Synthesis and fungicidal activity of alkyltrimethylammonium salicylate ionic liquids

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Abstract. Three alkyltrimethylammonium salicylates (C_nTASal) with different alkyl side chains were synthesized and characterized. Solubility, surface activity, and biological activity of the ionic liquids were determined. The results of solubility and surface activity showed that the synthesized ILs are amphiphilic surface-active compounds. Five common agricultural fungi, Valsa mali, Rhizoctonia solani, Fusarium graminearum, Phytophthora capsica and Alternaria solani were tested to identify the most sensitive strain and Valsa mali was selected for the structure-activity relationship (SAR) study. The results of the study revealed that the antifungal activity of the ILs is positively related to the alkyl chains length.

Keywords: Ionic liquid, Fungicide, Solubility, Surface activity, Alkyltrimethylammonium salicylate.

1 Introduction

Amphiphilic compounds usually consist of hydrophobic and hydrophilic groups, showing surface activity and forming aggregates in an aqueous solution[1]. Cationic surfactants are a typical kind of amphiphilic compounds that can be classified into an amine, quaternary ammonium salt (QAS), quaternary phosphorus salt, quaternary sulphur salt and so on[2]. Among them, QASs are the ones with the greatest applications and uses[3]. They are usually used in textile, agricultural, paper, paint, road construction, and many other industries due to their low toxicity, strong penetration, and excellent surface activity [3-6]. The surface activity and lipophilicity of QASs make them easily dissolve with the phospholipid layers on the cell membrane and produce distortion to membrane[7], which makes them be widely used as antimicrobial agents.

Ionic liquids (ILs) are consisting of organic cations or anions with melting points below 100°C[8,9]. ILs can be regarded as relatively new materials and continuous development has been observed in this field[10,11]. Since 2007, ILs can be roughly classified into three generations[9,12] according to their usage and structure. The concept of task-based ILs to biomedical fields was introduced by employing active pharmaceutical ingredients to give them biological activities[9,11-13]. ILs have many favourable properties, such as low vapor pressure, low toxicity, high thermal stability, and high solubility[14-16]. Furthermore, ILs are tunable, making them easy to combine different properties in a single compound[8,17,18].

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Salicylic acid is an essential plant growth regulator. Also, salicylic acid and its derivative, aspirin, are common anti-inflammatory drugs for humans[19]. For plants, researchers have found that salicylic acid possesses fungicidal activity and decreases fungus development[20,21]. It is a considerable choice to be the anion of fungicidal ILs. Alkyltrimethylammonium(C_nTA) salts are a large group of quaternary ammonium salts. However, researches on C_nTASal are not mainly focused on their fungicidal activity. Herein, we synthesized C_nTASal with different alkyl chains and studied their solubility and surface activity. Furthermore, we systematically studied their biological activities on common agricultural fungi for the first time to expand the germicidal spectrum and analyzed their structure and activity relationship combined with their solubility and surface activity.

2 Experimental

2.1 Materials

Butyltrimethylaminium(C_4TA) chloride, octyltrimethylammonium(C_8TA) chloride, dodecyltrimethylammonium (C_{12}TA) chloride, potassium hydroxide and salicylic acid were purchased from Maya Reagent (Jiaxing, China). All substances mentioned were used without further purification.

2.2 General information

^{1}H and ^{13}C-NMR spectra were obtained using a Bruker Avance Neo spectrometer operating at 500 MHz and 126 MHz, respectively, with the samples dissolved in deuterated methanol (methanol-d_4). IR spectra were obtained with a Nicolet iS10 FT-IR spectrometer. And a TA Instruments Discovery DSC 250 was used to determine melting points at a heating rate of 10 °C/min.

2.3 Synthesis

5g C_nTAC(n=4,8,12) was dissolved in 30mL methanol and an equal amount of KOH was added. The solutions were stirred at room temperature for 1 hour, and then filtered to remove the inorganic by-product KCl. Then, a stoichiometric amount of salicylic acid was added. The solutions were stirred overnight. Then the solvent was removed and 50 mL of acetonitrile was added to dissolve the residue. Then the solution was stirred for 30 min and the remaining KCl was filtered off [25,26]. This process was repeated three times. Then the solutions were evaporated until a large amount of white solid crude products precipitated. Next, the mixture was heated to 55°C to redissolve the solid and cooled to room temperature to produce crystal. And the crystal was obtained by vacuum filtration.

C_4TA salicylate(C_4TASal) (1): ^{1}H NMR (500 MHz, MeOD) δ = 0.98(t, J = 7.4 Hz, 3H; CH_3), 1.36(m, 2H; CH_2), 1.70(m, 2H; CH_2), 3.07(s, 9H; CH_3), 3.26(m, 2H; CH_2), 6.77(m, 2H, CH_2), 7.27(m, 1H; CH), 7.83(dd, J = 7.7, 1.5 Hz, 1H; CH); ^{13}C NMR (126 MHz, MeOD) δ = 12.48, 19.23, 24.44, 52.09, 66.29, 115.79, 117.60, 118.89, 130.28, 132.42, 161.29, 174.52; IR(cm^{-1}): 540.07, 671. 23, 709.80, 763.81, 810.10, 856.39, 910.40, 971.12, 1026.13, 1141.86, 1296.16, 1327.03, 1388.75, 1458.18, 1489.05, 1589.34, 1635.64, 2708.08, 2877.79, 2966.66, 3032.10, 3433.29; m.p: 24.82°C; yield: 55.58%

C_8TA salicylate(C_8TASal) (2): ^{1}H NMR (500 MHz, MeOD) δ = 0.90 (t, J = 6.9 Hz, 3H; CH_3), 1.31(m, 10H; CH_2), 1.73(m, 2H; CH_2), 3.07 (s, 9H; CH_3), 3.26(m, 2H; CH_2), 6.77(m, 2H; CH_2), 7.26(m, 1H; CH), 7.83(dd, J = 7.7, 1.6 Hz, 1H; CH); ^{13}C NMR (126 MHz, MeOD) δ = 13.03, 22.27, 22.52, 25.94, 28.77, 31.47, 52.04, 66.49, 115.78, 117.58, 118.91, 130.28,

132.37, 161.29, 174.54; IR(cm^{-1}): 536.12, 667.37, 705.95, 725.23, 759.95, 810.10, 860.25, 910.40, 968.27, 1029.99, 1138.00, 1253.73, 1334.74, 1388.75, 1458.18, 1589.34, 1631.78, 2858.51, 2927.94, 2954.95, 3402.43; m.p: 57.19°C; yield: 38.53%

C_{12}TA salicylate(C_{12}TASal)(3): ^{1}H NMR (500 MHz, MeOD) δ = 0.89 (t, J = 6.9 Hz, 3H; CH_3), 1.31(m, 18H; CH_2), 1.73(m, 2H; CH_2), 3.08 (s, 9H; CH_3), 3.26(m, 2H; CH_2), 6.76(m, 2H; CH_2), 7.25 (dd, J = 8.4, 1.4 Hz, 1H; CH), 7.83 (dd, J = 7.7, 1.7 Hz, 1H; CH); ^{13}C NMR (126 MHz, MeOD) δ = 13.07, 22.35, 22.53, 25.95, 28.81, 29.20, 31.68, 52.08, 66.49, 115.77, 117.57, 118.97, 130.29, 132.34, 161.28, 174.57; IR(cm^{-1}): 536.21, 597.93, 667.37, 705.95, 736.81, 759.95, 810.10, 860.25, 910.40, 968.27, 1026.13, 1138.00, 1253.73, 1330.88, 1388.75, 1458.18, 1485.19, 1627.92, 1651.07, 2854.65, 2924.09, 2954.95, 3039.81, 3402.43; m.p: 48.50°C; yield: 51.55%

2.4 Solubility

Solubility tests were performed according to the methods described in the literature[29]. Nine solvents (Table 1) were selected for the solubility tests according to their Snyder polarity index. 0.1 ± 0.0001 g of the tested compound were added to a specific volume of each solvent, and the results of dissolution were recorded and classified as "good", “limited” and “poor”, which refer to compounds that dissolved in 1 mL, 3mL and more than 3 mL of the solvents, respectively. And the experiment was carried out at two temperatures, 25 °C and 45 °C.

2.5 Surface activity

The surface activity was determined at 23.5°C by the platinum ring method with a JYW-200B automatic tensiometer. Measurements were repeated four times and average values are reported.

2.6 Fungicidal activity

Five common agricultural fungi were used to test fungicidal activities of the compounds *in vitro*. The method used to detect toxicity of the ILs was the mycelium growth rate method. The experiment is divided into two steps. 6-mm-diameter fungal colonization disks were taken from the edge of the colony and transferred to Petri dishes with a culture medium. 50.0 ppm of the ILs solutions were used for antifungal toxicity screening to select proper fungi strains. And these strains were used in a structure-activity relationship study with different concentrations of the ILs at 0.00, 1.56, 3.13, 6.25, 12.5, 25.0, 50.0ppm. Each experiment was conducted in three replicates. 10% DMSO without ILs was used as a control. The plates were placed in a fungal incubator at 28°C. The decussation method was used to measure the diameters of the colonies, and the inhibition rate was calculated according to Eq. (1). In this part, quaternary ammonium chloride of the ILs was used as references.

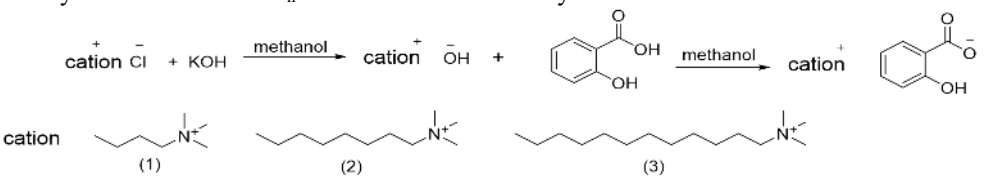
$$\text{growth inhibition} = \frac{(d_c - 0.6) - (d_e - 0.6)}{d_c - 0.6} \times 100\% \quad (1)$$

where d_c and d_e are the diameters (cm) of the colonies in the control group and the experimental group, respectively. The concentration values retain three significant digits and the inhibition rates were retained to two decimal places.

3 Results and discussion

3.1 Synthesis and characterization

The synthesis method of C_nTASal with different alkyl chains is shown in scheme 1:



Scheme 1. Synthetic routes of C_nTASal (n=4,8,12) ILs.

The C_nTA halides were converted to quaternary ammonium hydroxides. Then a stoichiometric amount of salicylic acid was added into the system and a neutralization reaction took place[25,26]. Hence, the crude products of the ILs were further purified to obtain a crystal in acetonitrile. The synthesized ionic salts were characterized by ^{1}H-NMR and ^{13}C-NMR spectra, FT-IR, and DSC.

3.2 Solubility

The solubility tests were conducted under 25 and 45°C as shown in Table 1. As shown from the results, C_4TASal, C_8TASal, and $C_{12}TASal$ had good solubility in most of the strong and weak polar solvents. However, it was different for water and ethyl acetate. Although all the three ILs could dissolve in water at 25°C, the state of the solutions after the dissolution was different, especially after $C_{12}TASal$ dissolved, the solution became oily. This indicates that the length of alkyl chains of the ILs and their hydrophilicity are inversely proportional. On the other hand, although none of the ILs dissolved in ethyl acetate at 25°C, their solubility changed as the temperature increased. And at 45°C, the solubility of $C_{12}TASal$, C_8TASal, and C_4TASal fell just within the range of "good", "limited" and "poor" respectively, demonstrating a direct relationship between the alkyl chain length and the solubility of the salts in weakly polar solvents. According to similarity-intermiscibility theory, the above phenomenon showed that the lipophilicity of ILs was enhanced while the hydrophilicity was weakened with the increase of the length of hydrophobic carbon chains.

Table 1. Solubilities of the synthesized ILs 1-3 at 25°C and 45°C.

IL	Temperature	Water	Methanol	DMSO	Acetonitrile	Acetone	2-propanol	Ethyl acetate	Chloroform	Hexane
C_4TASal	25°C	+	+	+	+	+	+	-	+	-
	45°C	+	+	+	+	+	+	-	+	-
C_8TASal	25°C	+	+	+	+	+	+	-	+	-
	45°C	+	+	+	+	+	+	+/-	+	-
$C_{12}TASal$	25°C	+ (oily)	+	+	+	+	+	-	+	-
	45°C	+	+	+	+	+	+	+	+	-

+: good;
+/-: limited;
-: poor

3.3 Surface tension

From the results of the solubility of the ILs, we've known that the C_nTASal ILs are amphiphilic substances. These kinds of compounds are likely to be surface active. Therefore, to explore their surface properties, surface activity studies were conducted and the data are presented in figure 1 and table 2.

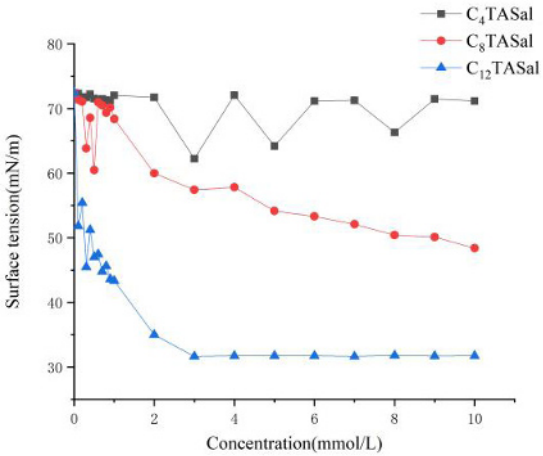


Fig. 1. Concentration-surface tension relationship of the prepared salts in water at 23.5°C.

Table 2. Surface activity parameters of C_4TASal, C_8TASal, C_{12}TASal.

ILs	CMC(mmol/L)	γ(mN/m)
C_4TASal	na	na
C_8TASal	3.28	57.59
C_{12}TASal	2.46	31.67

CMC: critical micelle concentration;
γ_{CMC}: surface tension at CMC.

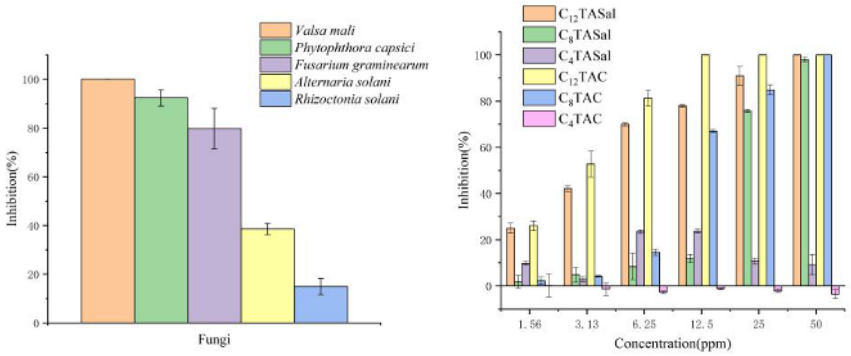
As shown in Figure 1, the surface tension of the aqueous solutions of C_8TASal and C_{12}TASal decreased with an increase in concentration. However, after the addition of C_4TASal, the surface tension of water fluctuated. Thus, C_nTASals were indeed surface-active and their surface activities were related to their alkyl chains length.

The critical micelle concentrations and their corresponding surface tension values were calculated according to the concentration-surface tension curve, as shown in Table 3. Comparing the CMC values and the corresponding surface tension of the three ionic salts showed that both C_8TASal and C_{12}TASal formed micelles in water but not C_4TASal, C_{12}TASal, with the longest carbon chain, had the smallest CMC value and surface tension. This suggests that alkyl chain length is an important factor influencing the surface activity of ILs. This is probably due to their hydrophobicity and ILs with longer alkyl chains tended to aggregate more easily and better reduce the surface tension of water.

3.4 Antifungal activity

The fungicidal activities of the synthetic ILs and C_{12}TA chloride(longest alkyl chain of the three original drugs) were tested on five common agricultural fungi, V*alsa mali, Rhizoctonia solani, Fusarium graminearum, Phytophthora capsici,* and *Alternaria solani* C_{12}TAC was

dissolved in DMSO to prepare a 50.0 ppm-concentration solution. Hypha diameter in each dish was measured and the inhibition was calculated using equation (1). And the results were displayed in figure 2a.



(a) (b)

Fig. 2. (a)Antifungal activity screening of C_{12}TAC; (b) SAR of different ILs.

As the bar graph shows (Figure 2a), both *Valsa mali* and *Phytophthora capsici* had inhibition rates over 80%, suggesting that the C_nTA salts may have excellent performance on *Valsa mali* (~100% inhibition rate). Thus, this strain was selected as the target strain for further studies. SAR study was conducted to find out the relationship between the structure of the synthetic ILs and their fungicidal activity. The series of three ILs were prepared using a two-fold dilution method. Their original drugs were used as control groups. The results were measured and presented in Figure 2b.

The fungicidal inhibition of the synthetic ILs and their original drugs remained at the same level for ILs with the same cations. Changing the counterions paired with cations didn't influence the fungicidal effect very much. When the anions were the same, the fungicidal effect of ionic salts was correlated with the carbon chain length of cations. C_{12}TA salts had the best fungicidal activities followed by C_8TA salts as shown in Figure 2b. Both of these two kinds of ILs showed excellent performance on *Valsa mali* with inhibition over 90% at the concentration of 50.0 ppm while C_4TA salts didn't show an ability to inhibit *Valsa mali*.

The antifungal activity of the ILs is correlated with the length of their alkyl side chains on the cations. Longer alkyl chains tend to possess better fungicidal activity. Of the anions and cations of which ILs have consisted, the cations play the role and the positive charge in ILs enables them to get attached to negatively charged phospholipid bilayer of cell membranes through electrostatic interactions[27,28]. As described in previous sections, the lipophilicity of ILs was enhanced when the alkyl chain length increased, and the lipophilic part in the ILs, being an alkyl chains, helps the ILs destroy the cell membranes of microorganisms and lead to their death

4 Conclusions

In this study, the C_nTA halides were converted into the alkaline hydroxide form. Then the C_nTA hydroxides were reacted with salicylic acid to obtain the desired C_nTA salicylate ILs. The structures and purity of the ILs were characterized and confirmed by ^{1}H-NMR and ^{13}C-NMR spectra, FT-IR, and DSC.

The solubility of the synthetic ILs in common solvents was tested. The C_nTASal ILs were able to dissolve in most solvents from strong to weak polarity. And it was observed that the solubilities of C_nTA salts were influenced by their alkyl chain. C_nTASal with longer alkyl

chains possesses better solubility in ethyl acetate, indicating an increase in lipophilicity of the ILs with the alkyl chains. The surface activity of the ILs was determined and their CMC values were obtained. C_4TASal caused the unstability of the surface tension of water while C_8TASal and C_{12}TASal effectively decreased the surface tension, and this effect increased as the carbon chain grew. The results of surface activity determination also suggested that longer alkyl chains are more lipophilic and more easily aggregate.

To obtain fungicidal activity test results, five common agricultural fungi were selected for screening. *Valsa mali*, being the most sensitive strain, was chosen. The SAR of the synthetic ILs and their original drugs was studied. The results of the SAR study demonstrated that the fungicidal activities of ILs with varying alkyl chains length were related to cations instead of anions. Salts with longer alkyl chains had better fungicidal activity, proving that the fungicidal activities of C_nTA salts were affected by the length of the alkyl chains. The results of solubility and surface activity tests showed that the positively charged C_nTA cations might get attached to negatively charged phospholipid bilayer of cell membranes through electrostatic interactions, and the lipophilic alkyl chains of the cations could further destroy the cell membranes and cause the death of the fungi.

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