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Design, synthesis and antimicrobial activity of 6-*N*-substituted chitosan derivatives

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ABSTRACT

Three novel 6-*N*-substituted chitosan derivatives were designed and synthesised and characterized by FTIR and NMR. The degree of substitution was calculated by elemental analysis results. The antimicrobial activities of the target compounds were evaluated by twofold serial broth dilution method and poisoned food technique. The antifungal activities of 6-aminoethylamino-6-deoxy chitosan (**3**), 6-butylamino-6-deoxy chitosan (**4**) and 6-pyridyl-6-deoxy chitosan (**5**) were significantly increased against *Rhizoctonia cerealis*, *Fusarium oxysporum* and *Botrytis cinerea*, and the inhibition rate ranged from 22.48% to 63.56% at the concentration of 0.2 mg/mL. The compound **3** had better antibacterial activities than chitosan, and the minimum inhibition concentration of which ranged between 6.25 and 25 mg/L against gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis* and *Bacillus anthracis*) and gram-negative bacteria (*Escherichia coli*, *Salmonella typhi*). The antibacterial activities of 6-*N*-substituted chitosan tended to increase with the increase of the number of $-NH_2$ group.

Chitosan (CS), as a kind of polysaccharide produced by partially or fully deacetylated of chitin, is the component of the cell wall of fungi, the exoskeleton of arthropods and insects and the biomass is just next to cellulose. Chitosan consists of primarily of 2-amino-2-deoxy-D-glucopyranose unit linked by β-(1-4) linkage, and small amount of N-acetyl-D-glucosamine residues. Chitosan has been found important and wide application in agriculture, medicine, food, cosmetics, also in antimicrobial field.[1] Many researches showed chitosan had antimicrobial activities on bacteria,[2] postharvest pathogens[3–5], plant phytopathogenic fungi[6,7], yeast[8] and virus.[9] Although chitosan is nontoxic, biocompatible and biodegradable, the insolubility in aqueous solution and lower antimicrobial activities than chemical biocides restrict its further application and development. It is generally believed that modifying the structure of CS is a helpful method to improve the antimicrobial activity. Since 2-NH_2 had higher reaction activity than 3-OH and 6-OH of CS, many chemical moiety was grafted on 2-NH_2 to get novel structure and their bioactivities were tested.[10–14] Furthermore, scientists contend that the amino protonated groups of chitosan are influence factors contributed to the antimicrobial activities of chitosan. The antimicrobial action was likely caused by the electrostatic interaction between $-NH_2$ of chitosan and phosphoryl groups of phospholipid components of cell membranes.[10,11,15,16] More amino groups or positive electronic groups may improve the antimicrobial activities of CS. Some tests have proved the thinking is reasonable by quaternizing modification of chitosan.[2,12,13] The 6-amino-6-deoxy chitosan derivatives which showed higher antibacterial activities were designed and synthesised with highly water solubility and higher positive surface charge than chitosan.[15]

On the bases of above hypothesis, for the sake of revealing the antimicrobial contributions of 2-NH_2 and 6-*N*-substitution of chitosan, we design and synthesize 6-aminoethylamino-6-deoxy chitosan (**3**), 6-butylamino-6-deoxy chitosan (**4**) and 6-pyridyl-6-deoxy chitosan (**5**), and test their antimicrobial activities.

Three 6-*N*-substituted chitosan derivatives were synthesised by multistep reactions (Scheme 1). The active amine of chitosan was protected by phthaloylation to give *N*-phthaloyl chitosan (**1**). Then the 6-bromo-6-deoxy-*N*-phthaloyl-chitosan (**2**) was obtained by nucleophilic substitution reaction with organonitrogen compound (1,2-diaminoethaneanhydrous, butylamine and pyridine). The target products (**3**–**5**) were obtained via deprotection of nucleophilic substituted products.

Phthaloylation of chitosan is an ideal and classic method to protect amine group. *N*-phthaloyl chitosan (NPC) was prepared

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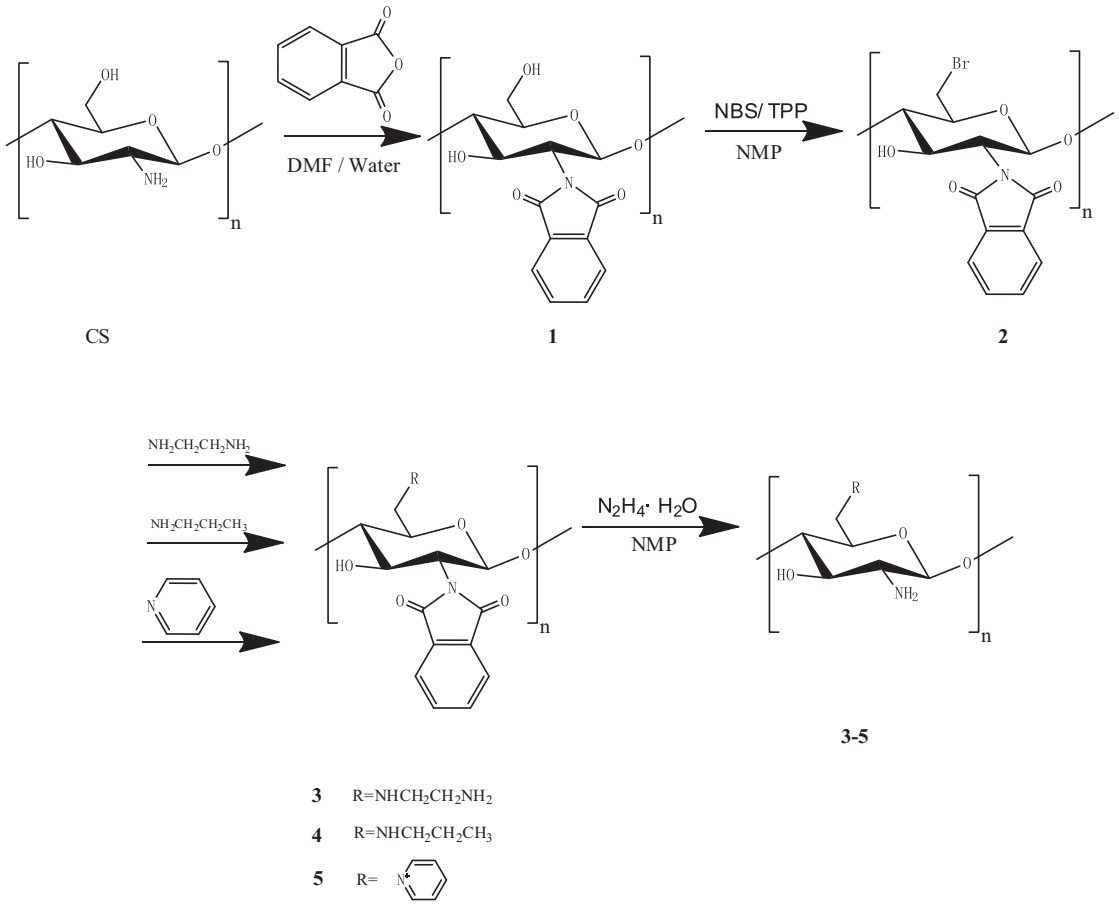

Scheme 1. Synthesis of 6-*N*-substituted chitosan derivatives.

according to Kurita's modified method.[15,16] Chitosan was added to a solution of 3 equiv of phthalic anhydride in *N*,*N*-dimethylformamide (DMF) containing 5% (v/v) water, and the mixture was heated in nitrogen at 120 °C for 8 h with stirring. Then the resulting pale mixture was cooled to room temperature and poured into ice water. The mixture was filtered, and the precipitate was washed with methanol, then dried to give *N*-phthaloyl chitosan (**1**).

Deoxyhalogenation of the C-6 positions of *N*-phthaloyl chitosan was conducted according to the literature method.[16] NPC was dispersed in *N*-methyl-2-pyrrolidone (NMP) and stirred consequently. Three equivalents of *N*-bromosuccinimide (NBS) and triphenyl phosphine (TPP) were added to the reaction solution in an ice/water bath, then stirred at 80 °C for 2 h. All mixture was poured into EtOH, and filtered. The precipitate was washed with EtOH untill the filtrate become colorless. After lyophilizing, the bromodeoxy derivative was obtained as 6-bromo-6-deoxy-*N*-phthaloyl-chitosan (**2**).

The typical procedure to prepare 6-*N*-subsituted-6-deoxy chitosan was as follows: 6-bromo-6-deoxy-*N*-phthaloyl-chitosan was dispersed in isopropanol added ethylendiamine. The mixture was stirred at 80 °C for 24 h. the product was dispersed in mixture of NMP and hydrazine hydrate, and stirred at 100 °C for 4 h. After cooling to room temperature, four times of EtOH was poured into the mixture. The precipitates were collected by centrifugation, and then washed with EtOH. After lyophilizing, a light yellow powder was obtained (**3**). The compound **4** (orange powder) and compound **5** (gray powder) were prepared by the same method.

The structure characters of the compounds were characterized by FTIR, ^{1}H NMR and elemental analysis. FTIR of chitosan and its derivatives are shown in Figure 1. The absorption band of compound **2** at wavenumbers 724, 799 and 876 cm^{-1} were corresponded to the benzene ring. And the absorption bands at 1777 and 1711 cm^{-1} were assigned to the carbonyl group of amide. The absorption band of compound **2** at wavenumber 534 cm^{-1} due to the C-Br group. The above characteristic absorption peaks disappeared or weakened in the FTIR spectrum of **3**, **4** and **5**, which means the nucleophilic substitution reaction of 6-Br of 6-bromo-6-deoxy-*N*-phthaloyl-chitosan and the deprotection of $-NH_2$ were processed.

The ^{1}H NMR of compounds **3**–**5** were shown in Figure 2. CS exhibited a single peak at 1.8 ppm (H-3), a single peak at 2.92 ppm (H-2), multiple peaks at 3.3–3.9 ppm (H-3 to H-6), and multiple peaks at 4.5 ppm (H-1). The spectrum of compound **3**–**5** contained all the peaks of CS. The spectrum of compound **3** showed a new peak at 3.1 ppm originated from the proton of $-NH-CH_2-CH_2-NH_2$, confirming again the successful grafting of ethylendiamine onto CS. A new peaks of compound **4** appeared at 3.2 ppm representing the protons of methylene of C-6, new peaks at 0.67 ppm, 1.13 ppm, 1.42 ppm and 2.14 ppm originated from the proton of methyl and methylene of *n*-butyl, confirming the structure of compound **4**. The peak signal of 6-CH_2 moved to 3.2 ppm in the spectrum of compound **5**. Signals appeared at 7.3 ppm, 7.8 ppm, 8.3 ppm and 8.6 ppm showed the protons peaks of pyridine ring, which confirming the successful synthesis of compound **5**.

The degree of substitution of CS derivatives was detected by elemental analysis. The degree of substitution of compound **3**, compound **4** and compound **5** were 48.5, 87.26 and 61.53, respectively (Table 1).

The in vitro antibacterial activities of chitosan and the synthesised compounds were carried out using three gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis* and *Bacillus anthracis*)