Synthesis and Preliminary Biological Studies of Novel Retinamine Derivatives Having a Phosphate Glucosamine Unit Functional Groups

Soung-Ryual Ryu[(1)], Ga-youn Ryu[(2)], Kyu-Yun Chae[(3)], Toshihiko Watanabe[(4)], Takeshi Mikami[(4)], Tatsuji Matsumoto[(4)]

[(1)]Department of Chemical Engineering, Dae Bul University, Chonnam 526-890, Korea
[(2)]Department of Chemistry, Sookmung women's University, Seoul 140-742, Korea
[(3)]Department of Chemistry, Won Kwang University, Iksan Chonbuk, Korea
[(4)]Department of Microbiology, Tohoku Pharmaceutical University, 4-4-1, Komatsushima, Aobaku, Sendai 981-8558, Japan.

E-mail: : ryusr@mail.daebul.ac.kr < S-R, Ryu>

Chitosan is widely used in agricultural and food-industries, cosmetics and medical fields. Special emphasis has been put on the chemical modification of chitosan to explore its full potential. We have descrive the synthesis and biological activity of novel retinamine derivatives. The retinamine derivatives were synthesized by introducing functional side chains into the monoglucosamine, triphosphated glucosamine etc. So, The poor aqueous solubility of retinamine derivatives hinders both pharmacological studies and pharmaceutical development. To make retinaminylglucosamine derivatives with improved biological effects and solubility, some attempts have been taken for consist of retinamine group like vitamin-A derivatives onto phosphated-glucosamine. The resulting substituted chitosan was characterized by solubility in various solvents, with enable facile characterized by ^{1}H-NMR, ^{13}C-NMR. These products were investigated for physical properties and bioactivities.

Key words: *retinamine chitosan, phosphated glucosamine, all-trans retinamine*

INTRODUCTION

As one of the most abundant natural resources, chitosan, (CTS), (poly-β-(1→4)-2-amino-2-deoxy-D-glucose) is obtained through partial deactylation of chitin, Recently CTS has attracted great attention since the range of its application has many expanded to medical, bioactivities [1-4]. But, because of its poor soluble ability in water, the use of chitosan is limited in many regions. And then many derivatives were synthesized in other to improve the solubility, and p-chitosan is the most important one. P-CTS is obtained by the reaction of chitosan and dietoxyphosphoryl chloride in nonpolar solvent under the present of argon. P-chitosan can dissolve in water and enlarge the fields of the use of chitosan.

The other hand, retinoids are natural and synthetic analogues of vitamin A that are involved in the regulation of several biological function such as cellular differentiation.

All-*trans*-retinoic acid (atRA), a hydrophobic anti-cancer drug, plays important roles in the regulation of proliferation and differentiation of epithelial tissues such as skin, bladder, lung, oral cavity, and mammary gland [5-7]. However, atRA is rapidly metabolized to inactive polar metabolites such all-trans-hydroxyl retinoic acid and all-trans-4-oxo-retinoic acid [8]. This rapid metabolism of atRA is due to the induction of the cytochrome P-450 by at RA [9]. To overcome such problem and to increase the therapeutic efficiency of atRA, various dosage forms have been reported such as liposomes and nanoparticles in this study, we aimed to synthesis of RA derivative coupled to water-soluble chitosan of RA-phosphate chitosan to afford RA-phosphate CTS as antibacterial agents. And its RA-phosphate chitosan derivatives were investigates their activity behavior at various

concentrations. The different states of RA-phosphated-chitosane derivatives were also determined using spectrum.

EXPERIMENTAL

Materials

Chitosan was isolate from crap was prepared in our laboratory as previously described method [9]. Briefly, chitin were treated with 40% NaOH at 100 °C for 2 h. filtered, and washed with water. The deacetylation procedure was repeated three more times to give chitosan. Other chemicals were purchased from Wako Chemical Co., Japan. Characterization of structural changes in chitosan and its derivatives were determined by the Nicolet 5DX FT-IR spectrophotometer and NMR experimental condition (at MNUCL): Spectrometer; Bruker Avance 400 NMR spectrometer. Solid-state nmr Prove : 4mm Double resonance MAS probe. Rotor: 4mm (o.d.) Zirconia with Kel-F cap. MAS rate: 13000 Hz for samples 2,3,4, and 5000 Hz.

Preparation of all-*trans*-retinoic-chitosan.

General procedure I: A Preparation of dietoxyphosphoryl chloride (DEPC).

Under an atmosphere of dry N_2, a stirred, in a mixture of Et_3N (100mg, 1.09 mmol, 2 equiv) and 30 ml of benzene was cooled to-10℃ in an ice/acetone bath and treated drop wise over 5 min with $POCl_3$ (0.10mL, 167 mg, 1.09 mmol) was added *via* hypodermic syringe. After 1h, the mixture was poured into 50 mL of ice/water, and the solution was neutralized (meter) by drop wise addition of cold acid, so it was monitored and adjusted for~40 min until it stabilized at pH 7, and then the solution was frozen and lyophilized overnight to give a paste of solid with Et_2O (50 mL) and filtration gave a hygroscopic white solid: wt 802 mg (>100% because of extra triethylammonium salts). And ether layer obtained.

Preparation of RA-CTS (RA-CTS)

RA-CTS was synthesized as follows (scheme 1). 3g of chitosan were dissolved in 30ml of 2% acetic acid and MeOH were added with 2.3g (8.0 mmol) of all trans retinal prepared by swern's method [10] was added and stirring at 25℃ for 5 hrs. The product was filtrated after 30 min. and the unreacted RA and the other inorganic mixture products were wash by aq-methanol and ether for 30 min. and it was dialyzed against deionized water for 24 h. and filtering to remove water. Swollen low polymeric schiff base RA-CTS gels were dried at room temperature for 3 day, and these samples were further dried in a vacuum oven for 1 day at 50℃.

Preparation of phosphate (CTS-retin-P)

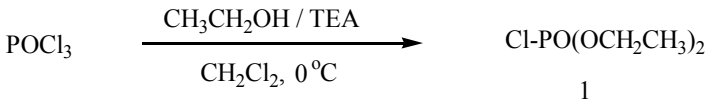
CTS-retin-P was synthesized according to the procedure by use of diethoxyphosphoryl chloride (DEPC). As shown in Scheme 1, RA-CTS[RA-(CTS)n] and DEPC were added to DMF: benzene (1:1) solution. The reaction mixture was stirred at 40℃ for 4hrs. Finally, the product was centrifuged, and filtering and its solid gel product was hydrolysis by 20% NaOH 30 ml was added at 80 □ and stirred for 40min. After stirring for 30 min at 25□. And neutralization the solution was precipitated by adding 10% HCl solution and 800 ml of MeOH, followed by centrifugation and it was dialyzed against deionized water for 24 h. and filtering to remove water. Swollen polymeric CTS-retin-P[CTS-retin-P']gels were dried at room temperature for 3 day, and these samples were further dried in a vacuum oven for 1 day at 50℃.

1. Antibacterial Test

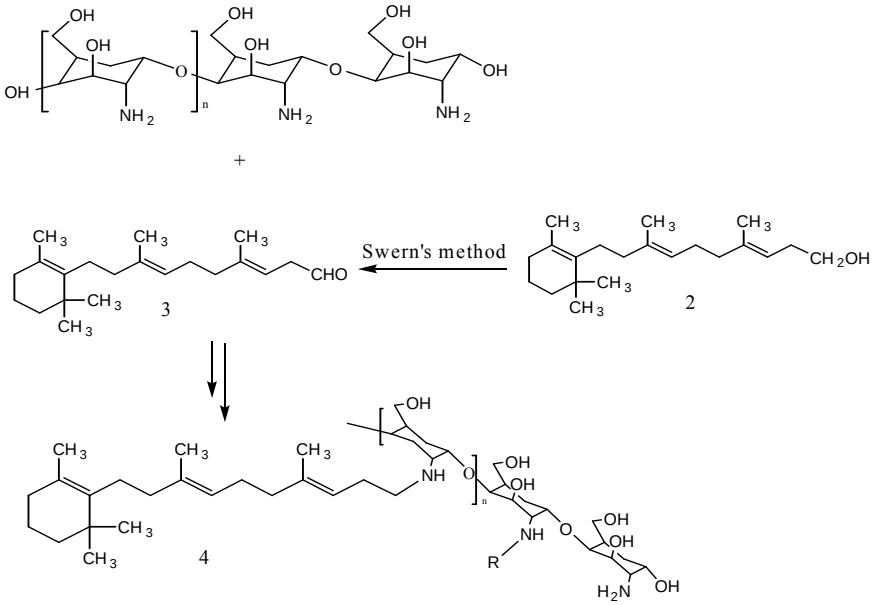
Microbiological Testing of the Chitosan Derivatives

The retinal conjugates of low molecular chitosan CTS-retin-P 13Kda and CTS-retin-P′ 36KDa the parent compound chitosan(RA-CTS). A method: The compound was tested against a panel of 3 organisms, including *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 10536.

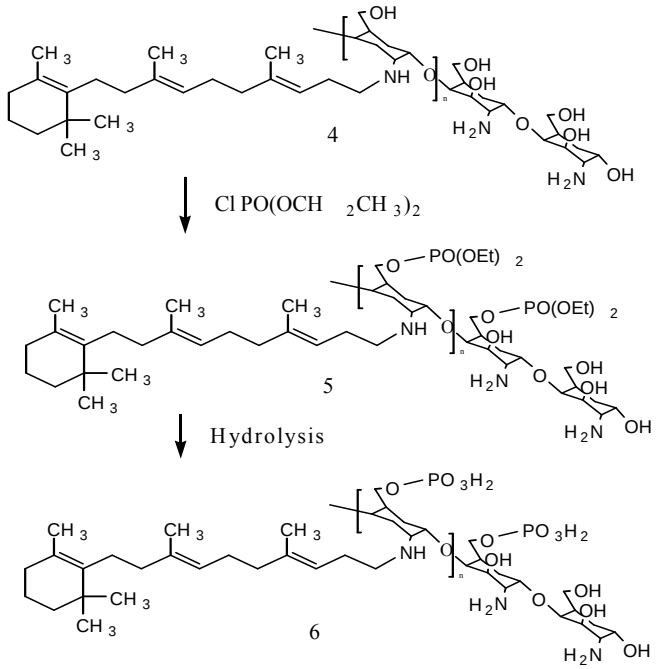
The nonresistant stock strains from the American Type Culture Collection (ATCC) were also included as controls. Susceptibility testing was performed by the standard agar dilution technique according to NCCLS guidelines. Results were read at 36-48 h of incubation and expressed in terms of minimum inhibition concentrations (MIC) in μg/mL as(Rroth Serial Dilution Method).



Scheme 1: Reaction pathway for synthesis of diethoxyphosphoryl chloride



Scheme 2: Reaction pathway for synthesis of *t*-retin-A chitosan derivatives containing O-phosphate.



Scheme 3: Reaction pathway for synthesis of *t*-retin-A chitosan derivatives containing O-phosphoric acid.

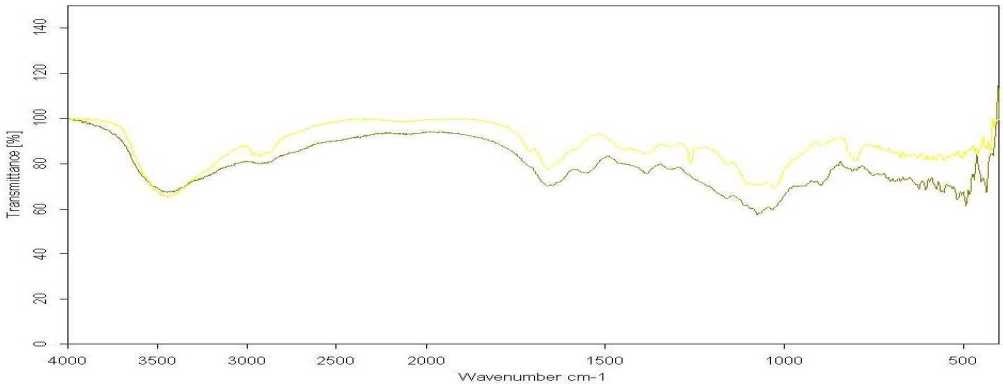


Fig 1. IR spectrum of chitosan(up) and RA-P-CTS polymer

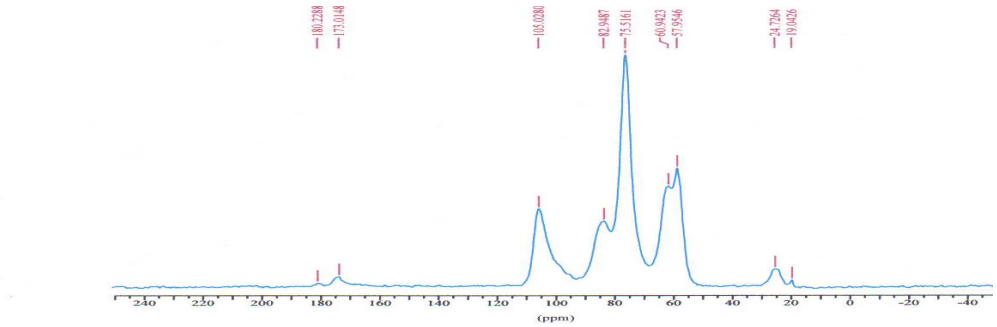


Fig 2. ^{13}C-SNMR Spectra of CTS

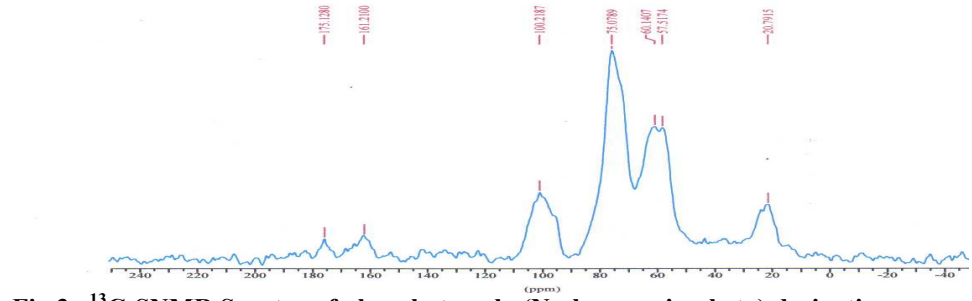


Fig 3. ^{13}C-SNMR Spectra of phosphate poly (N-glucosamine-keto) derivatives

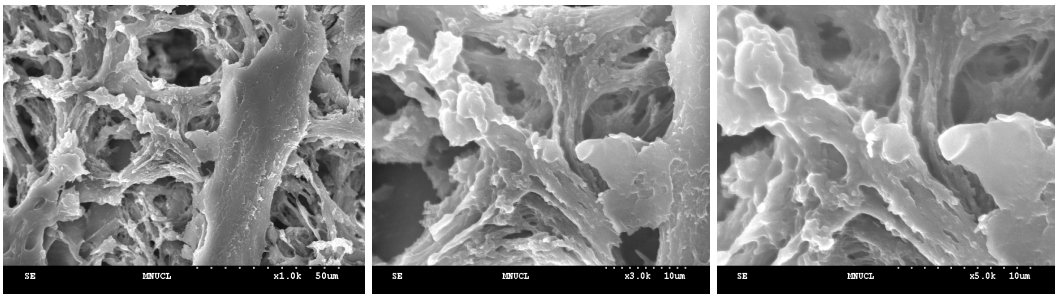


Fig 4. SEM photograph of RA-phosphate chitosan derivative; surface(X 300, 1000, 3000)

Antifungal Test (Effects of CTS-retin-P and CTS-retin-P′ on growth of *Candida albicans*)

B method: Fungus: *Candida albicans* NIH A207 strain was used in this assay.

2. Anti fungal activity:

C. albicans (1 x 10^5 cells/ml YPD*) was mixed with 1 mg/ml of CTS-retin-P and CTS-retin-P′, and these were incubated for 24 h at 27 ^{O}C. After the incubation, the culture medium was photographed with an IX51 microscope (Olympus, Japan). * YPD; Yeast peptone dextrose medium. It is used for cultivation of fungus.

3. Sustained release of antifungal agent from sample:

C. albicans (1 x 10^5 cells/ml) was planted on YPD agar, and samples CTS-retin-P and CTS-retin-P′ were put on the center of YPD agar. Twenty-four hours after the incubation, clear circle by growth inhibition of *C. albicans* was observed. These supernatant samples (0.01 ml) were mixed with 0.09 ml of *C. albicans* (1 x 10^5 cells/ml YPD) suspension. Twenty-four hours after the incubation, the amount of *C. albicans* cells was measured as OD620nm.

Result and Discussion

Structure of the derivatives

1. The elemental analysis results and the IR spectra of chitosan, with CTS-retin-P and and the schiff bases are shown in Fig 1, as shown in Fig 1, the schiff bases of RT-CTS have strong peaks at 1640 - 1670 cm^{-1}, which assigned to the characteristic absorbance of imines groups [-CH=N]. Moreover, there are strong peak at about 1500-1650 cm^{-1} corresponding to the conjugated double bond groups. These results indicated that the schiff bases of with CTS-retin-P and were obtained, and the analysis result of compounds indicated that chitosan was instituted by retinal and phosphoric acid.

2. The antibacterial activities were shown in Table 1. with CTS-retin-P and has better antibacterial activity than that of chitosan with (CTS)n-retin-P′ and. But antibacterial activity of (CTS)n-retin-P′ is not increased like that (CTS)-retin-P, It may be because of the all *trans* retinal group coupling to (CTS)n-retin-P′, and it can be identified also by the degreased antibacterial activity of high molecular chitosan as

(CTS)n-retin-P′

3. Antibacterial activity Results

Table 1. Antimicrobial Test on Chitosan Derivatives

No	Antibacterial Activity % Inhibition= (1-T/C) 100%, C=log CFU/ml of control and T= log CFU/mol of sample		
	Staphylococcus aureus ATCC 25923	*Pseudomonas aeruginosa* ATCC 9027	*Escherichia coli* ATCC 10536
(CTS)-retin-P	18.5	32.7	19.2
(CTS)n-retin-P′	- 62.4	47 81	34 92

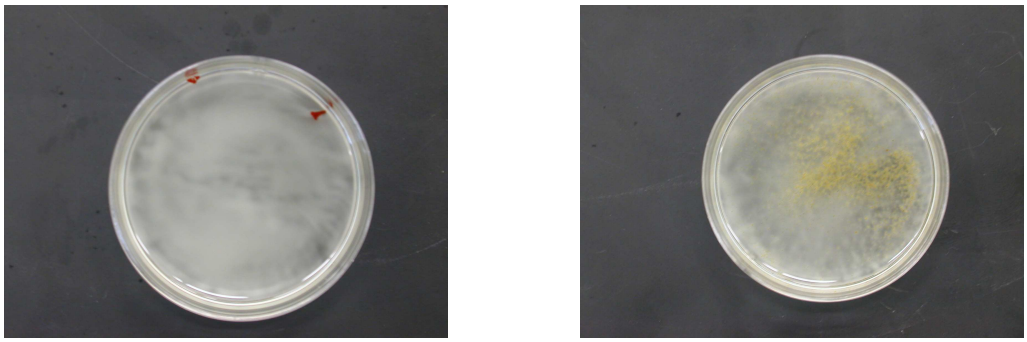
ATCC is an abbreviation for American Type Culture Collection.

% Inhibition= (1-T/C) 100%, C=log CFU/ml of control and T= log CFU/mol of sample

4. Anti fungal activity:

To confirm the anti-fungal activities of CTS-retin-P and CTS-retin-P , these samples were mixed with *C. albicans*. Twenty-four hours after the incubation, the culture medium was observed (Fig. 1). The cultured medium added with CTS-retin-P and CTS-retin-P′ did not exhibit the anti-fungal activity, thus, *C. albicans* could grow in this condition and these culture medium became to muddiness.

Sustained release of antifungal agent from sample: To confirm whether a soluble antifungal agent was released from these samples, the effects of samples (CTS-retin-P and CTS-retin-P′) on growth of *C. albicans* on YPD agar were monitored. CTS-retin-P and CTS-retin-P′ did not exhibit the anti fungal activity.



CTS-retin-P

CTS-retin-P’

Fig. 5. Effect of CTS-keto-P, CTS-retin-P and CTS-retin-P′ on growth of *Candida albicans*

CONCLUSION

Micro low molecular water-soluble chitosan products of Chitosan Medicine Tech Co. were synthesized with all RA derivatives, a well-known antibacterial agent. To improve the chemical compound's solubility and cellular permeability, phosphorous acid was mixed with carbohydrate. The study on this compound's minimum growth suppression concentration and inhabitation found the following conclusions:

1. The MIC value of two sample with different chitosan molecular weights was 18.5, 32.7, 19.2 for the all trans retinoic acid derivative with the molecular weight of (CTS)-retin-P, 13KDa and 62.4, 81, 92 for the all trans retinoic acid derivative with the molecular weight of (CTS)n-retin-p’ 36KDa. In result, these samples showed higher antifungal activity compared to chitosan derivative with higher molecular weight.
2. P-Chitosan derivative's MIC value for a *Staphylococcus aureus* ATCC 25923, was 18.5 and its antibacterial activity was very high compared to its MIC value for bacteria. In (CTS)-retin-P, the MIC value of the chitocan derivative with molecular weight of (CTS)n-retin-P’ 36KDa was 34 with the highest antibacterial activity than CTS.
3. The detailed structure of bacteria influenced by chitosan was observed under a microscope to examine the antibacterial activity of chitosan derivatives and it was found that cell wall and cell membranes had big injuries and interfered with the life activities of chitosan processing microorganisms.
4. As chitosan molecular weight affects its antibacterial and antifungal activities, chitosan derivative containing compounds with outstanding antibacterial activity and appropriate molecular weight would be considerably helpful to develop naturally high-molecular and anti-microorganism chitosan. In addition, the new development would be suitable for various industries and medicines.

In order to check the appropriateness for skin and pharmacological efficacy, chitosan was converted into sulfate. To be used as biological adhesive, all *trans* retinoic acid converted into phosphoric acid by synthesizing 2 with retinoic acid and the following result came out.

(1) Increment of water solubility (2) In the reaction of phosphate, the reaction of exothermic dietoxyphosphoryl chloride (DEPC) was desirable and the color varied considerably due to the decomposition by temperature.
(3) For DEPC: TEA: RA-CTS= 1: 2: 1(mole ratio), the temperature under 5°C was desirable.
(4) It seems necessary to check the behavior of electromagnetic wave scattering or gellation in order to obtain the physical constant that is minutely comparable.

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