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Research Article

Synthesis, Characterization and Antibacterial Properties of Dihydroxy Quaternary Ammonium Salts with Long Chain Alkyl Bromides

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Five *N*-methyl-*N*-R-*N*,*N*-bis(2-hydroxyethyl) ammonium bromides (*R* = -benzyl (chloride, BNQAS), -dodecyl (C12QAS), -tetradecyl (C14QAS), -hexadecyl (C16QAS), -octadecyl (C18QAS)) were prepared based on *N*-methyldiethanolamine (MDEA) and halohydrocarbon. Five QAS were characterized by FTIR, NMR, and MS. BNQAS, C12QAS, C14QAS, and C16QAS were confirmed by *X*-ray single-crystal diffraction. Their antibacterial properties indicated good antibacterial abilities against *E. coli*, *S. aureus*, *B. subtilis*, especially C12QAS with the best antibacterial ability (100% to *E. coli*, 95.65% to *S. aureus*, and 91.41% to *B. subtilis*). In addition, C12QAS also displayed the best antifungal activities than BNQAS and C18QAS against Cytospora mandshurica, Botryosphaeria ribis, Physalospora piricola, and Glomerella cingulata with the ratio of full marks. The strategy provides a facile way to design and develop new types of antibacterial drugs for application in preventing the fruit rot, especially apple.

Key words: antibacterial, antifungal, quaternary ammonium salt

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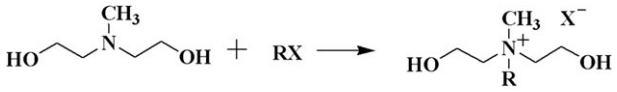
Over the past decades, many quaternary ammonium salts (QAS) due to the antibacterial activities were used for antiseptics, disinfectants, and a variety of clinical purposes, such as preoperative disinfection of unbroken skin, application to mucous membranes, and disinfection of non-critical surfaces (1–6). For example, alkyl-QAS, dialkyl-QAS, QAS-iodine, and BQAC [Bisquaternary ammonium compounds, such as dequalinium chloride (7)] have received increasing attentions due to their vital antibacterial activities (8–10). Therein, long chain alkyl-QAS shows unsurpassed antibacterial activity. Drug toxicity of shorter chain alkyl-QAS is weak than that of comparatively longer chain alkyl-QAS; benzyl-QAS has high drug toxicity than methyl-QAS; More importantly, the antibacterial activities are influenced by the alkyl chain length. As the growth of the alkyl chain, the antibacterial activities gradually increase, until they reach a reasonable limit (11,12). Many discrete QAS were reported with good antibacterial activities in the literature and wildly used because they were cheap and could disinfect quickly and exhaustively (13). In this study, *N*-methyldiethanolamine (MDEA) has been selected as activity donors to construct novel QAS because MDEA contains two flexible hydroxyl groups. It is expected that novel QAS with two flexible hydroxyl groups can more easily pass through cell membrane into the cell to passivate enzyme and damage bacteria (14,15).

Motivated by our interest in the antibacterial material based on dihydroxy QAS with long chain alkyl bromides, an effective strategy for getting novel QAS by designing and synthesizing novel dihydroxy QAS with long chain alkyl bromides is carried out and we report five *N*-methyl-*N*-R-*N*,*N*-bis(2-hydroxyethyl) ammonium bromides (*R* = -benzyl (BNQAS), -dodecyl (C12QAS), -tetradecyl (C14QAS), -hexadecyl (C16QAS), -octadecyl (C18QAS)) based on MDEA and halohydrocarbon (Scheme 1), respectively. And their antibacterial properties and antifungal properties were tested by the methods of qualitative test (inhibition zone method) and quantitative test (oscillation method).

Experimental

Materials

N-methyldiethanolamine (MDEA), dodecyl bromide, tetradecyl bromide, hexadecyl bromide, octadecyl bromide,



$R = -CH_2C_6H_5$ (BNQAS), $-C_{12}H_{25}$ (C12QAS), $-C_{14}H_{29}$ (C14QAS), $-C_{16}H_{33}$ (C16QAS), $-C_{18}H_{37}$ (C18QAS)

$X^- = Cl^-$ or Br^-

Scheme 1: The synthesis and structures of five QAS.

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benzyl bromide, and chlorhexidine acetate were purchased from Sinopharm Chemical Reagent Co Ltd. (Shanghai, China) and were used as obtained without further purification. *E. coli, S. aureus*, *B. subtilis,* and fungus (*Cytospora mandshuria*, *Botryosphaeria ribis*, *Physalospora piricola*, and *Glomerella cingulata*) were purchased from American Type Culture Collection, Manassas, VA, USA.

Methods

Infrared (IR) samples were prepared as KBr pellets, and spectra were obtained in the 400–4000 cm^{-1} range using a Nicolet MAGNA-IR 550 FTIR spectrometer. Elemental analyses were performed on a Perkin-Elmer Model 2400 analyzer (Waltham, MA, USA). ^{1}H NMR data were collected using a Bruker Avance 400 spectrometer. All ^{13}C NMR data were collected at 100 MHz using Bruker Avance-400 spectrometer. Chemical shifts are reported in δ relative to TMS. MS data were collected using an Agilent GC-MS 7890A-5975C.

Single-crystal structure determination

Suitable single crystals of BNQAS, C12QAS, C14QAS, and C16QAS were selected and mounted in air onto thin glass fibers. X-ray intensity data were measured at 293 K on a Bruker SMART APEX CCD-based diffractometer (Mo Kα radiation, λ = 0.71073 Å). The raw frame data were integrated into SHELX-format reflection files and corrected for Lorentz and polarization effects using SAINT (16). None of the crystals showed evidence of crystal decay during data collection. All structures were solved by a combination of direct methods and difference Fourier syntheses and refined against F^2 by the full-matrix least-squares technique. Crystal data, data collection parameters, and refinement statistics for BNQAS, C12QAS, C14QAS, and C16QAS are listed in Table 1. Relevant bond lengths, bond angles, and torsion angles are shown in Table S1.

Cytotoxicity testing with MTT method

3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was used to determine cellular mitochondrial dehydrogenase activity reflecting initial cell death (17). Mouse alveolar epithelial type II (AT2) cell line were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin at 37 °C in a 5% CO_2/95% air incubator MCO-15AC (SANYO). AT2 cells in 96-well plate were adjusted to 1.0 × 10^5 cells mL, cells were passed, and cell supernatant was pour out before plating on glass slide at 37 °C, 5% CO_2 for 24 h. Cells were pretreated with 100 μL QAS for 24 h, and cell supernatant with QAS was pour out and washed with PBS buffer system. Then, the cells were treated with the MTT solution (20 μL, 5 mg/mL) for 4 h. The dark blue formazan crystal formed in the intact cells was solubilized with dimethyl sulfoxide, and absorbance at 570 nm was measured with a microplate reader (Multiskan MK3-Thermo Labsystems, Marietta, GA, USA). The results were expressed as percentage of the control value from the normal cells without treatments of QAS.

Results and Discussion

Structural analysis

In this study, five *N*-methyl-*N*-R-*N*,*N*-bis(2-hydroxyethyl) ammonium bromides (*R* = -benzyl (chloride, BNQAS), -dodecyl (C12QAS), -tetradecyl (C14QAS), -hexadecyl (C16QAS), -octadecyl (C18QAS)), respectively) were prepared from MDEA and halohydrocarbon. The reaction conditions including temperatures and times of reactions were optimized to get pure products in high yields (Table 2).

Their structures were characterized by FTIR, ^{1}H NMR, MS, and elemental analysis. The FTIR and ^{1}H NMR spectra are shown in Figure S1–S25. From the FTIR spectra, strong

Table 1: Crystallographic data for BNQAS, C12QAS, C14QAS, and C16QAS

Formula	$C_{12}H_{20}ClNO_2$ BNQAS	$C_{17}H_{38}BrNO_2$ C12QAS	$C_{19}H_{42}BrNO_2$ C14QAS	$C_{21}H_{46}BrNO_2$ C16QAS
CCDC	983 195	1 007 475	983 196	1 007 476
fw	245.74	368.39	396.45	424.50
crystal size (mm)	0.20 × 0.14 × 0.10	0.14 × 0.10 × 0.10	0.17 × 0.12 × 0.12	0.15 × 0.10 × 0.08
crystal system	Orthorhombic	Monoclinic	Monoclinic	Monoclinic
a (Å)	9.595 (4)	19.217 (6)	20.952 (10)	22.767 (7)
b (Å)	9.894 (4)	7.305 (2)	7.322 (4)	7.331 (2)
c (Å)	27.420 (10)	14.552 (5)	14.465 (7)	14.479 (5)
β (°)	90	95.084 (6)	90.413 (8)	94.796 (5)
V (Å^3)	2603.1 (17)	2034.9 (11)	2219.2 (19)	2408.2 (13)
Space group	*Pbca*	*P2 (1)/c*	*P2 (1)/c*	*P2 (1)/c*
Z value	8	4	4	4
ρ calc. (g/cm^3)	1.254	1.203	1.187	1.171
μ (Mo *K*α) (mm^{-1})	0.281	2.025	1.862	1.720
Temp (K)	296 (2)	296 (2)	296 (2)	296 (2)
No. of observations (I > 3σ)	2413	3778	4115	4466
Final R indices [I > 2sigma (I)]: R1[a]; *wR2*[a]	0.0387, 0.0968	0.0503, 0.1173	0.0497, 0.1115	0.0399, 0.0908

[a]$R1 = \Sigma\ ||F_o| - |F_c||/\Sigma\ |F_o|$. $wR2 = \{\Sigma\ [w(F_o^2 - F_c^2)^2]/\Sigma\ [w(F_o^2)^2]\}^{1/2}$.

Table 2: The optimum conditions and yields for synthesis of QAS

QAS	Temperature/°C	Time/h	Yield/%
BNQAS	60	6	93.2
C12QAS	80	6	91.6
C14QAS	80	8	74.5
C16QAS	70	10	66.7
C18QAS	80	12	81.2

characteristic absorption band of hydroxy and stretching vibration band of C-O can be found, which demonstrates the existence of -OH of QAS. For example in FTIR spectra of BNQAS, the two bands lie in 3346 and 1076 cm^{-1}, respectively. In addition, the distinct band in 772 cm^{-1} belongs to the stretching vibration band of linear paraffin in FTIR spectra of BNQAS. In other QAS, the stretching vibration bands of linear paraffin are 722 cm^{-1} (C12QAS), 723 cm^{-1} (C14QAS), 721 cm^{-1} (C16QAS), and 721 cm^{-1} (C18QAS), respectively. In ^1H NMR spectra, the single bands of hydroxy of BNQAS can be found at δ 5.53–5.51 ppm, but the hydroxyl bands disappeared in ^1H NMR spectra by D_2O exchange (Figures S3, S8, S13, S18 and S23). This further proved the presence of hydroxyl in BNQAS.

X-Ray single-crystal analysis indicates that BNQAS crystallizes in an orthorhombic space group *Pbca* and C12QAS, C14QAS, C16QAS in monoclinic space group *P2(1)/c*. As shown in Figure 1, the asymmetric unit of BNQAS, C12QAS, C14QAS, and C16QAS contains one crystallographically independent molecule, respectively. The central N atoms of QAS bond one methyl, two 2-hydroxyethyl groups, and one benzyl group in BNQAS or a linear paraffin in other QAS, respectively. The C2-N1-C4 angels are 107.39(13) Å in BNQAS, 111.8(2) Å in C12QAS, 109.7(2) Å in C14QAS, and 111.72(18) Å in C16QAS, respectively. In the four QAS, the directions of two 2-hydroxyethyl compared to the central N atom in BNQAS are obviously different from that of C12QAS, C14QAS, and C16QAS, which can be proved by the torsion angles of N1-C2-C3-O1 (94.6(2) Å in BNQAS, 68.2(4) Å in C12QAS, 67.4(3) Å in C14QAS, and 68.0(3) Å in C16QAS) and N1-C4-C5-O2 (−79.7(2) Å in BNQAS, −175.8(3) in C12QAS, −174.5 (3) Å in C14QAS, and −175.8(2) Å in C16QAS). Another data also can help explain that point by the O1-N1-O2 angles (87.2(2) Å in BNQAS, 101.9(1) Å in C12QAS, 101.3 (2) Å in C14QAS, and 101.7(1) Å in C16QAS, respectively). In addition, in C12QAS, C14QAS, and C16QAS, there are long alkyl chains of $C_{12}H_{25}$, $C_{14}H_{29}$, and $C_{16}H_{33}$. In these long alkyl chains, the C-C-C angel (109.8(3)···117.5(3) Å) are similarly as well as the C-C-C-C torsion angles (175.4 (4)···180.0(3) Å), which well confirm the linear-type conformation of long alkyl chains in the solid state. Relevant bond lengths, bond angles, and torsion angles in BNQAS, C12QAS, C14QAS, and C16QAS are shown in Table S1. Two flexible hydroxyl groups and the linear-type alkyl chain of C14QAS could easily pass through cell membrane into the cell. It can be a structural advantage of five QAS to display their unsurpassed antibacterial activity.

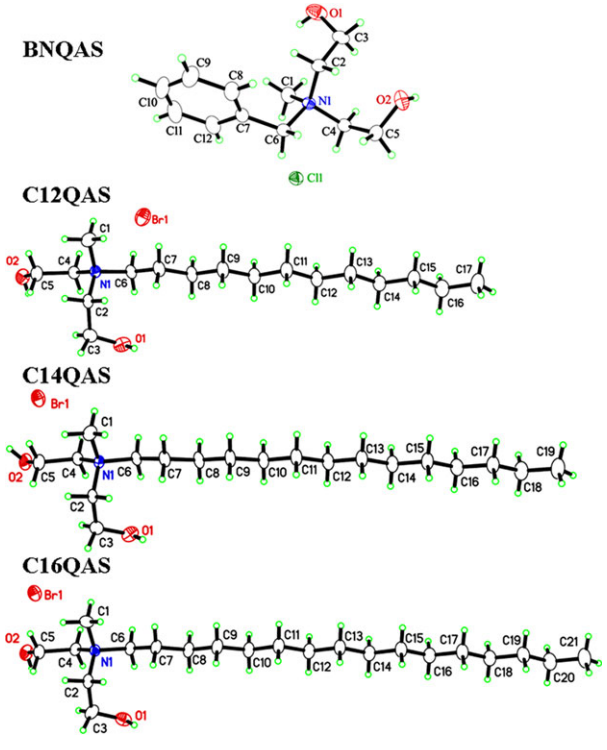

Figure 1: The ORTEP figures of BNQAS, C12QAS, C14QAS, and C16QAS (displacement ellipsoids with 30% probability).

Antibacterial properties

In this study, antibacterial activities of five QAS were detected through qualitative test and quantitative test. The data of diameter of inhibition zone for five QAS against *S. aureus*, *B. subtilis,* and *E. coli* were obtained. Chlorhexidine acetate was selected as positive controls. In qualitative test, the inhibition zone could be found during the concentration of 100, 500, 2500 ppm in Table 3. The diameters of inhibition zone of QAS were gradually increasing following the concentration gradient. The results demonstrate that all five QAS have good antibacterial activities against *S. aureus*, *B. subtilis,* and *E. coli*. Therein, BNQAS and C12QAS are the most sensitive to *E. coli*, C14QAS and C16QAS are the most powerful to *B. subtilis*, and C18QAS are the most potent to *S. aureus*.

In quantitative test, the curves of antibacterial activity of five QAS at different concentrations and at the same contact times were tested, respectively. Chlorhexidine acetate was selected as positive controls. We could see from Figure 2, the antibacterial ratios of five QAS went up along with the increasing of the concentration. It is interesting that the antibacterial ratios of BNQAS and C12QAS against *E. coli* were always greater than against *S. aureus* and *B. subtilis* at the same concentration (2500 ppm, Table 4 and Figure 2). Specifically, the ratios against *E. coli* could reach 90.52% and 100%, against *S. aureus* were 84.78% and 95.65%,

Table 3: Diameter of inhibition zone (mm) of five QAS

	S. aureus			*B. subtilis*			*E. coli*		
Concentration/ppm	100	500	2500	100	500	2500	100	500	2500
BNQAS	10.3	16.5	24.6	10.1	14.7	19.2	11.6	19.2	28
C12QAS	10.2	22.4	34.2	10.5	17	31.1	11.2	24.1	36.3
C14QAS	14	15.2	16.7	13.3	18	21.1	10.1	10.5	11.2
C16QAS	13	13.8	14.6	13.5	14.3	15.5	0	10.2	10.6
C18QAS	11.2	11.5	12.1	10.5	11	11.4	0	0	0
Chlorhexidine Acetate	16.1	25.2	37.5	12.8	21.2	35.4	13.4	27.5	41.3

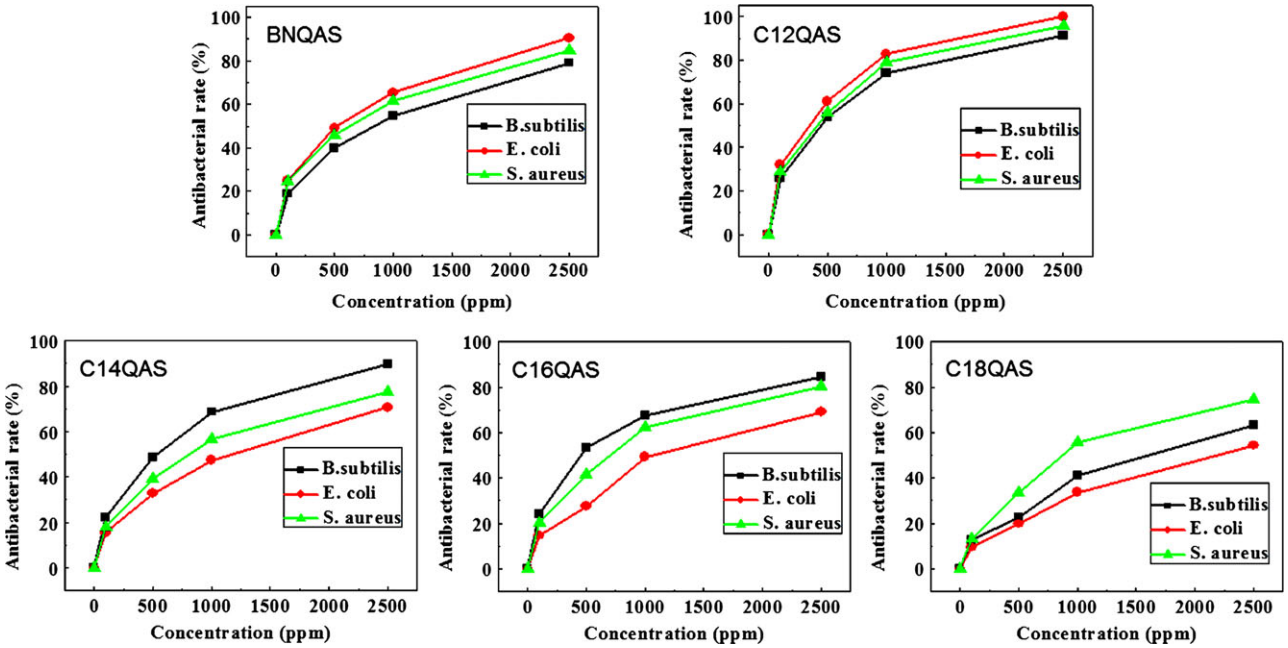

Figure 2: Antimicrobial activities of five QAS at different concentrations at the same contact times.

Table 4: Antibacterial ratios of five QAS against *S. aureus*, *B. subtilis,* and *E. coli* under the concentration of 2500 ppm, respectively

QAS	Antibacterial ratios against *S. aureus*	Antibacterial ratios against *B. subtilis*	Antibacterial ratios against *E. coli*
BNQAS	84.78	79.14	90.52
C12QAS	95.65	91.41	100
C14QAS	77.54	70.69	89.57
C16QAS	80.43	84.66	68.97
C18QAS	74.64	63.19	54.31
Chlorhexidine acetate	97.52	96.8	100

and against *B. subtilis* were 79.14% and 91.41% at the concentration of 2500 ppm for BNQAS and C12QAS. The antibacterial ratios of C12QAS against *E. coli*, *S. aureus,* and *B. subtilis* can approach chlorhexidine acetate at the most extent. Additionally, the antibacterial ratios of C14QAS and C16QAS against *B. subtilis* were always more than those of C14QAS and C16QAS against *S. aureus* and *E. coli* at the same concentration. The ratios against *B. subtilis* could reach 89.57% and 84.66%, against *S. aureus* were 77.54% and 80.43%, and against *E. coli* were 70.69% and 68.97% at the concentration of 2500 ppm for C14QAS and C16QAS. Lastly, the antibacterial ratios of C18QAS against *S. aureus* were always more than those of C18QAS against *B. subtilis* and *E. coli* at the same concentration. The ratio against *S. aureus* could reach 74.64%, against *B. subtilis* was 63.19%, and against *E. coli* was 54.31% at the concentration of 2500 ppm for C18QAS. The results showed the antibacterial ratios of C12QAS against *S. aureus*, *B. subtilis,* and *E. coli* were all bigger than those of the other four QAS. In other words, C12QAS displayed the best antibacterial abilities against *E. coli, S. aureus,* and *B. subtilis* under the concentration of 2500 ppm. The results suggest that the antibacterial activities of five QAS are influenced by the alkyl chain length (18).

Moreover, as shown in Figure 3, the antibacterial ratios of five QAS synthesized increased rapidly during the short

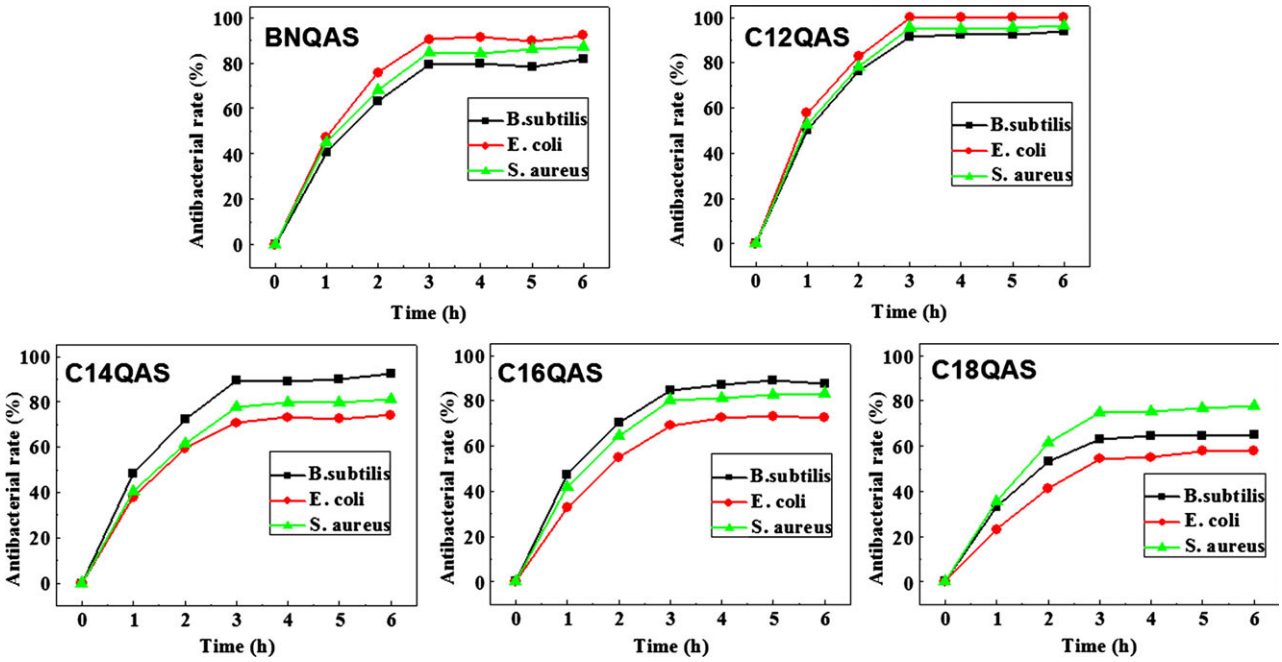

Figure 3: Antibacterial activities of five QAS under the concentration of 2500 ppm at different contact times.

time (1–2 h), but the ratios reached a maximum after touching for 3 h, which displayed that five QAS played potent antibacterial activity further.

Antifungal properties

To study the possibility as antifungal drugs of BNQAS, C12QAS, and C18QAS, four funguses, such as Cytospora mandshuria, Botryosphaeria ribis, Physalospora piricola, and Glomerella cingulata were selected to detect antifungal activity of BNQAS, C12QAS, and C18QAS. And the examination of antifungal activity of three QAS is performed by placing fungus with 5 mm diameter in contact with growth medium mixed QAS solution of 2500 ppm. The antifungal activity of QAS is judged based on the growth diameter of inhibition zone to fungus compared with the growth diameter of fungus without any QAS. The shorter diameter of inhibition zone to fungus proves the better antifungal activity of QAS. In other method, it is judged also based on the ratio between the growth diameter of fungus mixed QAS solution and that of fungus without any QAS. The bigger ratio proves the better antifungal activity of QAS. As shown in Figure 4, BNQAS displays barely antifungal activity, except against Cytospora mandshuria with the ratios of 77%. For C18QAS, the better antifungal activities can be found. The ratios of 84% against Cytospora mandshuria, 89% aganist Botryosphaeria ribis, and 100% against Physalospora piricola are higher than that of BNQAS. It happens that there is a special case that the ratio against Glomerella cingulata is only 56%, which demonstrates C18QAS can be as a good antifungal drug aganist Cytospora mandshuria, Botryosphaeria ribis, and Physalospora piricola, but not fit for Glomerella cingulata because of the bad antifungal activity. However, the best antifungal drug should display the highest antifungal activity against several funguses of apple, such as Cytospora mandshuria, Botryosphaeria ribis, Physalospora piricola, and Glomerella cingulata. Only then like this, application as antifungal drug for apple will only be possible. Fortunately, C12QAS displays the highest antifungal activity than BNQAS and C18QAS, which can be proved by the ratio of full marks against the four kinds of fungus. Moreover, good antifungal activity can be found under the concentration of 500 ppm and 100 ppm, except against Cytospora mandshuria.

Cytotoxicity

The cytotoxicity of QAS was evaluated for mouse alveolar epithelial type II (AT2) cell line using MTT assay. The results are showed in Figure 5 summarizing the corresponding cell viability (%) under the different concentrations of QAS. And IC_{50} values can be estimated. For example, IC_{50} values of C14QAS and C16QAS are about 4–8 ppm, while IC_{50} value of C12QAS is under the concentration of 2–4 ppm; however, the value of C18QAS can reach the concentration of 8–16 ppm. Through comparative analysis, all QAS except BNQAS were potent inhibitors of the growth of AT2 cell line, and the IC_{50} value of C12QAS is smallest compared with other QAS, demonstrating the biggest cytotoxicity for AT2 cell, which is corresponding to the highest antibacterial activity of C12QAS. In addition, BNQAS displays weaker cytotoxicity, which

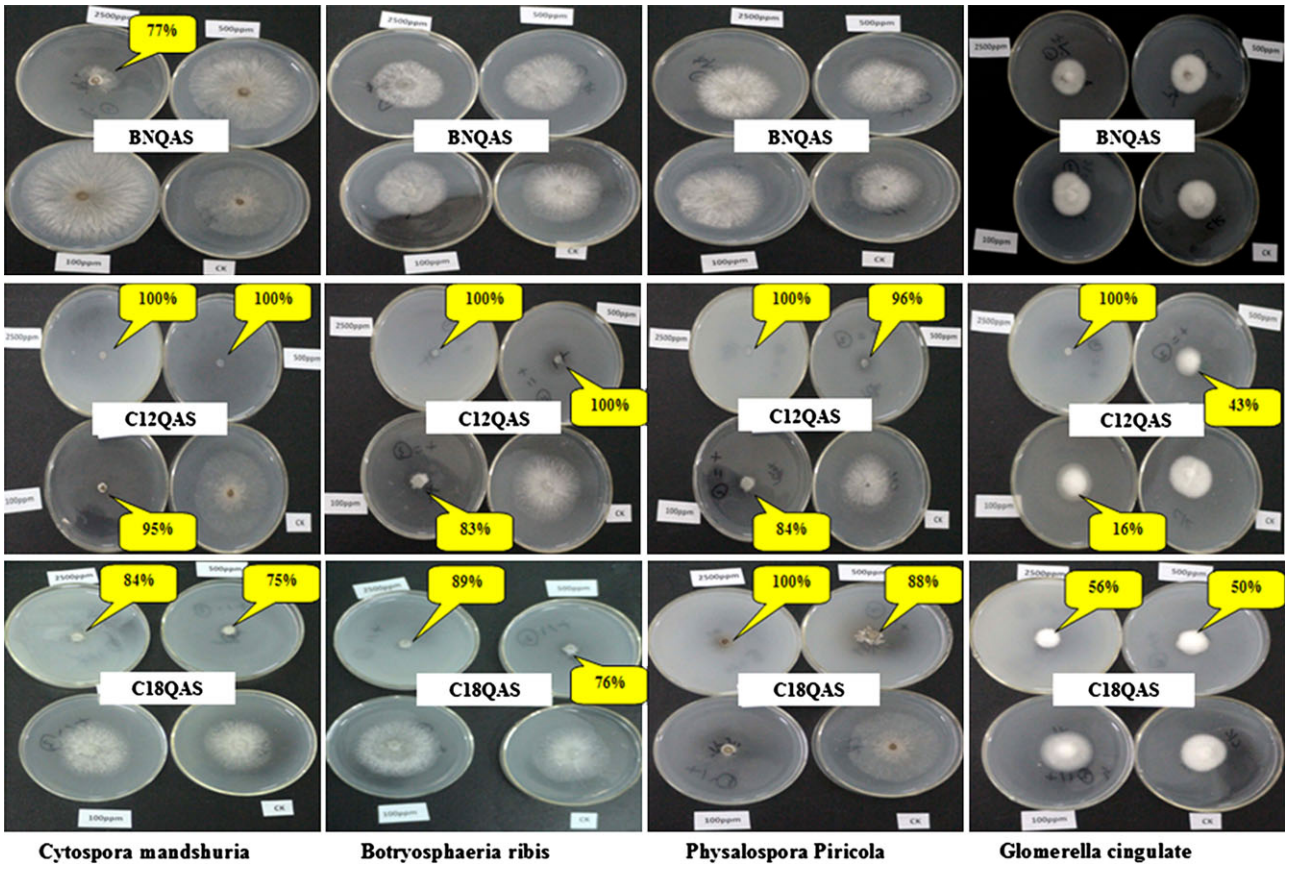

Figure 4: Antifungal activities of five QAS under different concentrations at the same contact time.

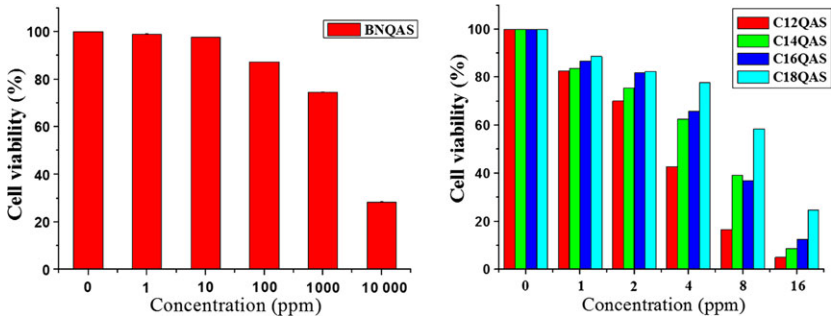

Figure 5: Cell viability of five QAS under different concentrations for AT2 cell.

can be proved by the IC_{50} value of 1000–10 000 ppm. The results display that the cytotoxicity of QAS of dodecyl QAS is stronger than that of comparatively longer chain alkyl-QAS and benzyl-QAS have very weak drug cytotoxicity than long chain alkyl-QAS.

Conclusions

In this study, five *N*-methyl-*N*-R-*N*,*N*-bis(2-hydroxyethyl) ammonium bromides (BNQAS (chloride), C12QAS, C14QAS, C16QAS, and C18QAS) were prepared based on MDEA and halohydrocarbon, and their structures were characterized by FTIR and NMR. BNQAS and C14QAS were confirmed by *X*-ray single-crystal diffraction. Their antibacterial properties were tested. The result displays that the antibacterial activity and antifungal activity of QAS are basically related to the introduction of the long chain alkyl group in MDEA. The long chain alkyl group in QAS can change the geometric arrangement of QAS, which can help two flexible hydroxyl groups of QAS to pass through cell membrane into the cell to passivate enzyme and damage bacteria. All the long chain alkyl-QAS show unsurpassed antibacterial activities, especially C12QAS

against *E. coli, S. aureus*, *B. subtilis,* and four funguses with the ratio of full marks. In addition, the cytotoxicity of QAS was evaluated for AT2 cell line using MTT assay. The results display that the cytotoxicity of QAS is influenced by the alkyl chain length, and the cytotoxicity and the antibacterial activity of C12QAS are stronger than other QAS. The strategy provides a facile way to design and develop new types of antibacterial and antifungal drugs for application in preventing the fruit rot, especially apple.

Acknowledgment

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Conflict of Interest

All authors declare no conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. ^{1}H NMR, ^{13}C NMR, GC-MS, IR data for five QAS and relevant bond lengths, bond angles and torsion angles of BNQAS, C12QAS, C14QAS and C16QAS.