layer of peptidoglycan, Gram negative bacteria are surrounded by additional hydrophobic membrane that may be less susceptible to be crossed by the TPA/PIL's compounds. As described here, there is no significant difference in the activity examined for the three compounds **9**, **10** and **11** considered above. They share the same molecular scaffold and only differ by the presence of a different counterion (Br^-, Cl^-, I^- respectively). Thus, we selected, one of them, compound **9** bearing a bromide ion for further studies. Compound **9** showed a complete inhibition of growth of *S. aureus* (strain CIP 7625) as shown in Figure 3A. This inhibition corresponds to a strong bactericidal activity (Fig. 3B) with a >3$\log_{10}$ CFU/mL inoculum reduction during the first 30 min. A second period of inoculum decrease is observed with a smaller slope after a 30 min time lag. Together, these two periods correspond to a >4$\log_{10}$ CFU/mL inoculum reduction during the first 2 h of incubation with **9** at 2 mg/L (MIC × 4 for this strain). We then were wondering if the bactericidal activity observed resulted from a rapid inhibition of metabolic activity (Fig. 3C). The dashed line curve shows the reduction rate of resazurin in resorufin after treatment for 15 min of bacteria with **9** in the same amount as in A and B. Compared to the untreated control (solid line), only a weak reduction rate is observed in the treated cells, demonstrating a strong inhibition of the bacterial metabolism.

This remarkable results encouraged us to undertake further experiments taking advantage of the intrinsic fluorescence of the TPA moiety of **9**. Intra-cellular accumulation of a drug is a key step in biocides mode of action. This process can be impaired by envelope impermeability and/or efflux transport. Together these processes can drive resistance of the bacteria by strongly decreasing the intra-cellular concentration of the drug, and consequently



Scheme 1. Synthesis of the target compounds **9**, **10** and **11**. Reagents and conditions: (a) $NaBH_4$, ethanol/chloroform, RT (quant.); (b) NaH, 18-crown-6, 78 °C and Br $(CH_2)_6$OTBDMS **3** (86%); (c) TBAF, THF, RT (quant.); (d) NBS, PPh_3, DCM, 0 °C to RT (61%); (e) CCl_4, $P(Ph)_3$, 0 °C to reflux (65%); (f) NaI, acetone, reflux (95%) (g) PBu_3, microwaves (200 W), 140 °C.