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Robust antimicrobial compounds and polymers derived from natural resin acids†

Jifu Wang,[ab] Yung Pin Chen,[b] Kejian Yao,[b] Perry A. Wilbon,[b] Wujie Zhang,[c] Lixia Ren,[b] Juhua Zhou,[d] Mitzi Nagarkatti,[d] Chunpeng Wang,[a] Fuxiang Chu,[a] Xiaoming He,[c] Alan W. Decho*[b] and Chuanbing Tang*[b]

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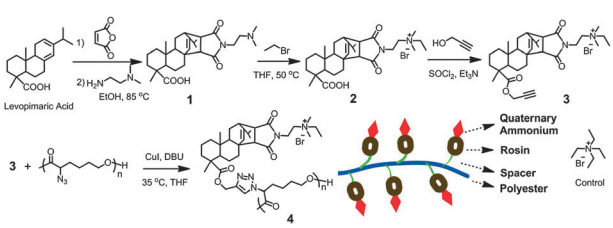
We report novel robust resin acid-derived antimicrobial agents that exhibit excellent antimicrobial activities against a broad spectrum of bacteria (6 Gram-positive and 7 Gram-negative) with selective lysis of microbial membranes over mammalian membranes. Our results indicate that hydrophobicity and unique structures of resin acids can be determining factors in dictating the antimicrobial activity.

Bacterial contamination of food, drinking water and medical implants and devices has posed serious threats to human health and, in some cases, has caused widespread outbreaks of infectious diseases.[1–3] Development of effective antibacterial agents has attracted much attention as they are capable of killing pathogenic microorganisms[4,5] or preventing biofouling of surfaces (*e.g.* coating to ship hulls).[6–8] Currently the majority of synthetic antimicrobial materials are compounds or polymers having cationic functional groups, which promote rapid sorption onto the negatively-charged cell surfaces of microorganisms.[9–13] Many synthetic and non-degradable polymers such as poly-norbornenes, polyacrylates, polyarylamides, polyesters, poly-(β-lactam) and pyridinium polymers have exhibited efficient antimicrobial activities.[12,14–20] Most recently, two groups reported biodegradable cationic quaternary ammonium-containing polymers including synthetic polycarbonates[21] and natural chitosan as effective antimicrobial materials.[22] However, many of these antimicrobial materials are either effective towards only certain types of bacterial strains or toxic towards mammalian cells, thus having a limited role in battling infections. In addition, most of the antimicrobial materials are very expensive, limiting their large-scale use. The development of robust, selective and efficient antimicrobial agents in large quantities and low cost is essential to prevent these infections.

Herein we report that natural resin acid-derived cationic compounds and polymers exhibit high antimicrobial activities against a broad spectrum of bacteria while maintaining selective lysis of bacterial cell membranes without inducing significant haemolysis of red blood cells over a wide range of concentrations. Our results suggest that the observed excellent antimicrobial activities are a combination of resin acids and their cationic charge.

Resin acids, abundant and low-cost natural resins (or rosin) from pine and conifer trees, consist of diterpene resin acids such as abietic, levopimaric, and pimaric acids that have a characteristic bulky hydrophenanthrene structure with the empirical formula $C_{20}H_{30}O_2$.[23–29] The hydrophenanthrene moiety, consisting of fused cycloaliphatic and aromatic structures, provides resin acids with substantial hydrophobicity, a property which has facilitated their use in marine antifouling coating materials for decades[30] and in biocides, though lacking rational design and investigation of selectivity and biocompatibility.[31] We have recently prepared various resin acid-derived monomers and polymers, which showed excellent hydrophobicity, biodegradability and biocompatibility.[24,26–29] We hypothesize that resin acids could be used as a hydrophobic component in antimicrobial agents, and be used to influence the relationship between antimicrobial activity and hydrophilic–hydrophobic balance.

As shown in Scheme 1, the synthesis started with a highly efficient Diels–Alder reaction between levopimaric acid and maleic anhydride to produce maleopimaric acid, followed by an amidation reaction with *N*,*N*-dimethylaminoethylamine to yield compound **1**. Quaternary ammonium-containing resin



Scheme 1 Synthesis of quaternary ammonium-containing resin acid-derived antimicrobial compounds and polymers.

[a] *Institute of Chemical Industry of Forestry Products, Chinese Academy of Forestry, Nanjing 210042, China*
[b] *Department of Chemistry and Biochemistry, Department of Environmental Health Sciences and USC Nanocenter, University of South Carolina, Columbia, South Carolina 29208, USA. E-mail: tang.c@chem.sc.edu, awdecho@mailbox.sc.edu; Fax: +1-803-777-8100; Tel: +1-803-777-3628*
[c] *Department of Biomedical Engineering, The Ohio State University, Columbus, Ohio 43210, USA*
[d] *Department of Pathology, Microbiology and Immunology, University of South Carolina School of Medicine, Columbia, South Carolina 29209, USA*
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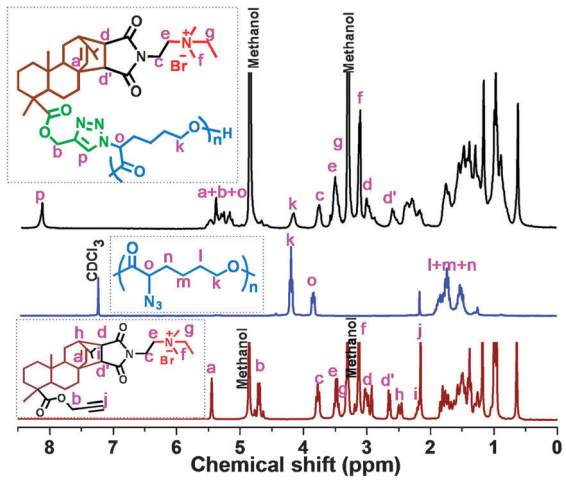

Fig. 1 ^{1}H NMR spectra of quaternary ammonium-containing resin propargyl ester **3** in methanol-d_4, azide-substituted PCL in $CDCl_3$, and quaternary ammonium-containing resin acid-substituted PCL **4** in methanol-d_4.

acid **2** was then prepared by a quaternization reaction between **1** and ethyl bromide. An esterification between compound **2** and propargyl alcohol led to the formation of quaternary ammonium-containing resin propargyl ester **3**. The structure and purity of **3** were characterized using ^{1}H NMR analyses (Fig. 1 and Fig. S9–S10, ESI†), and showed chemical shifts of vinyl protons at 5.4 ppm, while alkyne protons, methylene protons next to the alkyne group and protons next to the imide group were located at 2.1 ppm, 4.7 ppm and 3.7 ppm, respectively. In parallel, azide-substituted poly(ε-caprolactone) (PCL) (M_n (NMR) = 16 800 g mol^{-1}, M_w/M_n (GPC) = 1.15, Fig. S13, ESI†) was prepared in a multi-step route according to a previously-reported procedure.[20,32] According to our early report,[28] quaternary ammonium-containing resin acid-substituted PCL **4** was prepared *via* a click reaction between compound **3** and azide-substituted PCL in dimethylformamide with the use of CuI/DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) as catalysts. After the click reaction, the chemical shift at 8.1 ppm of polymer **4** was assigned to the characteristic proton from the triazole group. Vinyl protons and protons next to the triazole group were located at 5.1–5.7 ppm in NMR spectra, while protons next to the imide group and protons on the backbone –CH_2O– were also assigned. Integration of these characteristic protons demonstrated the quantitative reaction between alkyne-containing resin esters and azide-substituted PCL, indicating a high fidelity of the click reaction. FTIR spectra further confirmed the high efficiency of click reaction (Fig. S11, ESI†). The peak at ~2120 cm^{-1}, corresponding to the azide absorption, disappeared completely after the click reaction and a new absorption band at ~1650 cm^{-1} emerged, corresponding to absorption of the triazole group.

The antimicrobial activities of the resin acid-derived compounds (**2** and **3**) and polymer (**4**) were then tested against a range of pathogenic and non-pathogenic microorganisms, including Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Enterobacter agglomerans*, *Salmonella typhimurium*, *Alcaligenes faecalis*) and Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus cereus*, *Streptococcus pyogenes*, *Micrococcus luteus*, *Mycobacterium smegmatis*, *Corynebacterium xerosis*). Initially, broth dilution and disk-diffusion methods were compared in determining the minimal inhibitory concentrations (MICs) of **2**, **3** and **4** against *S. aureus*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa* as proxies for evaluating their antimicrobial activities. The results (Fig. S12, Tables S1 and S2, ESI†) showed that most MIC values for a given bacterium obtained by compounds **2** and **3** and polymer **4** showed significantly lower MIC obtained by the disk-diffusion method than the one by the broth dilution method.

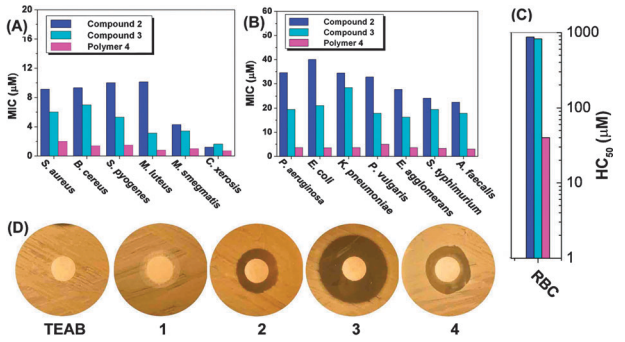

Fig. 2 (A) and (B) Resin acid-derived antimicrobial materials against various pathogens by a disk-diffusion method; (C) haemolysis of mouse red blood cells (RBCs) incubated with resin acid-derived antimicrobial materials; (D) images of disk diffusion assay results on agar plates showing inhibition zones (dark brown) of *S. aureus* bacterial growth (light brown) by various agents applied to a central disk (white).

Considering the potential applications of antimicrobial compounds and polymers as coatings for food packaging, medical implants and devices, and antifouling surfaces,[2,6,7,33] all subsequent MIC determinations were carried out using the disk-diffusion method. As shown in Fig. 2, results of the assays indicated that both resin acid-derived quaternary ammonium compounds (including acid-based **2** and ester-based **3**) and their polymers **4** exhibited strong antimicrobial activities against both Gram-positive bacteria with MICs ranging between 0.7–10.1 μM, and Gram-negative bacteria with MICs between 3–40 μM. These MIC values are comparable or better than many new systems developed recently.[21,22] Our results illustrated a highly efficient antimicrobial activity of these materials against a broad spectrum of bacteria (Table S2, ESI†).

The time-dependent efficiencies of antimicrobial activities of compound **3** and polymer **4** against *S. aureus* were then investigated (Fig. S13, ESI†). It was observed that antimicrobial effects of compound **3** were very rapid, with approximately 90% of *S. aureus* cells being killed within 1 h, while >75% strains were killed by polymer **4** in 6 h. We hypothesize that the excellent antimicrobial activities are derived from hydrophobicity of the hydrophenanthrene moiety, which likely enhanced the penetration of the compounds/polymers into cell membranes and subsequent killing of the bacteria. This was further confirmed with control experiments, in which a quaternary ammonium compound without the resin acid moiety, tetraethylammonium bromide (TEAB), showed no activities against both Gram-positive and Gram-negative bacteria (Table S2, ESI†). In addition, controls consisting of resin acid-derived compound **1** without quaternary ammonium also exhibited negligible activities against all bacterial strains, and highlighted the importance

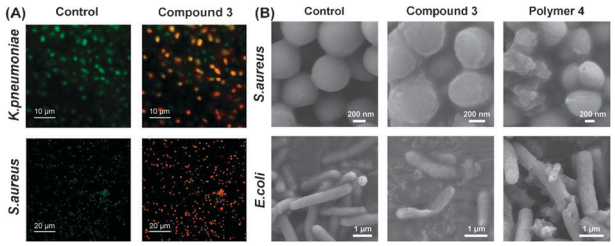

Fig. 3 Images of antimicrobial activities of **3** and **4**: (A) LIVE/DEAD viability assay of *K. pneumoniae* and *S. aureus* on **3**, imaged using CSLM; (B) morphology of *S. aureus* and *E. coli* cells exposed to **3** and **4**, and imaged using FE-SEM.

of the quaternary ammonium moiety in the observed antimicrobial activities. Fig. 2C clearly shows the visual effect of different materials against *S. aureus* using the Agar diffusion method.

To further confirm that the antimicrobial activities were not caused by residual copper catalysts and solvents (*i.e.* methanol), control experiments were carried out using these reagents against different bacterial strains. However, both copper catalysts and methanol did not exhibit significant toxicity, again indicating that the antimicrobial activities originated from resin acid-derived compounds and polymers.

BacLight LIVE/DEAD® bacterial viability assays were conducted with bacteria *K. pneumoniae* (Gram-negative) and *S. aureus* (Gram-positive) exposed to compound **3**. In this assay, bacterial cells were stained using Syto-16 and propidium iodide, to distinguish live (green fluorescence) from dead (red fluorescence) cells using confocal scanning laser microscopy (CSLM). Fig. 3A shows that the majority of *S. aureus* and *K. pneumoniae* cells were alive (*i.e.* green fluorescence) in controls, but dead (*i.e.* red fluorescence) when exposed to compound **3**. The morphology of *S. aureus* and *E. coli* cells exposed to **3** and **4** was also observed by field emission scanning electron microscopy (FE-SEM) (Fig. 3B), which indicated the disruption of cell membrane and subsequent killing of bacterial cells by absorption of **3** and **4**.

Haemolysis of mouse red blood cells (RBCs) was evaluated after incubation with resin acid-derived compounds **2**, **3** and polymer **4** (Fig. 2C). The HC_{50} (haemolytic concentration that resulted in 50% haemolysis of RBCs) of compound **2** was higher than 860 μM. Interestingly, compound **3** displayed a HC_{50} of 162 μM, however, a further increase in concentration to 810 μM did not induce the increase of haemolysis level. Polymer **4** showed that the HC_{50} was much higher than 30 μM. The high selectivity was manifested by the high ratios of HC_{50} to MIC, which were at least 21–500, 6–100, and 6–44 for compound **2**, compound **3** and polymer **4** respectively. This indicated that our antimicrobial materials are capable of selectively lysing microbial membranes, rather than mammalian cells.

In conclusion, we have developed quaternary ammonium-containing antimicrobial compounds and polymers, which utilized natural resin acids as an active hydrophobic component. These antimicrobial materials possessed excellent antimicrobial activity and high selectivity against bacteria over mammalian cells. It was suggested that the high antimicrobial activity was due to the hydrophobicity and unique structure of resin acids.

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