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Synthesis of novel N-acyl thiolated chitosan derivatives and their assessment for toxicity and antimicrobial activity *in vitro*

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ABSTRACT

Chitosan derivative was successively synthesized by initial preparation of acylthiourea reagent using ammonium thiocyanate and making it to react with at primary amino groups of chitosan and then reduced to yield thiolated chitosan. Due to the formation of disulfide bonds with mucus glycoproteins, mucoadhesiveness is augmented. The thiol groups were then quantified using Ellaman's reagent. The derivatives inherit good swelling property in neutral and alkaline media. The different derivatives containing thiol groups were formulated into tablets using reference drug for evaluation. The thiolated chitosan can guarantee prolonged controlled release of embedded therapeutic ingredients.

Key words: Thiolated Chitosan, acyl isothiocyanate, Isopropanolol, Mucoadhesion, Matrix tablet.

INTRODUCTION

Chitosan is devoid of pharmaceutical use because of poor or no water solubility and alkaline solubility is far impossible. Literature states that acylation of chitosan at amino position results in increase in its water solubility. Further, some chitosan conjugates with thiol containing moieties have shown swelling property in alkaline pH. The article deals with synthesis of some novel derivatives which contains thiol group obtained in stepwise reaction. The derivatives were prepared by applying a simple thiouride synthetic pathway which is then further reduced to form a Thiolated Chitosan derivative. The derivative shows a good mucoadhesion, water solubility, swelling in alkaline pH which can be used for controlled release for intestinal release. The derivatives retain the acid solubility inherited from the precursor. The derivatives thus have good pharmaceutical properties to be used as a polymer in intestinal delivery of drug.

MATERIAL AND METHODS

Materials

Chitosan (medium molecular mass: 400 KDa; degree of deacetylation: 83–85%) was purchased from Research Laboratories, Acylchlorides, ammonium thiocyanate, Elman's Reagent were obtained from Research Lab and aluminium isopropoxide was procured from SIGMA. All chemicals were of analytical grade. NMR analysis was performed at IIT (Powai).

Preparation of acylthiocyanate reagent

The solution of ammonium thiocyanate (0.011 mol) in dry acetone (25 ml) was prepared. Benzoyl chloride (0.01 mol) was added slowly in above solution with stirring. The reaction mixture was subjected to microwave irradiation for 3mins at 560 watts power to yield an acylthiocyanate reagent[1].

Synthesis of acylthiouride of chitosan

A solution of chitosan in 2%AcOH/MeOH (50 ml) was added slowly to the above solution so as to maintain reflux condition. After the addition was complete, the mixture was stirred for 90 min at room temperature, which is separated as solid precipitate on pouring in NaOH solution (pH 10).[2, 3]

Synthesis of Thiolated Chitosan derivative

The above precipitate was washed with acetone thoroughly to remove the traces of acylthiocyanate. The product was then treated with aluminium isopropoxide in isopropanaol which reduces the thioketone group from acylthiouride to form the thiol resulting in the formation of Thiolated Chitosan. The aluminium isopropoxide in isopropanaol solution is as specific reducing agent for ketones[4] (Fig. 1).

Procedure for Quantification of Sulfhydryl Groups

Prepare the dilution buffer (0.1 M Sodium phosphate, 1 mM EDTA, pH 8.0) and DTNB [(5,5'-dithio-*bis*-(2-nitrobenzoic acid)] working solution.

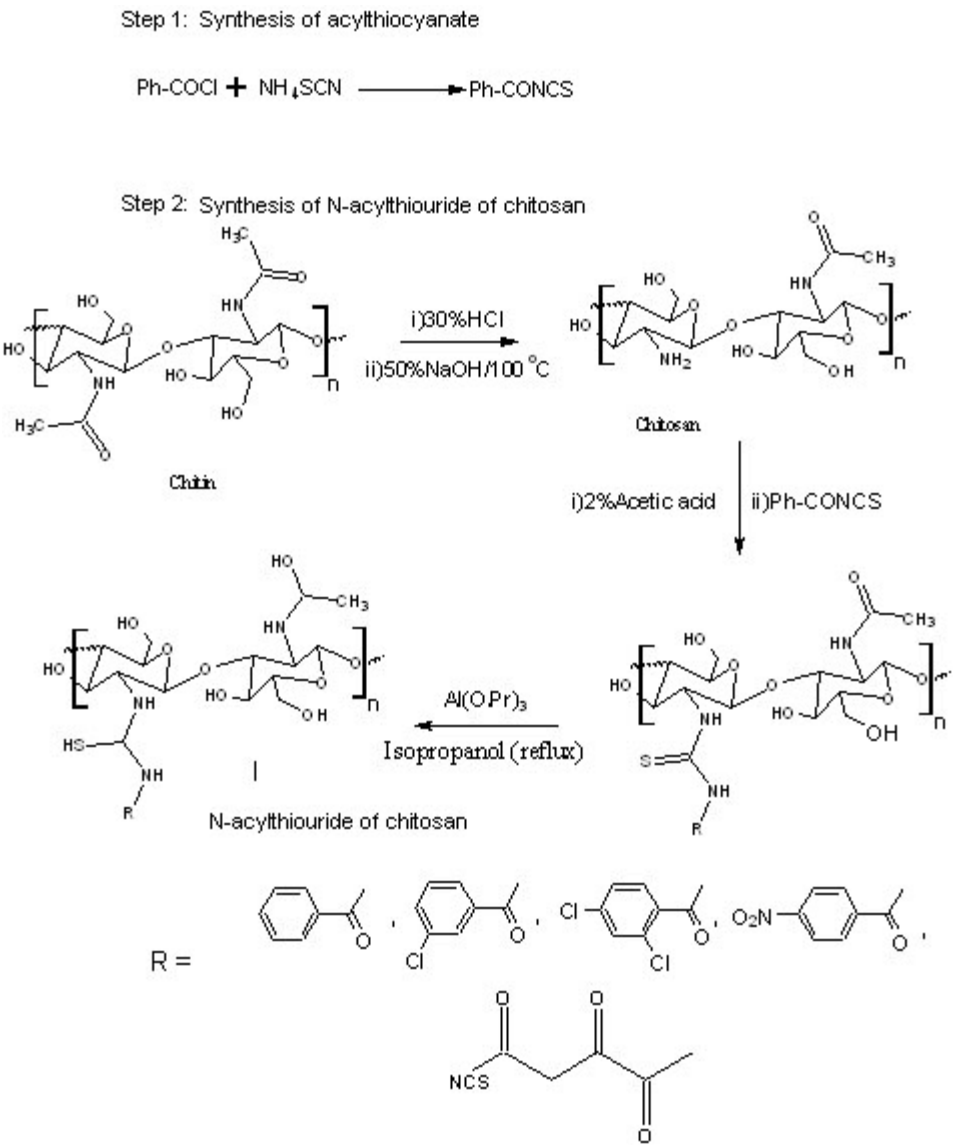


Fig. 1: Synthetic Scheme for Thiolated Chitosans

Table 1: Quantification of thiol group by Elman's reagent

S. No.	Sample*	Absorbance (412 nm)	Sulfhydryl content (moles)
1	TC1	0.3333	0.6608×10^{-7}
2	TC2	0.2341	0.462×10^{-7}
3	TC3	0.1611	0.3192×10^{-7}
4	TC4	0.1898	0.3752×10^{-7}
5	TC5	0.3113	0.6160×10^{-7}

*TC1: N-benzoylthiolurea derivative, TC2: N-(3-oxybenzoyl) thiolurea derivative, TC3: N-(2-chloro-4-oxybenzoyl) thiolurea derivative, TC4: N-(4-aminobenzoyl) thiolurea derivative TC5: N-Itaconoyl thiolurea derivative.

Store at +4°C. Dissolve 4mg of DTNB in 1 ml dilution buffer. Prepare a set of Sulfhydryl standard (Cysteine HCl) with sample dilution buffer (or distilled water). Dissolve Cysteine HCl (26.34 mg) to prepare 1.5 mM solution, then serial dilutions 1.25 mM, 1.0 mM, 0.75 mM, 0.5 mM and 0.25 mM and was used immediately. Add the following components to test tubes:

250 µl Sample/Standard
2.5 ml dilution buffer
50 µl DTNB reagent

The resulting solution was incubated for 15 minutes and absorbance was measured at the wavelength of 412 nm and concentration of

Table 2: The minimum Inhibitory Concentration (MIC) of the N-acyl Thiolated derivatives for Bacteria and Fungi

Organism	Concentrations in µg/ml				
	TCS1	TCS2	TCS3	TCS4	TCS5
E. coli.	20	30	20	35	15
C. albicans	225	150	200	175	125

Table 3: Dose level and response data for each animal

Compounds	No. of animal used (Rat)	Limit dose	Duration of effect	Mortality
Chitosan	1	2000 mg/Kg	0	NO
TCS1	1	2000 mg/Kg	0	NO
TCS2	1	2000 mg/Kg	0	NO
TCS3	1	2000 mg/Kg	0	NO
TCS4	1	2000 mg/Kg	0	NO
TCS5	1	2000 mg/Kg	0	NO

Sulfhydryl group was determined using Cysteine as standard solution. Molar extinction coefficient at 412 nm of DTNB is 14,150 $M^{-1}cm^{-1}$. [5, 6] (Table 1).

Evaluation of Chitosan derivatives

***In vitro* antibacterial assessment**

The antibacterial activity of the water soluble *N*-acyl crosslinked chitosan derivatives was studied by employing the Minimum Inhibitory Concentration (MIC) method. A MIC is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation.

In-vitro antifungal assessment

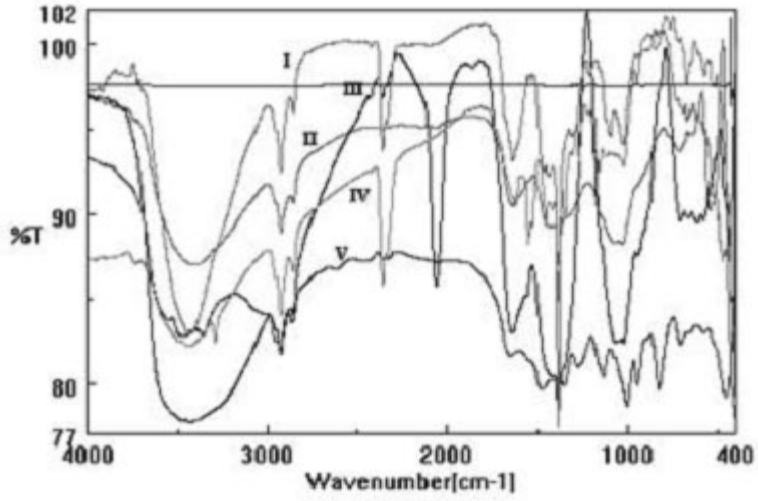
Briefly, the derivative was dissolved in water. Then, a solution of derivative was added to the sterilized sabrose dextrose agar to give a final

concentration of 10, 20, 30, 40, 50, 60 µg/mL. After the mixture was cooled, the mycelium of fungi was transferred to the test plate and incubated at 29 ^{0}C for 4 days. When the mycelium of fungi reached the edges of the control plate (without the added samples) the inhibited zone was observed.

Toxicity studies

OECD guideline for Testing of Chemicals: OECD/OCDE 423

Rats are fasted prior to dosing (food but not water is withheld for 3-4 hours). The fasted body weight of each animal is determined and the dose



I: N-benzoylthiolurea derivative, II: N-(4-aminobenzoyl) thiolurea derivative, III: N-(3-oxybenzoyl) thiolurea derivative, IV: N-itaconoyl thiolurea derivative, V: N-(2-chloro-4-oxybenzoyl) thiolurea derivative

Fig. 2: Ovelaid IR spectra for N-acyl Thiolated Chitosan derivatives

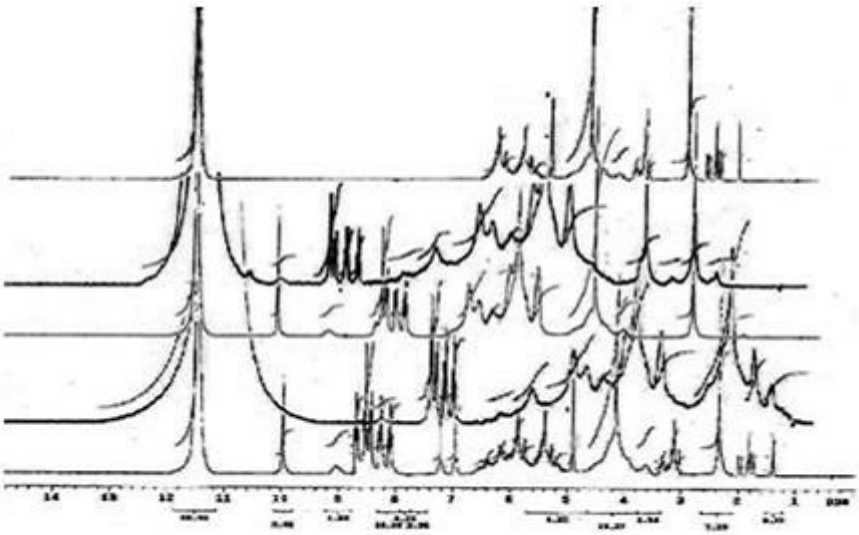


Fig. 3: Overlain NMR spectra for N-acyl Thiolated Chitosan derivatives

is calculated according to the body weight. The test substance is administered in a single dose by oral feeding needle. Dosing volume: not exceeded than 10ml/100g of body weight.

RESULTS AND DISCUSSION

Five thiolated derivatives were synthesized viz; N-Benzoyl thiolurea derivative, N-(3-oxybenzoyl) thiolurea derivative, N-(2-chloro-4-oxybenzoyl) thiolurea derivative, N-(4-aminobenzoyl) thiolurea derivative and N-Itaconoyl thiolurea derivative. The IR spectra (Fig. 2) and NMR spectra (Fig. 3) were assessed for structural confirmation.

Significant IR peaks

3435.56, O-H Stretch (H-bonded alcohols); 2064.42, C-O stretch overtone (aryl-alkyl ether); 1383.68, S-H stretch (aliphatic); 658.571, C-S Stretch.

Significant NMR peaks

5.067, tetrahydropyran methane; 4.414, 2 amine, 1 thiol of N-C(SH)-N ;4.161, tetrahydropyran methine C-O-, -N-C=O, -O from methane; 1.58, Thiol.

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