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Note

Synthesis of acyl thiourea derivatives of chitosan and their antimicrobial activities in vitro

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Abstract—Three different acyl thiourea derivatives of chitosan (CS) were synthesized and their structures were characterized by FT-IR spectroscopy and elemental analysis. The antimicrobial behaviors of CS and its derivatives against four species of bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Sarcina*) and four crop-threatening pathogenic fungi (*Alternaria solani*, *Fusarium oxysporum f.* sp. *vasinfectum*, *Colletotrichum gloeosporioides* (*Penz.*) *Saec*, and *Phyllisticta zingiberi*) were investigated. The results indicated that the antimicrobial activities of the acyl thiourea derivatives are much better than that of the parent CS. The minimum value of MIC and MBC of the derivatives against *E. coli* was 15.62 and 62.49 μg/mL, respectively. All of the acyl thiourea derivatives had a significant inhibitory effect on the fungi in concentrations of 50–500 μg/mL; the maximum inhibitory index was 66.67%. The antifungal activities of the chloracetyl thiourea derivatives of CS are noticeably higher than the acetyl and benzoyl thiourea derivatives. The degree of grafting of the acyl thiourea group in the derivatives was related to antifungal activity; higher substitution resulted in stronger antifungal activity.

Keywords: Antibacterial activity; Antifungal activity; Chitosan; Acyl thiourea derivatives

Chitosan (CS) is the N-deacetylated product of chitin and an attractive material because of its properties such as immunological activity, wound healing, biocompatibility, low toxicity, and biodegradability.[1–6] The antimicrobial properties of chitosan and its derivatives have been widely explored.[7–9] Studies have shown that the antimicrobial activity of chitosan depends on its molecular weight, deacetylation degree, pH of chitosan solution, and the target organism.[10–13] To improve the antimicrobial activity of chitosan, other researchers have prepared derivatives such as *N*,*N*-dimethyl-chitosan, *N*,*N*,*N*-trimethyl-chitosan iodide, and other quaternary ammonium salts of chitosan, as well as Schiff-base and sulfanilamide derivatives of chitosan.[14–19]

Thioureas have strong antifungal activities that are comparable to the activity observed for the common antifungal antibiotic ketoconazole.[20,21] Moreover, they have antibacterial and insecticidal properties.[22,23] Eweis et al.[24] have prepared a benzoyl thiourea derivative of chitosan and studied its antifungal efficacy against sugar-beet pathogens, and showed that the antifungal activity of the derivative was much better than that of native chitosan.

Although many chitosan derivatives showed higher antimicrobial activities than native chitosan, the antimicrobial activities of these materials are still lower than that of the antimicrobial agents currently used. As part of our search for new types of chitosan derivatives with much higher antimicrobial activity, we describe here the preparation of acetyl, chloracetyl, and benzoyl thiourea derivatives of chitosan (ATUCS, CATUCS, and BZTUCS, Fig. 1). The antimicrobial activities of these materials against four bacterial species (*Escherichia coli*,

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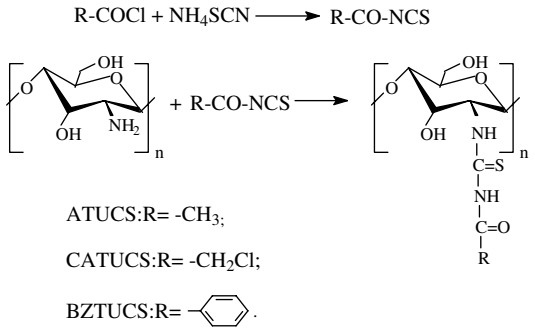

Figure 1. Synthesis of acyl thiourea derivatives of chitosan.

Pseudomonas aeruginosa, *Staphylococcus aureus*, and *Sarcina*) and four crop-threatening pathogenic fungi (*Alternaria solani*, *Fusarium oxysporum f.* sp. *vasinfectum*, *Colletotrichum gloeosporioides* (*Penz.*) *Saec*, and *Phyllisticta zingiberi*) are also described.

Figure 2 presents a comparison of the transmission FT-IR spectra for ATUCS, CATUCS, and BZTUCS with the parent CS. As for as the FT-IR spectra of ATUCS and CS are concerned, first, an obvious change between 3500 and 3200 cm^{-1} due to the O–H and N–H group stretching vibration is observed. In addition, the characteristic absorbance of $–NH_2$ at 1600 cm^{-1} disappeared; these results show that $–NH_2$ group had reacted with acetyl thiocyanate. Second, new peaks at ~1736 cm^{-1} and 1657 cm^{-1} appeared in the spectra of ATUCS, which is the characteristic absorbance of the C=O group in a secondary acylamide. Third, there are new strong peaks at 1519.97 cm^{-1} and 1076.13 cm^{-1} in the ATUCS spectra, which were assigned to the characteristic absorbance of –NH and C=S. All of the above results support the structure of ATUCS. In the spectrum of CATUCS, new peaks appeared at 1733 cm^{-1} (C=O), 1657 cm^{-1} (C=O), 1517 cm^{-1} (N–H), and 1077 cm^{-1} (C=S), which showed that CATUCS was obtained. For the same reason, new peaks at 1663 cm^{-1} (C=O), 1517 cm^{-1} (N–H), 1065 cm^{-1} (C=S), 1544 cm^{-1} (phenyl), and 795 cm^{-1} (phenyl) indicated that BZTUCS was formed.

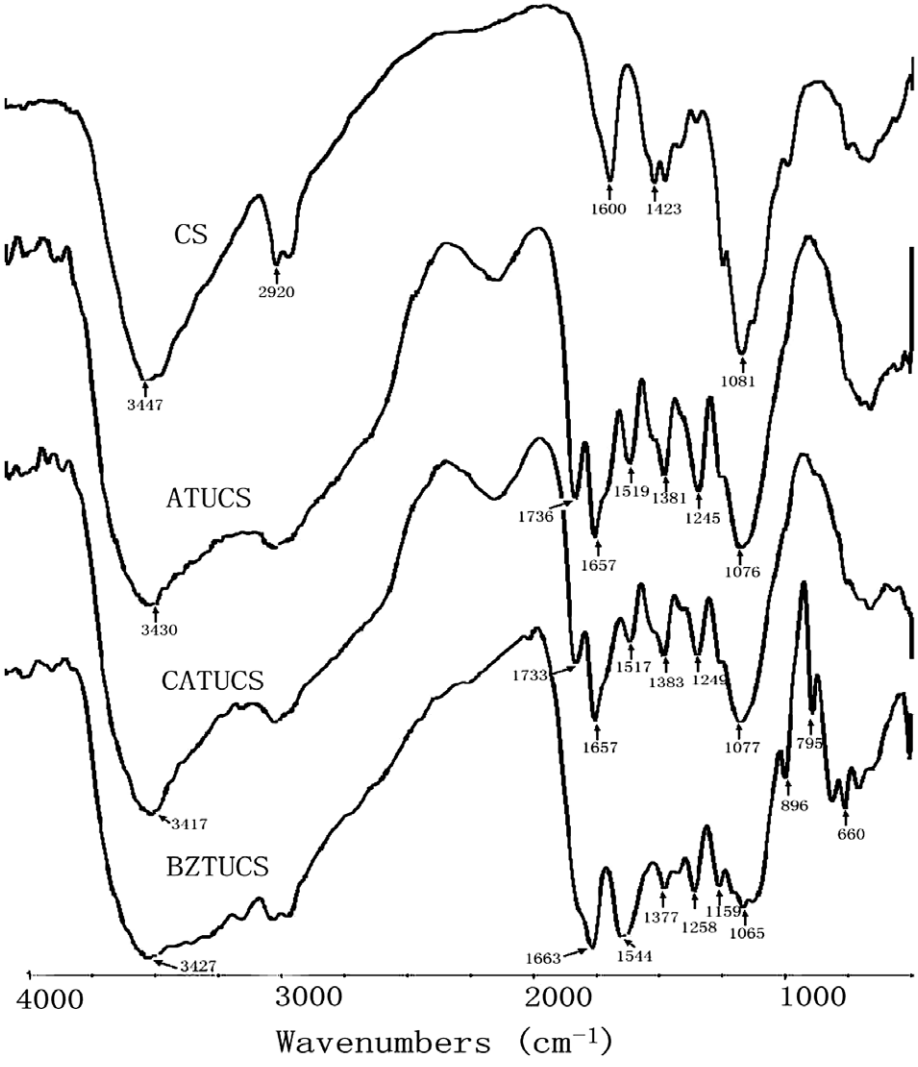

Figure 2. FT-IR spectra of chitosan and its acyl thiourea derivatives.

Table 1. Yield, elemental analysis, and grafting degree of chitosan derivatives

Compound	Yield (%)	Elemental analysis (%)				Grafting degree (%)
		C	N	H	S	
CS	—	44.28	8.52	7.36	—	—
ATUCS	75	43.40	10.01	6.22	10.76	89.2
CATUCS	81	38.92	9.00	5.26	9.83	91.5
BZTUCS	71	50.93	8.62	5.46	8.82	81.0

The results of elemental analysis, yield, and the grafting degree of the derivatives are listed in Table 1. From Table 1, the yield of ATUCS, CATUCS, and BZTUCS was 75%, 81%, and 71%, respectively. The elemental analysis results indicate that the substitution degree of ATUCS, CATUCS, and BZTUCS is ~89.2%, 91.5%, and 81.0%. The procedure used in this work for the preparation of BZTUCS is different from that of Eweis et al.[24] The toxicity of the solvent used in our experiment (dimethylformamide and acetic acid) was lower than that of their works (acetonitrile). The sulfur content (8.82%) is slightly higher than that of the BZTUCS (8.25%) prepared by Eweis et al., and so we speculate that the substitution degree of acyl thiourea group is also higher.

The antibacterial activity of ATUCS, CATUCS, and BZTUCS was compared with that of CS. Table 2 shows the MIC and MBC of the derivatives against various microorganisms. The results indicate that all of the derivatives showed strong antibacterial activity against the tested bacteria, and the minimum value of MIC and MBC of the derivatives against *E. coli* was 15.62 and 62.49 μg/mL, respectively. Moreover, the antibacterial activities of ATUCS, CATUCS, and BZTUCS are significantly higher than that of CS. In addition, the antibacterial activity of the derivatives against *E. coli* and *P. aeruginosa* was more effective than against *S. aureus* and *Sarcina*, so we can conclude that the derivatives showed stronger antibacterial activity against Gram-negative than Gram-positive bacteria.

This observation may be attributed to their different cell walls. *E. coli* and *P. aeruginosa* are typical Gram-negative bacteria, the cell wall of which is made up of a thin membrane of peptidoglycan and an outer membrane constituted of lipopolysaccharide, lipoprotein, and phospholipids.[25] Chitosan and its derivatives with large molecular weight can be expected to coat the cell surface and prevent the leakage of intracellular components. Helander et al.[12] have shown that chitosan disrupted the barrier properties of the outer membrane of Gram-negative bacteria, as chitosan is protonated at acidic conditions and the carboxyl and phosphate groups of the bacterial surface are anionic and offer potential sites for electrostatic binding. The C=O, C=S, and NH groups in the acyl thiourea derivatives of chitosan can be protonated under acidic conditions, so they can react with the carboxyl and phosphate groups of the bacterial surface and therefore show antibacterial activity against Gram-negative bacteria. On the other hand, *S. aureus* and *Sarcina*, typical Gram-positive bacteria, have cell walls composed solely of peptidoglycan, which does not allow the formation of a surface layer.

A. solani is the causal agent of early blight disease of tomato. Epidemics induced by this economically important pathogen can cause severe tomato seeding defoliation in areas with high humidity and frequent night dew. *F. oxysporum f.* sp. *vasinfectum* can result in cotton fusarium wilt, which is highly destructive and economically limiting to the production of good quality cotton. *C. gloeosporioides* (*Penz.*) *Saec* can cause anthracnose, which is a major post-harvest disease that affects many fruits and crops. *P. zingiberi* is the causal fungi of black spot disease in crops. It mainly appears on the leaves of crops leading to the formation of holes, which in serious cases can cause death.

The antifungal activities of these CS derivatives against *A. solani*, *F. oxysporum f.* sp. *vasinfectum*, *C. gloeosporioides* (*Penz.*) *Saec*, and *P. zingiberi* are shown in Table 3. These data indicate that the derivatives had effective activities against these fungi, with inhibitory indices ranging from 31.23% to 66.67% at 500 μg/mL. The inhibitory index of all of the compounds enhanced with an increase in concentration, and the highest antifungal activity was observed at 500 μg/mL. Moreover, the antifungal activities of CATUCS are noticeably higher than those of ATUCS and BZTUCS, which may be due to the presence of the chlorine atom in CATUCS. The chloro-group is used in many fungicides such as pentachloronitrobenzene and chlorothalonil.[26] However, these fungicides have pronounced toxicities and their residues in the environment have been serious problems. When these groups are grafted onto CS, they might be released slowly and may induce lower pollution to the environment.[27] As a final trend, a higher grafting degree of the acyl thiourea group in these derivatives resulted in stronger antifungal activity.

Table 2. MIC and MBC values of chitosan derivatives against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Sarcina*

Samples	MIC/MBC (μg/mL)			
	E. coli	*P. aeruginosa*	*S. aureus*	*Sarcina*
CS	62.49/124.99	62.49/249.98	249.98/499.95	124.33/249.98
ATUCS	15.62/62.49	62.49/124.99	62.49/124.99	62.49/124.99
CATUCS	15.62/62.49	15.62/124.99	62.49/124.99	62.49/124.99
BZTUCS	15.62/62.49	62.49/124.99	62.49/124.99	62.49/124.99

Table 3. Inhibitory indices of the chitosan derivatives against *Alternaria solani*, *Fusarium oxysporum f.* sp. *vasinfectum*, *Colletotrichum gloeosporioides* (*Penz.*) *Saec*, and *Phyllisticta zingiberi*

Samples	Concentration (μg/mL)	*A. solani*	*F. oxysporum f.* sp. *vasinfectum*	*C. gloeosporioides* (*Penz.*) *Saec*	*P. zingiberi*
CS	50	0	0	0	0
	100	3.57	19.51	5	0
	500	20.86	39.02	10	18.75
ATUCS	50	14.28	31.70	20	16.67
	100	21.43	40.78	30	25.33
	500	42.86	58.54	40	46.67
CATUCS	50	21.42	32.39	21.78	28.57
	100	27.86	46.82	48.33	33.58
	500	53.57	60.98	66.67	57.89
BZTUCS	50	13	14.63	8.33	11.05
	100	20	19.51	21.11	24.58
	500	32.14	48.78	31.23	36.84

1. Experimental

1.1. Materials and methods

Chitosan was supplied by Qingdao Baicheng Biochemical Corp. (China). Its deacetylation is 96% and its average molecular weight is 50 kDa. All other chemicals and reagents used were of analytical grade, and were used without further purification. The crop-threatening pathogenic fungi (*A. solani*, *F. oxysporum f.* sp. *vasinfectum*, *C. gloeosporioides* (*Penz.*) *Saec*, and *P. zingiberi*) and bacteria (*E. coli*, *P. aeruginosa*, *S. aureus*, and *Sarcina*) used for the antimicrobial assay were provided by Key Laboratory of Experimental Marine Biology (Institute of Oceanology, Chinese Academy of Sciences) and Qingdao Academy of Agricultural Sciences.

1.2. Analytical methods

Fourier transform infrared (FT-IR) spectra of the derivatives were measured in the 4000–400 cm^{-1} regions using a Nicolet Magna-Avatar 360 FT-IR spectrometer with samples prepared as KBr disks. Elemental analysis (C, H, N) was performed on a Carlo-Erba 1106 elemental analyzer and sulfur content was measured on a SC-132 sulfur meter (LECO). The average viscometric molecular weight of chitosan was estimated from the intrinsic viscosity determined in the solvent 0.1 M CH_3COOH–0.2 M NaCl using the Mark–Houwink parameter $\alpha = 0.96$, $K_\eta = 1.424$ at 25 °C when the intrinsic viscosity is expressed in mL/g.

1.3. Preparation of ATUCS, CATUCS, BZTUCS

Dry ammonium thiocyanate (NH_4SCN, 0.67 mol) was dissolved in CH_2Cl_2 (30 mL), then a CH_2Cl_2 solution containing 0.04 mol acetyl chloride, chloracetyl chloride, or benzoyl chloride was added. Polyethylene glycol-400 (PEG-400, 0.8 mL) was added dropwise as phase transfer catalyst. After stirring for 2 h at room temperature, the mixture of products was filtered through a Buchner funnel under reduced pressure. The filtrate (acyl thiocyanate) was added to a chitosan solution in dimethylformamide and acetic acid. The reaction medium was stirred at 100 °C for 5 h, cooled, and filtered. The yellowish white product was washed with methanol and dried at 50 °C.

1.4. Antifungal assays

Antifungal assays were performed based on the method of Jasso et al.[28] Briefly, the derivatives were dissolved in 1% (v/v) HCl. Then, a solution of each derivative (ATUCS, CATUCS, and BZTUCS) was added to the sterilized potato dextrose agar to give a final concentration of 50, 100, and 500 μg/mL. After the mixture was cooled, the mycelium of fungi was transferred to the test plate and incubated at 29 °C for 3 days. When the mycelium of fungi reached the edges of the control plate (without the added samples), the inhibitory index was calculated as follows:

$$\text{Inhibitory index } (\%) = (1 - D_a/D_b) \times 100,$$

where D_a is the diameter of the growth zone in the test plate and D_b is the diameter of growth zone in the control plate. Each experiment was performed three times, and the data were averaged. The Scheffe method was used to evaluate the differences in antifungal index in antifungal tests. Results with $P < 0.05$ were considered statistically significant.[29]

1.5. Antibacterial assays

The minimum inhibition concentration (MIC) of CS and its acyl thiourea derivatives was determined by a turbidimetric method.[30] In this method, a number of test tubes each containing 5.0 mL Muller–Hinton broth (MHB, Difco, England) were autoclaved for 20 min at

121 °C. The samples were accurately quantified and added to 1% HCl. To the first tube, 5.0 mL of chitosan or ATUCS (CATUCS or BZTUCS) (1 mg/mL) was added. After mixing, 5.0 mL of the mixture was transferred to the second tube, and similar transformations were repeated. Hence, each tube contained a test sample solution with half of the concentration of the previous one. The tubes were inoculated under aseptic conditions with 50 μL of the freshly prepared bacterial suspension. The blank control tubes were incubated at 37 °C for 24 h. The tubes were then studied for the visible signs of growth or turbidity. The lowest concentration of chitosan or its acyl thiourea derivatives that inhibited the growth of bacteria was considered as the minimum inhibitory concentration or MIC.

The minimum bactericidal concentration (MBC), or the lowest concentration of the samples that kills 99.9% of the bacteria, was determined by assaying the live organisms from those tubes that, in the MIC test, showed no growth.[31] A loopful from each of those tubes was inoculated on EMB (Eosin–Methylene Blue) agar, and signs of growth were monitored. The growth of bacteria demonstrates the presence of these germs in the original tube. In contrast, if no growth was observed, the original tube contained no live bacteria and the material was considered bactericidal at that concentration.

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