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PREPARATION FROM CHITIN OF (1→4)-2-AMINO-2-DEOXY-β-D-GLUCOPYRANURONAN AND ITS 2-SULFOAMINO ANALOG HAVING BLOOD-ANTICOAGULANT PROPERTIES*†

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ABSTRACT

Chitosan, prepared by total *N*-deacetylation of chitin, underwent complete and specific carboxylation at C-6 when oxidized, as the perchlorate salt **2**, with chromium trioxide in acetic acid. The resultant (1→4)-2-amino-2-deoxy-β-D-glucopyranuronan, obtained as its perchlorate (**3**), was *N*-sulfated with chlorosulfonic acid in pyridine to afford a (1→4)-2-deoxy-2-sulfoamino-β-D-glucopyranuronan, isolated as its amorphous sodium salt **4**; the latter displayed moderate blood-anticoagulant activity. The products **3** and **4** showed marked *in vitro* growth inhibition of leukemia L-1210 cells.

INTRODUCTION

A program in this laboratory is concerned with the preparation of artificial blood anticoagulants modeled after heparin, the natural blood anticoagulant, by synthetic modification of readily available polysaccharides. Key functional groups in the polysaccharide chain of heparin are the 2-sulfoamino and 6-carboxyl moieties. Simulation of this gross structure is achieved in the present work by converting chitosan, a β-D-(1→4)-linked polymer of 2-amino-2-deoxy-D-glucose residues, into its 6-carboxyl analog and then *N*-sulfating this polymer to afford a product having moderate anticoagulant activity. Although partial C-6 carboxylation of chitosan has been achieved[2] by use of dinitrogen tetraoxide or oxygen–platinum, only low degrees of substitution by the carboxyl group were attained, and there was some concomitant degradation of amino functionality and partial oxidative depolymerization. Reactions of higher specificity leading to higher degrees of carboxylation are of interest, not only for simulating more closely the uronic acid content of heparin (d.s. by CO_2H, 0.5) but also for obtaining fully carboxylated structures that would be synthetic analogs of microbial, extracellular polymers containing 2-amino-2-deoxy-D-glucuronic acid[3].

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There is considerable biological and technological interest in chitosan and its derivatives[4]. Chitosans sulfated to various extents have been prepared by use of sulfur dioxide–sulfur trioxide[5], chlorosulfonic acid–pyridine[6], sulfur trioxide–*N*,*N*-dimethylformamide[6], and sulfur trioxide–pyridine[2]; some products having moderate to good anticoagulant activities were obtained. Acute toxicities for some of these products were low, but some of them manifested delayed toxic behavior[7].

There exists the possibility that sulfated chitosan derivatives might display the antilipemic activity in the blood plasma shown by heparin. Chitin oligomers themselves are of interest as agents that promote the healing of wounds[8]. In recent reports, it has been shown that chitosan[9] and several of its *N*-acylated derivatives[10] can effect selective aggregation of cancer cells, notably cells of leukemia L-1210, sarcoma 37, and Ehrlich ascites; the possibility for further leads in this area provided additional impetus for the present study.

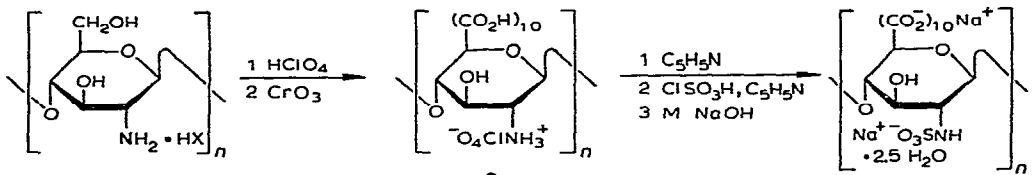
DISCUSSION

Chitin was *N*-deacetylated[11] by use of concentrated aqueous sodium hydroxide to afford a product that by the evidence of n.m.r. spectroscopy was completely *N*-deacetylated. The acetate salt (**1**) of this chitosan was obtained in solution in aqueous acetic acid and was precipitated by acetone.

In order to oxidize the chitosan to the 6-carboxyl analog, it was necessary to devise conditions that would (*a*) obviate the need for temporary substitution at the functional positions 2 and 3, (*b*) give specific reaction at C-6 without oxidation at C-2 or C-3, and (*c*) allow the polymeric structure to be maintained with the minimum of degradation. Chromium trioxide in acetic acid was selected as the oxidant, and the amino group was protected from oxidation by protonation to the ammonium ion by use of a strong acid (perchloric acid). This method of protection follows the general rationale used by Billman and coworkers[12] in a successful synthesis of α-amino acids through oxidizing β-amino alcohol precursors as their sulfate salts by the action of permanganate.

It has been reported[13] that primary alcohol groups are oxidized rapidly to aldehydes by chromium trioxide in acetic acid, but that the presence of water slows down the reaction markedly. In order to effect oxidation to the aldehyde stage at a maximal rate, it thus appeared desirable to initiate reaction in a water-free medium to minimize the time of exposure to the oxidant and concomitant possibility of partial depolymerization. As the subsequent oxidation of aldehydes to carboxylic acids appears[14] to require water, it was proposed to introduce water at the terminal phase of the oxidation step.

A solution of chitosan acetate (**1**) in aqueous acetic acid was freed from most of the water by evaporation and, by addition of perchloric acid, chitosan perchlorate (**2**) was precipitated as a fine dispersion suitably surface-activated for the oxidative step. The oxidation of **2** was performed under relatively anhydrous conditions in acetic acid with an excess of chromium trioxide, and water was introduced after 1 h of reaction at ~25°. The perchlorate salt (**3**) of the 6-carboxylated polymer was obtained



in good yield as a water-soluble, methanol-insoluble powder. Microanalysis indicated that no nitrogen had been lost from the product, and free amino groups were indicated by a positive ninhydrin reaction. The product gave a negative carbazole reaction[15], but this was anticipated, as positive tests for uronic acids are not given by 2-amino-2-deoxy-D-glucuronic acid[16]. As chitosan has been reported[9] to be an inhibitor of tumor growth, this C-6 carboxylated analog **3** was evaluated biologically. Tested *in vitro* against leukemia L-1210 cells, compound **3** was observed to inhibit cell growth by 50% at a concentration of 0.6 μM (300 μg/ml).

Titration of **3** with base to the point of neutralization of perchloric acid and the carboxylate groups indicated that the product had been fully converted (degree of substitution 1.0) into the 6-carboxyl derivative. Titration to the point of half-neutralization followed by dialysis yielded the free aminouronic acid polymer, whose solubility in water was much lower than either the perchlorate salt or the sodium carboxylate salt of the free base. The high recovery after dialysis indicated that there was little degradation of the polysaccharide to the level of dialyzable oligosaccharides. The infrared spectra[17] of the salt **3**, the neutral polysaccharide, and the sodium salt all indicated the absence of ketone-type absorption such as would have resulted from possible oxidation at C-3 to the ketone. It may be speculated that the charged group (NH_3^+) adjacent to HO-3 may help to protect the latter group from attack by the oxidant. The carboxylated chitosan derivative **3** was of high molecular weight (4×10^5 by viscometry, corresponding to d.p. 1,480), and this factor and the consequent low rate of tumbling of the molecules in solution, account for the broadened signals observed in the n.m.r. spectrum of **3**. The molecular weight of **3**, determined in water by light scattering, was 5.8×10^5.

By suspending it in pyridine, the perchlorate salt **3** of carboxylated chitosan was obtained in a form suitable for heterogeneous sulfation. Sulfation with chlorosulfonic acid–pyridine, by the procedure used by Wolfrom and Shen Han[6] for sulfation of chitosan, gave a water-soluble polymer formulated as disodium (1→4)-2-deoxy-2-sulfoamino-β-D-glucopyranuronan (**4**) of d.s. 1.0 by the sulfoamino group and by the carboxylate group. This formulation was supported by elemental analysis, which indicated that equimolar amounts of sulfur and nitrogen were present, by a positive Toluidine Blue[18] reaction for the sulfoamino group, and a negative ninhydrin reaction indicating the absence of free amino groups. The molecular weight of **4** (4.3×10^5; d.p., 1,440 by viscometry) indicates that little depolymerization accompanies the sulfation of **3**. The infrared spectrum of **4**, showing close similarity with the spectrum of commercial sodium heparinate, is given in Fig. 1.

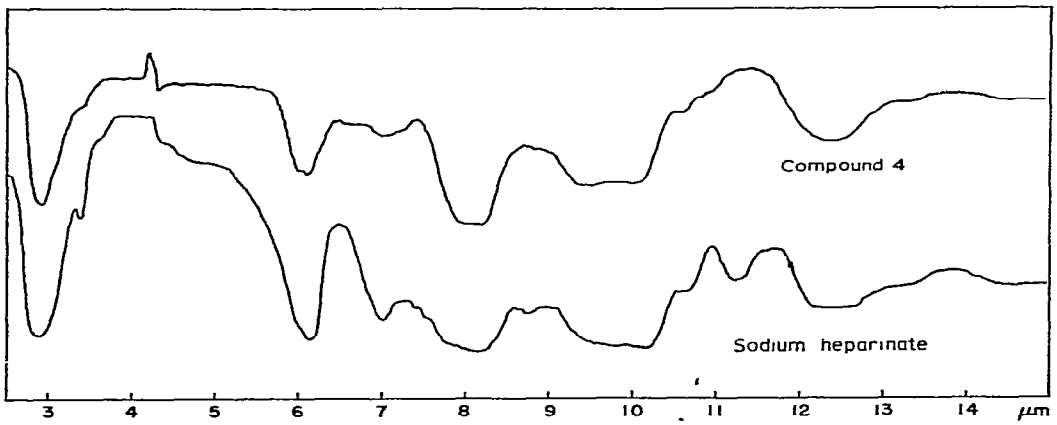


Fig. 1. The i.r. spectra of (upper trace) disodium (1→4)-2-deoxy-2-sulfoamino-β-D-glucopyranuronan (4) and that of (lower trace) sodium heparinate (Upjohn).

The chitin-derived polysaccharide **4** differs from heparin in that the latter is an *N*-sulfated, partially *O*-sulfated alternating polymer of 2-amino-2-deoxy-D-glucose and hexuronic acid residues[19]. Nevertheless, it is well established[6] that polymers having anticoagulant activity can be obtained by introducing sulfoamino and sulfate ester groups into polysaccharide chains having linkage-patterns different from that of heparin; the sulfoamino group appears to make an important contribution[2,6,20,21]. The product **4** was found to have an anticoagulant activity of 25.8 International Units per mg (as compared with 110–150 I.U./mg for commercial heparins). Its acute toxicity in the mouse (LD_{50}, intraperitoneal) was 237 mg/kg, that is, about three times that of heparin. At 0.2 μM concentration (100 μg/ml) it inhibited the growth of leukemia L-1210 cells by 50%.

Maximal anticoagulant activity of heparin preparations is known to depend to an important extent on fractionation to a fairly narrow range of molecular weight[22,23]. Heparin fractions of high molecular weight have been reported[23,24] to have low or variable anticoagulant activity, and the low activity of **4** may be ascribed to its high molecular weight relative to that of therapeutic grade heparins ($M \simeq 13{,}000$) derived from biological sources.

The chromium trioxide procedure here described for carboxylation of chitosan is simple and reproducible, and provides a cationic–anionic biopolymer of interest in several areas of biological chemistry.

EXPERIMENTAL

General methods. — Solutions were evaporated at a bath temperature below 50° under diminished pressure. N.m.r. spectra of preparations **1** and **3** were recorded with a Varian HA-100 spectrometer in the frequency-sweep mode; samples were lyophilized repeatedly with deuterium oxide and spectra were recorded at concentrations of

~10% in deuterium oxide with an external standard of tetramethylsilane. Molecular weights of **3** and **4** were estimated by viscometry for solutions 0.1M in sodium chloride; calculations employed the relationship given by Lasker and Stivala[23] ($[\eta] = 1.75 \times 10^{-5} M^{0.98}$) equating the intrinsic viscosity of heparins with molecular weight. Elemental analyses were performed by W. N. Rond.

Chitosan acetate (**1**). — Chitin (100 g) from crustacean shell (Pfanstiehl Laboratories, Waukegan, Illinois) was *N*-deacetylated with 40% aqueous sodium hydroxide by the two-step procedure already described[11]. The solution of the resultant chitosan in 10% aqueous acetic acid (3 l) was filtered and added to acetone (6 l). The resultant precipitate was filtered off, washed exhaustively with acetone and then ether with use of a blender, and then dried *in vacuo* to afford **1** as a fine, white powder (80 g, 73%); λ_{max}^{KBr} 2.91 (OH), 6.36, 7.10 μm (CO_2^-); n.m r. data: broad signals of 4-, 2- and 1-proton intensities centered at τ 6.20, 6.78 and 7.36, respectively.

Anal. Calc. for $C_6H_{11}NO_4(HOAc)_{0.5} \cdot 0.9H_2O$: C, 40.53; H, 7.19; N, 6.75. Found: C, 40.75; H, 6.97; N, 6.56.

A sample of the original chitosan, before treatment with acetic acid, was examined by n.m.r. spectroscopy (100 MHz, external tetramethylsilane) in deuterium oxide containing 0.1M hydrochloric acid-*d*. The signal of absorption by the *N*-acetyl group near τ 8.0 was of negligible intensity.

Chitosan perchlorate (**2**). — To a solution of chitosan acetate (**1**, 10 g, 45 2 mmol based on 2-amino-2-deoxy-D-glucose acetate residues) in water (50 ml) was added acetic acid (200 ml), and the solution was evaporated to a thick, viscous syrup. Acetic acid (200 ml) was added and the solution was again evaporated; this process was repeated several times to remove water until further addition of acetic acid resulted in incomplete dissolution of **1**. Acetic acid was added to bring the volume to 1 liter, and 60% aqueous perchloric acid (12 ml) was then added slowly with vigorous stirring to precipitate the finely divided salt **2**. This suspension was used directly in the following step.

(1→4)-2-Amino-2-deoxy-β-D-glucopyranuronan perchlorate (**3**). — To the rapidly stirred suspension of **2** (45 mmol) from the preceding experiment was added a solution of chromium trioxide (3 g, 30 mmol) in water (3 ml) and acetic acid (30 ml) After 30 min at ~25° a second, similar batch of oxidant solution was added, and after a further 30 min a final increment of chromium trioxide (2 g in 75 ml of water; total CrO_3 80 mmoles) was added. Stirring was continued for a further 1 h and the excess oxidant was then decomposed by addition of methanol (25 ml). After stirring for a further 15 min, the mixture was allowed to settle and the supernatant solution was decanted off. The precipitate was filtered off and washed exhaustively with methanol until both product and washings were colorless and free from acid. The product was washed with ether and dried under vacuum to give **3** as a fine white powder (9.7 g, 79%), $[\alpha]_D^{24}$ −19° (*c* 0.2, water); λ_{max}^{KBr} 2.89 (OH), 5.71 μm (CO_2H); n.m.r. data: broad signals of 2-, 2-, and 1-proton intensities centered at τ 5.10, 5.94, and 6.62, respectively.

Anal. Calc. for $[C_5H_6O_3(NH_3ClO_4^-)_{1.0}(CO_2H)_{1.0}]$: C, 26.14; H, 3.65; N, 5.08. Found: C, 25.97; H, 4.85; N, 5.13.

Product **3** gave a positive ninhydrin reaction. The carbazole test[14] was negative. Titration of **3** with 0.05M sodium hydroxide to an end-point of pH 7.85 (corresponding to formation of sodium perchlorate and sodium carboxylate) gave an equivalent weight of 275.7 g/mole per residue of 2-amino-2-deoxy-D-glucuronic acid perchlorate, corresponding to a d.s. by carboxyl in **3** of 0.99. Compound **3** had a weight-average molecular weight by viscometry of 4.0×10^5 corresponding to d.p. 1,480. By light scattering in water solution (determined by ArRo Laboratories, Joliet, Illinois), compound **3** had $\overline{M}_w$ 5.8×10^5.

Assay of compound **3** against cultures of leukemia L-1210 cells showed 50% inhibition of growth at a 0.6 μM concentration (300 μg/ml).

(1→4)-2-Amino-2-deoxy-β-D-glucopyranuronan. — A solution of the perchlorate salt **3** (0.5 g) in water (50 ml) was neutralized by titrating with 0.05M sodium hydroxide to pH 6.65 (corresponding to formation of sodium perchlorate and the free acid form of the polymer) resulting in the complete precipitation of the glucopyranuronan polymer. The mixture (90 ml) was dialyzed against distilled water for 2 days and lyophilized to afford a water-insoluble solid (0.29 g, 94%), λ_{max}^{KBr} 2.90 (OH), 6.16, 7.14 μm (CO_2^-).

Anal. Calc. for $[C_5H_6O_3(NH_3^+)_{1.0}(CO_2^-)_{1.0}]$: C, 37.66; H, 5.69; N, 7.32. Found: C, 37.89; H, 5.79; N, 6.94.

(1→4)-2-Deoxy-2-sulfoamino-β-D-glucopyranuronan, sodium salt (**4**). — The perchlorate salt **3** (2 g), still wet with ether, was neutralized and obtained in a form activated for substitution by suspending it at ~25° in anhydrous pyridine (100 ml). The gelatinous solid obtained in suspension was centrifuged off, washed twice with 100-ml portions of pyridine (centrifuge), and resuspended in dry pyridine (75 ml). This suspension was added to a chlorosulfonic acid–pyridine sulfating reagent[6] prepared by the dropwise addition of chlorosulfonic acid (10 ml) to pyridine (40 ml). The mixture was heated with exclusion of moisture for 1.5 h under reflux on a steam bath. The supernatant was decanted from the solid that had settled and was discarded. A solution of the residue in water (100 ml) was brought to pH 9.00 with M sodium hydroxide, dialyzed against distilled water for 2 days, and freeze-dried to afford *N*-sulfated, carboxylated chitosan (**4**) as a fluffy, light-brown solid (1.8 g, 72%), $[\alpha]_D^{24}$ −13° (*c* 0.2, water); λ_{max}^{KBr} 2.89 (OH), 6.06, 7.06 μm (CO_2^-); see also Fig. 1; weight-average molecular weight by viscometry 4.3×10^5, corresponding to d.p. 1,440.

Anal. Calc. for $[C_5H_6O_3(NHSO_3Na)_{1.00}(CO_2Na)_{1.00} \cdot 2.5H_2O]$: C, 20.93; H, 3.51; N, 4.07; Na, 13.35. Found: C, 21.02; H, 3.78; N, 3.82; Na (as Na_2SO_4), 13.51.

Product **4** was freely soluble in water, gave a negative ninhydrin test for free amino groups and a positive Toluidine Blue test for sulfoamino (or sulfate ester) groups. Its anticoagulant activity **4**, assayed with sheep plasma[25], was 25.8 I.U./mg. The concentration of **4** required for 50% growth inhibition of leukemia L-1210 cells was 0.2 μM (100 μg/ml) and the toxicity (LD_{50}, mouse, interperitoneal) 237 mg/kg.

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