Research Article

Synthesis, characterization, and the antifungal property of aminoethyl chitosan quaternary ammonium salts†

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Abstract
Chemical modification of chitosan is one of effective methods to improve the biological activity of chitosan. In this paper, aminoethyl chitosan quaternary ammonium salts, including \(N\)-trimethyl quaternary ammonium salt chitosan (TMC), 6-O-(aminoethyl)-2-trimethyl quaternary ammonium chitosan derivative (ONC), and 6-N-(aminoethyl)-2-trimethyl quaternary ammonium chitosan derivative (NNC), were successfully designed, synthesized, and characterized by FT-IR, \(^1\)H NMR, and elemental analyses. Moreover, three kinds of antifungal activities, including \(B.\ cinerea\ Pers.,\ Gibberella\ zea,\) and \(P.\ asparagi,\) were all tested by hyphal measurement \textit{in vitro}. The results showed that all chitosan derivatives had better antifungal activities than chitosan. And the antifungal activity decreased in the order: NNC > ONC > TMC > chitosan at 1.0 mg/mL. Furthermore, the inhibitory indices of NNC and ONC against three kinds of phytopathogens were higher than 75% at 1.0 mg/mL. Especially, the inhibitory index of NNC against \(B.\ cinerea\ Pers.\) attained even 90% at 1.0 mg/mL. These data demonstrated that the aminated chitosan derivative could remarkably improve the antifungal activity of chitosan. On the one hand, the higher positive charge would interact with the anionic components to damage the cell wall. On the other hand, based on the "permeability" point, the aminoethyl as lipophilic group might pass the oil film into the cell.
**Abbreviations:** FT-IR, Fourier Transform Infrared spectroscopy; $^1$H NMR, $^1$H Nuclear Magnetic Resonance spectrometer; TMC, $N$-trimethyl quaternary ammonium salt chitosan; TOSTMC, 6-tosyl-2-trimethyl quaternary ammonium salt chitosan; ONC, 6-O-(aminoethyl)-2-trimethyl quaternary ammonium-chitosan derivative; NNC, 6-$N$-(aminoethyl)-2-trimethyl quaternary ammonium-chitosan derivative; DMF, $N,N$-dimethylformamide; NMP, $N$-methyl-2-pyrrolidone; PDA, potato dextrose agar.
1 Introduction

Pathogenic fungi are one of the most important factors that causing serious diseases to plants, which would cause the crop yield losses worldwide, especially in developing countries.[1] Besides, the secondary metabolites of fungi might be deleterious, which would be detrimental to consumers.[2] Compared with the other groups of microorganisms, fungi have a worse effect on vegetables, fruits, and other crops. With the development of the science, chemical pesticides have been widely used to control the plant disease. However, the abuse of chemical pesticides would cause the environment pollution, and the pesticide residue would be bad for the health of human.[3] Therefore, it’s very necessary to develop safe, efficacious, and environmentally friendly natural alternatives to chemical pesticides.[4, 5] With the development of the green chemistry, chitosan as a potentially important renewable resource that is both non-toxic and biodegradable has gained more and more attention recently.[6-8] As the only natural cationic amino polysaccharide, chitosan has attracted much attention in the filed of food, agriculture, biotechnology, pharmaceutics, textiles, and environmental protection and so on.[9-13]

Chitosan is one of the most abundant natural polysaccharide after cellulose found on the earth, and it can be obtained through the deacetylation of chitin under alkaline conditions.[14] The main chain of chitosan is mainly composed of 1,4-β-d-glucopyranosamine.[15] According to the report, chitosan with less than 50 kDa is classified into water-soluble chitosan oligosaccharide, which can be degraded by chemical scission (acid and alkaline hydrolysis), physical scission (high
temperature) or enzymatic scission (chitosanase, chitinase, chitobiase, lysozyme, cellulase, pectinase, pepsine, papaine, lipase, pronase).[16-18] And there are some papers reported that the antimicrobial activity of chitosan is dependent on its molecular weight, degree of deacetylation, type of the substituent group, degree of the substitution and so on. And these factors influence the solubility of chitosan and the interaction with the cell walls of the target microorganisms.[19, 20] Besides, there are reported that water-insoluble chitosan can precipitate or absorb on the surface of the microbial cell, which could form a layer around the cell to block the channel on the microbial cell surface. Meantime, the layer can be proposed to destabilize the cell wall thereby causing serious leakage of cell constituents.[21] Qin and Seyfarth et al. have reported that the water-soluble chitosan has no/low significant antifungal activity, because it could not form such a layer on the microbial cell surface.[22, 23] According to the report, based on the truth that hydroxyl groups and primary amino groups on chitosan are all ideal modification site, the chemical modification of chitosan molecule plays an important role to improve the biological activity of chitosan based on the principle of active superposition. Owing to the biological and medicinal importance, the polysaccharide derivatives with amino groups have attracted great interest in recent years.[24] Aminated polysaccharides have remarkable functions including antioxidant activity, antifungal activity, cell-cell adhesion, cell motility, and prevention of thrombosis, which could be applied in the treatment of cancer, pathogens invasion, such as Lyme disease, maternal malaria and so on.[25-30] It was reported that the amino-containing carbohydrate compounds, especially glycosaminoglycans, have essential natural
biological functions, which is involved with the specific interactions with proteins, enzymes, RNA or the backbone phosphate of DNA.[24, 31-35] Moreover, bioactive compounds, such as antibiotic, and biopolymers, are all bearing the amino substituent at different sugar ring positions.[36, 37] Furthermore, there are reported that amino polysaccharide derivatives can be used as better gelators for various organic and inhibitory of glycosidase activities.[38-43] And the aminoalkyl chain in \( N \)-alklyglycosyamines not only facilitates the interaction between chitosan derivatives with microorganisms, but also enhances the biological activity of chitosan.[44] In addition, the natural aminopolysaccharide, chitosan, has demonstrated some benefits for biomedical and pharmaceutical applications, which might attribute to the primary amine probably.[45] Besides, as excellent antibaterial product, amino polysaccharide quaternary ammonium salt has been widely applied in medicine field, such as antibacterial trauma, anticoagulation, sustained-release drug delivery, skin induction and other fields. Its biological products of amino polysaccharide quaternary ammonium salt with high efficiency, safe sterilization and no drug resistance are remarkable characteristics, such as nasal lavage fluid, skin douche, vaginal lavage fluid, oral rinse fluid and hemorrhoid eliminating gel, non antibiotic bactericide, have no irritation to human body, no drug resistance, no toxic side effects. In this paper, it was proposed to investigate the synthesis of aminoethyl chitosan quaternary ammonium salts, including \( N \)-aminoethyl and \( O \)-aminoethyl at \( C_6 \) position of chitosan. As for \( N \)-aminoethyl derivative, toslyation chitosan at \( C_6 \) position which was a good intermediate was substituted by nucleophilic regent ethylenediamine. Meantime, 2-chloroethylamine hydrochloride was chose to give aminoethy group at \( C_6 \) positon of chitosan to form \( O \)-aminoethyl derivative. Furthermore, the impacts of chitosan derivatives against \( B. \) cinerea Pers., \( Gibberella \) zea, and \( P. \) asparagi which
were common fungi in agriculture were tested in this paper, respectively.2

Experimental

2.1 Materials

Chitosan (MW 10 kDa, the degree of deacetylation 97%) was purchased from Introduction of Jinhu Crust Product CO., LTD (China). Ethylenediamine (product code 10009518), 2-chloroethylamine hydrochloride (product code XW08702462), triethylamine (product code 80134318), 4-toluene sulfonyl chloride (product code 80131127), \(N,N\)-dimethylformamide (DMF) (product code 81007718), \(N\)-methyl-2-pyrrolidone (NMP) (product code 30121518), iodomethane (product code 80084117), sodium iodide (product code 20039818), and sodium hydroxide (product code 10019718) were purchased from Sinopharm Chemical Reagent Co., Ltd. The other reagents were all analytical grade and were used without further purification.

2.2 Analytical methods

Fourier Transform Infrared (FT-IR) spectrometers were recorded on a Jasco-4100 ranging from 4000cm\(^{-1}\) to 400 cm\(^{-1}\) (Japan, provided by JASCO Co., Ltd., Shanghai, China) at 25 °C with KBr disks. \(^1\)H Nuclear magnetic resonance (\(^1\)H NMR) was recorded on a Bruker AVIII 500 spectrometer (Fällanden, Switzerland, provided by Bruker Biospin CN / Bruker (Beijing) Tech. and Serv. Co., Ltd., Beijing, China), using D\(_2\)O as solvents with tetramethylsilane (TMS) as internal standard. Chemical shift values were given in \(\delta\) (ppm). The Degree of Substitution (DS) of chitosan derivatives were calculated based on elemental analyses. The results were processed by computer programs Excel (Microsoft, Redmond), OriginPro 8 (OriginLab,
Northampton, MA, USA), and MestReNova (Mestrelab Research S.L.) and reported as mean ± SD.

2.3 Synthesis of TMC

As shown in Scheme 1, 2.0 g chitosan was dispersed in 30 mL N-methyl-2-pyrrolidone (NMP), then 11 mL aqueous sodium hydroxide solution (15%, w/t), 4.8 g sodium iodide, and 11.5 mL iodomethane were added. The reaction was refluxed gently with stirring at 60 °C for 2 hours. Then the solution was precipitated by excess ethanol, and the precipitate was collected by filtration and washed by ethanol. Finally, the product was dried at 60 °C for 24 h. Yield: 89.0%; DS: 38.3% (Table 1).

2.4 Synthesis of ONC

0.4 g TMC was dissolved in 10 mL aqueous sodium hydroxide solution (15%, w/t) and stirred at 60 °C for 4 h. Then 0.35 g 2-chloroethylamine hydrochloride was added, and the reaction was continually stirring at 60 °C for 24 h. The mixture was poured into excess acetone. Then the precipitate was collected by filtration and washed by ethanol. Then the product was dialyzed against deionized water for 2 days, and it was freeze-drying. Yield: 90.5%; DS: 33.8% (Table 1).

2.5 Synthesis of NNC

0.6 g TMC was dissolved in 30 mL N,N-dimethylformamide (DMF) in 70 °C with stirring until homogenous. Then the solution was cooled to 0 °C, and 0.6 g Et3N was added. Next, a solution of 0.86 g p-toluenesulfonyl chloride in 10 mL DMF was added dropwise. After stirred at 0 °C for 11 h under nitrogen atmosphere, the reaction mixture was poured into excess acetone and the 6-tosyl-2-trimethyl
quaternary ammonium salt chitosan (TOSTMC) was precipitated easily. Then the precipitation was filtered off and washed carefully with enough acetone. The production was dried at 60 °C for 24 h.

0.2 g TOSTMC was added into 20 mL ethylenediamine at room temperature. Then the reaction was refluxed gently with stirring at 60 °C for 16 h. The mixture was poured into excess acetone, and the precipitation was filtered off, washed carefully with acetone. After dialyzed against deionized water for 2 days, the product was freeze dried. Yield: 93.62%; DS: 59.7% (Table 1).

**Scheme 1.** Synthetic pathway for TMC, NNC, and ONC.

### 2.6 Antibacterial assay

Antifungal assay was evaluated against *B. cinerea Pers*, *Gibberella zeae*, and *P. asparagi*. in vitro by measuring the growth rate of mycelium according to the method of Guo et al.[46] Briefly, the compounds (chitosan, TMC, NNC, and ONC) were dissolved in distilled water at a concentration of 5 mg/mL at room temperature. Then, the test sample solution was added to the sterilized potato dextrose agar (PDA) medium to get a final concentration of 0.1, 0.5, and 1.0 mg/mL respectively, and poured into the sterilized Petri dishes (9.0 cm). Identical volume distilled water substituting samples were poured into control plates. Finally, the fungi mycelia disk with a diameter of 5.0 mm was placed into the center of the PDA Petri dishes and incubated at 27 °C for 2-3 days. When the diameter of the fungi mycelium was reached to the edges of the control plate (without the sample), the inhibitory index was calculated as follows:
Inhibitory index (%) = \((1 - \frac{Da}{Db}) \times 100\)

where \(Da\) is the diameter of the growth zone in the test plates, and \(Db\) is the diameter of the growth zone in the control plate. The experiments are performed three times. And all the data are averaged and expressed as means ± SD.

The Scheffe’s multiple range test, a single-step multiple comparison procedure in analysis of variance, which was applied to the set of estimates of all possible contrasts among the factor level means, was used to evaluate the inhibitory indices differences in antifungal tests. The level of \(P < 0.05\) was considered statistically significantly.

3 Results and Discussion

3.1 Structure of the chitosan derivatives

The synthetic procedures of amine chitosan derivatives are shown in Scheme 1. Each step of the syntheses was followed by FT-IR or \(^1\)H NMR spectroscopy. The FT-IR spectra (thin film) of chitosan, TMC, TOSTMC, NNC, and ONC are shown in Figure 1, respectively. The spectrum of chitosan showed that saccharide mainly contained the following characteristic bands: \(\nu (O-H) \text{ or } \nu (N-H) 3428.81 \text{ cm}^{-1}\), \(\nu (C-H) 2927.41 \text{ cm}^{-1}\), \(\nu (O=C-NH \text{ I band}) 1635.34 \text{ cm}^{-1}\), \(\delta (C-H) 1423.21, 1384.64 \text{ cm}^{-1}\), \(\nu (O=C-NH \text{ III band}) 1261.22 \text{ cm}^{-1}\), \(\nu (C-O) 1072.23 \text{ cm}^{-1}\), and 898.67 cm\(^{-1}\) indicated the \(\beta\) glycosidic bond. After trimethyl quaternized at \(C_2-N\) position, a new peak of TMC appeared at 1469.49 cm\(^{-1}\) for C-H of trimethyl quaternary ammonium salt. In TOSTMC spectra, the absorption position of –SO\(_2\) was at about 1176.37, 1114.65, and 1380.78 cm\(^{-1}\), and 1481.06 cm\(^{-1}\) indicated C-H of trimethyl quaternary ammonium salt. Moreover, TOSTMC had new peaks at about 817.67 and 556.97 cm\(^{-1}\) corresponding to the benzene groups with 1,4-substitution. When the
tosyl group was substituted by the aminoethyl group, the new peak at 1600.63 cm\(^{-1}\) in NNC spectra was a typical absorption of primary amine, and the peaks of the tosyl group was disappeared. Besides, 1477.21 cm\(^{-1}\) meant the absorption of N-CH\(_3\). Meanwhile, in ONC spectra, new peaks at 2884.99 cm\(^{-1}\), 1608.34 cm\(^{-1}\), and 1072.23 cm\(^{-1}\) appeared compared with chitosan, which were assigned to the vibration of the -CH\(_2\), -NH\(_2\) and C-O, respectively, and 1461.78 cm\(^{-1}\) indicated N-CH\(_3\). Above results demonstrated preliminarily that chitosan derivatives were obtained respectively.

**Figure 1.** FT-IR spectra of chitosan, TMC, TOSTMC, NNC, and ONC.

Figure 2 showed the \(^1\)H NMR spectra of chitosan, TMC, NNC, and ONC, respectively. The position of each group absorption peaks was marked in the Figure 2 respectively. It was reported that the protons of glucose skeleton of chitosan were at about 5.12 to 3.81 ppm. As far as TMC, NNC, and ONC, the new peaks about 3.3 or 3.2 ppm was assigned to N-CH\(_3\). In \(^1\)H NMR spectrum of NNC, the new peaks appeared at about 3.1 ppm, 2.63 ppm, and 1.97 ppm were assigned to -NH, -NHCH\(_2\), -CH\(_2\)NH\(_2\), and -NH\(_2\), respectively. Besides, the new chemical shift at 3.44 ppm, 2.85 ppm, and 2.06 ppm was assigned to the protons of -OCH\(_2\), -CH\(_2\)NH\(_2\), and -NH\(_2\), respectively. These data further indicated that chitosan derivatives were successfully synthesized.

**Figure 2.** \(^1\)H NMR spectra of NNC, ONC, and TMC.

### 3.2 Antifungal activity

It was reported that the antifungal activity of water-soluble chitosan was limited. In this paper, all chitosan derivatives were synthesized successfully to improve the antifungal activity of chitosan remarkably. All aminoethy trimethyl quaternary chitosan derivatives were prepared as aqueous solutions at the concentration of 0.1 to
1.6 mg/mL. Here, we tested water-soluble chitosan, TMC, NNC, and ONC against three common plant-threatening fungi, *B. cinerea* Pers., *Gibberella zeae*, and *P. asparagi*. The results were shown in Figure 3-5.

Figure 3 showed the inhibitory indices of chitosan, TMC, NNC, and ONC against *B. cinerea* Pers. According to the graph, we concluded the results as follows: Firstly, all the samples inhibited the growth of *B. cinerea* Pers, and the inhibitory indices enhanced with increasing concentration. Besides, Compared with chitosan, all derivatives had better antifungal activity. The inhibitory indices of chitosan, TMC, NNC, and ONC were listed as 22.8%, 80.2%, 90%, and 82.4% at 1.0 mg/mL, respectively. It was apparent that TMC, NNC, and ONC, which all had trimethyl quaternary groups, had better antifungal ability than chitosan. The above were in accord with the conclusion that the higher positive charge density in TMC, NNC, and ONC could contribute to the antifungal action, which might be related to the disruption of the charge balance on the surface of the cell.[47, 48] It was reported that there were some anionic components on the cell surface, such as glucan, mannan, protein and lipid and so on. And the positive charge would interact with these anionic components to form an impervious layer around the cell. On the one hand, the impervious layer could prevent the essential nutrients from entering the cell. On the other hand, the interaction might damage the cell wall, which would cause the cell death because of the leakage of cell constituents.[49, 50] Furthermore, the antifungal activity of NNC and ONC were better than TMC, which might be due to the introduction of aminoethyl functional groups. In the antimicrobial mechanism, there
was a "permeability" point of view that the oil film outside the cell wall only allowed lipid soluble substances to pass, so the lipophilic characteristic of aminoethyl might be other reason to explain the mechanism of the increased antifungal activity.[51] Furthermore, the antifungal ability of NNC was a little better than ONC, which was possibly because of the electronegativity of N element is weaker than O element. And N element lone pair electron as an electron donor at the C\textsubscript{6}-N position might interact with the cationic component, which might break the balance of the ion pump on the cell surface and led to the cell death.

Besides, the antifungal property of chitosan and chitosan derivatives against *Gibberella zeae* and *P. asparagi* were shown in Figure 4 and 5, respectively, which were similar to the antifungal activity against *B. cinerea Pers.* Firstly, the inhibitory indices of all samples were mounted up with increasing concentration. Secondly, the inhibitory indices of chitosan, TMC, NNC, and ONC against *Gibberella zeae* were 31.4%, 70.3%, 80.5%, and 75.6% at 10mg/mL, respectively. And the inhibitory indices of chitosan, TMC, NNC, and ONC against *P. asparagi* were 30.4%, 80.3%, 87.3%, and 84.6%, respectively. It could conclude that all the samples showed antifungal activities against *Gibberella zeae* and *P. asparagi*. And compared with natural chitosan, three chitosan derivatives get much stronger antifungal activity. Besides, the inhibitory indices of NNC and ONC were higher than TMC at the same concentration, and NNC was also better than ONC at against fungi *Gibberella zeae* and *P. asparagi*. The results further concluded that the introduction of trimethyl quaternary ammonium salt at C\textsubscript{2}-NH\textsubscript{2} and aminoethyl groups at C\textsubscript{6} position of
chitosan would increase the antifungal activities of chitosan derivatives.

According to the results mentioned above, the antifungal activities of products against *Gibberella zeae*, *P. asparagi*, and *B. cinerea Pers.* were almost in order of NNC > ONC > TMC > chitosan at 1.0 mg/mL, which could conclude that the antifungal ability might associate with the density of the positive charge and the aminoethyl groups. Further comprehensive investigation to ascertain the antifungal mechanism and the structure-activity relationship would be studied in the future.

**Figure 3.** The antifungal activity of chitosan, TMC, NNC, and ONC against *B. cinerea Pers.*

**Figure 4.** The antifungal activity of chitosan, TMC, NNC, and ONC against *Gibberella zeae*.

**Figure 5.** The antifungal activity of chitosan, TMC, NNC, and ONC against *P. asparagi*.

### 4 Conclusions

In this paper, aminoethyl chitosan quaternary ammonium salts were successfully designed and synthesized. The antifungal activities against three kinds of phytopathogens were estimated by hyphal measurement *in vitro*. All chitosan derivatives exhibited higher inhibitory indices than water-soluble natural chitosan. On the one hand, it would be reasonable to assume that the positive charge would interact with the anionic components of the cell wall, which would break the balance of the cell wall component and ultimately lead the death of the cell. On the other hand, based on the point the "permeability", aminoethyl with lipophilic characteristic might
pass through the oil film outside the cell wall into the cell, which might be another reason to explain the mechanism of the increased antifungal activity. Further comprehensive investigation to the mechanism of the antifungal activity and the structure-activity relationship need to be developed in the future.

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Conflict of interest statement

The authors have declared no conflicts of interest.

References


346, 121-126.


Figure legends

Scheme 1. Synthetic pathway for TMC, ONC, and NNC.

Figure 1. FT-IR spectra of chitosan, TMC, TOSTMC, NNC, and ONC.

Figure 2. $^1$H NMR spectra of TMC, NNC, and ONC.

Figure 3. The antifungal activity of chitosan, TMC, NNC, and ONC against *B. cinerea* Pers.

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\[\text{Inhibitory Index (\%)}\]
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Figure 5. The antifungal activity of chitosan, TMC, NNC, and ONC against *P. asparagi.*
Table 1. The elemental analyses, yields, and the degrees of substitution of chitosan derivatives.

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<th>Degrees of Substitution (%)</th>
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