Regioselective Synthesis of Curdlan Derivatives

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Abstract

Curdlan, a (1→3) linked linear homopolysaccharide composed of β-D-glucan, is produced by the bacterium *Alcaligenes faecalis var.* myxogenes. Several strategies to synthesize chemically modified curdlan derivatives have been reported, but there have been few reports of regioselective functionalization at specific positions of the curdlan backbone, especially of aminated curdlan derivatives which have remarkable potential in biomedical and pharmaceutical applications. We demonstrate herein the design, synthesis and characterization of a family of regioselectively aminated curdlan derivatives including 6-deoxy-6-(bromo/azido/amino/amido/ammonium) curdlans starting from 6-bromo/azido-6-deoxycurdlan.

A key reaction that enabled the whole synthesis of new curdlan derivatives at C-6 described in this dissertation was the highly selective bromination of curdlan. The resultant 6-bromo-6-deoxycurdlan, prepared with high regioselectivity, was treated with trialkylamines or heterocyclic amines to produce a range of water-soluble curdlan ammonium salts. The bromide was then nucleophilically displaced by sodium azide to produce the versatile precursor 6-azido-6-deoxycurdlan. Its water solubility was enhanced either by the incorporation of hydrophilic trioxadecanoate esters into *O*-2/4 positions or by the borohydride reduction to afford 6-amino-6-deoxycurdlan. The iminophosphorane intermediate generated during Staudinger reactions was further investigated for subsequent syntheses: i) 6-amino or 6-amido-6-deoxycurdlan *by in situ* reaction with water or excess carboxylic anhydride, ii) 6-monoalkylamino curdlan by reductive amination using aldehydes and sodium cyanoborohydride, and iii) 6-dialkylamino-/tri-alkylammoniocurdlans by reacting with methyl iodide. Such derivatives could have properties useful for a range of biomedical applications, including interactions with proteins, encapsulation of drugs, and formulation with genes or other biological compounds.

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The completion of my dissertation and subsequent Ph.D. has been a long journey. It is true that “Life is like a box of chocolates, you never know what you are gonna get” when I am completing my dissertation. I never thought of coming aboard to pursue a Ph.D. when I was a kid but this is happening right now. At any rate, I have finished, but not alone, and am elated. I could not have succeeded without the support from my advisor and committee members, my friends and my family.

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Attribution

One colleague aided with sample analysis and another two colleagues aided with writing and research behind two of my chapters presented as part of this dissertation. A brief description of their contributions is included here.

Chapter 5: S. Liu, currently a PhD candidate of Chemistry at Virginia Tech, served as a co-author on this paper and helped to characterize the samples with 2D NMR spectroscopy.

Chapter 7: Dr. Zheng served as a co-author on this paper for the pullulan and glucomannan experimental data. J. Kuang, currently a senior of Bioscience at Virginia Tech helped to run some curdlan experiments.

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Chapter 1. Dissertation overview

Due to the worsening environment and limited fossil fuel resources, there has been growing interest in how to fully exploit the biocompatible and biodegradable natural polymers, in particular the renewable polysaccharides. Aminated polysacchraides have important natural functions especially specific interactions with proteins in human bodies. Though the natural cationic aminopolysaccharide chitin and its *N*-deacetylated derivative chitosan have shown excellent benefits in biomedical and pharmaceutical areas, it becomes increasingly important to synthesize chitosan analogs by modifying other polysaccharides in an effort to avoid disadvantages as well as better understand the structure-property relationships among different polysaccharides.

Glucans, the essential members in polysaccharides family, are composed of D-glucose monomers connected by glycosidic linkages. Among the (1→3)-β-glucans, the bacterial exopolysaccharide curdlan has attracted more attention due to its rheological, solubility and biomedical properties. Though the biosynthesis, structural analysis, general biomedical/food application of curdlan have been reported in previous literatures, chemical modifications in a regioselective pattern studied herein not only alter the physical properties of curdlan but also expand the range of applications.

My doctoral research in this dissertation presents a complete investigation on the synthesis of a family of aminated curdlan derivatives including amino-, trialkylammonio- and amidocurdlans regioselectively substituted at the less hindered C-6 position for potential biomedical applications such as tight junction opening and anionic drug delivery. Detailed spectroscopic and property analysis of those derivatives are described as well.

Chapter 2 gives a comprehensive literature review on properties, chemistry and applications of the bioactive polysaccharide curdlan.

Chapter 3 presents the synthesis of curdlan derivatives *O*-(*N*)-acylated 6-amino-6-deoxycurdlan via three steps, bromination at C-6, nucleophilic displacement by sodium azide and Staudinger reduction using triphenylphosphine.

Chapter 4 then reports the synthesis of two water-soluble aminocurdlans by chemoselective azide reduction using sodium borohydride, which are promising candidates for biomedical applications. The hydrophilic oligo(ethylene oxide) ester trioxadecanoate was incorporated into curdlan backbone to better enhance the aqueous solubility.

Chapter 5 describes the synthesis of cationic 6-deoxy-6-(*N*,*N*,*N*-trialkylammonio)curdlan derivatives by reacting 6-bromocurdlan and its 2,4-diesters with trialkylamines or heterocyclic amines. The resultant water-soluble tertiary amines provided access to quaternized curdlan derivatives with permanent positive charge, which indicated potential application on permeation enhancement and delivery of anionic drugs.

Chapter 6 further explores subsequent reactions of Staudinger reduction on aminated curdlans including reductive amination and alkylation of aminocurdlan. The importance of triphenylphosphonium intermediate generated during Staudinger reaction in the regioselective synthesis of curdlan aminates was well discussed.

Chapter 7 covers glycan ester deacylation by tetrabutylammonium hydroxide/fluoride with regard to regioselectivity *vs.* polysaccharide (amylose, curdlan, dextran, pullulan and glucomannan) structures. Regioselectivity and degree of substitution of those derivatives were determined by means of 1D and 2D NMR techniques.

Chapter 8 summarizes the research results for each chapter in this dissertation and talk about future work.

Chapter 2. Literature Review: Properties, Chemistry, and Applications of the Bioactive Polysaccharide Curdlan

Zhang, R.; Edgar, K. J. *Biomacromolecules* **2014**, *15*, 1079-1096. Used with permission of American Chemical Society, 2014.

2.1 Introduction

Polysaccharides constitute an incredibly diverse family of natural polymers, with much higher complexity (or, put another way, information content) per repeating unit than other important natural polymers like proteins and poly(nucleic acids). Given the finite nature of fossil fuel resources, it becomes increasingly important for us to learn how to exploit the natural abundance and variety of renewable polysaccharides. Progress has been rather slow to date; cellulose, starch, chitin, and some natural gums are reasonably well understood and utilized, but the great majority of natural polysaccharides are much less well understood and exploited. There has been growing interest in bacterial exopolysaccharides in recent years, since they can be prepared in bulk through biotechnological routes from inexpensive, renewable feedstocks, and are readily harvested.

Glucans are polysaccharides composed of D-glucose monosaccharides connected by glycosidic linkages. Members of the family of (1→3)-β-glucans, including the linear glucans, the side-chain branched (1→3, 1→2)-β-glucans, and the (1→3, 1→6)-β-glucans, are found in both prokaryotes and eukaryotes.[1] Among the (1→3)-β-glucans, the bacterial exopolysaccharide curdlan is of special interest due to its biomedical, solubility, and rheological properties, and to the simplicity of its structure which is conducive to synthetic manipulation. Curdlan is a linear glucan, so named due to its ability to "curdle" when heated. Curdlan is neutral, and is composed exclusively of (1→3)-β-glucosidic linkages, without branching or substituents. Curdlan was first discovered in 1966.[2] While investigating succinoglucan production by *Alcaligenes faecalis* var. *myxogenes* 10C3 strain, Harada and coworker found that curdlan was produced by a mutant bacterium.[3] With increasing market demand, mutant *Agrobacterium* strains that provide high fermentation yield have been selected for curdlan production.[4]

Curdlan is also of special interest due to its advantageous solubility properties in comparison with some other commonly used polysaccharides, like cellulose and chitin; it is insoluble in water but soluble in alkaline media. Curdlan also has enhanced organic solubility vs. many other natural polysaccharides, facilitating chemical reactions and processing. Its rheological properties are especially interesting with regard to utilization in applications; once a curdlan aqueous suspension is heated, two types of gels can form depending on the heating temperature, one of which is a high-set thermal non-reversible gel (~80 °C) while another is a low-set thermal reversible gel (~55 °C).[5] This gelation property has been attributed to curdlan's helical structure transformation at different temperatures[6], from a mixture of single helices and loose inter-twined triple helices at room temperature to more condensed rod-like triple helices at higher temperatures. Curdlan's remarkable rheological and thermal behaviors have led to its application as a thickening agent or fat-mimic substitute in the food industry. Recently, the immunostimulatory effects of β-glucans have triggered investigations of biomedical applications of curdlan and its derivatives. Some aminated and sulfated curdlans have been synthesized and identified as biological response (anti-tumor, wound repair or anti-HIV)[7] modifiers to enhance or adapt immune responses. Chemical derivatization is especially promising for refining natural properties, enhancing ease of processing, and imparting new properties of value, due to the relatively good solubility and simple structure of curdlan.

In recent years, several reviews have focused on the biosynthesis, structural analysis and general biomedical/food applications of curdlan[8]. It is our intention to present a more comprehensive review, including emphasis on the wide variety of chemical modifications that have been discovered for curdlan, and with particular attention to relation of structure/property/application relationships of curdlan and its derivatives. This is of importance since curdlan and derivatives have found such a diverse range of interesting applications in the relatively short time since curdlan became commercially available. Therefore we present this review of the production, physiochemical properties, food/biomedical application and chemical modifications of the renewable, bacterial exopolysaccharide curdlan, in order to enhance the understanding of this promising bacterial polysaccharide and to facilitate further studies of its fascinating structure, properties, and potential applications.

2.2 Curdlan Background

2.2.1 Chemical structure determination

Curdlan is a linear homopolymer of the monosaccharide D-glucose (**Fig. 2.1**). Harada *et al.*[9] first reported the chemical structure of curdlan as (1→3)-β-D-glucan using methylation and periodate oxidation methods as early as 1968. Curdlan was first methylated using dimethyl sulfate in sodium hydroxide and then hydrolyzed to yield only 2,4,6-tri-*O*-methyl-D-glucose, indicating the lack of branching and the exclusive 1→3 linkage of the D-glucose residues. The curdlan infrared absorption peak at 890 cm^{-1} (β-configuration) rather than 840 cm^{-1} (α-configuration) and its low specific optical rotation provided evidence for the β-configuration and pyranose ring structure. To determine the degree of polymerization (DP), curdlan was first oxidized with sodium metaperiodate and then reduced with sodium borohydride. Because of the 1→3 linkages there is no vicinal diol on the interior monosaccharides, so only the ends of the chain are oxidized, consuming a total of 5 moles of periodate and releasing 3 moles of formic acid per mole of polysaccharide. Since the periodate consumption was 0.04 mol/anhydroglucose unit (AGU) and the liberation of formic acid was 0.03 mol/AGU, the DP of curdlan was calculated as approximately 125 using **Eq. 2.1**.[3] Saito *et al.*[10] produced curdlan in four different culture batches and by the same periodic acid oxidation-borohydride reduction method determined their DP values to be between 135 and 455. Nakata *et al.*[11] reported that the average molecular weight of curdlan is between 5.3×10^4 and 2.0×10^6 in 0.3 M NaOH solution by light scattering and viscosity measurements.

$$DP\ (curdlan) = \frac{consumption\ of\ periodate\ per\ mol\ of\ curdlan}{consumption\ of\ periodate\ per\ AGU}$$

$$= \frac{5\ mol}{0.04\ mol/AGU} = 125\ AGU/mol \qquad \textbf{(Eq. 2.1)}$$

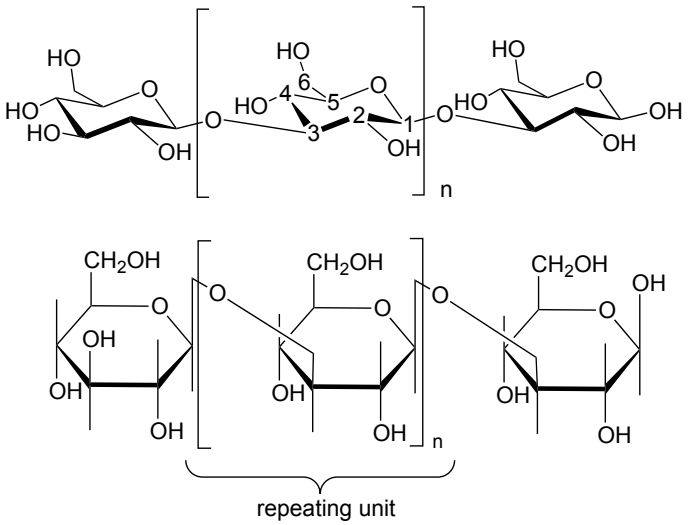

Fig. 2.1. Molecular structure of curdlan showing β-(1→3) linkages.

2.2.2 Resources and biosynthesis

Members of the (1→3)-β-glucan polysaccharide family are present in various living organisms, including protozoa, algae, yeast, fungi and higher plants. In this family, curdlan was the first to be detected by Harada and colleagues[2] in 1966. It is a neutral polymer produced by *Alcaligenes faecalis* var. myxogenes 10C3 strain. Subsequent research on curdlan production focused on selection of bacterial strains (**Table 2.1**) and optimization of the culture media.

Table 2.1.

Occurrence of bacterial curdlan[1 a]

Bacterial source	References
Agrobacterium sp. 10C3 and derivatives	Harada and Harada (1996)[12], Nakanishi *et al.* (1976)[13]
Agrobacterium sp. ATCC 31749 and derivatives	Phillips *et al.* (1983)[14], Stasinopoulos *et al.* (1999)[15], Kim *et al.* (2003)[16], West (2009)[4c]
Agrobacterium sp. Biovar I GA-27 and GA-33	Kanegae *et al.* (1995)[17]
A. radiobacter IFO 12607 *et al.*	Nakanishi *et al.* (1976)[13]

A. rhizogenes IFO 13259	Nakanishi *et al.* (1976)[13]
Rhizobium trifolii J60	Ghai *et al.* (1981)[18]
Rhizobium sp. TISTR 64B	Footrakul *et al.* (1981)[19]
Rhizobium Niftal 600	Mamaril *et al.* (1989)[20]

[a]Note: Adapted with kind permission from McIntosh *et al.*; Stone, B. A.; Stanisich, V. A. Curdlan and other bacterial (1→3)-β-D-glucans. *Appl. Microbiol. Biotechnol.* **2005**, *68*, 163-173. Copyright 2005 Springer-Verlag. With kind permission from Springer Science and Busineess Media.

Curdlan is an extracellular polysaccharide, believed to be biosynthesized in three steps; 1) substrate processing, 2) intracellular metabolism, and 3) excretion from the cell.[21] Kai *et al.*[22] used ^{13}C-labeled glucose in the culture medium to study curdlan biosynthesis. The results indicated that biosynthesis was mostly by direct polymerization of glucose (60%) rather than glycolysis from cleaved triose. Only 3% and 8% of curdlan were synthesized by C-1→C-3 rearrangement and C-6→C-1 isomerization via Embden-Meyerhof pathway. The metabolic pathway for curdlan biosynthesis is described in **Fig. 2.2**.[23] Zhan and co-workers[8d] used *Agrobacterium* sp. ATCC 31749 as an example to analyze the proposed metabolic pathway of curdlan production in great detail. Many essential factors influence curdlan production, including carbon, nitrogen, oxygen, and phosphate sources, as well as pH.

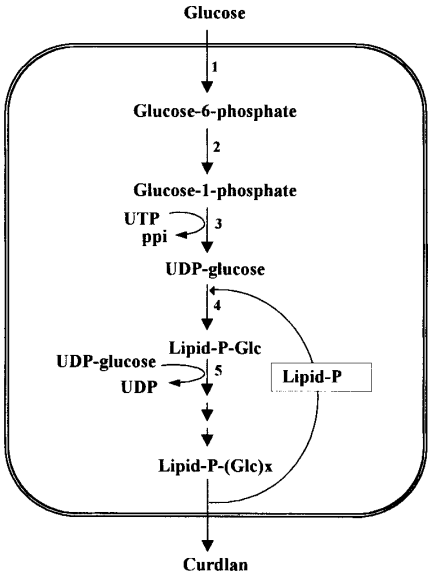

Fig. 2.2. Metabolic pathway for curdlan biosynthesis.[23] Enzymes (numbers correspond to those over arrows): (1) hexokinase; (2) phosphoglucomutase; (3) UDP-glucose

pyrophosphorylase; (4) transferase; (5) polymerase. Lipid-P represents isoprenoid lipid phosphate. Lee, I.-Y. Curdlan. *Biopolymers* **2005**, 135-158. Copyright 2005 Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

2.2.3 Solution behavior

Curdlan is comprised of as many as 12,000 glucose units, as measured by viscometry.[24] It is insoluble in water and the majority of organic solvents while it is soluble in dilute alkali (0.25 M NaOH), dimethylsulfoxide (DMSO), formic acid, and some aprotic solvents such as *N*-methylmorpholine *N*-oxide (NMMO) and dimethylacetamide containing lithium chloride (DMAc/LiCl). The enhanced solubility of curdlan vs. other linear glucans like cellulose, the strong dependence of solution behavior on factors like temperature, pH, and DP, and the technical importance of this behavior for applications like food thickening have led many investigators to study curdlan solution behavior.

Modeling using the worm-like chain model suggested that soluble curdlan molecules contained three conformers; single helices, triple helices and random coils. In 1972, Ogawa *et al.*[25] used optical rotatory dispersion, viscosity and flow birefringence to study curdlan conformational behavior in alkaline solution. As NaOH concentration increased from 0.19M to 0.24 M, curdlan conformation changed from the helical (ordered) form to the random coil (disordered) form. Curdlan conformation was shown to be sensitive to DP; a disordered form was present in both neutral and alkaline solutions when DP < 25, while an ordered structure formed in 0.1M NaOH at DP up to 200.[26] In 1973, Ogawa *et al.* reported a curdlan conformational transition from a flexible disordered form to a rigid, ordered structure in DMSO with the addition of non-solvents such as water, dioxane, or 2-chloroethanol[27] and in aqueous NaOH with addition of increasing mole fractions of NaCl[28].

Kasai and Harada identified three forms of curdlan regenerated at different temperatures (**Fig. 2.3**). One form was obtained from a 4% aqueous suspension of curdlan at room temperature. Based on X-ray diffraction data, the structural models of this form were variously interpreted as a mixture of 7/1-single and 7/1-triple helix[6] or an exclusive 7/1-triple helix[29]. A hydrated form was prepared by heat-treatment above 120 °C in a sealed bomb under humid atmosphere and was converted to an anhydrous form by drying under

vacuum. Both of the two allomorphs were highly crystalline and composed of right-handed 6/1 helices.[30] In the hydrated state, the dimensions of the unit cell increased with incorporation of a great deal of water (18~36 molecules of water per unit cell) that contributed to H-bonding in the helical conformation.[31]

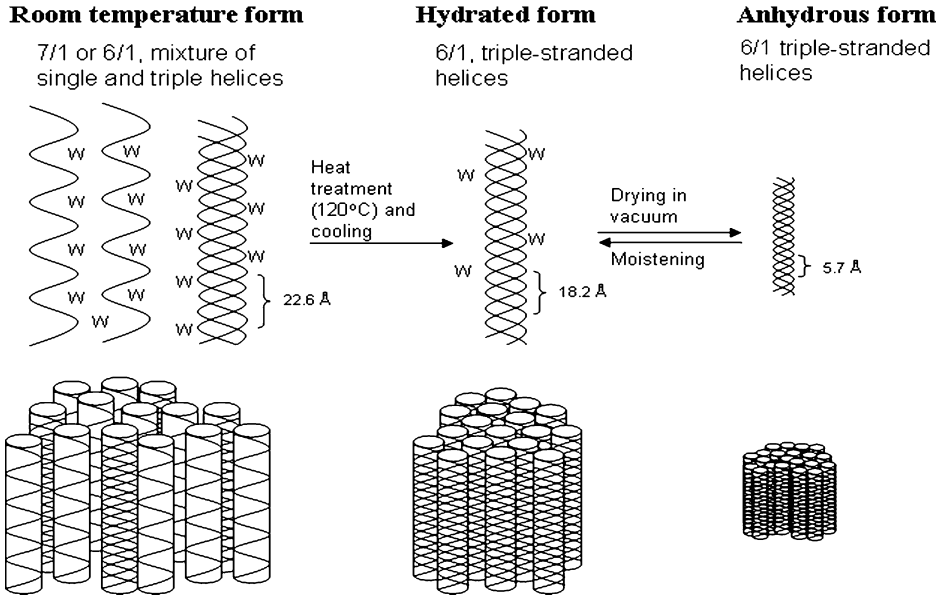

Fig. 2.3. Schematic illustrations of structural changes among three forms of curdlan at room temperature (left) and high temperature (middle and right).[1, 6] Reprinted with permission from Kasai, N.; Harada, T.; Ultrastructure of curdlan. *ACS Symp. Ser.* **1980**, *141*, 363-383. Copyright 1980 American Chemical Society. Reprinted from McIntosh, M.; Stone, B. A.; Stanisich, V. A. Curdlan and other bacterial (1→3)-β-D-glucans. *Appl. Microbiol. Biotechnol.* **2005**, *68*, 163-173. Copyright 2005 Springer-Verlag. With kind permission from Springer Science and Business Media.

2.2.4 Gel structure and mechanism

The unique gelation properties of curdlan were first reported by Harada and coworkers when they discovered curdlan in 1966[2] and have been well studied thereafter. In 1967 Maeda *et al.*[32] observed that curdlan aqueous neutral or dilute alkaline solutions formed gels when heated above 55 °C. This thermo-reversible gel, referred to in the literature as "low-set", can disassociate upon cooling. This gelation process involves the aggregation of rod-like triple helices by non-covalent association. As temperature increases, the triple-helical strands

unwind to give single chains that can anneal to reform a triple helical structure upon cooling. On the other hand, curdlan aqueous suspensions form a thermo-irreversible gel on heating to 80~145 °C, referred to as a "high-set" gel. Watase *et al.* found that adding DMSO, a good curdlan solvent, to aqueous curdlan suspensions can increase curdlan gelation temperature.[33] Curdlan gels can alternatively be obtained non-thermally by neutralizing a 0.1 M aq NaOH curdlan solution with acid followed by dialysis to purify the gel suspension.[34]

A number of reports have investigated curdlan gelation behavior and mechanism using various characterization methods. Saito *et al.*[35] used ^{13}C nuclear magnetic resonance spectroscopy (NMR) to study single- and multiple-helical formation during curdlan gelation. The linewidths of C-1 to C-6 carbon peaks after gelation were very broad, ascribed to the immobility of triple-helical structures. They sharpened as the alkali concentration increased, attributed to the formation of random coils. Differential scanning calorimetry (DSC) of this gel showed two sharp endothermic peaks at 56 °C and 154 °C, attributed to the gelation and melting temperatures, respectively.[36] The band intensity ratio (I_{1080}/I_{1045}) in the attenuated total reflection infrared spectroscopy (ATR-IR) spectra of curdlan gel was found to be not only characteristic for gel type (1.12 ± 0.01 for high-set gels (triple helix) *vs.* 1.22 ± 0.03 for low-set gels (single helix)), but indicative of the single helix as an intermediate in the formation of triple helix conformation.[37] Electron microscopy (EM) was carried out to image the ultrastructure of curdlan with low to high DP that had been subjected to various heat-treatments. Curdlan low-set gels obtained by heating (~60 °C) or neutralization showed similar rod-like fibrillar structures. The microfibrils were wider for curdlan gels (DP=455) obtained by heating at 120 °C (300~400 Å wide) than for those treated at 90 °C (100~200 Å wide), a difference attributed by the authors to decreased crystallinity.[34a]

Ikeda *et al.*[38] investigated curdlan conformation in NaOH solutions, from a single helix at a lower concentration to a disordered chain at a higher concentration. These results indicated that heat-induced gelation involved two steps, in which partial single chains first dissociate from aggregates followed by crosslinking into triple helices via hydrophobic interactions. This was consistent with single-molecule force spectroscopy (SMFS) results that elucidated the unwinding transition from triple helices to single helices and then to random coils in the concentration of NaOH from 0.19 to 0.24 M.[39] Longer holding time at high temperature

allowed imperfect triplexes to unwind completely, promoting annealing and increasing the resilience and thermoirreversibility of curdlan gels.[40]

2.2.5 Degradation

Polysaccharides can undergo hydrolysis under acidic conditions. The mechanism of acid-catalyzed hydrolytic cleavage of curdlan involves three steps (**Fig. 2.4**). First, the glycosidic bond is protonated at the bridging oxygen. A new non-reducing end group is subsequently formed together with a carbocation at C-1, which is then rapidly attacked by water to form a reducing end group.

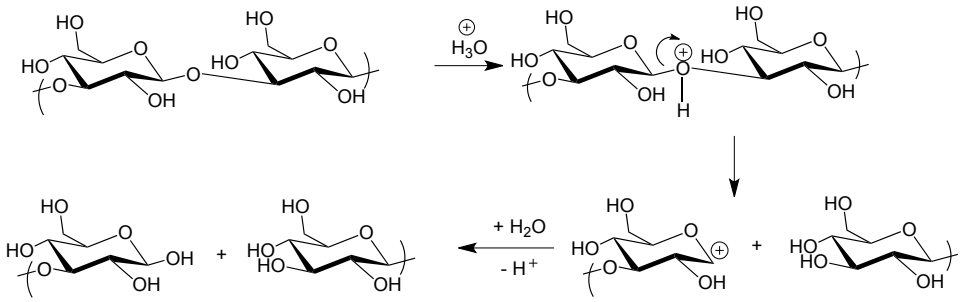

Fig. 2.4. Mechanism of curdlan acid-catalyzed hydrolysis.

Acid-catalyzed hydrolysis is widely used to obtain curdlan oligomers, which are of great interest for biomedical applications. Formic acid, sulfuric acid, trifluoroacetic acid (TFA) and HCl have been used to catalyze curdlan hydrolysis. Ohno *et al.*[41] used 90% HCO_2H to hydrolyze curdlan for 20 min at 100 °C, providing low molecular weight (MW) derivatives as antagonists for a β-glucan receptor. Curdlan hydrolyzates degraded by HCO_2H were also used as immunostimulants to promote proliferation of B- and T-lymphocytes.[42] Conversion of curdlan to glucose was achieved by hydrolysis with 70% TFA at 100 °C for 2.3 h.[43]

Some glucanase and glucosidase enzymes produced by fungi, yeast or bacteria are able to degrade curdlan. Kusama *et al.* investigated a β-(1,3)-glucanase system of *Streptomyces* sp. K27-4. Under different conditions, this enzyme hydrolyzed curdlan into two types of disaccharides, laminaribiose (31% yield) and gentiobiose (29.4 % yield).[44] Gentiobiose was produced in two steps, in which β-(1,3)-glucanase first hydrolyzed curdlan into glucose and

laminari-oligosaccharides, and then laminari-oligosaccharides were transglucosylated by β-glucosidase into gentiobiose.[45] *Streptomyces* sp. W19-1 can produce a curdlan-degrading enzyme as well.[46] β-Laminaribiose octaacetate was prepared in > 50% yield by curdlan hydrolysis (degraded by kitalase, a yeast cell-wall catalytic enzyme) and subsequent acetylation.[47]

Grandpierre *et al.*[48] compared the kinetics of curdlan acidolysis and enzymatic hydrolysis in great detail using high performance anion exchange chromatography (HPAEC). Enzymatic extracts from *Trichoderma harzianum* completely hydrolyzed curdlan within 90 h under mild conditions to afford products with low polydispersity, while acid-catalyzed degradation (H_2SO_4 or TFA (1M) at 60 °C) left more than 75% non-degraded mass. Zhan and coworkers[49] combined acidic and enzymatic hydrolyses in order to obtain target curdlan oligomers with reduced cost/energy consumption and environmental pollution under moderate reaction conditions.

In addition, vacuum pyrolysis can degrade curdlan[50] by a "peeling" mechanism that has been demonstrated for alkaline degradation (shown in **Fig. 2.5**). As each AGU is removed from the polysaccharide chain, the next unit is exposed and undergoes the same degradation process. Since it reduces the DP of curdlan one by one, this reaction does not rapidly reduce molecular weight.

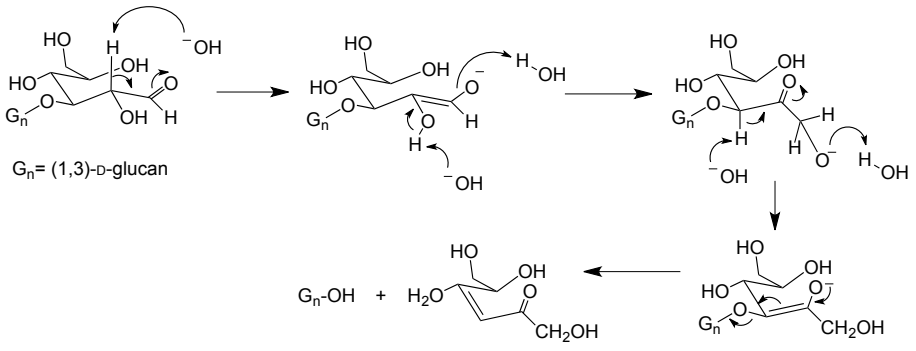

Fig. 2.5. "Peeling" mechanism for alkaline degradation of curdlan beginning at the reducing end of the chain.

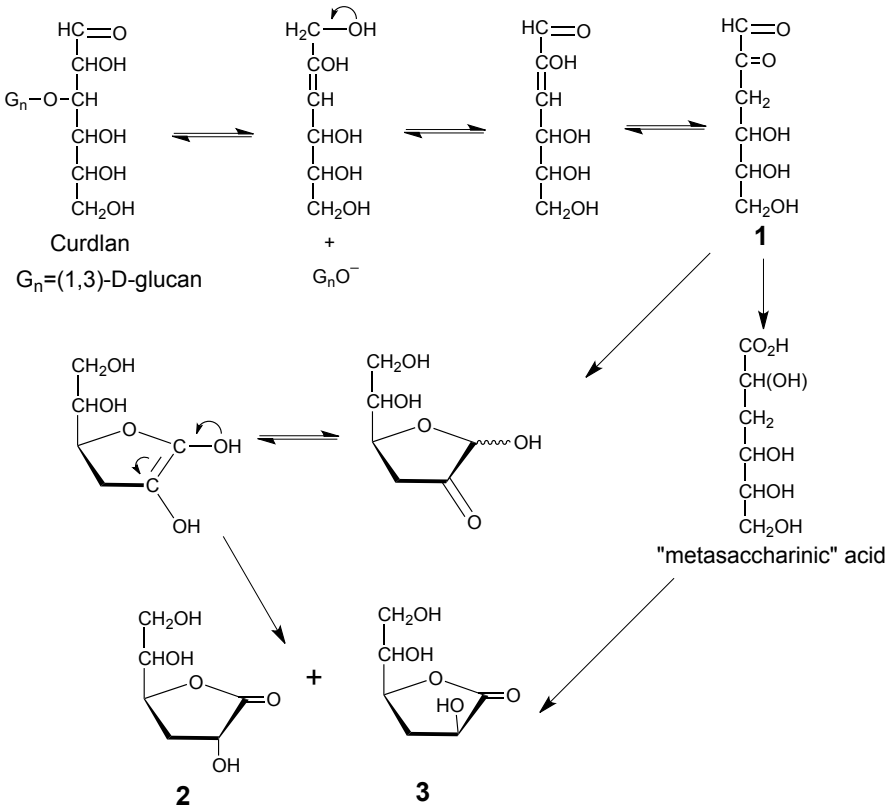

Fig. 2.6. "Peeling" mechanism for pyrolysis degradation of curdlan.[50] Reprinted with permission from Richards, G. N.; Shafizadeh, F. Formation of "glucometasaccharinolactones" in the pyrolysis of curdlan, a (1→3)-β-D-glucan. *Carbohydr. Res.* **1982**, *106*, 83-91. Copyright 1982 Elsevier.

This study demonstrated the effect of sodium chloride (NaCl) on curdlan degradation (**Fig. 2.6**). In the absence of NaCl, intermediate **1** underwent benzilic acid rearrangement to metasaccharinic acid in 49% yield. Water, provided by competing thermal degradation reactions, was required for this rearrangement. With increasing proportion of NaCl, curdlan was converted via **1** into volatile lactones **2** and **3** in 38% yield.

2.2.6 Application

2.2.6.1 Food industry

Curdlan was approved for food usage in Korea, Taiwan, and Japan as early as 1989 and registered in the United States in 1996 by the Food and Drug Administration (FDA) as a food additive (formulation aid, processing aid, stabilizer and thickener or texturizer)[51]. It is

useful in food due to its remarkable rheological properties. It has also been evaluated for safety and non-toxicity by a series of animal studies and *in vitro* tests. Curdlan is tasteless, colorless and odorless and can form gels with different textural qualities, physical stabilities and water-uptake capacities depending on the heat-treatment. There are two types of applications for curdlan in the food industry: i) direct addition to existing food systems at low levels (< 1 %) and ii) to create the food structure of new types of food products at higher levels.[5] Curdlan gelation can take place across a wide range of pH values, from 2 to 10, making it suitable for use in both dressings and dips (pH ~ 3.5), as well as in nutraceutical ingredients (pH ~ 8).[32] Often curdlan is used for its ability to form high-set, thermo-irreversible gels upon heating at high temperature, taking advantage of its stability during retorting, deep-fat frying, and freeze-thaw cycles.[1]

Owing to the helical structure of the curdlan molecule, it can absorb up to 100 times its weight of water, enabling for example formation of a moist food product. When hydrated and heated, curdlan can mimic the "mouthfeel" of fat-containing products. Aqueous suspensions of curdlan display thixotropic properties, which can imitate the rheological behavior of fat in low-fat products in dressings, sauces, dairy products and gravy. Curdlan has been used in vegetarian food formulations, such as seafood delicacies (crabmeat, fish tofu and shrimp ball) and vegetarian meat analogues.[5] In Japan, curdlan is also used to improve the elasticity and strength of freezable noodles. In brief, the versatile applications of curdlan together with its health benefits make it valuable in the design of innovative food systems.

2.2.6.2 Biomedical and pharmaceutical application

Apart from applications based on its physicochemical properties, curdlan also shows potential in biomedical and pharmaceutical applications. Curdlan has been used as a sustained release suppository for gel encapsulation of indomethacin, prednisolone or salbutamol sulfate. Compared with other rectal suppository systems that release drug by melting or dissolving, curdlan drug-impregnated gels prepared by various heat treatments stayed intact in the rectum, allowing localized drug diffusion and delivery to the lower rectum. In this way the drug migration into the colon that occurs with other suppository types was avoided, thereby avoiding drug uptake into the portal vein and subsequent first-

pass clearance in the liver.[52] Often in oral drug delivery systems, zero-order release kinetics are preferred to enable once-daily administration. *In vitro* drug release from curdlan tablets prepared from spray-dried curdlan/theophylline particles was diffusion-controlled, with the drug release rate remaining constant over the first 8 hours.[53] Kim's research demonstrated that the gelling potential of curdlan makes it promising as a protein drug vehicle.[54] However, the severe temperature and/or pH conditions of traditional curdlan gelation processes may limit its potential, since they may denature proteins. Bioavailability (the amount of drug that reaches the systemic circulation intact) was not considered in these studies.

Curdlan belongs to the class of biological response modifiers that enhance or restore normal immune defenses, including anti-tumor, anti-infective, anti-inflammatory and anti-coagulant activities.[7] The effectiveness of curdlan derivatives depends on the chemical structure (functionality and DS), molecular weight, and adjustable conformation (triple or single helices). Detailed descriptions of various curdlan derivatives in biomedical and pharmaceutical applications are provided in **Section 2.3**.

2.3 Curdlan derivatization

Curdlan has been chemically modified to create a large variety of derivatives intended for the food industry and biomedical applications. Solubility, reactivity and characterization are three crucial factors to be considered when designing new synthetic strategies. Curdlan is insoluble but swells in water, resulting in heterogeneous aqueous suspensions. For homogeneous reactions, an appropriate organic solvent should be selected that is compatible with reagent selection for modification. The degree of curdlan solubility in different solvents can affect the substitution pattern achieved. Each curdlan AGU has three chemically non-equivalent hydroxyl groups, one primary OH at C-6 and two secondary OH groups at C-2 and C-4. As a primary alcohol, O-6 is the most easily approachable position for modification, especially useful in regioselective reactions. Regioselectivity refers to the selectivity of substitution among the position(s) within each AGU (6 *vs.* 2 *vs.* 4). Perfect regioselectivity would provide a highly structurally regular polymer with each AGU containing the same substituents at the same positions. In contrast, the minor reactivity differences between O-2 and O-4 make it challenging to discriminate between them for selective modification. Reagents that can cause curdlan degradation should be avoided as well. To understand the substitution pattern,

structure-property relationships, and reaction mechanisms of obtained curdlan derivatives in detail, characterization is critical. In view of the achieved progress on curdlan derivatization, we herein highlight the essential chemistries reported in the literature to date.

2.3.1 Esterification of curdlan

Esterification has substantial precedent in polysaccharide chemistry, having been applied successfully to cellulose, starch, dextran, chitin, alginate and many others. In nature, the three free hydroxyls on the curdlan backbone are not acylated, limiting its solubility and applications.

Toga and coworkers[55] synthesized curdlan triacetate (**Fig. 2.7**) using an acid-catalyzed esterification technique. Dry curdlan powder was first suspended in warm water for 1 h to activate it towards esterification. Afterwards, the pretreated curdlan was agitated in a mixture of acetic acid, acetic anhydride and perchloric acid at room temperature for 60 h followed by 45 °C for several hours. In the ^{1}H NMR spectrum (**Fig. 2.8**), there are three peaks at 2.0 ~ 2.2 ppm, corresponding to the acetyl (-CH_3) groups at the C-2/4/6 positions. Based on the integration of acetyl protons, the degree of substitution (DS) was determined to be 2.9. Moreover, the researchers studied the chiral recognition ability of curdlan triacetate using high-performance liquid chromatographic (HPLC) resolution with various test racemates. They found that high temperature heat-treatment and solvent contact remarkably changed the chiral recognition ability of curdlan triacetate.

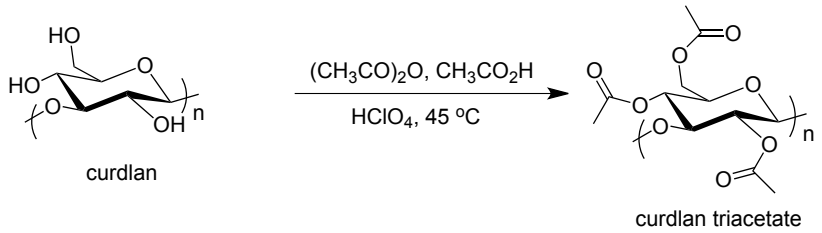

Fig. 2.7. Synthetic scheme for curdlan triacetate.

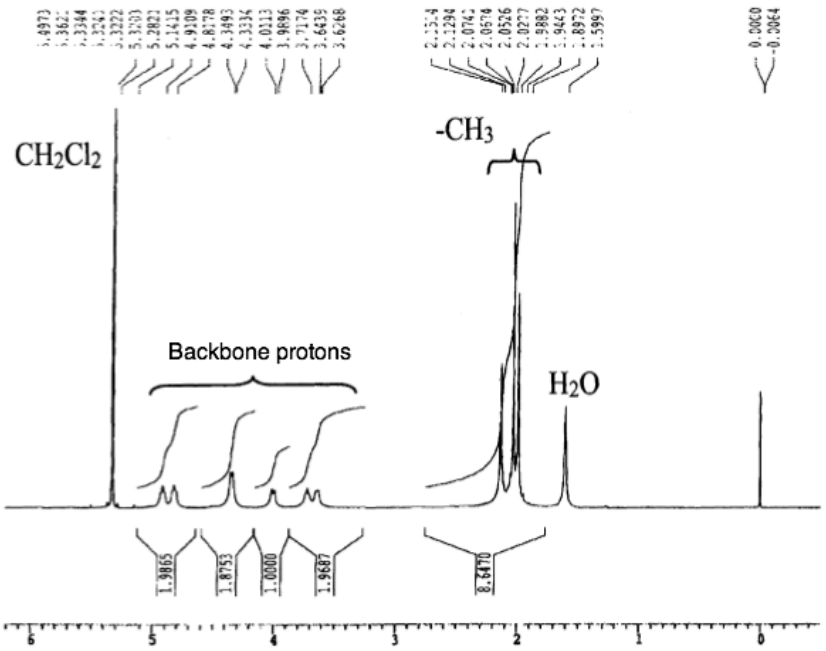

Fig. 2.8. [1]H NMR spectrum of curdlan triacetate.[55] Reprinted with permission from Toga, Y.; Ichida, A.; Shibata, T.; Tachibana, K.; Namikoshi, H. Chiral recognition ability of curdlan triacetate: solvent and temperature effects. *Chirality* **2004**, *16*, 272-6. Copyright 2004 Wiley-Liss, Inc., A Wiley Company.

Okuyama *et al.*[56] investigated the single helical structure of curdlan triacetate using XRD. Each unit cell contained six repeating units correlated by 6/1 helical symmetry with dimensions $a = b = 11.00$ Å, c (fiber axis) = 22.91 Å and $\gamma = 120°$ (**Fig. 2.9**). The terminal methyl in each acetyl moiety was constrained to the *trans* conformation and in particular, the primary acetyl is in a skew, *gauche*, *trans* conformation. There was one water molecule found per glucose residue, and the most plausible model showed that the water oxygen atom was linked to two C=O oxygens in adjacent molecules by H-bonds.

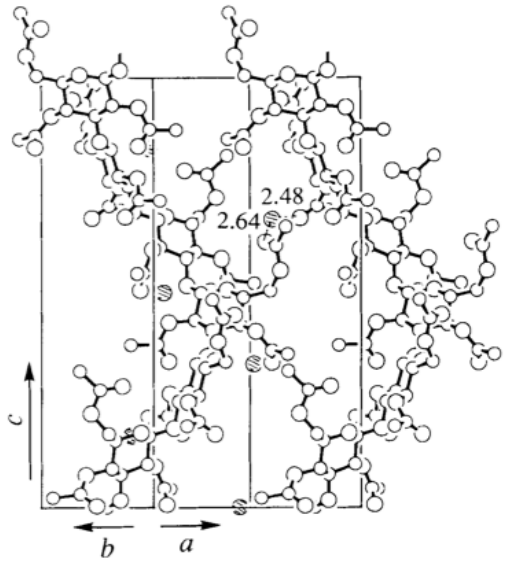

Fig. 2.9. ORTEP drawing of the crystal structure of curdlan triacetate looking normal to *c* (fiber axis). Hatched circles denote the water oxygen atoms.[56] Reprinted with permission from Okuyama, K.; Obata, Y.; Noguchi, K.; Kusaba, T.; Ito, Y.; Ohno, S. Single helical structure of curdlan triacetate. *Biopolymers* **1996**, *38*, 557-66. Copyright 1996 John Wiley & Sons, Inc.

A curdlan furoate ester with DS_{fur} 1.59 was synthesized by Hesse *et al.*[57] by reacting curdlan with *N, N*-carbonyldiimidazole (CDI) and furan-2-carboxylic acid in DMSO (**Fig. 2.10**). AFM and SEM (**Fig. 2.11**) were applied to investigate its supermolecular structure regarding its surface roughness and the macro-pore size and distribution. Curdlan furoates yielded self-supporting films with small macro-pore size, low roughness value and low maximum height, described as having potential utility as membrane biomaterials.

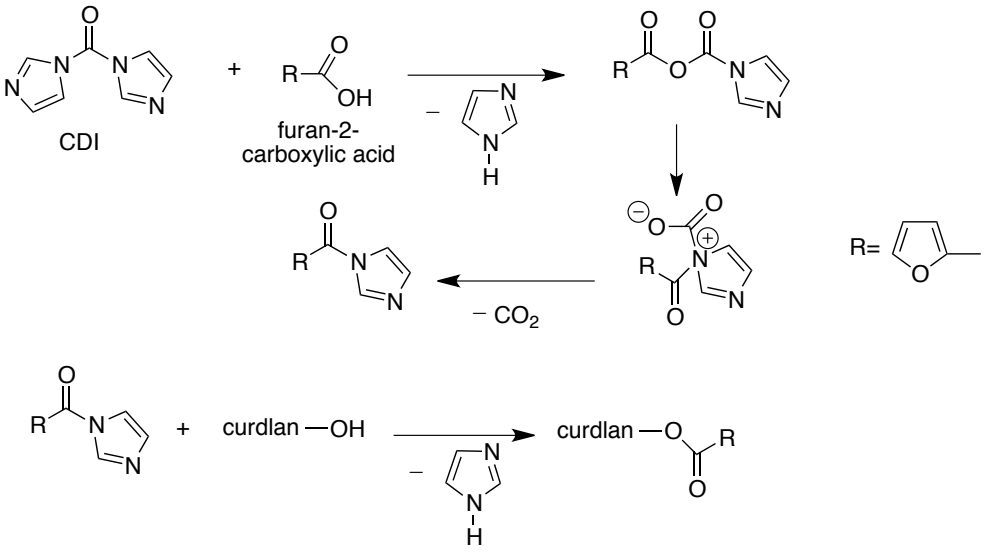

Fig. 2.10. Esterification of curdlan with furan-2-carboxylic acid.[57] Adapted with permission from Hesse, S.; Liebert, T.; Heinze, T., Studies on the film formation of polysaccharide based furan-2-carboxylic acid esters. *Macromol. Symp.* **2006**, *232*, 57-67. Copyright 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

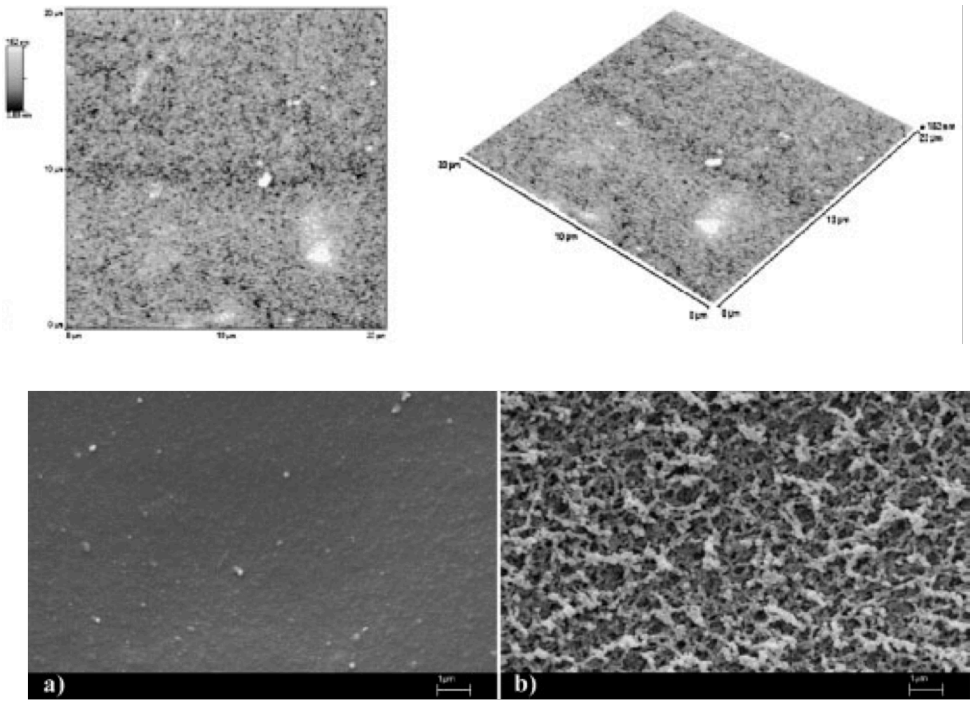

Fig. 2.11. Curdlan furan-2-carboxylic acid ester (DS_{fur} 1.59) film[57]: AFM image (contact mode; scan range 20 × 20 μm) of the supermolecular structure on mica surface (top); SEM image a) glass-film contact side, b) film-solvent contact side (bottom). Reprinted with

permission from Hesse, S.; Liebert, T.; Heinze, T. Studies on the film formation of polysaccharide based furan-2-carboxylic acid esters. *Macromol. Symp.* **2006**, *232*, 57-67. Copyright 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Recently Marubayashi and coworkers[58] synthesized a series of curdlan ester derivatives with varying alkyl chain lengths (C2-C12) by heterogeneous reaction in trifluoroacetic anhydride, conditions which more effectively preserve polysaccharide DP than other esterification methods. As the ester carbon number increased, fully acylated curdlan esters (DS = 3) with high molecular weight (Mw $\geq 6\times10^5$) demonstrated lower Young's modulus and tensile strength, larger elongation at break and reduced crystallinity. The results clearly illustrated the structure-property relationship among curdlan esters.

2.3.2 Carboxymethylation of curdlan

Curdlan has antitumor activity[59] and was found to inhibit growth of tumor S-180 by 99 - 100% with doses ranging from 10 to 20 mg/kg. This antitumor action was described as host mediated because of decreased effectiveness *in vitro,* as well as increased effectiveness when animals were pretreated by injection prior to tumor transplant. However, the water-insolubility of curdlan has limited its *in vivo* administration. Introduction of hydrophilic carboxymethyl groups has been explored to enhance aqueous solubility. Jin *et al.*[60] confirmed successful introduction of the carboxymethyl group by FTIR and NMR spectroscopy and compared carboxymethylated curdlan (CM-curd) with native curdlan. Based on rheological, DSC and AFM results, they drew three conclusions: i) the gelling characteristic was entirely lost due to the destruction of H-bonds between water and the curdlan chain, ii) the structural conformation changed from micellar aggregation to triple-strand helices and iii) the hydrophobic interactions related to the methylene groups at C-6 disappeared. In 1979, Sasaki *et al.*[61] first synthesized CM-curd (**Fig. 2.12**) and studied the relationship between its antitumor activity and DS_{CM}. The findings indicated that CM-curd with DS 0.47 strongly inhibited the growth of the *sarcoma 180* tumor; its antitumor activity decreased as DS increased.

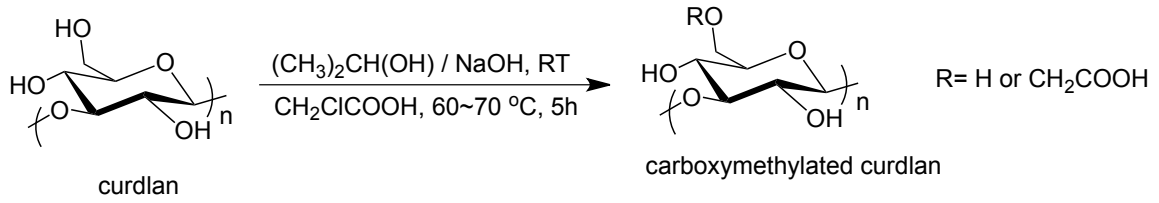

Fig. 2.12. Curdlan carboxymethylation synthetic scheme.

Carboxymethylated curdlan has been widely employed to prepare nanoparticles for biomedical applications acting as release-controlled drug vehicles and biological imaging agents. Na and colleagues[62] synthesized self-assembled hydrogel nanoparticles from CM-curd (**Fig. 2.13**) as a drug carrier for the treatment of liver cancer. The polar carboxylic acid group provided solubility improvement, while the sulfonylurea (SU) provided the hydrophobic inner core for enhanced self-assembly, forming an amphiphilic core-shell structure for encapsulating hydrophobic drugs. The mechanism involves first inducing immunological enhancement, binding to hepatic carcinoma cells and finally releasing the anti-cancer drug in a controlled fashion. The average nanoparticle diameter decreased as the degree of sulfonylurea (SU, hydrophobic moiety) substitution increased. Lee *et al.*[63] synthesized superparamagnetic iron oxide nanoparticles (SPION) coated by CM-curd for cellular and *in vivo* imaging. SPION showed improved stability and dispersity in water due to the introduction of the CM-curd moiety (**Fig. 2.14**). Silver nanoparticles decorated with CM-curd were studied using Surface Enhanced Raman Scattering (SERS) to detect more scattering peaks and enhanced peak intensities.[64] The particle size and size distribution were positively correlated with the concentrations of $AgNO_3$ and CM-curd (**Fig. 2.15**).

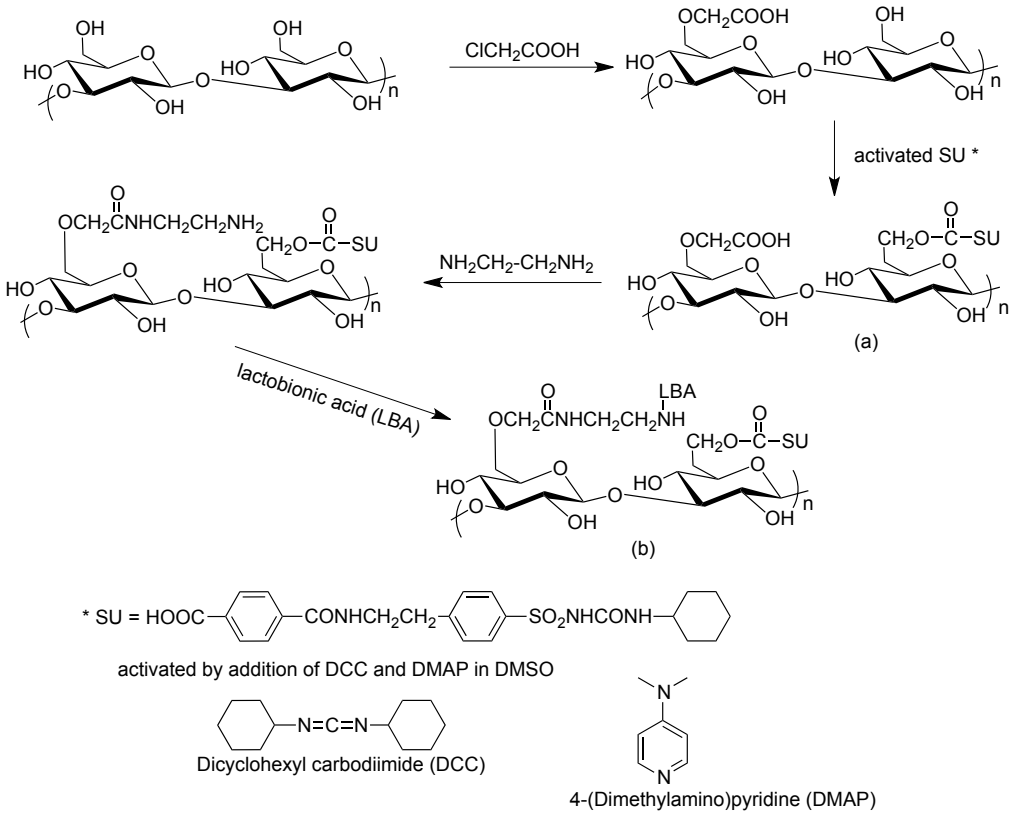

Fig. 2.13. Synthetic schemes of CM-curd/SU (a) and LBA/CM-curd/SU (b).[62] Adapted with permission from Na, K.; Park, K.-H.; Kim, S. W.; Bae, Y. H. Self-assembled hydrogel nanoparticles from curdlan derivatives: characterization, anti-cancer drug release and interaction with a hepatoma cell line (HepG2). *J. Control. Release. 69 (2)*, 225-236. Copyright 2000 Elsevier.

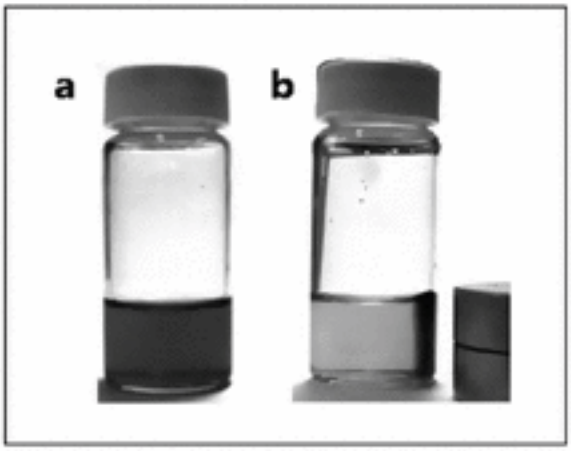

Fig. 2.14. Photographs of the CM-curd-coated SPION (a) dispersed in water and (b) dragged by magnetic force.[63] Reprinted with kind permission from Lee, C.-M.; Jeong, H.-J.; Kim, E.-M.; Cheong, S.-J.; Park, E.-H.; Kim, D. W.; Lim, S. T.; Sohn, M.-H. Synthesis and characterization of iron oxide nanoparticles decorated with carboxymethyl curdlan. *Macromol. Res.* **2009**, *17*, 133-136. Copyright 2009 Springer Science and Business Media.

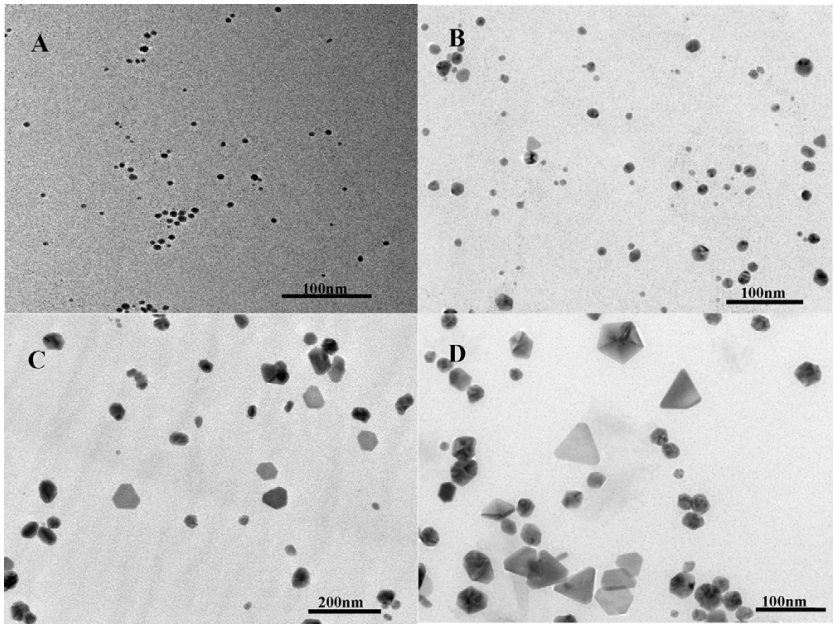

Fig. 2.15. TEM images of Ag nanoparticles prepared by 2 mM $AgNO_3$ and CM-curd with different concentrations: (A) 0.05 mg/ml, (B) 0.1 mg/ml, (C) 0.3 mg/ml, and (D) 0.5 mg/ml.[64] Reprinted with permission from Wu, J.; Zhang, F.; Zhang, H. Facile synthesis of carboxymethyl curdlan-capped silver nanoparticles and their application in SERS. *Carbohydr. Polym.* **2012**, *90 (1)*, 261-269. Copyright 2012 Elsevier.

2.3.3 Phosphorylation of curdlan

In addition to carboxymethylation, phosphorylation of curdlan can improve the water solubility as well. The synthesis of phosphorylated curdlan with a molar mass of 178,000 g/mol was recently described by Suflet *et al.*[65] who also investigated its polyelectrolyte behavior. **Fig. 2.16** shows the reaction scheme, in which curdlan was reacted with phosphorous acid in molten urea. Fully water-soluble phosphorylated curdlan was attained with DS 1±0.11 regardless of the reaction time. The ^{31}P decoupled NMR spectrum suggested that the introduction of phosphate groups occurred not only at C-6, but also at C-

2 and C-4 positions. Its polyelectrolyte behavior was studied by potentiometric and conductometric titration with monovalent bases (KOH or LiOH), as well as by viscometry. The potentiometric inflection points corresponded to the dissociation of one acid group, which was also supported by results of conductometric titration, confirming that the product is a monobasic curdlan phosphate. Compared with other phosphorylated polysaccharides, the higher chain flexibility of phosphorylated curdlan measured by viscometry was attributed to its (1→3)-glucosidic linkage.

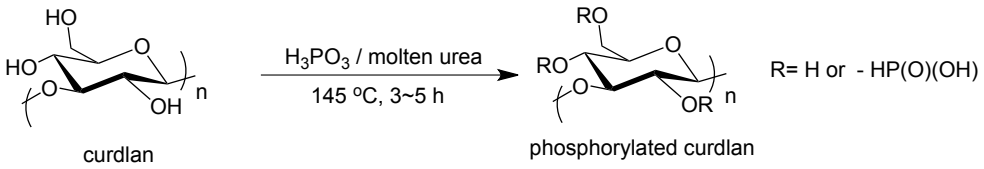

Fig. 2.16. Curdlan phosphorylation scheme.

With respect to applications of phosphorylated curdlan, Popescu *et al.*[66] compared monobasic curdlan phosphate microgels (MG) and curdlan microgels for controlled drug release. Using the water-in-oil inverse emulsion technique, both curdlan MG and phosphorylated curdlan MG were chemically cross-linked with epichlorohydrin to form anionic and neutral microgels, respectively (**Fig. 2.17**). SEM micrographs showed that curdlan MG displayed a smooth surface with small pores, versus a wrinkled surface for phosphorylated curdlan MG, ascribed to the higher cross-linking degree, higher density and lower swelling degree of curdlan MG. Drug loading and release experiments for phosphorylated curdlan MG indicated that the release rates increased with higher pH and ionic strength. Phosphorylated curdlan MG did not degrade during the release process and showed higher biocompatibility than curdlan MG based on evidence of better cell seeding and spreading around phosphorylated curdlan MG.

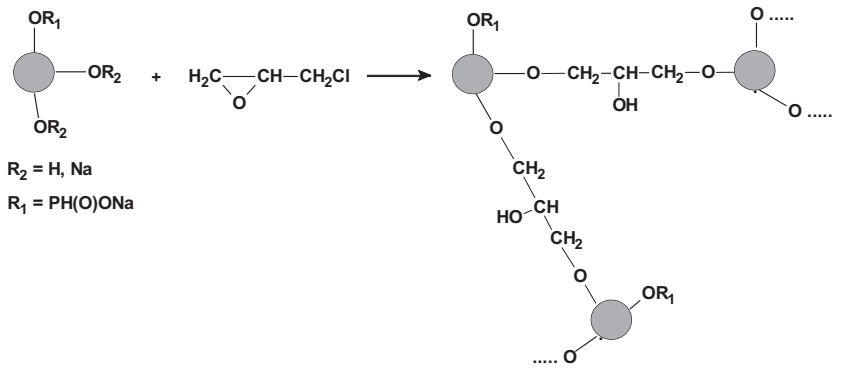

Fig. 2.17. Synthesis of curdlan MG and phosphorylated curdlan MG by chemical cross-linking with epichlorohydrin.[66] Reprinted with permission from Popescu, I.; Pelin, I. M.; Butnaru, M.; Fundueanu, G.; Suflet, D. M. Phosphorylated curdlan microgels. Preparation, characterization, and in vitro drug release studies. *Carbohydr. Polym.* **2013**, *94 (2)*, 889-898. Copyright 2013 Elsevier.

2.3.4 Regioselective reaction at C-6 of curdlan

As well as the degree of substitution, the distribution of functional groups strongly influences the properties of polysaccharide derivatives.[67] By controlling the sites at which substituents are attached to curdlan, researchers can modify its physical properties, such as solubility, crystallinity and thermal characteristics of the regioselectively substituted derivatives, thus permitting detailed studies of structure-property relationships. Regioselectively modified curdlan derivatives have many potential applications including as food additives, in drug and gene delivery, and as anti-tumor, anti-infective or anti-inflammatory agents[7a]. Two strategies have been applied for regioselective synthesis of polysaccharide derivatives. The protecting group strategy is somewhat labor intensive, first blocking the most reactive hydroxyl sites followed by modifying the remaining free hydroxyls, and then removing the protecting groups so that the other hydroxyls can be further derivatized. The other strategy is activation of C-6 by introducing a substituent specifically at that location, since it is most susceptible to modification due to steric hindrance, and then in some cases displacing or modifying the substituent in subsequent steps. The latter is more commonly practiced in curdlan regioselective synthesis and will be discussed in detail in this section.

2.3.4.1 Curdlan C-6 oxidation by TEMPO

The stable nitroxyl radical 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) has been use to catalyze oxidation of the C-6 primary hydroxyl group of polysaccharides, including cellulose[68], pullulan, starch and curdlan[69]. TEMPO mediated curdlan oxidation can produce water-soluble β-(1→3)-polyglucuronic acid, with significant potential in biotechnological fields.

Delattre *et al.*[70] reported curdlan oxidation using the TEMPO/NaBr/NaOCl system under basic conditions. Oxidation of 1 mol of primary alcohol to the carboxylic acid requires 2 mol of NaOCl, used to regenerate the TEMPO radical that is the actual curdlan oxidant. The proposed catalytic cycle comprised of TEMPO radical (**a**), *N*-oxoammonium ion (**b**) and hydroxylamine (**c**) is shown in **Fig. 2.18** (**Step 1**). Reaction at pH 11 for 1 h produced pure, water-soluble β-(1→3)-polyglucuronic acid with a degree of oxidation (DO) 100%. SEC-MALLS analysis confirmed some depolymerization during oxidation, presumably due to the propensity of uronic acids to undergo 5,4-elimination reactions, but the molecular weight of the final products was not significantly reduced from that of the starting curdlan. In the ^{13}C NMR spectrum, the new signal of the carboxyl group around 175 ppm together with the absence of a C-6 resonance at 61 ppm for native curdlan was indicative of total oxidation of the primary 6-OH. Low molecular weight β-(1→3)-polyglucuronic acid sodium salts (LMW-PGU, DP up to 25) were produced in two steps: i) radical depolymerization (1% H_2O_2, w/w) or thermal depolymerization (120 °C, 40 min) and ii) subsequent *O*-sulfation ($DS_{sulfate}$ 1.35) or *O*-acetylation ($DS_{acetate}$ 2.55; **Fig. 2.18 Step 2**).[71] The resulting polymers were utilized for transcriptomic analysis to predict the impact of carbohydrates on biological processes. Preliminary results identified the levels of gene expression induced by anionic β-(1→3)-glucans and their acetylated and sulfated derivatives across the human genome.

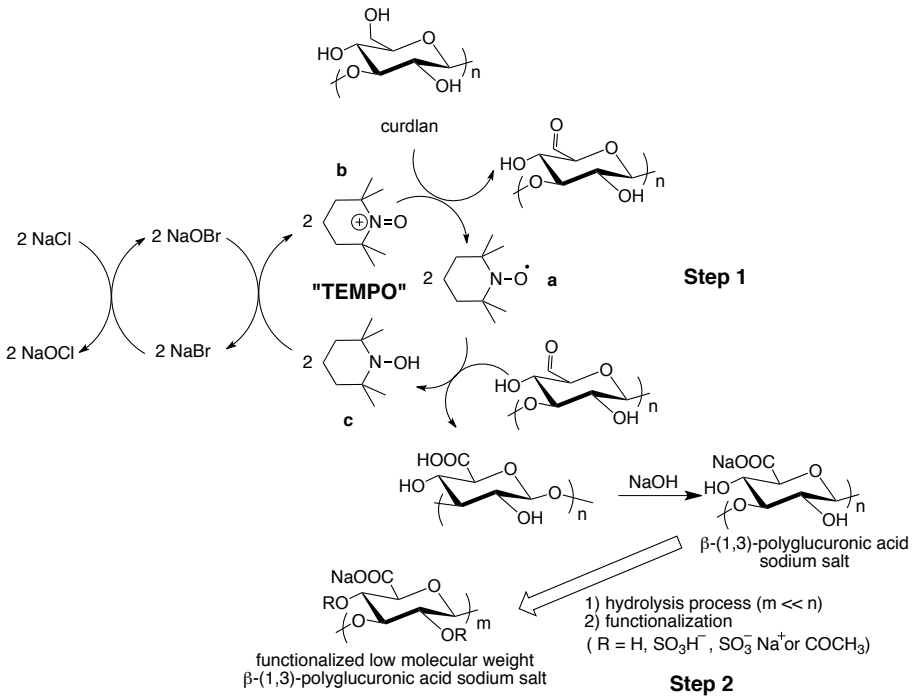

Fig. 2.18. Simplified scheme of curdlan regioselective TEMPO oxidation (**Step 1**) and subsequent functionalization (**Step 2**).[71] Adapted with permission from Delattre, C.; Michaud, P.; Chaisemartin, L.; Berthon, J. Y.; Rios, L. A transcriptomic approach to predict the impact of β-(1,3)-polyglucuronic acid sodium salt and derivatives in the main biological processes. *Carbohydr. Polym.* **2012**, *87 (2)*, 1828-1836. Copyright 2011 Elsevier.

Another system, 4-acetamido-TEMPO/NaClO/$NaClO_2$ (**Fig. 2.19**) was used to perform regioselective C-6 oxidation of curdlan (DO 100%) under acidic conditions (pH 4.7, 35 °C). By varying the reaction time, temperature, reagent ratios and pH, a family of β-(1,3)-polyglucuronic acid sodium salts with different DOs was obtained. Tamura *et al.*[69] showed that curdlan with DO 95% had a weight-average DP more than 1000, much higher than that prepared by the conventional TEMPO/NaBr/NaOCl oxidation at pH 10. Compared with the C-6 oxidation of starch, amylose and pullulan under the same conditions, curdlan derivatives had higher carboxyl content, indicating that the C-6 hydroxyls on α-(1,4)- and α-(1,6)-glucans were less susceptible to oxidation than those on β-(1,3)-glucan. Using COSY and HSQC spectra, Watanabe *et al.*[72] assigned all ^{1}H and ^{13}C chemical shifts of the polyglucuronic acid. SEC-MALLS results demonstrated that depolymerization during the oxidation took place randomly along the curdlan main chains. Based on optical rotatory

dispersion (ORD) spectra, Lien and colleagues[73] found that C-6 oxidized curdlan behaved as a random coil in aqueous media at various pH values, advantagously for complexation with polymeric guests. They investigated three types of pH-responsive complexes between β-(1,3)-polyglucuronic acid and single-walled carbon nanotubes (SWNTs), cationic water-soluble polythiophenes (PT-1) or polycytidylic acid (poly (C)). The interaction with SWNTs afforded a water-soluble complex, stable in aqueous solution for several months The wrapping of the hydrophobic SWNT by the hydrophobic interior of the C-6 oxidized curdlan, combined with the hydrophilic exterior of the complex provided by the ionized carboxyl groups, afforded the desired water disperibility. The PT-1 complex became CD-active only when pH ≥ 7 and the positive Cotton effect suggested that the conjugated curdlan main chain was twisted in the right-handed direction. The binding and release of poly (C) complex was dependent on salt concentrations.

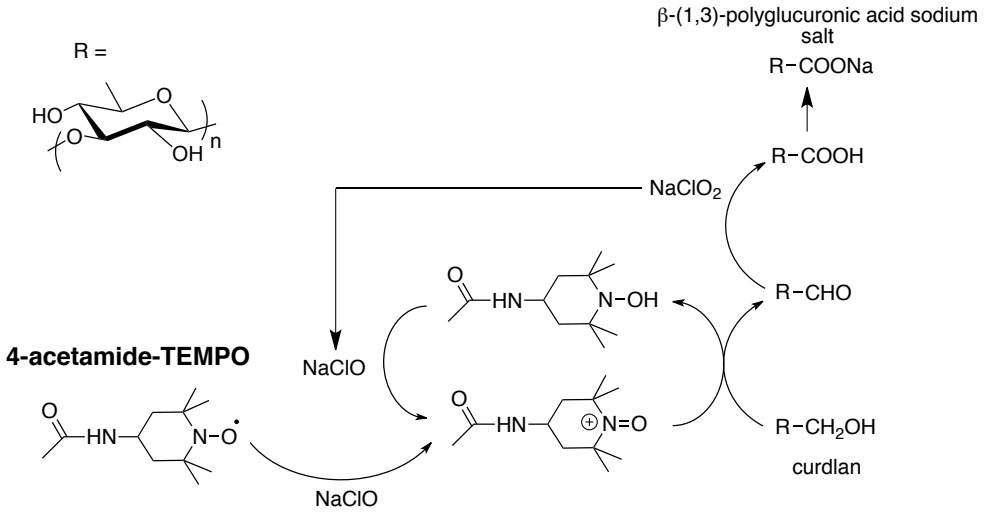

Fig.2.19. Oxidation of primary hydroxyls by 4-acetamide-TEMPO/NaClO/$NaClO_2$ system under acid-neutral condition.[69] Reprinted with permission from Tamura, N.; Hirota, M.; Saito, T.; Isogai, A. Oxidation of curdlan and other polysaccharides by 4-acetamide-TEMPO/NaClO/$NaClO_2$ under acid conditions. *Carbohydr. Polym.* **2010**, *81 (3)*, 592-598. Copyright 2010 Elsevier.

2.3.4.2 Curdlan C-6 bromination and azidation

Borjhan *et al.*[74] reported the direct regioselective azidation of curdlan with triphenylphosphine (Ph_3P), carbon tetrabromide (CBr_4) and lithium azide (LiN_3) in DMF at

room temperature to synthesize 6-azido-6-deoxycurdlan in 87% yield (**Fig. 2.20-A**). The IR spectrum showed a strong absorption at 2100 cm^{-1} indicating N=N=N stretching vibration. The ^{13}C NMR spectrum showed the expected upfield C-6 shift from 61 ppm (OH) to 51 ppm (N_3) and the DS_{azide} was estimated to be 0.84 by quantitative ^{13}C NMR. Hasegawa *et al.*[75] described a two-step method to produce azido curdlan in 100% yield by first reacting curdlan with a halogenation agent, then displacing the halogen with the azide group (**Fig. 2.20-B**). The disappearance of C-6-OH peak at 61 ppm and the presence of a new resonance around 44 ppm in the ^{13}C NMR spectrum of bromo curdlan indicated that the bromination took place exclusively at the C-6 position (6-bromo-6-deoxycurdlan).

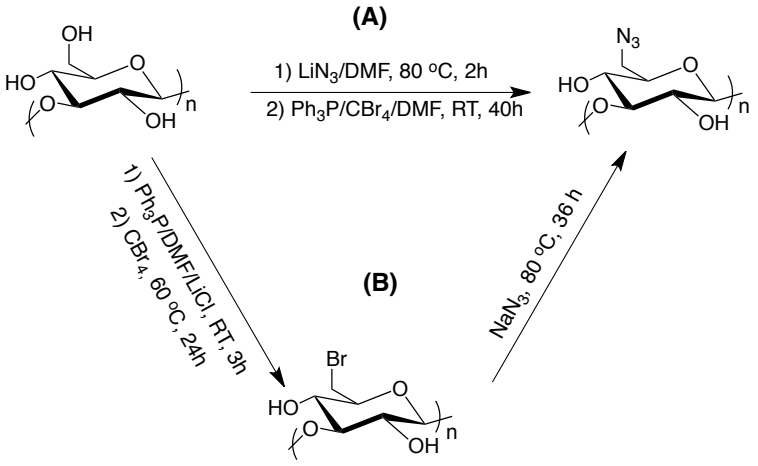

Fig. 2.20. Synthesis of 6-azido-6-deoxycurdlan via **(A)** direct synthesis and **(B)** two-step method with 6-bromo-6-deoxycurdlan as the intermediate.

2.3.4.3 Chemoselective coupling of 6-azido-6-deoxy-curdlan and alkyne-terminated functional modules by "click chemistry"

Hasegawa *et al.*[76] reported Cu(I)-catalyzed coupling between 6-azido-6-deoxycurdlan and alkyne-terminated functional modules to synthesize a series of artificial β-(1,3)-glucans with various functional appendages. Native curdlan was first converted to 6-azido-6-deoxycurdlan following the two-step approach described in section 3.5.2. Alkyne-terminated modules bearing various other functional groups (**Fig. 2.21**) were coupled with azido curdlan ($CuBr_2$, ascorbic acid, base (triethylamine, n-propylamine or ammonia), DMSO solvent), to append C-6 branches to curdlan. Those bulky molecules are difficult to incorporate into polysaccharides via chemo-enzymatic monosaccharide polymerization (bottom-up strategy).

This Cu(I)-catalyzed cycloaddition is an example of 'click chemistry'[77] with high yield, wide scope, stereospecificity, and rapid, clean kinetics. In the IR spectra of all obtained curdlan derivatives, the characteristic absorption of N_3 at 2100 cm^{-1} disappeared after modification. The ^{13}C NMR spectra provided evidence of conversion from azido curdlan to targeted derivatives by 1) absence of N_3 signal at 51 ppm, 2) two new resonances at 145 and 124 ppm assigned to the 1,4-triazole-linker, and 3) all new peaks assignable to functional appendages. Advantageously, this reaction can be monitored *in situ* by ATR-IR spectroscopy to tune the reaction conditions. Based on the disappearance of the N_3 peak, all chemoselective couplings occurred with quantitative conversion within 1 h. Furthermore, this reaction can be performed in several polar organic solvents, such as DMSO, DMF and NMP, enabling use with various bulky functional groups.

The resultant curdlan derivatives had low cytoxicity and long blood circulation time, providing potential promise for biomedical applications. Interactions between curdlan derivatives and poly(C) have been carried out to form stable macromolecular complexes that showed strong and specific lectin-affinity, beneficial for use as polynucleotide carriers.

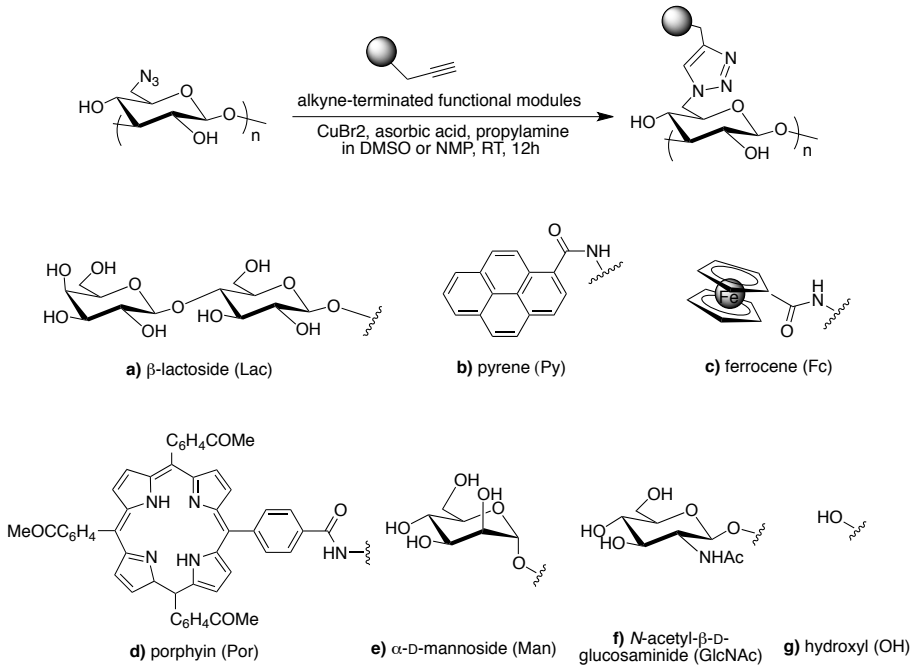

Fig. 2.21. Chemoselective coupling between 6-azido-6-deoxycurdlan and alkyne-terminated functional modules.[76]

2.3.5 Sulfation of curdlan

Acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus (HIV) is a severe disease that can destroy the body's immune system, so the discovery of methods to prevent AIDS infection is of great importance. There have been a number of reports stating that curdlan sulfates with high DS exhibit high anti-HIV activity *in vitro* since 1990, as part of a broader research effort on anionic polysaccharide derivatives as candidate components of anti-HIV contraceptive foams.

Yoshida *et al.*[78] synthesized curdlan sulfates using piperidine-*N*-sulfonic acid (PSA) in DMSO (**Fig. 2.22(A)**). Depending on molar ratios, reaction time and temperature, curdlan sulfates with DS ranging from 0.35 to 1.6 were obtained. From the ^{1}H, ^{13}C and COSY NMR spectra, sulfation took place almost exclusively at O-6 at lower DS, then at O-2 as DS increased. Even at DS(SO_3H) of 1.6, very little substitution at O-4 was observed. Anti-HIV assays were carried out on MT-4 cells with activity determined by prevention of HIV-induced cytopathic effects. Curdlan sulfates with DS 1.6 could inhibit HIV infection at a concentration as low as 3.3 μg/mL.

Osawa *et al.*[79] performed curdlan sulfation with an SO_3-pyridine complex in DMF (**Fig. 2.22(B)**) and curdlan sulfopropylation with propane sultone in DMSO (**Fig. 2.22(C)**) to investigate the effects of chain length of the sulfate-containing group and sulfating condition (homogeneous or heterogeneous phase) on anti-HIV activity. EC_{50} and SI (selectivity index, **Eq. 2.2**) were used to evaluate anti-HIV activity. Based on the experimental results, sulfopropyl curdlan inhibited HIV infection more weakly than standard curdlan sulfate (**Fig. 2.22 (A)**) since the latter showed lower EC_{50} and higher SI. The substitution pattern of curdlan sulfates obtained in homogeneous reactions indicated that the reactivity of the three hydroxyls was O-6 > O-2 > O-4. In contrast, under heterogeneous sulfation conditions, as the total DS increased, the O-4 position was sulfated as well as O-2. Since the insoluble and self-associated portion of heterogeneously-prepared curdlan sulfates was shown to denature MT-4 cells, heterogeneous sulfation appears undesirable due to unwanted cytotoxicity.

$$SI = CC_{50}/EC_{50}, \qquad \textbf{(Eq. 2.2)}$$

where EC_{50} - 50% effective concentration of polysaccharide solution

CC_{50} - 50% cytotoxic concentration of polysaccharide solution

Gao and coworkers synthesized three regioselectively substituted curdlan sulfates with medium molecular weight to investigate the relationship between substitution distribution and anti-HIV activity.[80] Commercial high MW curdlan was first hydrolyzed to medium MW and then sulfated with SO_3-pyridine/DMSO to produce curdlan sulfate 62S (see **Fig. 2.23(A)** for schemes and abbreviations). Curdlan sulfate 42S and 642S were prepared by a protecting-deprotecting group strategy. Curdlan was first pivaloylated to block the C-6 position, followed by sulfation and depivaloylation (**Fig. 2.23(B)**). The regioselectivity of substitution was confirmed by the chemical shifts of C-2/4/6 peaks in ^{13}C NMR spectra. The anti-HIV and anticoagulant activities of the resulting curdlan sulfates were assayed by the MTT method with the MT-4 cell line and activated partial thromboplastin time (APTT) calculation *in vitro*, respectively. It was revealed that these curdlan sulfates with average MW ranging from 6.2×10^3 to 10.8×10^3 showed high anti-HIV activity characterized by low EC_{50} (0.04~0.4 μg/mL) and low cytotoxicity as shown by high CC_{50} (> 1000 μg/mL). Furthermore, they concluded that the anti-HIV activities of curdlan sulfates depended on DS rather than on the position of sulfation.

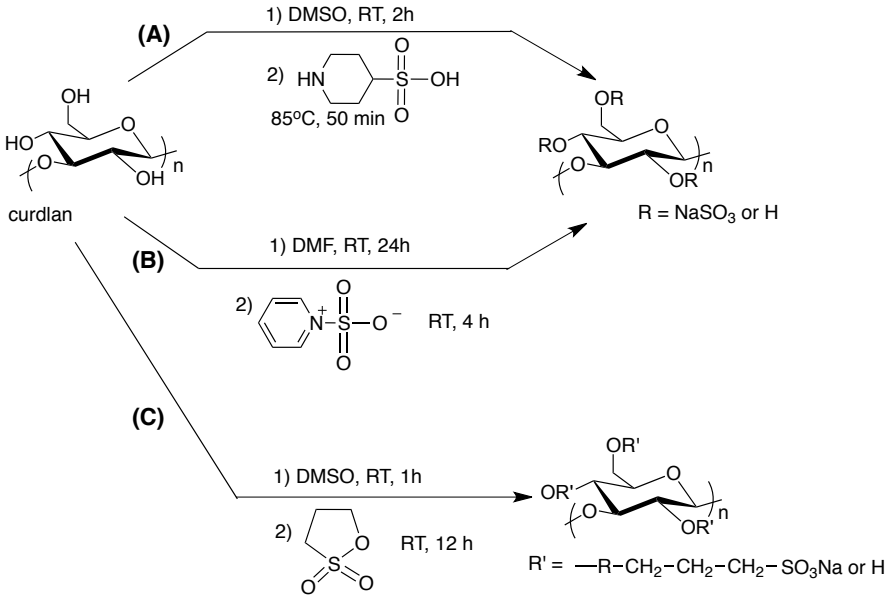

Fig. 2.22. Synthetic schemes for curdlan sulfates: **A)** piperidine-*N*-sulfonic acid in DMSO, **B)** SO_3-pyridine complex in DMF and **C)** propane sultone in DMSO.

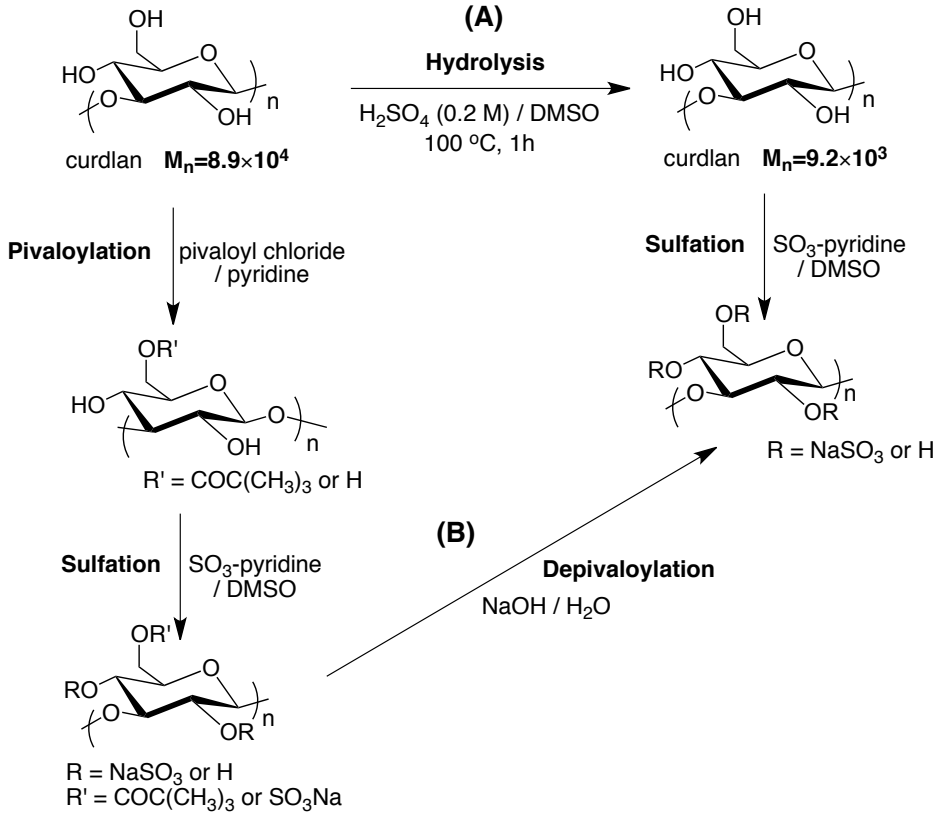

Fig. 2.23. Synthetic routes of regioselective substituted curdlan sulfates[80]: **(A)** sulfated at all C-6 and some C-2 positions (S62); **(B)** sulfated at all C-4 and some C-2 positions (42S) and some C-6, C-4 and C-2 positions (642S). Adapted with permission from Gao, Y.; Fukuda, A.; Katsuraya, K.; Kaneko, Y.; Mimura, T.; Nakashima, H.; Uryu, T. Synthesis of regioselective substituted curdlan sulfates with medium molecular weights and their specific anti-HIV-1 activities. *Macromolecules* **1997**, *30*, 3224-3228. Copyright 1997 American Chemical Society.

Jagodzinski *et al.*[81] proposed a mechanism for the anti-HIV activity of curdlan sulfates *in vitro*. When present for more than 24 h, curdlan sulfate was found to impede the membrane fusion process during HIV infection but had no effect on the virions. It neither accumulated inside the cells to reach concentrations sufficient to inhibit HIV infection nor blocked cellular receptors. Inhibition of virus entry into the target cells was also attributed to the

interactions of curdlan sulfates with both the continuous epitopes on the V3 loop and the discontinuous CD4 binding site of gp120.

Polysaccharide sulfates have been studied as components of intravaginal hydrogel formulations to provide long-term prevention of HIV transmission. Some recent clinical results have cast doubt on the utility of sulfated polysaccharides for prevention of HIV infection, and have even revealed potential, unexpected negative effects on anti-HIV contraception. Carraguard, a sulfated polysaccharide hydrogel formulation, has been found to prevent HIV-infected cells from migrating across vaginal epithelia. Although it passed the phase I and phase II safety tests for both healthy and HIV-negative humans, a placebo-controlled phase III trial revealed that the rates of incident HIV infections were similar in Carraguard and placebo groups.[82] Ushercell (sodium cellulose sulfate) was applied to two phase III efficacy trials versus placebo in Africa and India. However, both studies were halted because the researchers found out that the HIV seroincidence was higher in women receiving Ushercell than those receiving placebo gel.[83]

2.3.6 Synthesis of branched curdlan derivatives

The antitumor activity of (1→3/1→6)-β-glucans was initially attributed to their ordered structures (single or triple helices) and high molecular weights. Demleitner and coworkers[84] synthesized a series of branched curdlan derivatives and investigated the effects of sugars attached to the C-6 position on antitumor activity. They used trityl as the protecting group and designed a protecting-deprotecting strategy to selectively introduce side chain sugars to C-6 (**Fig. 2.24**). With 2,6-dimethylpyridinium perchlorate as the catalyst, condensation between sugar ortho esters and 2,4-di-*O*-phenylcarbamoylcurdlan was the most essential step for branching. Products with various degrees of branching (DB), determined by methylation analysis, were obtained by varying the reaction time and reactant molar ratios. By forming complexes with Congo Red, the curdlan derivative was stable in NaOH (< 0.1 M), indicating the presence of single helices. Interestingly, antitumor activity tests showed that the rhamnosyl and arabinosyl derivatives were the most active (**Table 2.2**). These findings suggested that the antitumor activity of these polysaccharides was not necessarily correlated with the degree of tertiary structural order.

Table 2.2.

Antitumor activity of branched curdlan derivatives against *Sarcoma* 180[84 a]

branch	D.b.[b] (%)	dose[c] (mg/kg)	tumor weight (g)	inhibition[c] ratio (%)	significance[d] (p <)
control			5.40		0.02
glucose	29	5	0.79	85	n.s.
		25	2.20	59	n.s.
gentiobiose	29	5	1.72	68	0.02
		25	1.26	77	0.01
rhamnose	33	5	0.39	93	0.01
		25	0.04	99	0.01
arabinose	33	5	0.07	99	0.01
		25	0.04	99	0.01

[a]Note: Adapted with permission from Demleitner, S.; Kraus, J.; Franz, G. Synthesis and antitumour activity of derivatives of curdlan and lichenan branched at C-6. *Carbohydr. Res.* **1992**, *226 (2)*, 239-246. Copyright 1992, Elsevier. [b]Degree of branching. [c]Treatment was performed daily from day 1-10, i.p. [d] $\% = (C - {}^{T}/_{C}) \times 100$, where C is the average tumor weight of the control group an dT is the average tumor weight of the treated group. [e]Evanluated according to the Student's *t* test (p>0.05=n.s.).

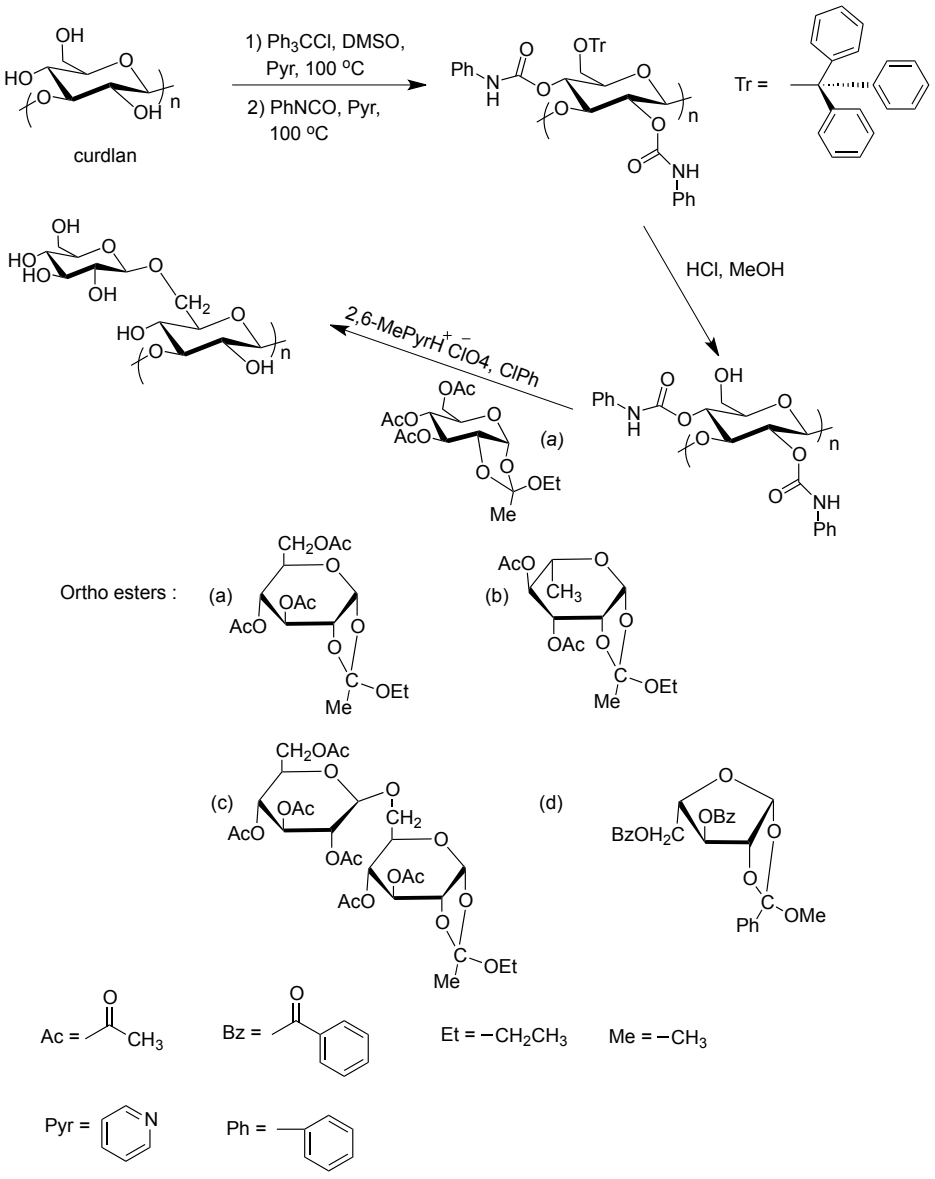

Fig. 2.24. Synthetic scheme for branching glycosylation of curdlan with: (a) 3,4,6-tri-*O*-acetyl-α-D-glucose 1,2-(ethyl orthoacetate); (b) 3,4-di-*O*-acetyl-β-L-rhamnose 1,2-(ethyl orthoacetate); (c) 3,4,2',3',4',6'-hexa-*O*-acetyl-α-gentiobiose 1,2-(ethyl orthoacetate); (d) 3,5-di-*O*-benzoyl-β-L-arabinofuranose 1,2-(methyl orthobenzoate).[84] Adapted with permission from Demleitner, S.; Kraus, J.; Franz, G. Synthesis and antitumour activity of derivatives of curdlan and lichenan branched at C-6. *Carbohydr. Res.* **1992**, *226 (2)*, 239-246. Copyright 1992 Elsevier.

In addition, curdlans with glycosyl side chains were sulfated with piperidine-N-sulfonic acid to produce branched curdlan sulfates for an anti-AIDS virus infection study (**Fig. 2.25**).[85] To increase the proportion of branches, Yoshida and coworkers repeated the condensation reaction between curdlan and glucosyl/mannosyl orthoacetates three times and finally obtained 17~39 mol % branched curdlan derivatives containing of D-glucosyl, L-glucosyl, D-mannosyl and L-mannosyl side chains, respectively. After subsequent sulfation, all branched curdlan sulfates were soluble in water, with the mannosyl-branched derivatives having higher DS than glucosyl-branched derivatives (1.8 *vs.* 1.3).

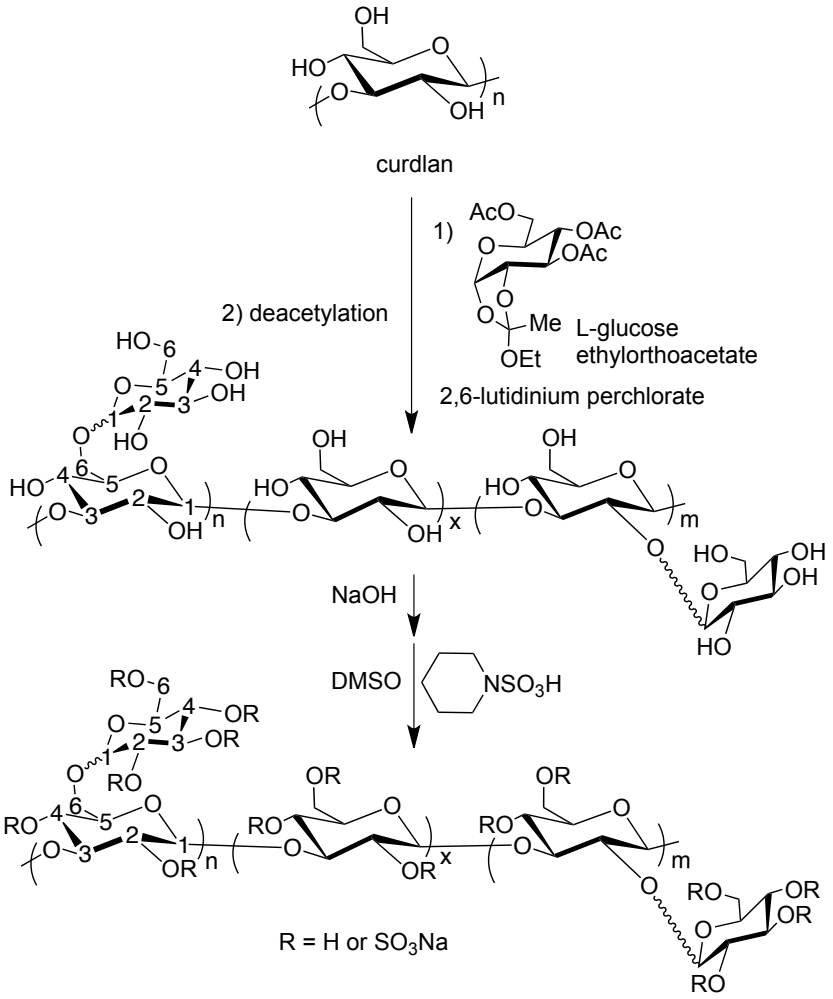

Fig. 2.25. Synthesis of L-glycosyl-branched curdlan sulfate.[85] Reprinted with permission from Yoshida, T.; Yasuda, Y.; Uryu, T.; Nakashima, H.; Yamamoto, N.; Mimura, T.; Kaneko, Y.; Synthesis and in vitro inhibitory effect of L-glycosyl-branched curdlan sSulfates

on AIDS virus infection. *Macromolecules* **1994**, *27*, 6272-6276. Copyright 1994 American Chemical Society.

Compared with curdlan sulfates, the branched curdlan sulfate derivatives inhibited the cytopathic effects of AIDS virus infection at lower concentrations. They exhibited high anti-AIDS activity in the EC_{50} range of 0.3~1.2 μg/mL *in vitro* using MT-4 cells. Low cytotoxicity and low anticoagulant activity were observed, favorable for preventing side effects during protection against AIDS virus infection. Furthermore, a clearance time study of glycosyl-branched curdlan sulfates in the rat indicated that only a third of the initial concentration was detected 3 h after injection and the disappearance was ascribed to absorption mainly in the liver, bone marrow, kidney and lymph nodes.

2.4 Conclusions and perspectives

Recent advances in understanding of the secondary structure of curdlan – single and triple helix – have provided opportunities to explain its "conspicuously unusual" rheological properties and gelation behaviors. The natural extracellular polysaccharide curdlan is non-toxic and has been shown to be biocompatible to mammals. Both properties support its application in foods as a bio-thickener, gelling agent, and food additive. However, the limited solubility of curdlan in aqueous and organic solvents hampers its application to other specific areas. The development of chemically modified curdlan derivatives creates significant potential for tailoring curdlan materials for those targeted applications, especially in the biomedical and pharmaceutical fields. The development of efficient methods to introduce functional groups onto the curdlan backbone, enabling measurement of physicochemical properties of the derivatives, helps us to illuminate the structure-property relationships of curdlan derivatives. Preparation of water-soluble curdlan derivatives has been achieved by carboxymethylation, phosphorylation and sulfation. Apart from the degree of substitution, the substitution pattern plays an essential role as well in determining the resultant properties of curdlan derivatives. Oxidation by TEMPO at the C-6 position has provided water-soluble curdlan carboxylates useful for transcriptomic analysis. Additionally, the growing interest in aminated polysaccharides, driven by their positive interactions with proteins, nucleic acids and other biological compounds, has led to research into the introduction of amine groups onto the curdlan polymer chain. Regioselective bromination

followed by displacement with an azide group have provided 6-azido curdlan, a promising precursor for chemoselective coupling with alkyne-terminated functional molecules by click-chemistry to produce a series of artificial β-(1,3)-glucans with various functional appendages. In the near future, we can anticipate that researchers will employ azidocurdlan to synthesize a wide variety of potentially useful aminocurdlans with desirable solubility. *In vivo* cell line tests of curdlan derivatives for biomedical applications are the topic of much recent research. Due to curdlan's unique helical structure, gel-forming capacity and potent pharmacological properties, work on curdlan derivatization has emphasized two aspects: one being the use of gels to develop nanostructures for encapsulation and release of biologically active reagents, and the other being the creation of previously inaccessible curdlan derivatives for immunotherapy to treat cancers, heal wounds and inhibit microbes, fungi and viruses. These focused efforts point the way to much broader, enhanced prospects for curdlan and its derivatives as promising renewable biomaterials.

2.5 Acknowledgements

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2.6 References

1. McIntosh, M.; Stone, B. A.; Stanisich, V. A., Curdlan and other bacterial (1→3)-β-D-glucans. *Appl. Microbiol. Biotechnol.* **2005,** *68*, 163-173.

2. Harada, T.; Masada, M.; Hidaka, H.; Takada, M., Production of firm, resilient gel-forming polysaccharide in natural medium by a mutant of Alcaligenes faecalis var myxogenes 10C3. *Hakko Kogaku Zasshi* **1966,** *44*, 20-4.

3. Harada, T.; Misaki, A.; Saito, H., Curdlan. Bacterial gel-forming β-1,3-glucan. *Arch. Biochem. Biophys.* **1968,** *124*, 292-8.

4. (a) Kalyanasundaram, G. T.; Doble, M.; Gummadi, S. N., Production and downstream processing of (1→3)-β-D-glucan from mutant strain of Agrobacterium sp. ATCC 31750. *AMB Express* **2012,** *2*, 31; (b) Karnezis, T.; Fisher, H. C.; Neumann, G. M.;

Stone, B. A.; Stanisich, V. A., Cloning and characterization of the phosphatidylserine synthase gene of Agrobacterium sp. strain ATCC 31749 and effect of its inactivation on production of high-molecular-mass (1→3)-β-D-glucan (curdlan). *J. Bacteriol.* **2002,** *184*, 4114-4123; (c) West, T. P., Elevated curdlan production by a mutant of Agrobacterium sp. ATCC 31749. *J. Basic Microbiol.* **2009,** *49*, 589-592; (d) Yu, L.; Wu, J.; Zheng, Z.; Zhan, X., Involvement of ntrB gene in regulation of exopolysaccharide biosynthesis in Agrobacterium sp. ATCC 31749. *Gongye Weishengwu* **2011,** *41*, 26-33.

5. Yotsuzuka, F., Curdlan. *Food Sci. Technol. (N. Y., NY, U. S.)* **2001,** *113*, 737-757.

6. Kasai, N.; Harada, T., Ultrastructure of curdlan. *ACS Symp. Ser.* **1980,** *141*, 363-83.

7. (a) Bohn, J. A.; BeMiller, J. N., (1→3)-β-D-Glucans as biological response modifiers: a review of structure-functional activity relationships. *Carbohydr. Polym.* **1995,** *28*, 3-14; (b) Stone, B. A.; Clarke, A. E., *Chemistry and biology of (1→3)-β-D-glucans*. La Trobe University: Melbourne, 1992.

8. (a) Cui, S. W.; Wang, Q.; Zhang, M., Chapter 11 β-Glucans. In *Renewable Resources for Functional Polymers and Biomaterials: Polysaccharides, Proteins and Polyesters*, The Royal Society of Chemistry: 2011; pp 319-345; (b) Lehtovaara, B. C.; Gu, F. X., Pharmacological, Structural, and Drug Delivery Properties and Applications of 1,3-β-Glucans. *Journal of Agricultural and Food Chemistry* **2011,** *59* (13), 6813-6828; (c) Nishinari, K.; Zhang, H.; Funami, T., Handbook of hydrocolloids

Curdlan. 2009; pp 567-591; (d) Zhan, X.-B.; Lin, C.-C.; Zhang, H.-T., Recent advances in curdlan biosynthesis, biotechnological production, and applications. *Applied Microbiology & Biotechnology* **2012,** *93* (2), 525-531.

9. Harada, T.; Misaki, A.; Saito, H., Curdlan: A bacterial gel-forming β-1, 3-glucan. *Archives of Biochemistry and Biophysics* **1968,** *124* (0), 292-298.

10. Saito, H.; Misaki, A.; Harada, T., A comparison of the structure of curdlan and pachyman. *Agr. Biol. Chem. (Tokyo)* **1968,** *32*, 1261-9.

11. Nakata, M.; Kawaguchi, T.; Kodama, Y.; Konno, A., Characterization of curdlan in aqueous sodium hydroxide. *Polymer* **1998,** *39*, 1475-1481.

12. Harada, T.; Harada, A., Curdlan and succinoglycan. In *Polysaccharides in medical applications*, Dumitri, S., Ed. Dekker: New York, 1996; pp 21-58.

13. Nakanishi, I.; Kimura, K.; Suzuki, T.; Ishikawa, M.; Banno, I.; Sakane, T.; Harada, T., Demonstration of curdlan-type polysaccharide and some other β-1,3-glucans in microorganisms with aniline blue. *J. Gen. Appl. Microbiol.* **1976,** *22*, 1-11.

14. Phillips, K. R.; Pik, J.; Lawford, H. G.; Lavers, B.; Kligerman, A.; Lawford, G. R., Production of curdlan-type polysaccharide by Alcaligenes faecalis in batch and continuous culture. *Can. J. Microbiol.* **1983,** *29*, 1331-1338.

15. Stasinopoulos, S. J.; Fisher, P. R.; Stone, B. A.; Stanisich, V. A., Detection of two loci involved in (1→3)-β-glucan (curdlan) biosynthesis by Agrobacterium sp. ATCC31749, and comparative sequence analysis of the putative curdlan synthase gene. *Glycobiology* **1999,** *9*, 31-41.

16. Kim, M.-K.; Ryu, K.-E.; Choi, W.-A.; Rhee, Y.-H.; Lee, I.-Y., Enhanced production of (1→3)-β-D-glucan by a mutant strain of Agrobacterium species. *Biochem. Eng. J.* **2003,** *16*, 163-168.

17. Kanegae, K.; Yutani, A.; Nakatsui, I. Agrobacterium mutants for manufacture of β-1,3-glucan. JP07067679A, 1995.

18. Ghai, S. K.; Hisamatsu, M.; Amemura, A.; Harada, T., Production and chemical composition of extracellular polysaccharides of Rhizobium. *J. Gen. Microbiol.* **1981,** *122*, 33-40.

19. Footrakul, P.; Suyanandana, P.; Amemura, A.; Harada, T., Extracellular polysaccharides of Rhizobium from the Bangkok MIRCEN collection. *J. Ferment. Technol.* **1981,** *59*, 9-14.

20. Mamaril, J. C.; Paner, E. T.; Palacpac, E. S., The production of gel-forming polysaccharides by Rhizobium sp. and curdlan by a mutant cultured in coconut water. *Trans. Natl. Acad. Sci. Technol., Repub. Philipp.* **1989,** *10*, 339-49.

21. Sutherland, I. W., Microbial exopolysaccharide synthesis. *ACS Symp. Ser.* **1977,** *45*, 40-57.

22. Kai, A.; Ishino, T.; Arashida, T.; Hatanaka, K.; Akaike, T.; Matsuzaki, K.; Kaneko, Y.; Mimura, T., Biosynthesis of curdlan from culture media containing 13C-labeled glucose as the carbon source. *Carbohydrate Research* **1993,** *240* (0), 153-159.

23. Lee, I.-Y. In *Curdlan*, Wiley-VCH Verlag GmbH: 2005; pp 135-158.

24. Futatsuyama, H.; Yui, T.; Ogawa, K., Viscometry of curdlan, a linear (1→3)-β-D-glucan, in DMSO or alkaline solutions. *Biosci., Biotechnol., Biochem.* **1999,** *63*, 1481-1483.

25. Ogawa, K.; Watanabe, T.; Tsurugi, J.; Ono, S., Conformational behavior of a gel-forming (1.far.3)-β-D-glucan in alkaline solution. *Carbohyd. Res.* **1972,** *23*, 399-405.

26. Ogawa, K.; Tsurugi, J., Dependence of the conformation of a (1 .far. 3)-β-D-glucan on chain length in alkaline solution. *Carbohyd. Res.* **1973,** *29*, 397-403.

27. Ogawa, K.; Miyagi, M.; Fukumoto, T.; Watanabe, T., Effect of 2-chloroethanol, dioxane, or water on the conformation of a gel-forming β-1,3-D-glucan in dimethyl sulfoxide. *Chem. Lett.* **1973**, 943-6.

28. Ogawa, K.; Tsurugi, J.; Watanabe, T., Effect of salt on the conformation of gel-forming β-1,3-D-glucan in alkaline solution. *Chem. Lett.* **1973**, 95-8.

29. Fulton, W. S.; Atkins, E. D. T., The gelling mechanism and relationship to molecular structure of microbial polysaccharide curdlan. *ACS Symp. Ser.* **1980,** *141*, 385-410.

30. Okuyama, K.; Otsubo, A.; Fukuzawa, Y.; Ozawa, M.; Harada, T.; Kasai, N., Single-helical structure of native curdlan and its aggregation state. *J. Carbohydr. Chem.* **1991,** *10*, 645-56.

31. Chuah, C. T.; Sarko, A.; Deslandes, Y.; Marchessault, R. H., Packing analysis of carbohydrates and polysaccharides. Part 14. Triple-helical crystalline structure of curdlan and paramylon hydrates. *Macromolecules* **1983,** *16*, 1375-82.

32. Maeda, I.; Saito, H.; Masada, M.; Misaki, A.; Hara, T., Properties of gels formed by the heat treatment of curdlan, a bacterial β-1,3-glucan. *Agr. Biol. Chem. (Tokyo)* **1967,** *31*, 1184-8.

33. Watase, M.; Nishinari, K. In *Rheology and DSC of curdlan - DMSO - water systems*, Plenum: 1993; pp 125-9.

34. (a) Harada, T.; Koreeda, A.; Sato, S.; Kasai, N., Electron microscopic study on the ultrastructure of curdlan gel: assembly and dissociation of fibrils by heating. *J. Electron Microsc.* **1979,** *28*, 147-53; (b) Kanzawa, Y.; Harada, T.; Koreeda, A.; Harada, A., Curdlan gel formed by neutralizing its alkaline solution. *Agric. Biol. Chem.* **1987,** *51*, 1839-43.

35. Saito, H.; Ohki, T.; Sasaki, T., A carbon-13 nuclear magnetic resonance study of gel-forming (1 → 3)-β-D-glucans. Evidence of the presence of single-helical conformation in a resilient gel of a curdlan-type polysaccharide 13140 from Alcaligenes faecalis var myxogenes IFO 13140. *Biochemistry* **1977,** *16*, 908-14.

36. Konno, A.; Harada, T., Thermal properties of curdlan in aqueous suspension and curdlan gel. *Food Hydrocolloids* **1991,** *5*, 427-34.

37. Gagnon, M.-A.; Lafleur, M., From curdlan powder to the triple helix gel structure: an attenuated total reflection-infrared study of the gelation process. *Appl. Spectrosc.* **2007,** *61*, 374-378.

38. Ikeda, S.; Shishido, Y., Atomic force microscopy studies on heat-induced gelation of Curdlan. *J. Agric. Food Chem.* **2005,** *53*, 786-791.

39. Zhang, L.; Wang, C.; Cui, S.; Wang, Z.; Zhang, X., Single-Molecule Force Spectroscopy on Curdlan: Unwinding Helical Structures and Random Coils. *Nano Lett.* **2003,** *3*, 1119-1124.

40. Zhang, H.; Nishinari, K.; Williams, M. A. K.; Foster, T. J.; Norton, I. T., A molecular description of the gelation mechanism of curdlan. *Int. J. Biol. Macromol.* **2002,** *30*, 7-16.

41. Ohno, N.; Terui, T.; Chiba, N.; Kurachi, K.; Adachi, Y.; Yadomae, T., Resistance of highly branched (1→3)-β-D-glucans to formolysis. *Chem. Pharm. Bull.* **1995,** *43*, 1057-60.

42. Kajikawa, A.; Kameno, M.; Murosaki, S.; Kusaka, H. Immunostimulants containing linear carbohydrates having β-1,3-glucoside linkage. JP10194977A, 1998.

43. Prieto, M. A.; Vazquez, J. A.; Murado, M. A., Hydrolysis optimization of mannan, curdlan and cell walls from Endomyces fibuliger grown in mussel processing wastewaters. *Process Biochem. (Amsterdam, Neth.)* **2011,** *46*, 1579-1588.

44. (a) Kusama, S.; Kusakabe, I.; Murakami, K., Studies on the β-1,3-glucanase system of Streptomyces. Part II. A preparation method of gentiobiose from curdlan using the enzyme system from Streptomyces sp. *Agric. Biol. Chem.* **1984,** *48*, 2655-62; (b) Kusama, S.; Kusakabe, I.; Zama, M.; Murakami, K.; Yasui, T., Studies on the β-1,3-glucanase system of Streptomyces. Part I. Enzymic preparation of crystalline laminaribiose from curdlan. *Agric. Biol. Chem.* **1984,** *48*, 1433-40.

45. Kusama, S.; Kusakabe, I.; Murakami, K., Studies on the β-1,3-glucanase system of Streptomyces. Part III. Mechanism of formation of gentiobiose from curdlan by enzymes of Streptomyces sp. *Agric. Biol. Chem.* **1985,** *49*, 2055-61.

46. Watanabe, S.; Kusakabe, I.; Murakami, K., Induction of β-1,3-glucanase system from Streptomyces sp. W 19-1. *Kiyo - Seitoku Eiyo Tanki Daigaku* **1990,** *21*, 29-32.

47. Wang, L.; Sakairi, N.; Kuzuhara, H., Peracetylated laminaribiose: preparation by specific degradation curdlan and its chemical conversion into N-acetylhyalobiuronic acid. *Carbohydr. Res.* **1991,** *219*, 133-48.

48. Grandpierre, C.; Janssen, H. G.; Laroche, C.; Michaud, P.; Warrand, J., Enzymatic and chemical degradation of curdlan targeting the production of β-(1 → 3) oligoglucans. *Carbohydr. Polym.* **2008,** *71*, 277-286.

49. Zhan, X.; Zhu, L.; Wu, J.; Zheng, Z. Preparation of β-1,3-glucan oligosaccharide by acidolysis and enzymic hydrolysis. CN102660607A, 2012.

50. Richards, G. N.; Shafizadeh, F., Formation of "glucometasaccharinolactones" in the pyrolysis of curdlan, a (1 → 3)-β-D-glucan. *Carbohydr. Res.* **1982,** *106*, 83-91.

51. Food additives permitted for direct addition to food for human consumption; curdlan. *Fed. Regist.* **1996,** *61*, 65941-65942.

52. Kanke, M.; Tanabe, E.; Katayama, H.; Koda, Y.; Yoshitomi, H., Application of curdlan to controlled drug delivery. III. Drug release from sustained-release suppositories in vitro. *Biol. Pharm. Bull.* **1995,** *18*, 1154-8.

53. Kanke, M.; Koda, K.; Koda, Y.; Katayama, H., Application of Curdlan to Controlled Drug Delivery. I. The Preparation and Evaluation of Theophylline-Containing Curdlan Tablets. *Pharmaceutical Research* **1992,** *9* (3), 414-418.

54. Kim, B.; Jung, I.; Kim, J.; Lee, J.-h.; Lee, I.; Lee, K., Curdlan gels as protein drug delivery vehicles. *Biotechnology Letters* **2000,** *22* (14), 1127-1130.

55. Toga, Y.; Ichida, A.; Shibata, T.; Tachibana, K.; Namikoshi, H., Chiral recognition ability of curdlan triacetate: solvent and temperature effects. *Chirality* **2004,** *16*, 272-6.

56. Okuyama, K.; Obata, Y.; Noguchi, K.; Kusaba, T.; Ito, Y.; Ohno, S., Single helical structure of curdlan triacetate. *Biopolymers* **1996,** *38*, 557-66.

57. Hesse, S.; Liebert, T.; Heinze, T., Studies on the film formation of polysaccharide based furan-2-carboxylic acid esters. *Macromol. Symp.* **2006,** *232*, 57-67.

58. Marubayashi, H.; Yukinaka, K.; Enomoto-Rogers, Y.; Takemura, A.; Iwata, T., Curdlan ester derivatives: Synthesis, structure, and properties. *Carbohydrate Polymers* **2014,** *103*, 427-433.

59. Sasaki, T.; Abiko, N.; Sugino, Y.; Nitta, K., Dependence on chain length of antitumor activity of (1 → 3)-β-D-glucan from Alcaligenes faecalis var. myxogenes, IFO 13140, and its acid-degraded products. *Cancer Res.* **1978,** *38*, 379-83.

60. Jin, Y.; Zhang, H.; Yin, Y.; Nishinari, K., Comparison of curdlan and its carboxymethylated derivative by means of Rheology, DSC, and AFM. *Carbohydrate Research* **2006,** *341* (1), 90-99.

61. Sasaki, T.; Abiko, N.; Nitta, K.; Takasuka, N.; Sugino, Y., Antitumor activity of carboxymethylglucans obtained by carboxymethylation of (1 → 3)-β-D-glucan from Alcaligenes faecalis var. myxogenes IFO 13140. *Eur. J. Cancer* **1979,** *15*, 211-15.

62. Na, K.; Park, K.-H.; Kim, S. W.; Bae, Y. H., Self-assembled hydrogel nanoparticles from curdlan derivatives: characterization, anti-cancer drug release and interaction with a hepatoma cell line (HepG2). *Journal of Controlled Release* **2000,** *69* (2), 225-236.

63. Lee, C.-M.; Jeong, H.-J.; Kim, E.-M.; Cheong, S.-J.; Park, E.-H.; Kim, D. W.; Lim, S. T.; Sohn, M.-H., Synthesis and characterization of iron oxide nanoparticles decorated with carboxymethyl curdlan. *Macromol. Res.* **2009,** *17*, 133-136.

64. Wu, J.; Zhang, F.; Zhang, H., Facile synthesis of carboxymethyl curdlan-capped silver nanoparticles and their application in SERS. *Carbohydrate Polymers* **2012,** *90* (1), 261-269.

65. Suflet, D. M.; Nicolescu, A.; Popescu, I.; Chitanu, G. C., Phosphorylated polysaccharides. 3. Synthesis of phosphorylated curdlan and its polyelectrolyte behaviour compared with other phosphorylated polysaccharides. *Carbohydr. Polym.* **2011,** *84*, 1176-1181.

66. Popescu, I.; Pelin, I. M.; Butnaru, M.; Fundueanu, G.; Suflet, D. M., Phosphorylated curdlan microgels. Preparation, characterization, and in vitro drug release studies. *Carbohydrate Polymers* **2013,** *94* (2), 889-898.

67. (a) Fox, S. C.; Li, B.; Xu, D.; Edgar, K. J., Regioselective Esterification and Etherification of Cellulose: A Review. *Biomacromolecules* **2011,** *12* (6), 1956-1972; (b) Kamitakahara, H.; Funakoshi, T.; Nakai, S.; Takano, T.; Nakatsubo, F., Synthesis and Structure/Property Relationships of Regioselective 2-O-, 3-O- and 6-O-Ethyl Celluloses. *Macromolecular Bioscience* **2010,** *10* (6), 638-647.

68. Tanaka, R.; Saito, T.; Isogai, A., Cellulose nanofibrils prepared from softwood cellulose by TEMPO/NaClO/NaClO2 systems in water at pH 4.8 or 6.8. *Int. J. Biol. Macromol.* **2012,** *51*, 228-234.

69. Tamura, N.; Hirota, M.; Saito, T.; Isogai, A., Oxidation of curdlan and other polysaccharides by 4-acetamide-TEMPO/NaClO/NaClO2 under acid conditions. *Carbohydrate Polymers* **2010,** *81* (3), 592-598.

70. Delattre, C.; Rios, L.; Laroche, C.; Le, N. H. T.; Lecerf, D.; Picton, L.; Berthon, J. Y.; Michaud, P., Production and characterization of new families of polyglucuronic acids from TEMPO-NaOCl oxidation of curdlan. *Int. J. Biol. Macromol.* **2009,** *45*, 458-462.

71. Delattre, C.; Michaud, P.; Chaisemartin, L.; Berthon, J. Y.; Rios, L., A transcriptomic approach to predict the impact of β-(1,3)-polyglucuronic acid sodium salt and derivatives in the main biological processes. *Carbohydrate Polymers* **2012,** *87* (2), 1828-1836.

72. Watanabe, E.; Tamura, N.; Saito, T.; Habu, N.; Isogai, A., Preparation of completely C6-carboxylated curdlan by catalytic oxidation with 4-acetamido-TEMPO. *Carbohydrate Polymers* (0).

73. Lien, L. T. N.; Shiraki, T.; Dawn, A.; Tsuchiya, Y.; Tokunaga, D.; Tamaru, S.-i.; Enomoto, N.; Hojo, J.; Shinkai, S., A pH-responsive carboxylic β-1,3-glucan polysaccharide for complexation with polymeric guests. *Org. Biomol. Chem.* **2011,** *9*, 4266-4275.

74. Borjhan, G.; Hu, R.-c.; Liu, G.-g.; Zhong, G.-y., Direct regioselective synthesis of 6-azido-6-deoxy-curdlan. *Gaodeng Xuexiao Huaxue Xuebao* **2001,** *22*, 706-708.

75. Hasegawa, T.; Abe, H. Preparation of 6-position-modified deoxyglucose. JP2012180328A, 2012.

76. (a) Hasegawa, T.; Numata, M.; Okumura, S.; Kimura, T.; Sakurai, K.; Shinkai, S., Carbohydrate-appended curdlans as a new family of glycoclusters with binding properties both for a polynucleotide and lectins. *Org. Biomol. Chem.* **2007,** *5*, 2404-2412; (b) Hasegawa, T.; Umeda, M.; Numata, M.; Fujisawa, T.; Haraguchi, S.; Sakurai, K.; Shinkai, S., Click chemistry on curdlan: A regioselective and quantitative approach to develop artificial β-1,3-Glucans with various functional appendages. *Chem. Lett.* **2006,** *35*, 82-83; (c) Hasegawa, T.; Umeda, M.; Numata, M.; Li, C.; Bae, A.-H.; Fujisawa, T.; Haraguchi, S.; Sakurai, K.; Shinkai, S., 'Click chemistry' on polysaccharides: a convenient, general, and monitor approach to develop (1→3)-β-D-glucans with various functional appendages. *Carbohydr. Res.* **2005,** *341*, 35-40.

77. Kolb, H. C.; Finn, M. G.; Sharpless, K. B., Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angewandte Chemie International Edition* **2001,** *40* (11), 2004-2021.

78. Yoshida, T.; Hatanaka, K.; Uryu, T.; Kaneko, Y.; Suzuki, E.; Miyano, H.; Mimura, T.; Yoshida, O.; Yamamoto, N., Synthesis and structural analysis of curdlan sulfate with a potent inhibitory effect in vitro of AIDS virus infection. *Macromolecules* **1990,** *23*, 3717-2.

79. Osawa, Z.; Morota, T.; Hatanaka, K.; Akaike, T.; Matsuzaki, K.; Nakashima, H.; Yamamoto, N.; Suzuki, E.; Miyano, H.; et, a., Synthesis of sulfated derivatives of curdlan and their anti-HIV activity. *Carbohydr. Polym.* **1993,** *21*, 283-8.

80. Gao, Y.; Fukuda, A.; Katsuraya, K.; Kaneko, Y.; Mimura, T.; Nakashima, H.; Uryu, T., Synthesis of Regioselective Substituted Curdlan Sulfates with Medium Molecular Weights and Their Specific Anti-HIV-1 Activities. *Macromolecules* **1997,** *30*, 3224-3228.

81. Jagodzinski, P. P.; Wiaderkiewicz, R.; Kurzawski, G.; Kloczewiak, M.; Nakashima, H.; Hyjek, E.; Yamamoto, N.; Uryu, T.; Kaneko, Y.; et, a., Mechanism of the inhibitory effect of curdlan sulfate on HIV-1 infection in vitro. *Virology* **1994,** *202*, 735-45.

82. Cutler, B.; Justman, J., Vaginal microbicides and the prevention of HIV transmission. *The Lancet Infectious Diseases* **2008,** *8* (11), 685-697.

83. Rohan, L. C.; Sassi, A. B., Vaginal drug delivery systems for HIV prevention. *The AAPS journal* **2009,** *11* (1), 78-87.

84. Demleitner, S.; Kraus, J.; Franz, G., Synthesis and antitumour activity of derivatives of curdlan and lichenan branched at C-6. *Carbohydrate Research* **1992,** *226* (2), 239-246.

85. Yoshida, T.; Yasuda, Y.; Uryu, T.; Nakashima, H.; Yamamoto, N.; Mimura, T.; Kaneko, Y., Synthesis and in vitro Inhibitory Effect of L-Glycosyl-Branched Curdlan Sulfates on AIDS Virus Infection. *Macromolecules* **1994,** *27*, 6272-6.

Chapter 3. Synthesis of Curdlan Derivatives Regioselectively Modified at C-6: *O*-(*N*)-Acylated 6-Amino-6-Deoxycurdlan

Zhang, R.; Edgar, K. J. *Carbohydrate Polymers* **2014**, *105*, 161-168. Used with permission of Elsevier, 2014.

3.1 Abstract

There has been growing interest in aminopolysaccharide synthesis over the last two decades due to the critical natural functions of aminopolysaccharides, and their potential in biomedical applications. Regioselective introduction of amino groups into polysaccharide backbones is a challenge. Natural curdlan is a linear β-(1→3)-glucan that is of interest both for its physical properties and its biomedical applications. Aminated curdlan derivatives were synthesized in three steps. First, curdlan was regioselectively brominated at the C-6 position in lithium bromide-*N*,*N*-dimethylacetamide (DMAc/LiBr). Second, the bromide of the product 6-bromo-6-deoxycurdlan was displaced by nucleophilic substitution with sodium azide (NaN_3) in dimethyl sulfoxide (DMSO). Third, O-acylated 6-amido-6-deoxycurdlan was produced by a one-pot method. 6-Azido-6-deoxycurdlan was subjected to Staudinger reduction, followed by reaction *in situ* with excess carboxylic anhydride, without isolation of the 6-amino-6-deoxycurdlan intermediate. Regioselectivity and degree of substitution (DS) of these derivatives were confirmed by ^{1}H and ^{13}C NMR spectroscopy, FTIR spectroscopy, and elemental analysis.

3.2 Introduction

Aminated polysaccharides, in particular glycosaminoglycans, have essential natural functions, largely involving highly specific interactions with proteins. Exemplary functions include cell-cell adhesion[1], cell motility[2], and prevention of thrombosis[3]. These natural functions can be co-opted in disease processes ranging from cancer[4] to pathogen invasion, for example in Lyme disease[5] and in maternal malaria[6]. Other aminopolysaccharides, especially the natural polymer chitin and its partially *N*-deacetylated derivative chitosan, have demonstrated significant benefits for biomedical and pharmaceutical applications[7]. As a polycationic compound, chitosan can encapsulate anionic drugs including fatty acids, nucleic acids, and other biological compounds. In nutrition, chitosan is capable of binding fatty acids and bile

acids to reduce fat absorption in the intestine, contributing to a reduction of cholesterolemia[8]. In gene delivery, chitosan-based formulations of plasmid DNA encoding the beta-galactosidase reporter gene showed gene expression when transported into the intestines[9]. Additionally, the ability of chitosan to interact with proteins at the tight junctions between enterocytes of the gastrointestinal epithelium, temporarily opening up tight junctions, is of particular importance. Opening tight junctions in transient fashion creates the opportunity for enhanced paracellular permeability of drugs that otherwise have very poor ability to permeate from the gastrointestinal tract into the bloodstream, including important drug candidates like polypeptides[10]. Amino groups on the chitosan backbone that are protonated at physiological pH are responsible for these desirable properties; indeed, peralkylation of the amine groups of chitosan to create a permanent positive charge greatly enhances the ability to promote tight junction opening[11]. Despite many advantages, chitin and chitosan formulations have some drawbacks. Complexes of chitosan and nucleic acids have shown poor transfection efficiency[12]. Moreover, it is difficult to completely remove proteins from chitin when it is extracted from crustacean shells[13]. It is also not possible to control the sequence of amino groups on the polymer chain during the brutal reaction conditions required for chitin *N*-deacetylation to produce chitosan.

In an effort to avoid these problems, some researchers have taken the approach of modifying other polysaccharides to mimic the chemical structure of chitosan. The natural, neutral, linear homopolysaccharide, β-(1→3)-glucan (curdlan) is a promising candidate. It is produced by the bacterium *Alcaligenes faecalis var.* myxogenes[14]. Curdlan has excellent gelation properties and can form two different types of gels. One is a thermally reversible low-set gel formed at temperatures close to 55 °C while the other is a nonreversible high-set gel formed at temperatures greater than 80 °C[15]. This ability to gel has been utilized in the food industry, where curdlan is employed as a biothickening/gelation agent[16] and a fat/meat substitute[15], and mimics the rheological properties of sauces and dairy products[17], as well as in other applications. Sulfated curdlan derivatives have also been explored as anti-HIV agents[18]. One approach to aminocurdlans has been the application of Huisgen azide-alkyne 'click chemistry'[19] to attach amine-containing side chains to the curdlan backbone[20]. However, there are very few reports in the literature of attempts to replace the hydroxyl groups of curdlan with amine substituents. With its β-(1→3) linkages, the curdlan anhydroglucose unit

(AGU) has three hydroxyl groups, a primary hydroxyl at C-6 and two secondary hydroxyls at C-2 and C-4. The primary 6-OH is most reactive hydroxyl group in reactions where it plays the role of nucleophile.

There have been a number of studies detailing regioselective C-6 amination of other polysaccharides including cellulose[21] and amylose[22]. The precursors for these reactions are typically 6-azido polysaccharides. Early reports demonstrated a one step reaction to form azido derivatives of cyclodextrin[23], amylose and pullulan[24] using carbon tetrabromide (CBr_4), lithium azide (LiN_3) and triphenylphosphine (Ph_3P) in dimethylformamide (DMF). However, inadequate control over the degree of substitution as well as side reactions limited the utility of those reactions. Thus, separating the bromination from the azidation step is essential. Hasegawa et al. synthesized 6-bromo-6-deoxy-curdlan in DMF/LiCl with CBr_4.[20, 25] The long reaction time (> 24 hours) and partial substitution at C-6 of chloride from LiCl were major concerns. Furuhata et al.[26] and Tseng et al.[27] later successfully used *N*-bromosuccinimide (NBS) and Ph_3P in DMAc/LiBr to accomplish regioselective bromination of cellulose and chitin, respectively.

Herein, we report our attempts to achieve regioselective bromination of curdlan at C-6 using NBS and Ph_3P in DMAc/LiBr under homogeneous conditions. Our intention was to use this 6-bromo-6-deoxy derivative as a substrate for azide (NaN_3) displacement[28] to introduce nitrogen-containing functionality to C-6. We anticipated that Staudinger reduction of this azide, optionally with controlled *N*,*O*-acylation, might serve as an entry to new families of 6-amino-6-deoxycurdlan derivatives, which could be of significant interest for biomedical applications and for studying the specificity of their interactions with proteins. We report herein the results of these studies.

3.3 Experimental

3.3.1 Materials

β-(1→3)-Glucan (curdlan, $(-C_6H_{10}O_5-)_n$) was obtained from Wako Chemicals and dried under vacuum at 40°C overnight prior to use. LiBr (laboratory grade, Fisher) and NaN_3 (99%, Alfa Aesar) were dried under vacuum at 125 °C. *N*-Bromosuccinimide (99%, Acros)

was recrystallized from boiling water and dried for two days under reduced pressure over anhydrous calcium chloride. Triphenylphosphine (Ph_3P, 99%, Acros), acetic anhydride (Ac_2O, 99+%, Acros) and propionic anhydride (Pr_2O, 97%, Sigma-Aldrich) were used as received. DMAc (reagent grade, Fisher) and DMSO (HPLC grade, Fisher) were kept over 4 Å molecular sieves and stored under dry nitrogen until used. Acetone (HPLC grade, Fisher), methanol (HPLC grade, Fisher) and ethanol (HPLC grade, Fisher) were used as received.

3.3.2 Measurements

^{13}C NMR spectra were acquired on INOVA 400 or Bruker Avance 500 spectrometers with a minimum of 10,000 scans at 50 °C. Samples were analyzed as solutions in $CDCl_3$ or DMSO-d_6 (ca. 10 mg/mL) in standard 5 mm o.d. tubes. Chemical shifts are reported relative to the solvent peaks. Infrared spectroscopic analyses of samples as pressed KBr pellets were performed on a Thermo Electron Nicolet 8700 instrument. Elemental analyses (EA) to determine carbon, nitrogen and bromine contents were performed by Micro Analysis Inc. using a Perkin Elmer 2400 II analyzer. Carbon and nitrogen contents were determined by flask combustion followed by ion chromatography and bromine content was measured with a thermal conductivity detector.

3.3.3 Methods

3.3.3.1 Dissolution of curdlan in DMAc/LiBr

The method was adapted from a method previously reported for cellulose dissolution[29]. Dried curdlan (4.00 g, 24.7 mmol AGU) was weighed into a 250 mL, three-necked round-bottom flask. The flask was fitted with a nitrogen inlet, short-path distillation unit and overhead stirrer. Next, 150 mL DMAc was added to the flask, and the contents were stirred. The flask was flushed with dry nitrogen and heated in an oil bath at 150 °C for 26 min. LiBr (36.00 g, 42.4 mmol) was added to the flask and the mixture was heated at 170 °C for 8 min. The distillate (40 mL) was collected under a stream of nitrogen at 170 °C. The reaction mixture was allowed to cool to room temperature while being stirred continuously. Curdlan dissolved within 3 h to form a transparent solution.

3.3.3.2 Regioselective bromination of curdlan

Ph_3P (25.96 g, 4 eq per AGU) was dissolved in 50 mL of dry DMAc. A second solution of NBS (17.58 g, 4 eq per AGU) was prepared in an additional 50 mL of dry DMAc. The Ph_3P solution was added dropwise via a liquid addition funnel to a solution of 4.00 g curdlan in DMAc/LiBr, prepared as described above. Subsequently, the NBS solution was added in similar fashion. The reaction solution was then heated at 70 °C for 1 h. The reaction mixture was added slowly to 1 L of a 50:50 mixture of methanol and deionized water. The reaction mixture was held overnight at room temperature. The mixture was then filtered to recover the precipitate. The isolated product was re-dissolved in DMSO and re-precipitated in ethanol twice. The sample was dried under vacuum at 40 °C overnight to yield 6-bromo-6-deoxycurdlan. IR (KBr): ν 3445 (O-H), 2906 (C-H), 541 (C-Br). ^{13}C NMR (DMSO-d_6): δ 103.2 (C-1), 84.9 (C-3), 74.4 (C-5), 73.6 (C-2), 70.1 (C-4), 34.6 (C-6-Br) ppm. Elemental analysis: Br %=28.41 (Theoretical Br % 35.53 if DS(Br)=1). Yield: 4.75 g (86%).

3.3.3.3 Synthesis of 6-azido-6-deoxy-(2,4-di-*O*-acyl-)curdlan

Dry 6-bromo-6-deoxycurdlan (1.00 g, 4.44 mmol) was dissolved in 25 mL of anhydrous DMSO in a 100 mL flask. Then, NaN_3 (1.44 g, 5 eq per AGU) was added to the flask and dissolved. The solution was heated to 80 °C and stirred for 24 h under nitrogen. The product was isolated by pouring the reaction mixture into 300 mL of deionized water and the precipitate was collected by filtration. The product was re-dissolved in acetone and then re-precipitated in deionized water, followed by filtration. The sample was dried under vacuum at 40 °C overnight to yield 6-azido-6-deoxycurdlan. IR (KBr): ν 3460 (O-H), 2916 (C-H), 2109 (N_3). ^{13}C NMR (DMSO-d_6): δ 103.4 (C-1), 84.9 (C-3), 74.9 (C-5), 73.9 (C-2), 69.4 (C-4), 51.7 (C-6-N_3) ppm. Elemental analysis: N % = 18.84 (Theoretical N % = 22.45 if DS(N_3) = 1). Yield: 0.76 (92%).

Under nitrogen, dry 6-azido-6-deoxycurdlan (1.00 g, 5.34 mmol) and 4-dimethylaminopyridine (DMAP, 20 mg) were weighed into a 50 mL round-bottom flask. Pyridine (4.3 mL, 10 eq per AGU) and 10 eq of a carboxylic anhydride (acetic or propionic anhydride) were then added dropwise to the solution. The mixture was reacted at 80 °C for 24 h under nitrogen. The homogeneous mixture was then added slowly to 200 mL deionized

water. The crude product was collected by filtration, and then re-dissolved in 10 mL chloroform. This solution was added slowly with rapid stirring to 200 mL of ethanol. After filtration and washing with ethanol and water several times, the sample was dried under vacuum at 40 °C overnight to yield 6-azido-6-deoxy-2,4-di-*O*-acyl-curdlan. ^{13}C NMR (DMSO-d_6): δ 169.6 (C=O), 99.4 (C-1), 78.0 (C-3), 72.1 (C-5), 71.1 (C-2), 68.5 (C-4), 50.5 (C-6-N_3), 20.6 (CH_3) ppm. Yield: 1.40 g (6-azido-6-deoxy-2,4-di-*O*-acetyl-curdlan, 97%), 1.48 g (6-azido-6-deoxy-2,4-di-*O*-propionyl-curdlan, 93%).

3.3.3.4 Synthesis of (*O*-acylated-) 6-amino-6-deoxycurdlan

In a representative example, 0.20 g 6-azido-6-deoxy-(2,4-di-*O*-acyl-)curdlan was dissolved in 20 mL of DMAc, followed by the dropwise addition of 0.10 mL of deionized water. Ph_3P (0.56 g for 6-azido-6-deoxy-curdlan, 2 eq per AGU; 0.39 g for 6-azido-6-deoxy-2,4-di-*O*-acetyl-curdlan, 2 eq per AGU) was then added to the 50 mL round-bottom flask and the reaction was run under ambient conditions in a flask with a stopper to prevent solvent loss. After allowing the reaction to proceed for 12 h, the solution was transferred to 3,500 g/mol MWCO dialysis tubing that was then placed in a large beaker containing ethanol. As the dialysis of the reaction solution progressed, a light brown precipitate slowly formed within the tubing. After three days of dialysis, the contents of the tubing were removed. The precipitate was isolated by filtration and then dried under vacuum at 40 °C overnight to yield 6-amino-6-deoxy-(2,4-di-*O*-acyl-)curdlan. IR (KBr): ν 3450 (O-H), 2945 (C-H), 2590 (N-H). Yield: 0.11 g (6-amino-6-deoxy-curdlan, 64%), 0.11 g (6-amino-6-deoxy-2,4-di-*O*-acetyl-curdlan, 61%).

3.3.3.5 Synthesis of 6-amido-6-deoxy-2,4-di-*O*-acyl-curdlan

In a 100 mL round-bottom flask, 6-azido-6-deoxycurdlan (0.50 g, 2.67 mmol) was dissolved in 50 mL DMAc under dry nitrogen, followed by the dropwise addition of 0.25 mL of deionized water. As Ph_3P (1.41 g, 2 eq per AGU) was added to the flask, the reaction began as evidenced by solution color change from dark brown to light brown and appearance of some small bubbles. The reaction was run under ambient conditions in a flask with a stopper to prevent solvent loss. The reaction remained homogeneous for 12 h. Then, 50 eq per AGU of a carboxylic anhydride was added to the flask followed by 20 mg of DMAP and 5 mL of pyridine. The solution was stirred for 24 h at 80 °C under dry nitrogen. The solution

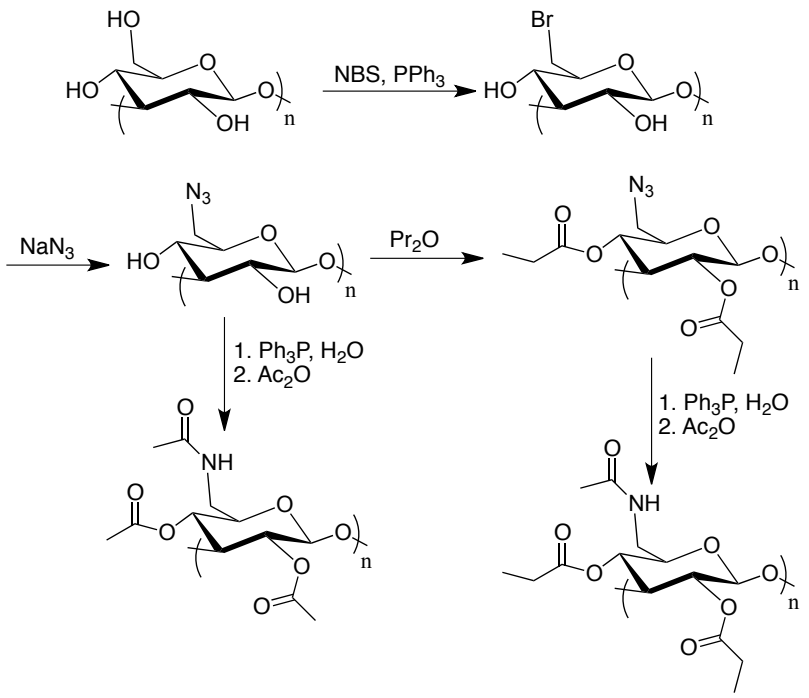
was transferred to 3,500 g/mol MWCO dialysis tubing (prewet with water) that was placed in a large beaker containing deionized water. As the dialysis of the reaction solution progressed, a precipitate slowly formed within the tubing. After one day of dialysis, the contents inside the tubing were removed, and the precipitate was isolated by filtration. The precipitate was further purified by Soxhlet extraction with ethanol for 1 day, and then dried in a vacuum oven at 40 °C overnight.

3.4 Results and discussion

3.4.1 Regioselective bromination of curdlan

DMAc/LiBr[26-27] was employed as the solvent system for curdlan dissolution and reaction, due to its proven ability to dissolve polysaccharides, interrupt polysaccharide H-bonding, and its compatibility with a wide variety of synthetic methods. We initially attempted to brominate curdlan in DMSO since it is a solvent for curdlan, NBS and Ph_3P. However, after adding NBS and Ph_3P, the reaction was exothermic and difficult to control, resulting in foaming out of the flask. Clearly the rather reactive DMSO is not a suitable solvent for this transformation. Other investigators[30] have reported exotherms upon DMSO/NBS mixing, although to our knowledge no detailed mechanistic studies have been done. The powerful mixed solvent system (DMAc/LiBr) is known to be effective for dissolution and chemical reactions of the recalcitrant cellulose; the use of LiBr rather than LiCl prevents halogen exchange in the bromopolysaccharide product. In the event, curdlan did dissolve in this solvent system successfully and it was suitable for the further reactions we had in mind.

Curdlan bromination (**Scheme 3.1**) with NBS and Ph_3P took place regioselectively at C-6 in homogeneous solution in DMAc/LiBr by procedures analogous to those previously used to brominate cellulose[26] and chitin[27]. It is believed that substitution of hydroxyl by bromide involves two consecutive S_N2 reactions, thereby ensuring virtually complete selectivity for the less hindered and more nucleophilic 6-OH. First, PPh_3 is brominated by NBS to form a bromotriphenylphosphonium ion, followed by S_N2 displacement of Br- from this intermediate by the 6-OH group to produce an alkoxyphosphonium salt intermediate.[31] Backside attack by bromide anion on C-6 displaces triphenylphosphine oxide as leaving group, affording 6-bromo-6-deoxycurdlan.



Scheme 3.1. Conversion of curdlan to 6-amido-6-deoxy-2,4-di-*O*-acyl-curdlan

Spectroscopic analysis of the 6-bromo-6-deoxycurdlan product confirmed that the reaction was highly chemo- and regioselective. The FTIR spectrum (**Fig. 3.1**) shows a broad hydroxyl stretching absorption near 3500 cm^{-1}, and C-H stretching absorption around 2900 cm^{-1} from the curdlan backbone. The relatively weak peak near 550 cm^{-1} is attributed to the C-Br stretching absorption. More conclusive evidence of conversion to the 6-bromo derivative is provided by the ^{13}C NMR spectrum (**Fig. 3.2**). Distinct, single peaks for C-1 to C-5 of the curdlan backbone are present between 65 and 105 ppm.[20, 25] The strong upfield chemical shift for C-6 upon substitution by bromine to 34 ppm is diagnostic for the postulated bromination. No peak is observed at 60 ppm where the original C-6-OH of pure curdlan would resonate, indicating essentially complete conversion. Previous curdlan brominations[20, 25] were carried out in DMF/LiCl using CBr_4, but DS values were not reported. The DS of bromide was calculated to be 0.80 by elemental analysis. This, however, is almost certainly a significant underestimate, due to the presence of residual triphenylphosphine and triphenylphosphine oxide that are very difficult to remove quantitatively (*vide infra*). All NMR

data on this and subsequent intermediates and products indicate that bromination was complete and that actual 6-Br DS approaches 1.0.

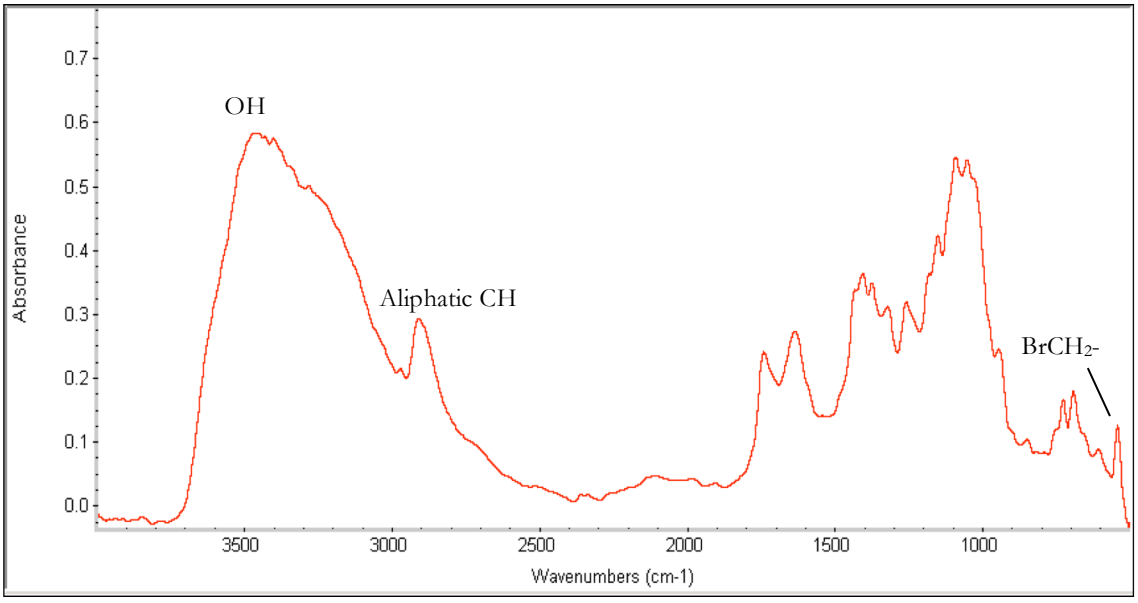


Fig. 3.1. FTIR spectrum of 6-bromo-6-deoxycurdlan (**A**).

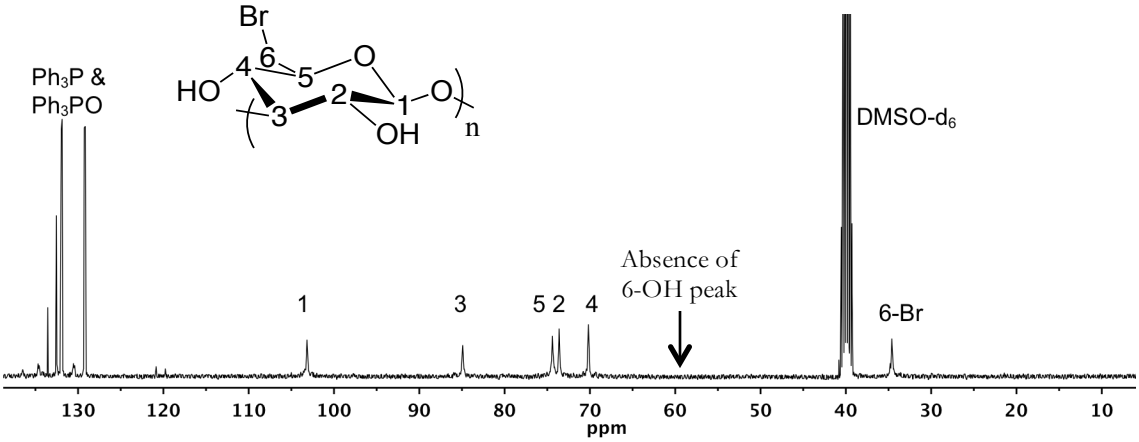


Fig. 3.2. ^{13}C NMR spectrum of 6-bromo-6-deoxycurdlan (**A**).

3.4.2 6-Azido-6-deoxycurdlan derivatives by azide displacement

Hasegawa, et al. have shown that 6-bromo-6-deoxycurdlan is an effective intermediate for subsequent S_N2 displacement by azide. These researchers carried out this substitution reaction by reacting the 6-bromo product with NaN_3 in DMSO at 80 °C for 36 h.[20, 25] We

first followed this procedure to obtain the azido product and confirmed its chemical structure by FTIR (**Fig. 3.3a**) and ^{13}C NMR spectra (**Fig. 3.4**). The strong -N_3 absorption at 2108 cm^{-1} indicates successful incorporation of azide. In the ^{13}C NMR spectrum the C-6 chemical shift appears at 51 ppm, shifted significantly downfield from the C-6 of the bromodeoxy starting material (34 ppm). Since different substituents at C-2 are known to cause multiplicity of signals for the anomeric carbons (C-1), the absence of such multiplicity here is additional evidence for the selectivity of the bromination/azidation sequence for substitution at C-6. Moreover, no peak at 60 ppm for C-6-OH was observed, indicating that no hydrolysis at C-6 occurred during azide substitution. There is also no trace of the brominated carbon at 34 ppm.

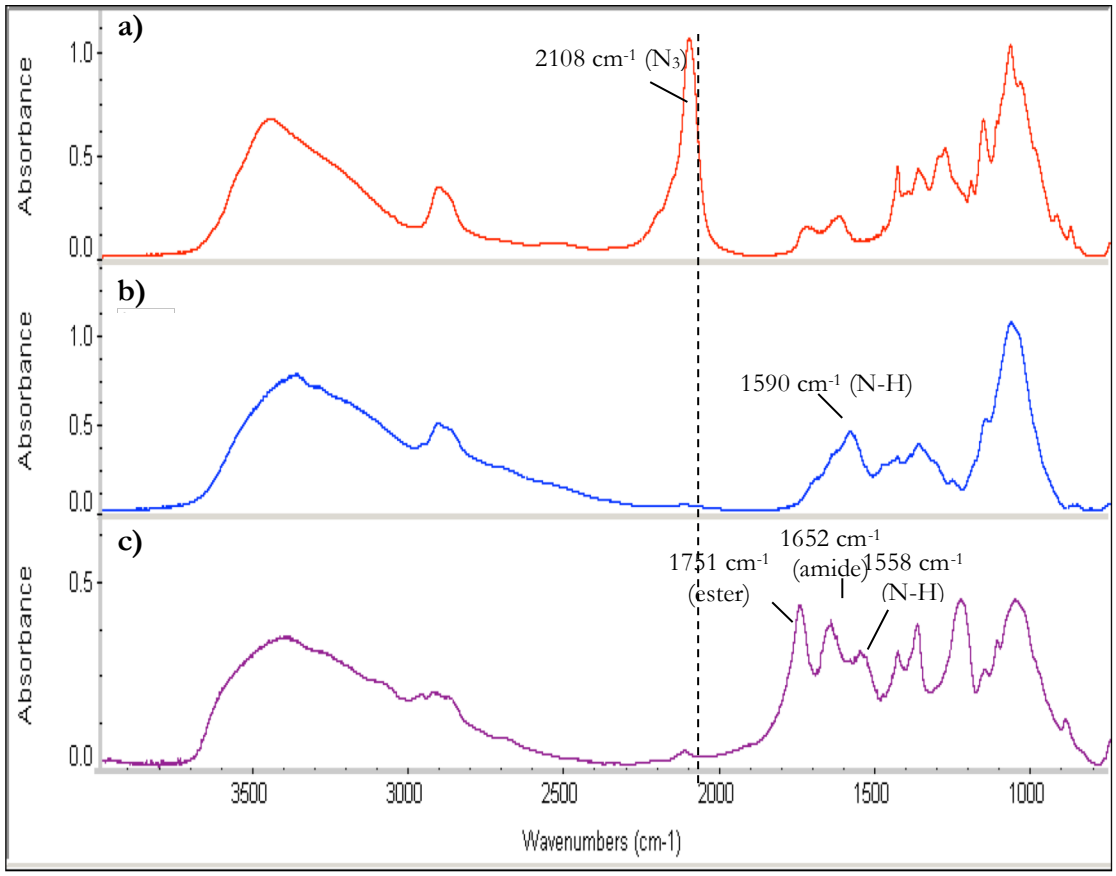


Fig. 3.3. FTIR spectra of **a)** 6-azido-6-deoxycurdlan, **b)** 6-amino-6-deoxycurdlan and **c)** 6-amino-6-deoxy-2,4-di-*O*-acetyl-curdlan synthesized by Staudinger reduction.

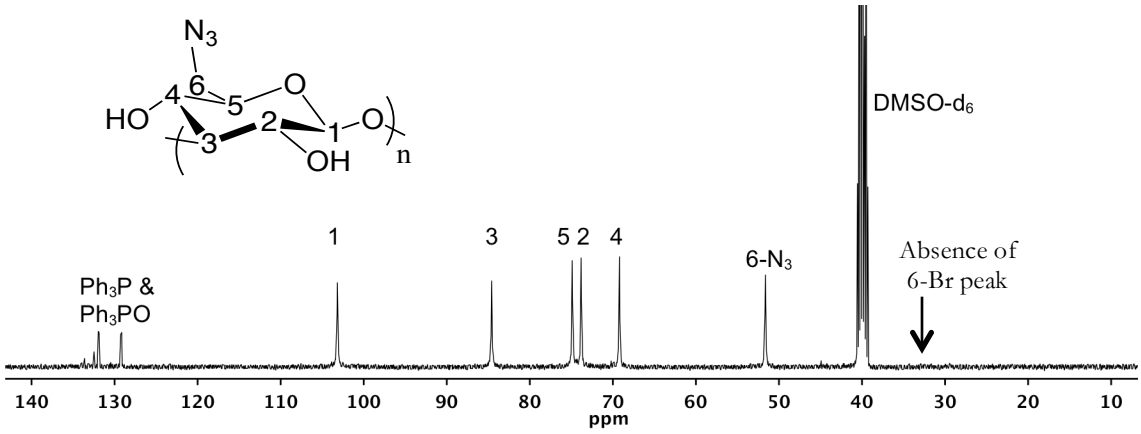


Fig. 3.4. ^{13}C NMR spectrum of 6-azido-6-deoxycurdlan (**B**).

In order to figure out the optimal reaction time for this azide displacement, we monitored the process by removal of aliquots of the reaction solution at predetermined lengths of time. Each product was isolated for elemental analysis and the results are shown in **Table 3.1**. Since the apparent DS of the azide levels off around 0.89 after 1 h, we shortened this displacement reaction time from 36 h to 1 h. Product elemental analysis indicated no Br content after 1 h, indicating complete bromide displacement. The ^{13}C NMR spectrum of the 1 h product (not shown) also displays only a single peak for C-6-N_3 at 51 ppm, confirming the clean and rapid nature of this reaction.

Table 3.1.

Kinetics of azide displacement on 6-bromo-6-deoxycurdlan in DMSO at 80 °C [*]

Reaction time (h)	**0.50**	**0.75**	**1**	**2**	**3**	**4**	**5**	**10**	**24**	**36**
N_3 wt%	12.47	16.87	19.93	19.93	19.94	19.94	19.12	19.04	18.84	19.07
DS (N_3)	0.555	0.751	0.887	0.887	0.888	0.888	0.851	0.847	0.838	0.846

[*] Measured by elemental analysis.

Conversion of pure 6-azido-6-deoxycurdlan to its peracylated derivatives was simple and straightforward. 6-Azido-6-deoxycurdlan was reacted with acetic or propionic anhydride (pyridine, DMAP, 80 °C, 24h) to fully esterify the free 2- and 4-hydroxyls. Product structures

were confirmed by ^{13}C NMR spectroscopy, in which the 2,4-diacetate carbonyl peaks appear around 170 ppm. In the ^{1}H NMR spectrum of the 2,4-diacetate, there are two peaks around 2.1 ppm for the acetyl methyl groups. The FTIR spectrum shows a strong ester carbonyl stretch around 1750 cm^{-1}. These derivatives are effective precursors for the subsequent synthesis of 6-amido-6-deoxycurdlan-2,4-*O*-esters, in which the *N*-acyl group differs from the ester acyl groups.

3.4.3 Synthesis of (*O*-acylated-) 6-amino-6-deoxycurdlan and 6-amido-6-deoxy-2,4-di-*O*-acyl-curdlans

Our first approach to aminocurdlan derivatives of interest was to synthesize (*O*-acylated-) 6-amino-6-deoxycurdlan, then carry out N-acylation (potentially with a different acyl group) to obtain 6-amido-6-deoxy-2,4-di-*O*-acyl-curdlan. A Staudinger reduction (PPh_3, H_2O, DMSO, room temperature) was used to reduce the azide to amine due to the mild conditions and high selectivity permitted by this approach[28]. Our previous work indicated that Staudinger reduction of polysaccharide azides to amines is sufficiently mild to preserve ester bonds against reduction[21a]. The azide reduction begins with formation of a phosphazide by reaction between 6-azido-6-deoxy-(2,4-di-*O*-acyl-)curdlan and Ph_3P. An iminophosphorane forms after the loss of nitrogen gas, followed by aqueous hydrolysis to produce, in this case, 6-amino-6-deoxy-(2,4-di-*O*-acyl-)curdlan and triphenylphosphine oxide (Ph_3PO)[21a].

After overnight Staudinger reduction at room temperature, the reaction mixture was dialyzed against ethanol for at least three days in order to obtain the 6-amino-6-deoxy-(2,4-di-*O*-acyl-)curdlan. Interestingly, once isolated, the products were found to be insoluble in any common NMR solvents including water, DMSO, chloroform and acetone, preventing characterization by solution NMR. **Fig. 3.3b** and **3.3c** shows the FTIR spectra of 6-amino-6-deoxycurdlan and 6-amino-6-deoxy-2,4-di-*O*-acetyl-curdlan, respectively, prepared by such Staudinger reduction. Small residual azide absorptions are evident at 2108 cm^{-1}, though peak intensity is greatly reduced (compare with **Fig. 3.3a**). Absorptions at 1590 and 1558 cm^{-1} are assigned to N-H bending of the free amine, confirming azide reduction by Ph_3P. An absorption band at 1652 cm^{-1}, typically associated with amide carbonyl stretches, is present in 6-amino-6-deoxy-2,4-di-*O*-acetyl-curdlan but not in 6-amino-6-deoxycurdlan. This is

probably the result of partial amide formation, resulting from the nucleophilic attack of the nitrogen of the iminophosphorane upon the carbonyl carbons of the 2,4-*O*-ester groups present on the curdlan chain[21a]. The poor solubility of these isolated amino products is likely due to intermolecular hydrogen bonding between the 6-amino groups, and would be limiting with regard to their further application as drug or gene delivery agents in biomedical fields. Therefore, we designed the synthesis of amido-derivatized curdlans with enhanced solubility.

To produce amido curdlan esters with identical *N*-acyl and *O*-acyl groups, we directly added excess carboxylic anhydride to the reaction mixture of 6-azido-6-deoxycurdlan after Ph_3P reduction without isolating the intermediate 6-amino-6-deoxycurdlan. In order to target derivatives in which the *N*-acyl group differed from the *O*-acyl groups, esterified 6-azido curdlans were used as starting materials (**Scheme 3.1**). They were first reduced through Staudinger reaction and then a carboxylic anhydride (with acyl moieties distinct from those of the *O*-acyls) was added to form an amide selectively while preserving the 2,4-*O*-ester groups. All amido products were soluble in $CDCl_3$, permitting ready acquisition of solution ^1H and ^{13}C NMR spectra (**Fig. 3.5**).

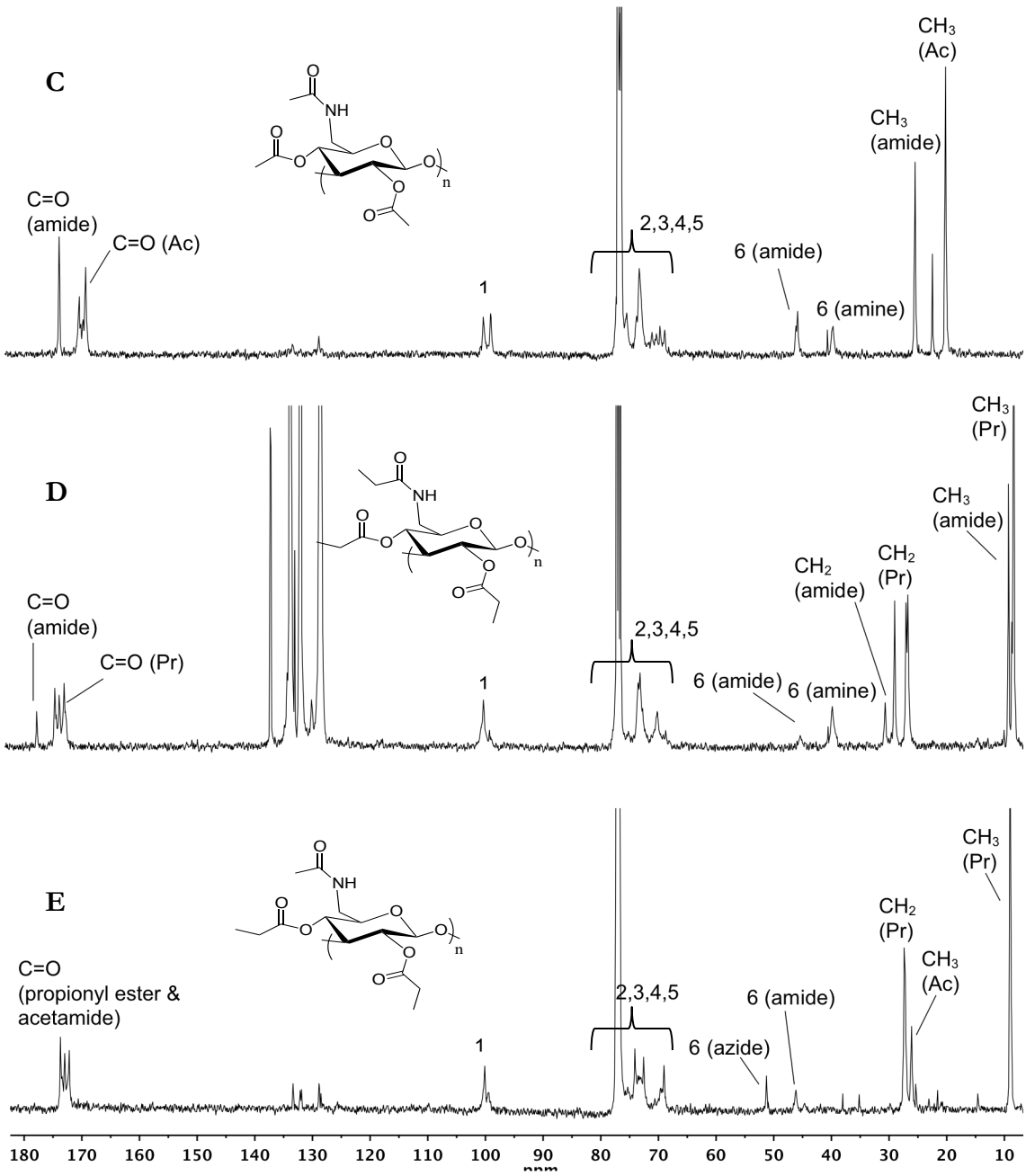


Fig. 3.5. ^{13}C NMR spectra of 6-acetamido-6-deoxy-2,4-di-*O*-acetyl-curdlan (**C**), 6-propionamido-6-deoxy-2,4-di-*O*-propionyl-curdlan (**D**) and 6-acetamido-6-deoxy-2,4-di-*O*-propionyl-curdlan (**E**).

In each case, the chemical shift for C-6 with a pendant amide group is 46 ppm. An aminated C-6 peak at 40 ppm shows up in **C** and **D** while a residual C-6-azide peak at 51 ppm appears in spectrum **E**, indicating incomplete *N*-acylation and Staudinger reduction, respectively.

Compared with those of ester substituents (acetate or propionate), the aliphatic and carbonyl carbons are shifted downfield in the amide groups (acetamide or propionamide). **Table 3.2** summarizes the chemical shifts for carbons in esters and amides of 6-amido-6-deoxy-curdlan esters. Despite washing the products with excess water and ethanol several times, there are some small peaks evident in the range of 128 to 135 ppm, attributed to residual aryl phosphorus reagent and by-product (Ph_3P & Ph_3PO). Soxhlet extraction was the most effective method (vs. for example reprecipitation) for further purification of the amidocurdlan products, significantly reducing (but not entirely eliminating) the intensity of aromatic peaks in ^{13}C NMR spectra.

Table 3.2.

^{13}C NMR chemical shift assignments for acetyl and propionyl groups in 6-amido-6-deoxycurdlan esters*

Carbon	δ (ppm)	Carbon	δ (ppm)
Acetyl		Propionyl	
CH_3 (ester)	20.6	CH_3 (ester)	27.3
CH_3 (amide)	26.2	CH_3 (amide)	31.3
C=O (ester)	170.1	CH_2 (ester)	8.9
C=O (amide)	173.6	CH_2 (amide)	9.8
		C=O (ester)	173.6
		C=O (amide)	177.6

* All spectra were determined using $CDCl_3$ solutions.

3.5 Conclusions

We have developed useful methods for synthesis of a variety of regioselectively substituted aminocurdlan derivatives. Curdlan dissolution in DMAc/LiBr has been demonstrated, along with regioselective, safe bromination with NBS and Ph_3P in DMAc/LiBr to obtain 6-bromo-6-deoxycurdlan, with high DS(Br) and no evidence of side products or reaction incompletion. We have determined the kinetics of the subsequent azide displacement reaction in DMSO, establishing that it is virtually complete within 1h, again with no spectroscopic evidence of incompletion or side reactions. We have demonstrated multiple

routes for conversion of the azide to amine or amides, creating the option of appending the same acyl group to form both ester and amide groups, or alternatively different *N*- and *O*-acyl groups forming dissimilar 6-amide and 2,4-diester groups. The mild Staudinger reaction is useful for selective reduction of azide to amine in the presence of ester groups, and is also tolerant of the presence of acylating reagent *in situ* for capturing the iminophosphorane intermediate as it is generated, in order to avoid migration of acyl groups from the ester moieties to the iminophosphorane nitrogen. We found that amide products have much improved solubility vs. 6-amino-6-deoxycurdlan or its *O*-esters. It is interesting to note that 6-aminopolysaccharide derivatives generally appear to have poor solubility in both water and organic solvents; we have made this observation in our lab for derivatives of curdlan, cellulose [21a], and pullulan [32]. It is necessary to further derivatize these 6-aminopolysaccharides in order to obtain solubility and thereby processability; herein we present *N*- and *O*-acylation as methods for achieving this goal, and will report others in future publications. The synthetic methods we report here open doors to a wide variety of potentially useful aminocurdlan and amidocurdlan polymers. The biomedical properties of these and related curdlan derivatives are of great interest to us and will be the topics of further study in our laboratory.

3.6 Supplemental material

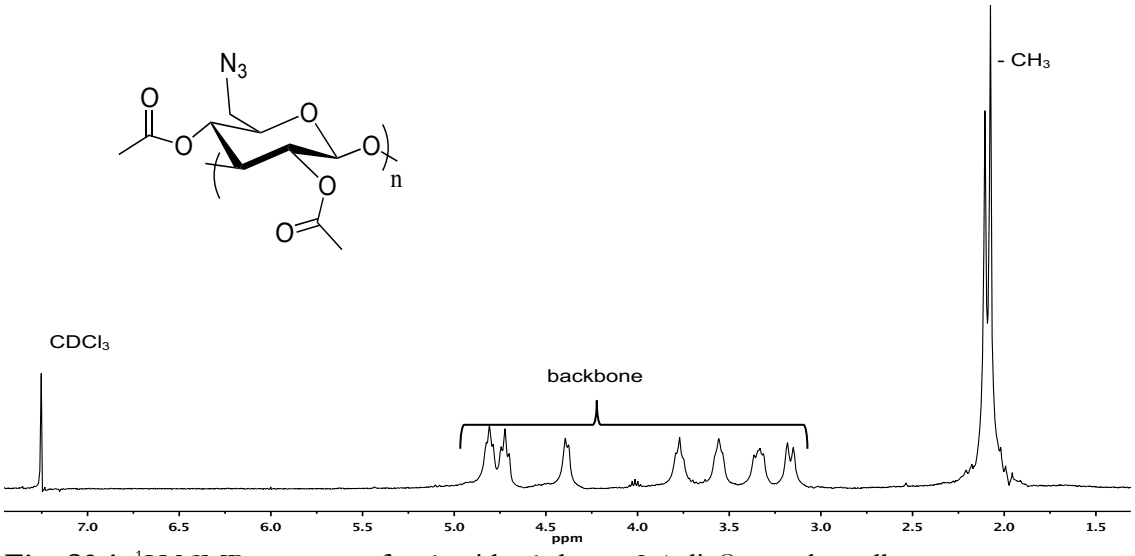


Fig. S3.1. ^{1}H NMR spectrum for 6-azido-6-deoxy-2,4-di-*O*-acetyl-curdlan.

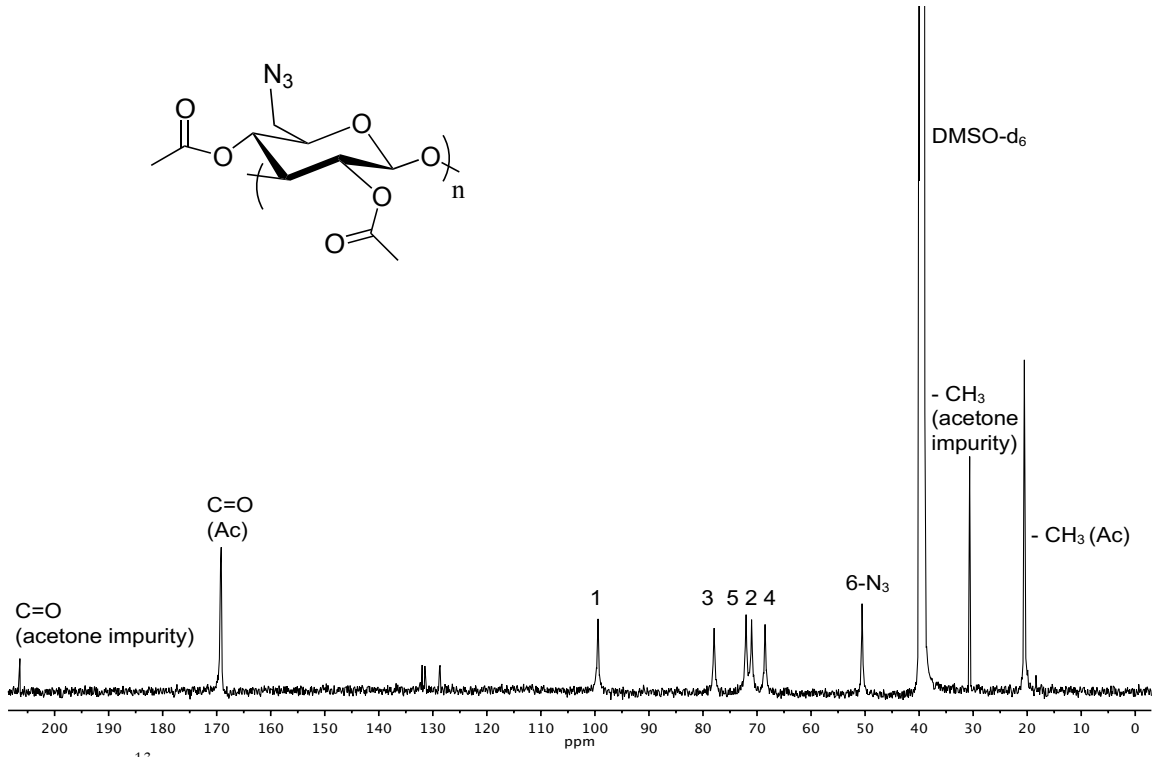


Fig. S3.2. ^{13}C NMR spectrum for 6-azido-6-deoxy-2,4-di-*O*-acetyl-curdlan.

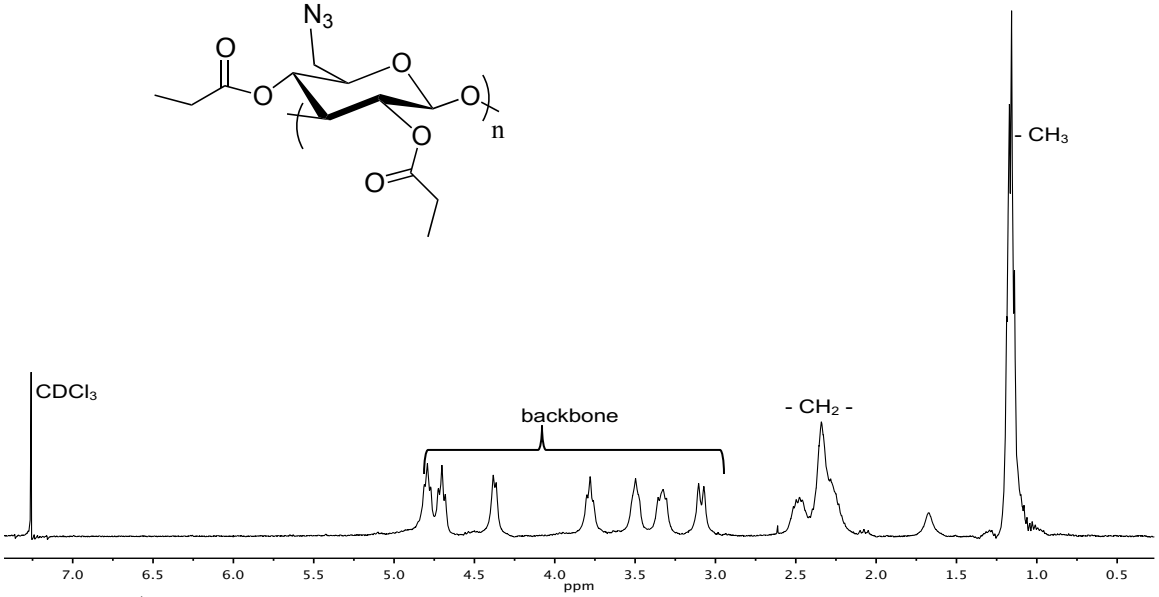


Fig. S3.3. ^{1}H NMR spectrum for 6-azido-6-deoxy-2,4-di-*O*-propionyl-curdlan.

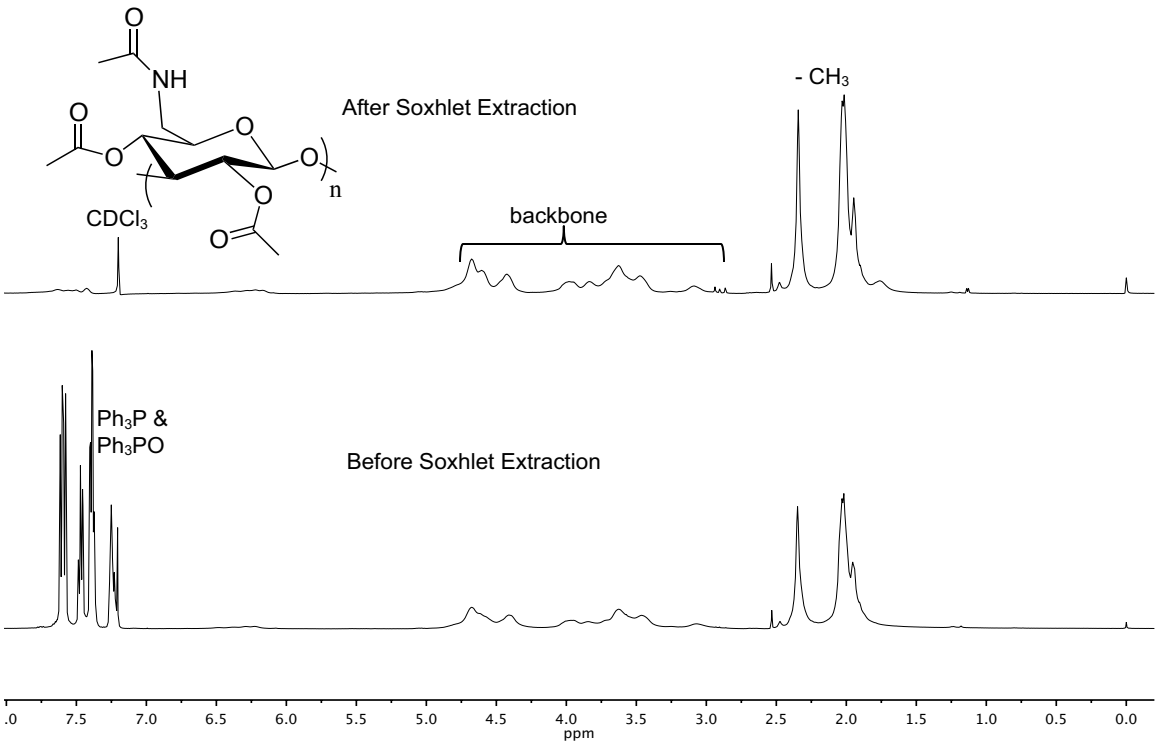


Fig. S3.4. ^{1}H NMR spectra for 6-acetamido-6-deoxy-2,4-di-*O*-acetyl-curdlan after and before Soxhlet Extraction purification.

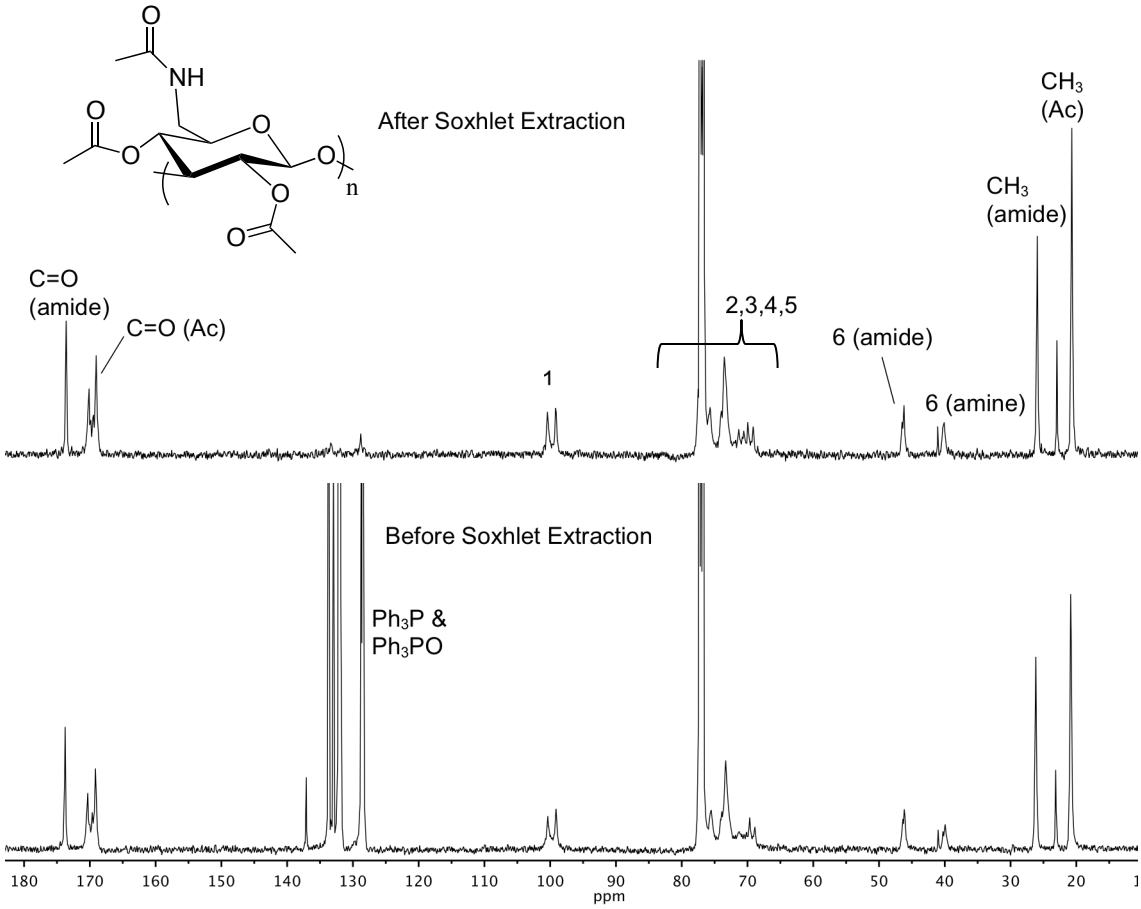


Fig. S3.5. ^{13}C NMR spectra for 6-acetamido-6-deoxy-2,4-di-*O*-acetyl-curdlan after and before Soxhlet Extraction purification.

3.7 Acknowledgements

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3.8 References

1. Lokeshwar, V. B.; Fregien, N.; Bourguignon, L. Y., Ankyrin-binding domain of CD44(GP85) is required for the expression of hyaluronic acid-mediated adhesion function. *The Journal of Cell Biology* **1994,** *126* (4), 1099-1109.

2. Kim, Y.; Lee, Y.-S.; Choe, J.; Lee, H.; Kim, Y.-M.; Jeoung, D., CD44-Epidermal Growth Factor Receptor Interaction Mediates Hyaluronic Acid-promoted Cell Motility by Activating Protein Kinase C Signaling Involving Akt, Rac1, Phox, Reactive Oxygen Species, Focal Adhesion Kinase, and MMP-2. *J. Biol. Chem.* **2008,** *283* (33), 22513-22528.

3. Shriver, Z.; Raman, R.; Venkataraman, G.; Drummond, K.; Turnbull, J.; Toida, T.; Linhardt, R.; Biemann, K.; Sasisekharan, R., Sequencing of 3-O sulfate containing heparin decasaccharides with a partial antithrombin III binding site. *Proc. Nat. Acad. Sci. U.S.A.* **2000,** *97* (19), 10359-10364.

4. Luo, Y.; Ziebell, M. R.; Prestwich, G. D., A Hyaluronic Acid–Taxol Antitumor Bioconjugate Targeted to Cancer Cells. *Biomacromolecules* **2000,** *1* (2), 208-218.

5. Leong, J. M.; Robbins, D.; Rosenfeld, L.; Lahiri, B.; Parveen, N., Structural Requirements for Glycosaminoglycan Recognition by the Lyme Disease Spirochete, Borrelia burgdorferi. *Infection and Immunity* **1998,** *66* (12), 6045-6048.

6. Buffet, P. A.; Gamain, B.; Scheidig, C.; Baruch, D.; Smith, J. D.; Hernandez-Rivas, R.; Pouvelle, B.; Oishi, S.; Fujii, N.; Fusai, T.; Parzy, D.; Miller, L. H.; Gysin, J.; Scherf, A., Plasmodium falciparum domain mediating adhesion to chondroitin sulfate A: A receptor for human placental infection. *Proc. Nat. Acad. Sci. U.S.A.* **1999,** *96* (22), 12743-12748.

7. Ranaldi, G.; Marigliano, I.; Vespignani, I.; Perozzi, G.; Sambuy, Y., The effect of chitosan and other polycations on tight junction permeability in the human intestinal Caco-2 cell line. *Journal of Nutritional Biochemistry* **2002,** *13* (3), 157-167.

8. Muzzarelli, R. A. A., Recent results in the oral administration of chitosan. *Adv. Chitin Sci.* **2000,** *4*, 212-216.

9. Köping-Höggård, M.; Tubulekas, I.; Guan, H.; Edwards, K.; Nilsson, M.; Vårum, K. M.; Artursson, P., Chitosan as a nonviral gene delivery system. Structure–property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo. *Gene Therapy* **2001,** *8* (14), 1108.

10. Yeh, T.-H.; Hsu, L.-W.; Tseng, M. T.; Lee, P.-L.; Sonjae, K.; Ho, Y.-C.; Sung, H.-W., Mechanism and consequence of chitosan-mediated reversible epithelial tight junction opening. *Biomaterials* **2011,** *32* (26), 6164-6173.

11. Thanou, M.; Verhoef, J. C.; Junginger, H. E., Chitosan and its derivatives as intestinal absorption enhancers. *Advanced Drug Delivery Reviews* **2001,** *50, Supplement 1* (0), S91-S101.

12. Bowman, K.; Leong, K. W., Chitosan nanoparticles for oral drug and gene delivery. *Int. J. Nanomed.* **2006,** *1*, 117-128.

13. Raabe, D.; Sachs, C.; Romano, P., The crustacean exoskeleton as an example of a structurally and mechanically graded biological nanocomposite material. *ACTA MATERIALIA* **2005,** *53* (15), 4281-4292.

14. Harada, T.; Harada, A., Curdlan and succinoglycan. In *Polysaccharides in Medicinal Applications*, Dumitriu, S., Ed. Dekker: New York, 1996; pp 21-57.

15. McIntosh, M.; Stone, B. A.; Stanisich, V. A., Curdlan and other bacterial (1→3)- β -d-glucans. *Applied Microbiology & Biotechnology* **2005,** *68* (2), 163-173.

16. Lee, I.-Y., Curdlan. In *Polysaccharides I: Polysaccharides from Prokaryotes*, Vandamme, E. J.; De, B. S.; Steinbuechel, A., Eds. Wiley: Biopolymers, 2002; Vol. 5, pp 135-158.

17. Funami, T.; Yada, H.; Nakao, Y., Curdlan Properties for Application in Fat Mimetics for Meat Products. *Journal of Food Science* **1998,** *63* (2), 283-287.

18. (a) Borjihan, G.; Zhong, G.; Baigude, H.; Nakashima, H.; Uryu, T., Synthesis and anti-HIV activity of −6-amino-6-deoxy-(1→3)- β -D-curdlan sulfate. *Polym. Adv. Technol.* **2003,** *14* (3-5), 326-329; (b) Gao, Y.; Fukuda, A.; Katsuraya, K.; Kaneko, Y.; Mimura, T.; Nakashima, H.; Uryu, T., Synthesis of Regioselective Substituted Curdlan Sulfates with Medium Molecular Weights and Their Specific Anti-HIV-1 Activities. *Macromolecules* **1997,** *30* (11), 3224-3228; (c) Osawa, Z.; Morota, T.; Hatanaka, K.; Akaike, T.; Matsuzaki, K.; Nakashima, H.; Yamamoto, N.; Suzuki, E.; Miyano, H.; Mimura, T.; Kaneko, Y., Synthesis of sulfated derivatives of curdlan and their anti-HIV activity. *Carbohydrate Polymers* **1993,** *21* (4), 283-288.

19. Huisgen, R., 1,3-Dipolar Cycloadditions. Past and Future. *Angewandte Chemie International Edition in English* **1963,** *2* (10), 565-598.

20. Hasegawa, T.; Umeda, M.; Numata, M.; Li, C.; Bae, A.-H.; Fujisawa, T.; Haraguchi, S.; Sakurai, K.; Shinkai, S., 'Click chemistry' on polysaccharides: a convenient, general, and monitorable approach to develop (1 → 3)- β -d-glucans with various functional appendages. *Carbohydr. Res.* **2006,** *341* (1), 35-40.

21. (a) Fox, S. C.; Edgar, K. J., Staudinger Reduction Chemistry of Cellulose: Synthesis of Selectively O-Acylated 6-Amino-6-deoxy-cellulose. *Biomacromolecules* **2012,** *13* (4), 992-1001; (b) Heinze, T.; Koschella, A.; Brackhagen, M.; Engelhardt, J.; Nachtkamp, K., Studies

on Non-natural Deoxyammonium Cellulose. *Macromolecular Symposia* **2006,** *244* (1), 74-82; (c) Liu, C.; Baumann, H., Exclusive and complete introduction of amino groups and their N-sulfo and N-carboxymethyl groups into the 6-position of cellulose without the use of protecting groups. *Carbohydrate Research* **2002,** *337* (14), 1297-1307; (d) Matsui, Y.; Ishikawa, J.; Kamitakahara, H.; Takano, T.; Nakatsubo, F., Facile synthesis of 6-amino-6-deoxycellulose. *Carbohydrate Research* **2005,** *340* (7), 1403-1406.

22. Cimecioglu, A. L.; Ball, D. H.; Kaplan, D. L.; Huang, S. H., Preparation of Amylose Derivatives Selectively Modified at C-6. 6-Amino-6-deoxyamylose. *Macromolecules* **1994,** *27* (11), 2917-2922.

23. Boger, J.; Corcoran, R. J.; Lehn, J.-M., Cyclodextrin chemistry. Selective modification of all primary hydroxyl groups of α- and β-cyclodextrins. *Helvetica Chimica Acta* **1978,** *61* (6), 2190-2218.

24. Cimecioglu, A. L.; Ball, D. H.; Huang, S. H.; Kaplan, D. L., A Direct Regioselective Route to 6-Azido-6-deoxy Polysaccharides under Mild and Homogeneous Conditions. *Macromolecules* **1997,** *30* (1), 155-156.

25. Hasegawa, T.; Umeda, M.; Numata, M.; Fujisawa, T.; Haraguchi, S.; Sakurai, K.; Shinkai, S., Click chemistry on curdlan: A regioselective and quantitative approach to develop artificial β-1,3-Glucans with various functional appendages. *Chem. Lett.* **2006,** *35*, 82-83.

26. Furuhata, K.-i.; Koganei, K.; Chang, H.-S.; Aoki, N.; Sakamoto, M., Dissolution of cellulose in lithium bromide-organic solvent systems and homogeneous bromination of cellulose with N-bromosuccinimide-triphenylphosphine in lithium bromide-N,N-dimethylacetamide. *Carbohydrate Research* **1992,** *230* (1), 165-177.

27. Tseng, H.; Furuhata, K.-i.; Sakamoto, M., Bromination of regenerated chitin with N-bromosuccinimide and triphenylphosphine under homogeneous conditions in lithium bromide-N,N-dimethylacetamide. *Carbohydrate Research* **1995,** *270* (2), 149-161.

28. Scriven, E. F. V.; Turnbull, K., Azides: their preparation and synthetic uses. *Chemical Reviews* **1988,** *88* (2), 297-368.

29. Edgar, K. J.; Arnold, K. M.; Blount, W. W.; Lawniczak, J. E.; Lowman, D. W., Synthesis and properties of cellulose acetoacetates. *Macromolecules* **1995,** *28*, 4122-8.

30. (a) Dalton, D. R.; Dutta, V. P.; Jones, D. G., Bromohydrin formation in dimethyl sulfoxide. *J. Am. Chem. Soc.* **1968,** *90* (20), 5498-5501; (b) Dalton, D. R.; Rodebaugh, R. K.;

Jefford, C. W., Bromohydrin formation in dimethyl sulfoxide. V. Reaction of norbornene. *The Journal of Organic Chemistry* **1972,** *37* (3), 362-367.

31. Fox, S. C.; Edgar, K., Synthesis of regioselectively brominated cellulose esters and 6-cyano-6-deoxycellulose esters. *Cellulose* **2011,** *18* (5), 1305-1314.

32. Pereira, J. M., Synthesis of New Pullulan Derivatives for Drug Delivery. *Unpublished PhD dissertation. Virginia Tech, Blacksburg, VA, USA* **2013**.

Chapter 4. Water-soluble Aminocurdlan Derivatives by Chemoselective Azide Reduction Using $NaBH_4$

Zhang, R.; Edgar, K. J. *Carbohydrate Polymers* **2015**, *122*, 84-92. Used with permission of Elsevier, 2015.

4.1 Abstract

Water-solubility can often enhance the utility of polysaccharide derivatives, for example in pharmaceutical and biomedical applications. Synthesis of water-soluble aminopolysaccharides, particularly those bearing other sensitive functional groups, can be a challenging endeavor. Curdlan is a bioactive β-1,3-glucan with considerable promise for biomedical applications. Aminocurdlans are intriguing target molecules for study of, for example, their interactions with the proteins that form tight junctions between enterocytes. Herein we report the preparation of two water-soluble 6-aminocurdlans starting from 6-bromo-6-deoxycurdlan. The 6-bromide was first displaced by nucleophilic substitution with sodium azide in dimethyl sulfoxide. The *O*-2, 4 groups were acylated with hydrophilic oligo(ethylene oxide) esters, so as to enhance aqueous solubility. The resultant 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoylcurdlan was then treated with excess sodium borohydride to reduce the azide; unexpectedly, the water-soluble product proved to be the amide, 6-trioxadecanamido-6-deoxycurdlan. Regioselectivity and degree of substitution (DS) of those derivatives were characterized by means of ^{1}H-, ^{13}C- NMR and FTIR- spectroscopy, elemental analysis, and titration. Alternatively, direct borohydride reduction of the parent 6-azido-6-deoxycurdlan afforded 6-amino-6-deoxycurdlan that was also water-soluble.

4.2 Introduction

Natural polysaccharides are always hydrophilic; they contain many hydroxyl groups and in some cases carboxylate, sulfate, amide and amino groups as well, providing a plethora of hydrogen bond donor and acceptor groups for interaction with water molecules. However, self-association through hydrogen bonding, and in some cases crystallization, prevents many polysaccharides from dissolving in water; important examples of these phenomena include cellulose, chitin, and curdlan. Considerable attention has been devoted to the study of water-soluble derivatized polysaccharides in pharmaceutical and biomedical applications, such as in

drug delivery[1], and for use as antimicrobial agents[2]. The natural polysaccharide chitin and its conversion to partially *N*-deacetylated, cationic, more soluble chitosan derivatives are exemplary; chitosan having been shown to possess for example the ability to encapsulate anionic drugs, nucleic acids and other biological compounds[3]. Chitosan can induce reversible opening of tight junctions between enterocytes that line the gastrointestinal (GI) tract by interacting with the proteins that help form these junctions, thereby increasing intestinal permeability and allowing paracellular absorption of otherwise poorly absorbed drugs, such as large and hydrophilic proteins[4]. The protonated amine groups of chitosan drive the necessary interactions with the tight junction proteins; permanently charged *N*-peralkylated derivatives are even more effective[5]. However, chitosan has its limitations for such uses. Its precursor, chitin, is isolated from matrices that contain large amounts of shellfish proteins; quantitative separation is essential since these proteins provoke severe immune responses in some individuals[6]. Ranaldi *et al.*[7] have found that chitosan has an irreversible effect on tight junctions at the highest concentration tested (0.01%), which could lead to degradation of the intestinal mucosal barrier function, permitting potentially toxic or allergenic molecules to enter the circulatory system.

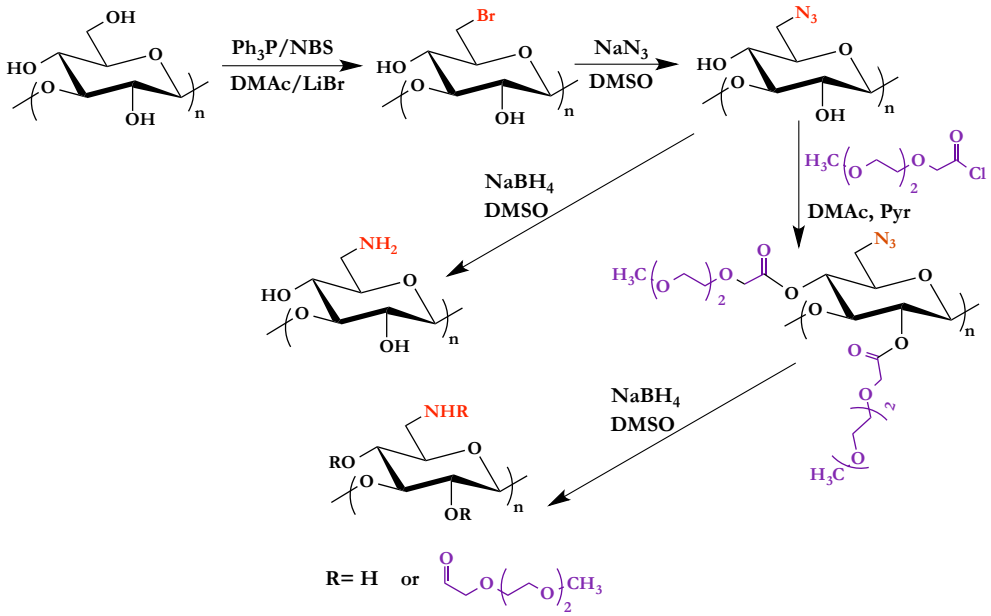
Recently researchers have explored amination of other polysaccharides including cellulose[8], curdlan[9], amylose[10], and pullulan[11] in an attempt to understand structure-property relationships, and ultimately to synthesize cationic chitosan analogs that would provide controlled interactions with proteins, for example short-term, reversible GI tight junction opening. Many of these approaches employ highly regioselective C-6 bromination of polysaccharides by triphenylphosphine in combination with a brominating agent (e.g. N-bromosuccinimide (NBS))[12]. This chemistry involves a double S_N2 mechanism[13] that provides essentially perfect C-6 selectivity for polysaccharides[14] that possess a C-6 primary alcohol; this is perhaps the most regioselective reaction known in polysaccharide chemistry[15]. The 6-halo-6-deoxy polysaccharides are converted to azides by nucleophilic displacement of bromide using a one-pot ($CBr_4/LiN_3/Ph_3P$/DMF) or two-step (NBS/Ph_3P in DMAc/LiBr; NaN_3/DMSO) approach. These approaches used the remarkably chemoselective and versatile Staudinger reaction[8, 10, 16] to reduce azido precursors to the desired amines, even in the presence of ester groups appended to the other polysaccharide OH groups. However, these Staudinger products had solubility limitations; the isolated aminopolysaccharides were

found to be insoluble in water or any common organic solvent. Alternatively, in a case where there were no other sensitive functional groups present, Matsui *et al.*[17] applied sodium borohydride to reduce the azide group, producing water-soluble 6-amino-6-deoxycellulose. While it is known that ester group reduction by sodium borohydride is relatively slow in comparison with that of more easily reduced groups like aldehydes and ketones, it is also known that sodium borohydride will effectively reduce ester groups to primary alcohols under more forcing conditions, such as in the presence of excess reagent[18]. Rapoport observed[18] "it is evident that esters are not resistant to reduction by sodium borohydride, although the rate of reduction is much slower than for aldehydes or ketones." Since reactions of polysaccharides are very frequently slower than those of analogous small molecules, and do require more forcing conditions, one could not be confident about the possibility of chemoselective reduction of azides in the presence of ester groups. Indeed, we have found no such examples of chemoselective borohydride reductions of azides in the presence of esters in the polysaccharide literature. Baumann *et al.*[19] described a clever approach to completely regioselectively substituted 6-amino-6-deoxycellulose with $DS_{C\text{-}6}$ 1.0 via tosylcellulose (DS_{tosyl} = 2.02, $DS_{6\text{-}tosyl}$ = 1.0). Nucleophilic displacement of tosyl groups by sodium azide at 50°C occurred only at C-6, while the tosyl groups at C-2/3 resisted displacement presumably due to the restricted approach angles available to these secondary tosylates. Subsequent complete reduction of azido groups to amino by use of lithium aluminium hydride ($LiAlH_4$) was accompanied by simultaneous reductive cleavage of the 2,3-O-tosyl groups, affording free cellulose hydroxyl groups at C-2, 3.

Curdlan (β-D-1,3-glucan) is a linear homopolysaccharide extracellularly produced by *Alcaligenes faecalis* var. myxogenes[20]. Its propensity to undergo thermal gelation has proven valuable in foods[21], including as a thickener, fat substitute, food additive, and other functions[22]. However, there are few reports in the literature describing how to prepare water-soluble aminocurdlans for biomedical applications, regio- and/or chemoselectively, or otherwise. Our own previous work indicated that synthesis of 6-amino-6-deoxycurdlan and its 2,4-*O*-diesters using the bromination/azidation/Staudinger reduction approach was successful, but afforded derivatives with essentially no water solubility[9].

The Heinze[23], Zhang[24], and Edgar[25] groups have investigated the syntheses and water-

solubility of cellulose trioxadecanoates (TOD) with a range of DS values by different esterification approaches including reaction of cellulose with 3,6,9-trioxadecanoyl chloride (TODCl), 3,6,9-trioxadecanoic acid (TODA)/1,1'-carbonyldiimidazole (CDI), TODA/*p*-toluenesulfonyl chloride (TosCl) and TODA/oxalyl chloride/DMF. We describe herein our attempts to prepare 6-amino-6-deoxycurdlan derivatives, rendered water-soluble by incorporation of hydrophilic ester (TOD) substituents, by using sodium borohydride to reduce their azido precursors, and exploring aspects of regioselectivity and chemoselectivity of the preparative reactions (**Scheme 4.1**). We also describe unexpected benefits from borohydride as opposed to Staudinger reduction of the 6-azido-6-deoxy polysaccharides.



Scheme 4.1. Synthetic scheme for 6-amino-6-deoxycurdlans.

4.3 Experimental

4.3.1 Materials

Curdlan (β-(1→3)-glucan, $(-C_6H_{10}O_5-)_n$, DP~6173) was obtained from Wako Chemicals and dried under vacuum at 40 °C overnight prior to use. Lithium bromide (LiBr, laboratory grade, Fisher) and sodium azide (NaN_3, 99%, Alfa Aesar) were dried under vacuum at 125°C. N-Bromosuccinimide (NBS, 99%, Acros) was recrystallized from boiling water and dried for two days under reduced pressure over anhydrous calcium chloride. *N,N*-Dimethylacetamide (DMAc, reagent grade, Fisher) and dimethyl sulfoxide (DMSO, HPLC grade, Fisher) were

stored over 4 Å molecular sieves. Hydrochloric acid (HCl, 37%, ACS reagent, Fisher) was used to make 1M HCl solution. 4-Dimethylaminopyridine (DMAP, Acros), triphenylphosphine (Ph_3P, 99%, Acros), pyridine (anhydrous, 99%, AcroSeal®), sodium borohydride ($NaBH_4$, 98+%, powder, Acros), 2-[2-(2-methoxyethoxy)ethoxy]acetic acid (3,6,9-trioxadecanoic acid, TODA, technical grade, Sigma-Aldrich), acetic anhydride (Ac_2O, 99+%, Sigma-Aldrich), sodium bicarbonate (reagent grade, Fisher) and regenerated cellulose dialysis tubing (MW 3500, Fisher) were used as received.

4.3.2 Measurements

4.3.2.1 NMR and IR Spectroscopy

^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance II 500 MHz spectrometer in $CDCl_3$, DMSO-d_6 or D_2O at room temperature or 50 °C, employing 32 and 15,000 scans, respectively. Infrared spectroscopic analyses of samples as pressed KBr pellets were performed on a Thermo Electron Nicolet 8700 instrument with 64 scans and 4 cm^{-1} resolution.

4.3.2.2 Ester saponification and back-titration

6-Azido-6-deoxy-*O*-trioxadecanoylcurdlan (238 mg, ~0.47 mmol) or its borohydride reduction product (228 mg, ~0.71 mmol) was carefully weighed and charged into a 50 mL 3-neck flask. An accurately measured volume of 0.1 N NaOH (20 mL) was added and stirred vigorously in a closed system at room temperature for 24 h. The mixture was then titrated with 0.1 N HCl solution. A potentiometric probe was inserted into the flask. Each measurement was carried out by adding a 100 μL aliquot of HCl solution, allowing the mixture to stir for 20 seconds and then collecting the pH data point. The precipitate was isolated by filtration, washed extensively with water, vacuum dried and then analyzed by infrared spectroscopy.

DS calculation after saponification and back-titration:

$$DS_{TOD\ (azido)} = \frac{\Delta V \times 0.1 \times (187 + 160 DS_{TOD})}{1000\, m} \Rightarrow DS_{TOD(azido)} = \frac{18.7}{1000 \frac{m}{\Delta V} - 16} \qquad \textbf{(Eq. 4.1)}$$

or

$$DS_{TOD\ (amino)} = \frac{\Delta V \times 0.1 \times (161 + 160 DS_{TOD})}{1000\ m} \Rightarrow DS_{TOD(amino)} = \frac{16.1}{1000 \frac{m}{\Delta V} - 16}$$ (**Eq. 4.2**)

Where $\Delta V = V_1 - V_2$ (V_1, V_2 are the volumes (mL) of 0.1 N NaOH and 0.1 N HCl solution used during the titration), and m (g) is the mass of added 6-azido or 6-amino curdlan trioxadecanoate.

4.3.2.3 Elemental analysis (EA)

Amino curdlans were purified and vacuum dried at 40 °C for 5 h prior to EA to determine carbon, hydrogen and nitrogen contents, carried out by Micro Analysis Inc. using a Perkin Elmer 2400 II analyzer. Carbon and nitrogen contents were determined by flask combustion followed by ion chromatography.

4.3.3 Methods

4.3.3.1 Dissolution of curdlan in DMAc/LiBr (referred to in examples as "standard cellulose solution in DMAc/LiCl")[26]

Dissolution of curdlan in DMAc/LiBr was performed by a procedure reported in our previous paper[9]. A mixture of dried curdlan (4.00 g, 24.7 mmol AGU) and DMAc (150 mL) was kept at 165 °C for 26 min with vigorous stirring under nitrogen. LiBr (36.00 g, 42.4 mmol) was added, and the mixture was stirred at 160 °C for 8 min. DMAc (40 mL) was distilled off to facilitate water removal. The slurry was allowed to cool to room temperature while being stirred continuously overnight, during which time dissolution occurred to form a transparent solution.

4.3.3.2 Regioselective bromination and azide displacement of curdlan

Solutions of Ph_3P (25.96 g, 4 eq per AGU) and NBS (17.58 g, 4 eq per AGU) were prepared, each in 50 mL of dry DMAc. The Ph_3P solution was added dropwise via a liquid addition funnel to a solution of 4.00 g curdlan in DMAc/LiBr prepared as described above, followed by dropwise addition of the NBS solution. The resulting solution was then heated at 70 °C for 1 h. The solution was cooled and then added slowly to 1 L of a 50:50 mixture of methanol and deionized water, then filtered to recover the precipitate. The isolated product was re-dissolved in DMSO and re-precipitated in ethanol, then the dissolution and

reprecipitation process was repeated. The product was dried under vacuum at 40 °C overnight to yield 6-bromo-6-deoxycurdlan. ^{13}C NMR (DMSO-d_6): δ 103.2 (C-1), 84.9 (C-3), 74.4 (C-5), 73.6 (C-2), 70.1 (C-4), 34.6 (C-6-Br). Yield: 86%.

Dry 6-bromo-6-deoxycurdlan (1.00 g, 4.44 mmol) was dissolved in 25 mL of anhydrous DMSO in a 100 mL flask. Then NaN_3 (1.44 g, 5 eq per AGU) was added to the flask and dissolved. The solution was heated to 80 °C and stirred for 24 h under nitrogen. The product was isolated by cooling the reaction mixture, then pouring into 300 mL of deionized water. The precipitate was collected by filtration. The product was re-dissolved in acetone and then re-precipitated in deionized water, followed by filtration. The sample was dried under vacuum at 40 °C overnight to yield 6-azido-6-deoxycurdlan. ^{13}C NMR (DMSO-d_6): δ 103.4 (C-1), 84.9 (C-3), 74.9 (C-5), 73.9 (C-2), 69.4 (C-4), 51.7 (C-6-N_3). Yield: 92%.

4.3.3.3 Reaction of 6-azido-6-deoxycurdlan with 3,6,9-trioxadecanoyl chloride (TODCl)

TODA (71.2 g, 61.3 mL, 0.4 mol) was first weighed into a 250 mL three-neck round-bottom flask equipped with a dropping funnel and a gas outlet connecting to a 5 M $NaHCO_3$ solution. Thionyl chloride (31.2 mL, 0.44 mol) was added dropwise into the flask with magnetic stirring at room temperature. It was stirred for 30 min and then heated to 70 °C for an additional 2 h until gas release stopped. The unreacted thionyl chloride was removed by rotary evaporation. The residual liquid (TODCl) was used for subsequent esterification without further purification.

Dry 6-azido-6-deoxycurdlan (0.5 g, 2.67 mmol) was dissolved in 25 mL of DMAc in a 100 mL flask. Pyridine (2.15 mL, 10 eq per AGU) was injected all at once, then TODCl (4.6 mL, 10 eq per AGU) was added dropwise from an addition funnel at room temperature. The mixture was heated to 80 °C and stirred at that temperature for 24 h. The homogeneous solution was cooled to room temperature and transferred to 3500 g/mol MWCO dialysis tubing (prewet with water) that was placed in a large beaker containing deionized water. After 5 days of dialysis, the brown solution was then freeze-dried to yield 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoyl-curdlan. ^{13}C NMR ($CDCl_3$): δ 169.1 (C=O), 100.1 (C-1), 72.9-67.2 (C-2~5 & C-8~14), 58.9 (C-16), 50.9 (C-6-N_3). Yield: 78 %.

4.3.3.4 Peracetylation of 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoylcurdlan

Dry 6-azido-6-deoxy-*O*-trioxadecanoylcurdlan (0.2 g) and DMAP (20 mg) were weighed into a 50 mL round-bottom flask. Pyridine (0.32 mL, 10 eq per AGU) and acetic anhydride (0.74 mL, 20 eq per AGU) were then added dropwise to the solution. The mixture was stirred at 80 °C for 24 h and then cooled and added slowly to 200 mL deionized water. The crude product was collected by filtration, and then re-dissolved in 10 mL chloroform. This solution was added slowly with rapid stirring to 200 mL of ethanol. After filtration, the sample was dried under vacuum at 40 °C.

4.3.3.5 Synthesis of 6-trioxadecanamido-6-deoxycurdlan using sodium borohydride from 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoylcurdlan

Dry 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoylcurdlan (0.2 g) was dissolved in 20 mL of DMSO in a 100 mL flask. Then $NaBH_4$ (0.30 g, 20 eq per AGU) was added to the flask and dissolved. The solution was heated to 100 °C and stirred for 24 h. It was cooled to 0 °C, then HCl (1M) was added dropwise to the mixture at 0 °C in an ice bath until gas generation ceased. The mixture was neutralized using saturated $NaHCO_3$ solution and then transferred to 3500 g/mol MWCO dialysis tubing. After 3 days of dialysis, the colorless solution was then freeze-dried to yield 6-trioxadecanamido-6-deoxycurdlan. ^{13}C NMR (D_2O): δ 173.3 (C=O amide), 103.1 (C-1), 85.0-69.2 (C-2~5 & C-8~14), 58.6 (C-16), 41.6 (C-6-amide), Yield: 79 %.

4.3.3.6 Synthesis of 6-amino-6-deoxycurdlan using sodium borohydride

Dry 6-azido-6-deoxycurdlan (0.2 g) was dissolved in 20 mL of DMSO in a 100 mL flask. Then $NaBH_4$ (0.81 g, 20 eq per AGU) was added to the flask and dissolved. The solution was heated to 100 °C and stirred for 24 h. It was cooled to 0°C, then HCl (1M) was added dropwise to the mixture at 0 °C in an ice bath until gas generation ceased. The mixture was neutralized using saturated $NaHCO_3$ solution and then transferred to 3500 g/mol MWCO dialysis tubing. After 3 days of dialysis, the colorless solution was then freeze-dried to yield 6-amino-6-deoxycurdlan. ^{13}C NMR (D_2O): 6-amino-6-deoxycurdlan: δ 105.4 (C-1), 86.1 (C-3), 77.4 (C-5), 76.4 (C-2), 72.4 (C-4), 44.1 (C-6-NH_2), Yield: 71%.

4.3.3.7 Synthesis of 6-acetamido-6-deoxycurdlan by sodium borohydride reduction of 6-azido-6-deoxy-2,4-di-*O*-acetylcurdlan

Under nitrogen, dry 6-azido-6-deoxycurdlan (0.5 g, 2.67 mmol) and 4-dimethylaminopyridine (DMAP, 20mg) were weighed into a 50 mL round-bottom flask. Pyridine (2.15 mL, 10 eq per AGU) and acetic anhydride (2.73 mL, 10 eq per AGU) were then added dropwise to the solution. The mixture was reacted at 80 °C for 24h under nitrogen. The homogeneous mixture was then added slowly to 200 mL deionized water. The crude product was collected by filtration, and then re-dissolved in 10 mL chloroform. This solution was added slowly with rapid stirring to 200 mL of ethanol. After filtration and washing with ethanol and water several times, the sample was dried under vacuum at 40 °C overnight to yield 6-azido-6-deoxy-2,4-di-*O*-acetyl-curdlan. ^{13}C NMR (DMSO-d_6): δ 169.6 (C=O), 99.4 (C-1), 78.0 (C-3), 72.1 (C-5), 71.1 (C-2), 68.5 (C-4), 50.5 (C-6-N_3), 20.6 (CH_3-Ac), Yield: 97%.

Dry 6-azido-6-deoxy-2,4-di-*O*-acetylcurdlan (0.2 g, 0.74 mmol) was dissolved in 20 mL of DMSO in a 100 mL flask. Then $NaBH_4$ (0.56 g, 20 eq per AGU) was added to the flask and dissolved. The solution was heated to 100 °C and stirred for 24 h. It was cooled to 0 °C, then HCl (1M) was added dropwise to the mixture at 0°C in an ice bath until gas generation ceased. The mixture was neutralized using saturated $NaHCO_3$ solution and then transferred to 3500 g/mol MWCO dialysis tubing. After 3 days of dialysis, the colorless solution was then freeze-dried to yield 6-acetamido-6-deoxycurdlan. ^{1}H NMR (D_2O): δ 3.87-3.30 (curdlan backbone protons), 2.03 (CH_3-Ac), Yield: 72%.

4.4 Results and discussion

As we have previously reported[9], curdlan can be brominated with complete regioselectivity at C-6 and the resulting 6-bromo-6-deoxycurdlan can then be reacted with NaN_3 in DMSO at 80 °C to produce 6-azido-6-deoxycurdlan with a DS_{azide}~1.0, which can be easily esterified to produce 2,4-*O*-diester derivatives readily soluble in a range of organic solvents. The azido curdlans have been found to be useful precursors for regioselective synthesis of several new curdlan derivatives, including (*O*-acylated-)6-amino-6-deoxycurdlan by Staudinger reduction,

as well as a series of 6-amido-6-deoxy-2,4-di-*O*-acyl-curdlans. Even though the mild Staudinger reaction chemoselectively reduces the 6-azide to 6-amine while leaving the esters at C-2/4 untouched, those aminodiester products showed poor solubility in any common organic solvent once isolated from the reaction mixture. We originally hypothesized that their insolubility could be attributed to intermolecular hydrogen bonding between 6-amino groups, and/or to the small amount of residual triphenylphosphine-related impurities that could be seen in NMR spectra of the derivatives[8-10]. Such poor solubility could be limiting with regard to applications as drug or gene delivery agents, and especially with respect to oral protein delivery via tight-junction opening.

We planned to impart water solubility to 6-amino-6-deoxycurdlan derivatives by esterifying the O-2/4 curdlan hydroxyl groups with 3,6,9-trioxodecanoate groups, by reacting 6-azido curdlan with 3,6,9-trioxadecanoyl chloride in dimethylacetamide. Therefore, in spite of our concerns about chemoselectivity and potentially competing ester reduction, we explored the use of sodium borohydride as azide reducing agent in an attempt to circumvent these solubility problems.

4.4.1 Synthesis of 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoyl-curdlan

Acylation of 6-azido-6-deoxycurdlan with the appropriate acid chloride seemed like a promising approach to the 2,4-di-*O*-trioxodecanoyl esters; therefore TODCl (**Fig. S4.2**) was synthesized by reaction of TODA with thionyl chloride just prior to its use, then was reacted with 6-azido-6-deoxycurdlan in DMAc in the presence of pyridine catalyst.

Identity and DS of the resulting 6-azido-6-deoxy-2,4-*O*-bis(trioxadecanoyl)curdlan were established by FTIR and NMR spectroscopy, peracetylation, and saponification followed by back-titration. FTIR analysis showed that, in comparison with the 6-azido starting material (**Fig. 4.5a**), a characteristic ester carbonyl absorption at 1771 cm^{-1} was present (**Fig. 4.1a**), indicating successful TOD ester introduction. Proton NMR is normally very useful for DS determination of polysaccharide derivatives, but peaks for the trioxadecanoate methylene groups at around 3.3-4.1 ppm overlap with the curdlan backbone proton resonances (3.7-4.8 ppm, **Fig. 4.2**) preventing DS_{TOD} calculation by this method (unsurprisingly since all are protons attached to carbons that also bear an electron-withdrawing oxygen atom). The ^{13}C

NMR spectrum (**Fig. 4.3a**) contained a TOD carbonyl signal at δ 169.1 ppm and a single C-1 signal at δ 100.1 ppm. Resonances assigned to the carbons of the TOD moiety and the curdlan C-2, 3, 4, 5 carbons are visible at δ = 59.0 (C-16) and δ = 65.7-73.8 (C-2 through 5, C-8 through 14), with similar issues of signal overlap as observed in the ^{1}H NMR. Therefore, we treated the putative 6-azido-6-deoxy-2,4-*O*-bis(trioxadecanoyl)curdlan with acetic anhydride in pyridine to yield products in which the proton NMR should show acetyl methyl signals at 1.9-2.1 ppm if $DS_{TOD} < 2$. However, no apparent acetyl methyl resonances were observed (**Fig. S4.3**), indicating complete TOD esterification (DS_{TOD}~2) at C-2/4 within the ^{1}H NMR detection limits.

Alternatively, we employed saponification of 6-azido curdlan trioxadecanoate with aqueous NaOH and back-titration of the excess base with HCl solution to determine DS_{TOD}. This approach has long been used for DS determination of polysaccharide esters[27]. Complete trioxadecanoate hydrolysis under mild conditions (0.1 N aq NaOH, 25 °C, 24 h) can be confirmed by FTIR (**Fig. S4.4b**), in which the ester carbonyl band at 1771 cm^{-1} disappeared while the hydroxyl stretching absorption near 3400 cm^{-1} increased. Aqueous HCl (0.1 N) was employed to back-titrate the excess NaOH and the volume of added acid was plotted against the pH change (**Fig. S4.5**). The peak at 10.8 mL indicated the equilibrium point that was used to calculate the DS_{TOD} = 1.89 by **Eq. (4.1)**.

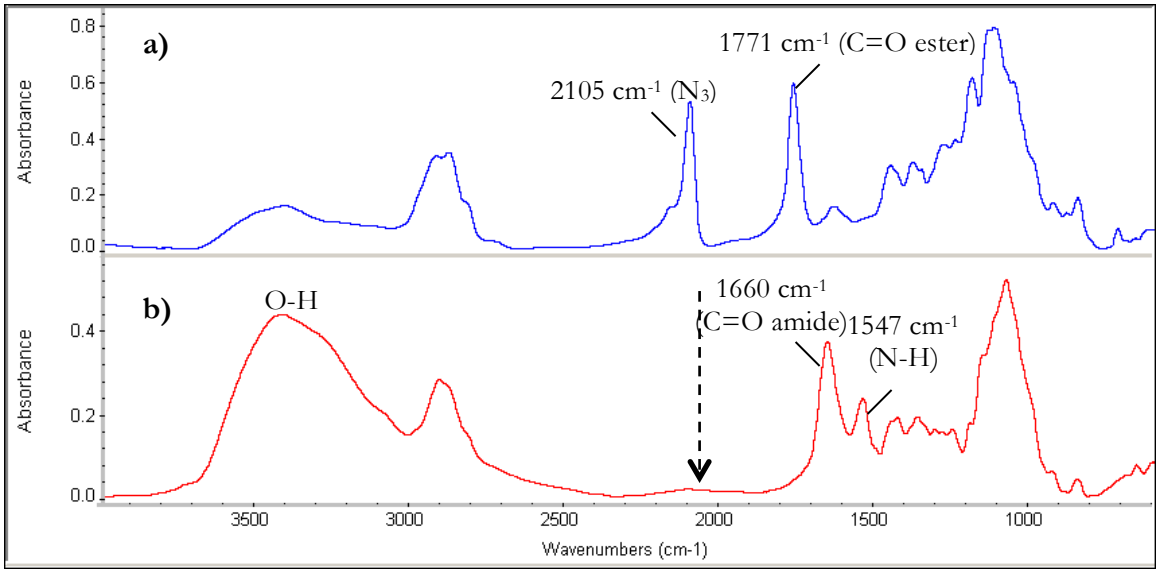


Fig. 4.1. FTIR spectra of **a)** 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoyl-curdlan and **b)** 6-trioxadecanamido-6-deoxycurdlan synthesized by $NaBH_4$ reduction.

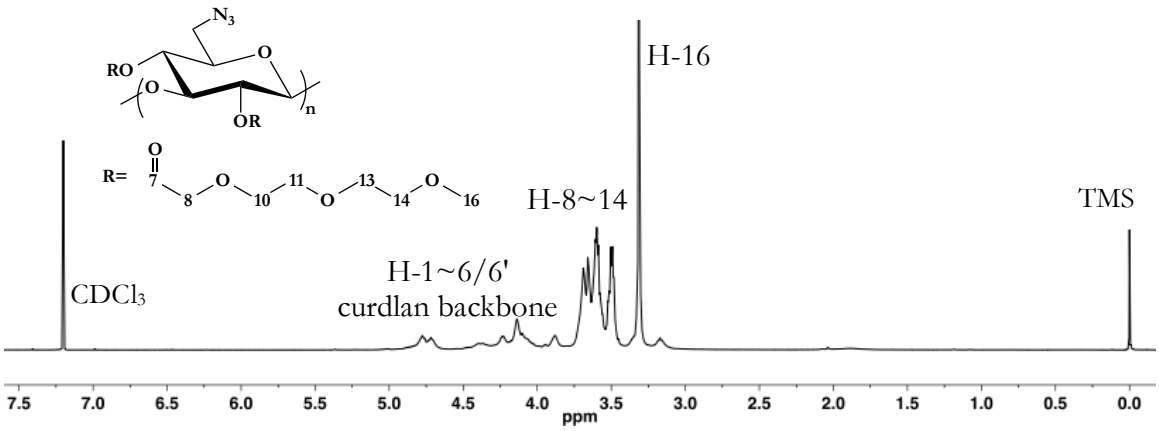


Fig. 4.2. ^{1}H NMR spectrum of 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoylcurdlan.

4.4.2 Borohydride reduction of 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoylcurdlan

Because of the solubility problems observed previously with the products of azide reduction using the Staudinger reaction, we wished to pursue an alternative strategy. Borohydride is a useful reagent for reduction of azides[28], but as mentioned previously we had concerns about partial or full concomitant reduction of the ester moieties under the rather strong conditions that we thought would probably be needed to reduce these polysaccharide azides. Indeed, preliminary experiments indicated that reaction with sodium borohydride at 100°C would be required for complete azide reduction. 6-Azido-6-deoxy-2,4-di-*O*-trioxadecanoyl-curdlan was treated with 20 molar equivalent of sodium borohydride at 100 °C for 24 h followed by dialysis and lyophilization. The product, gratifyingly, did indeed have excellent water solubility. In **Fig. 4.1b**, the typical absorption of azide around 2100 cm^{-1} was absent after reduction while N-H bending appeared at 1547 cm^{-1}. However, instead of the expected ester carbonyl absorptions, a carbonyl absorption was observed at 1660 cm^{-1}, an area that is typically associated with amides. No ester carbonyl absorption was observed in the range of 1735 to 1750 cm^{-1}, indicating no remaining trioxadecanoate after reduction. Additionally, broadened absorptions in the range of 3300-3500 cm^{-1} were assigned to O-H stretch of curdlan backbone hydroxyls resulting from ester reductive cleavage. It was vital to confirm whether concomitant partial or complete reduction of the TOD esters had occurred. More

conclusive evidence of conversion to the 6-trioxadecanamido-6-deoxycurdlan is provided by the ^{13}C NMR spectrum (**Fig. 4.3b**). The chemical shift for C-6 has moved upfield from 50.9 to 41.6 ppm, in accord with spectral changes observed with other polysaccharide azide to amide conversions.[9, 11] More importantly, only one carbonyl resonance from the TOD moiety was observed; it exhibited a downfield shift of about 4 ppm and changed from the two carbonyl resonances of the starting diester (δ 169) to a single resonance (δ 173, **Fig. 4.3**). From this spectroscopic evidence, we hypothesize that under the harsh conditions required to completely reduce the azide (100 °C, 24 h), the liberated amine reacted with one or both of the 2,4-*O*-ester groups, with the result of acyl transfer to the more nucleophilic nitrogen, and amide formation. Such O to N acyl transfers are well-known in organic chemistry[29] and even in carbohydrate chemistry[30]. Intramolecular acyl transfer from 4-O to 6-N is geometrically reasonable, but we cannot rule out the possibility that at least some of the acyl transfer occurred intermolecularly. The excess sodium borohydride reductively cleaved the remaining TOD esters at C-2/4, affording the observed amide product. Such acyl group migrations from O to N have been observed by us before, for 6-amino-6-deoxycellulose 2,3-*O*-esters[8], and for the analogous derivatives of pullulan[31]. However, in those cases Staudinger reduction was used to reduce azide to amine, so that acyl migration occurred from ester oxygen to a negatively charged nitrogen of the Staudinger ylide intermediate. In order to confirm this hypothesis, we subjected 6-azido-6-deoxy-2,4-acetyl-curdlan to the same $NaBH_4$ reduction conditions (since the acetyl methyl resonates upfield of the curdlan backbone hydrogens and is readily observed and quantified). IR and NMR analyses (**Fig. S4.7**) of this reduction product show acetyl migration to form the amide and reductive cleavage of the residual *O*-acetate esters, in exactly the same fashion observed for the TOD esters. It is interesting to note that the 6-acetamido-6-deoxycurdlan product, an isomer of chitin (which is insoluble in water and simple organic solvents), is soluble in water. This sharp difference in properties is worthy of further exploration.

In order to quantify residual trioxadecanoate following borohydride reduction of the TOD ester, the product was saponified with aqueous NaOH (0.1 N) followed by back-titration using HCl (0.1 N). Two inflection points were evident in the titration curve (**Fig. S4.6**); the first one for titration of excess NaOH, and the second for titration of the weakly basic 6-NH_2 groups. Based on the titrant consumed at the first inflection point (12.6 mL), DS_{TOD}

was calculated to be 1.09 by **Eq. (4.2)**. Combined with previous spectrometric evidence, almost all ester groups had been reduced to hydroxyls during azide reduction (compare with DS_{TOD} of 1.89 in the starting 6-azido curdlan trioxadecanoate) and a new trioxadecanamide was formed at C-6.

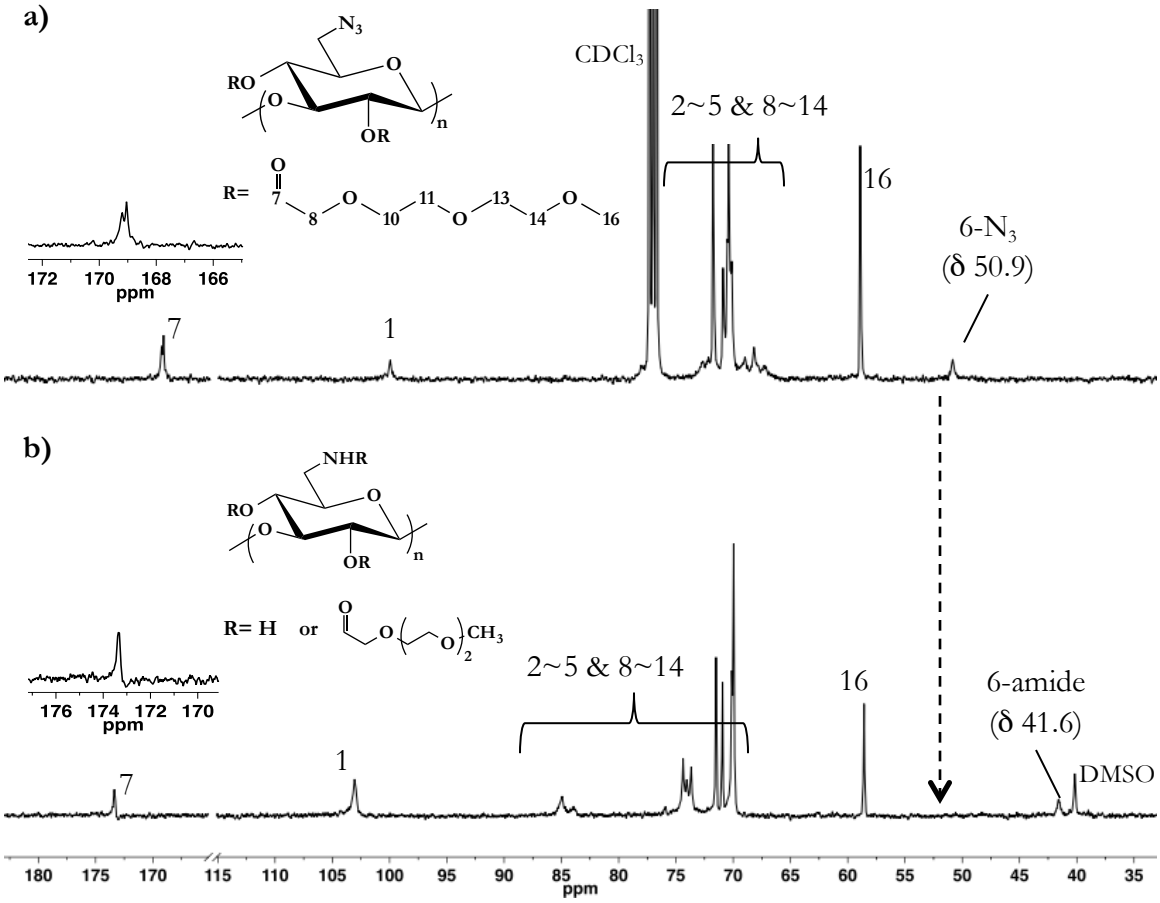


Fig. 4.3. ^{13}C NMR spectra of **a)** 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoyl-curdlan and **b)** 6-amino-6-deoxy-2,4-di-*O*-trioxadecanoyl-curdlan.

4.4.3 Synthesis of water-soluble 6-amino-6-deoxycurdlan

Given these results, it was of interest to investigate the reduction of 6-azido-6-deoxycurdlan to determine whether the poor water solubility previously observed for the parent amine (6-amino-6-deoxycurdlan) could be improved by using borohydride rather than Staudinger reduction. We employed a large excess of borohydride (20 eq $NaBH_4$, 60 °C, 24 h) as used by Matsui and co-workers, believing that it may be necessary to efficiently reduce recalcitrant

polysaccharide azides. In the product ^{13}C NMR spectrum (**Fig. 4.4a**), residual azide could be clearly observed as indicated by the azide-bearing C-6 resonance at 51 ppm. Partial conversion to the desired amine product could be confirmed by appearance of a resonance for the amine-bearing C-6, shifted upfield to 44 ppm. In order to improve the extent of reduction, we doubled the reaction time (48 h, **Fig. S4.1b**), but this was ineffective as the DS_{amine} decreased from previous 0.80 to 0.76 (determined by elemental analysis). Increases in reaction temperature proved more useful; at 80 °C, we observed significant decrease in intensity of the azide-bearing C-6 resonance at 51 ppm (**Fig. S4.1a**). Borohydride reduction at 100 °C gave complete disappearance of the azide peak, and appearance of a single C-6 peak at 41 ppm, indicating complete reduction of the azide to the amine (**Fig. 4.4b**). No residual Ph_3P or Ph_3PO peaks were observed (ca. 130 ppm), indicating complete removal of these impurities left over from the bromination (and of course no such impurities are generated during borohydride reduction, in contrast to the Staudinger reduction). **Figure 4.5** shows the FTIR spectra of 6-azido- and 6-amino-6-deoxycurdlan. The strong azide absorptions at 2107 cm^{-1} (**Fig. 4.5a**) present in the starting material are virtually completely eliminated in the borohydride reduction product (6-amino-6-deoxycurdlan, **Fig. 4.5b**). The product absorption at 1575 cm^{-1} is assigned to N-H bending of the free amine, confirming successful reduction. 6-Amino-6-deoxycurdlan prepared in this way is fully soluble in water (NMR spectra measured in D_2O); its $DS(NH_2)$ was calculated to be 0.95 by elemental analysis.

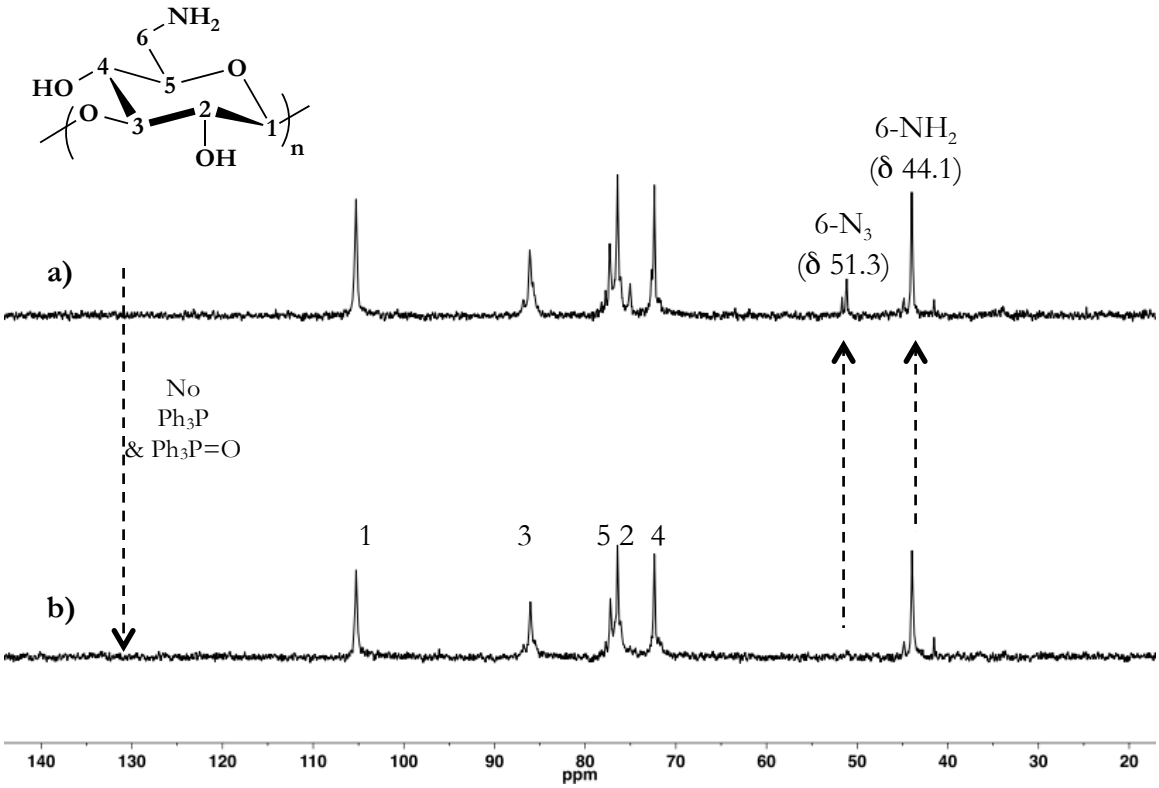


Fig. 4.4. ^{13}C NMR spectra of 6-amino-6-deoxycurdlan: **a)** 60 °C, 24 h and **b**) 100 °C, 24 h.

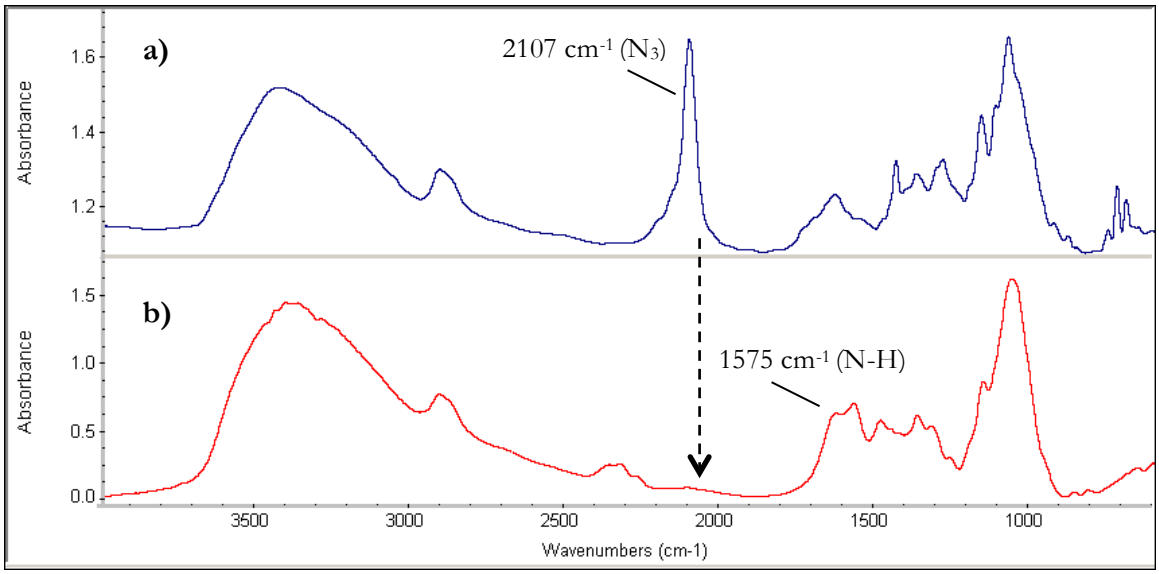


Fig. 4.5. FTIR spectra of **a)** 6-azido-6-deoxycurdlan and **b)** 6-amino-6-deoxycurdlan synthesized by $NaBH_4$ reduction.

4.5 Conclusions

We have developed approaches to two water-soluble curdlan derivatives that are characterized by very high regioselectivity. One approach involves appending hydrophilic, uncharged 3,6,9-trioxodecanoate groups to the 2- and 4-OH groups of curdlan, by initial bromination of curdlan, azide displacement of the bromide, and acylation of the 6-azido-6-deoxycurdlan at both 2- and 4-OH groups, affording a water-soluble, regioselectively substituted derivative. Interestingly, upon borohydride reduction of the 6-azido group to the 6-amine, acyl group migration occurred, along with concomitant reduction of residual ester groups, providing the *N*-TOD amide, which is also water soluble. The ability to synthesize water soluble 6-amido-6-deoxycurdlan derivatives in this way is of interest for the study of the biological properties of these 6-substituted, 1→3 β-linked analogues of natural chitin.

We were pleasantly surprised by the discovery that the parent polysaccharide, 6-amino-6-deoxycurdlan, has good water solubility when synthesized by sodium borohydride reduction of the corresponding azide, as opposed to the water insolubility observed for the Staudinger reduction product of the same azide. This is surprising given the close spectroscopic similarity of the two products, differing only in the small quantity of arylphosphorus containing impurities evident in the Staudinger product. Clearly borohydride reduction is preferred in order to obtain the most soluble aminocurdlan products; it has the additional benefits of being simpler and being compatible with easily handled solvents. Access to these families of water-soluble, regioselectively substituted curdlan derivatives should permit investigations of structure-property relationships for a variety of biomedical applications. Studies of the properties of these water-soluble analogs of chitosan with regard to their interactions with tight junction proteins are under way.

4.6 Supplemental material

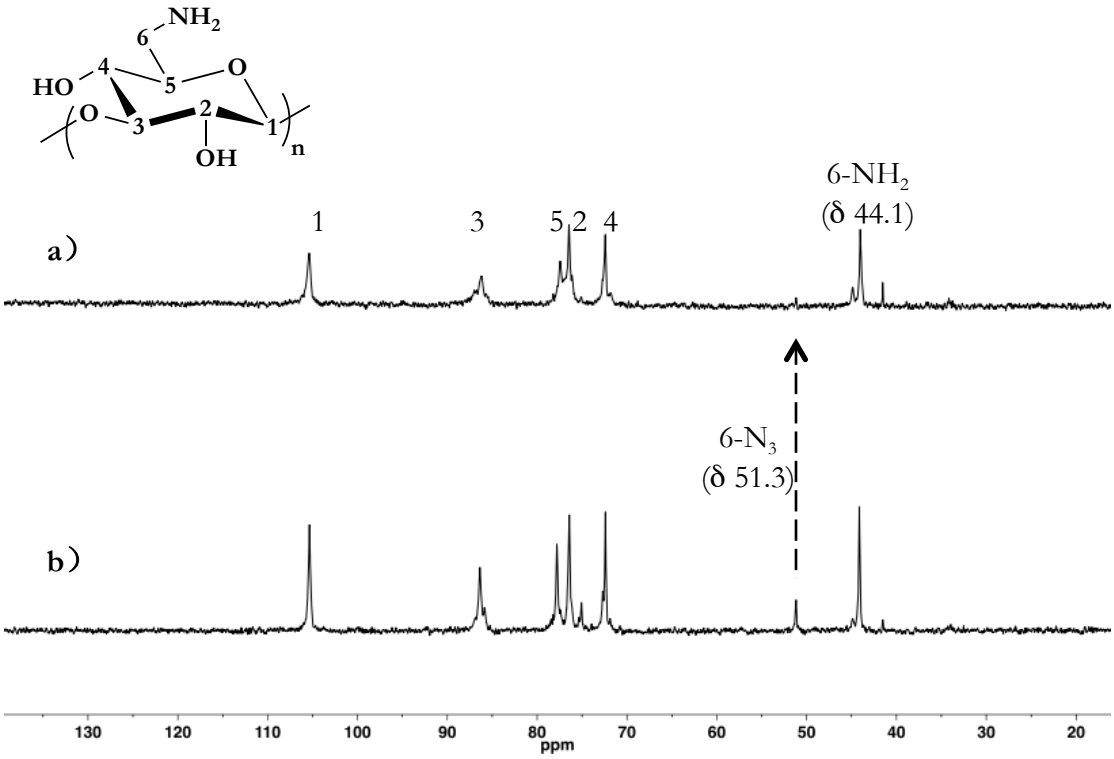


Fig. S4.1. ^{13}C NMR spectra of 6-amino-6-deoxycurdlan: **a)** 80 °C, 24 h and **b)** 60 °C, 48 h.

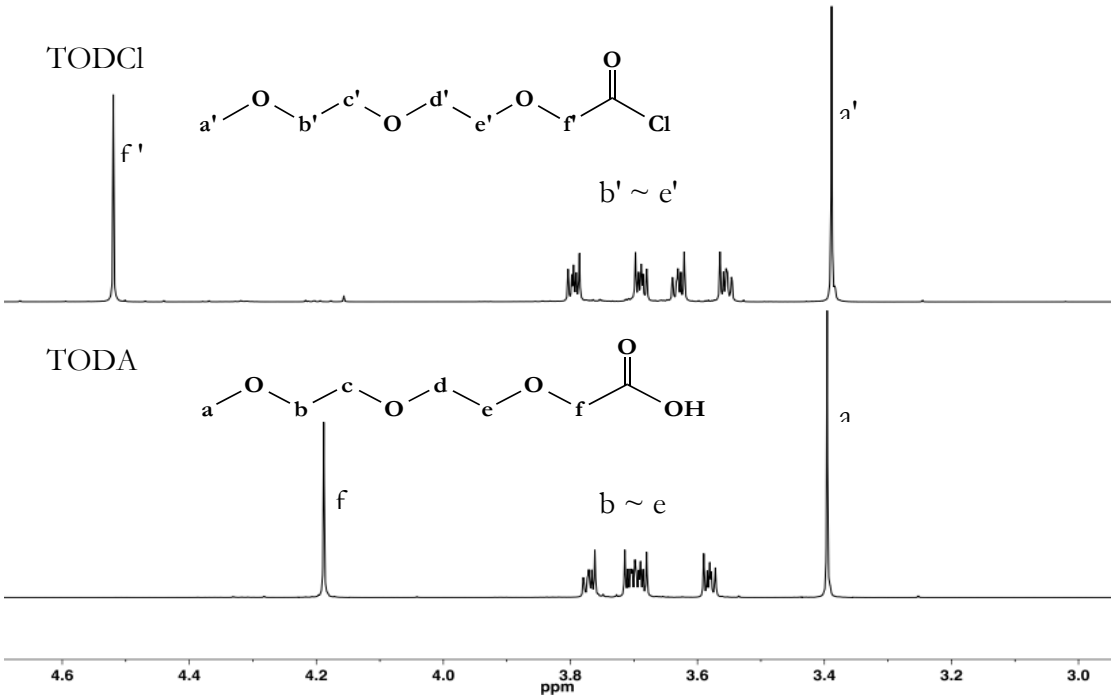


Fig. S4.2. [1]H NMR spectra of 3,6,9-trioxadecanoic acid (TODA) and 3,6,9-trioxadecanoyl chloride (TODCl).

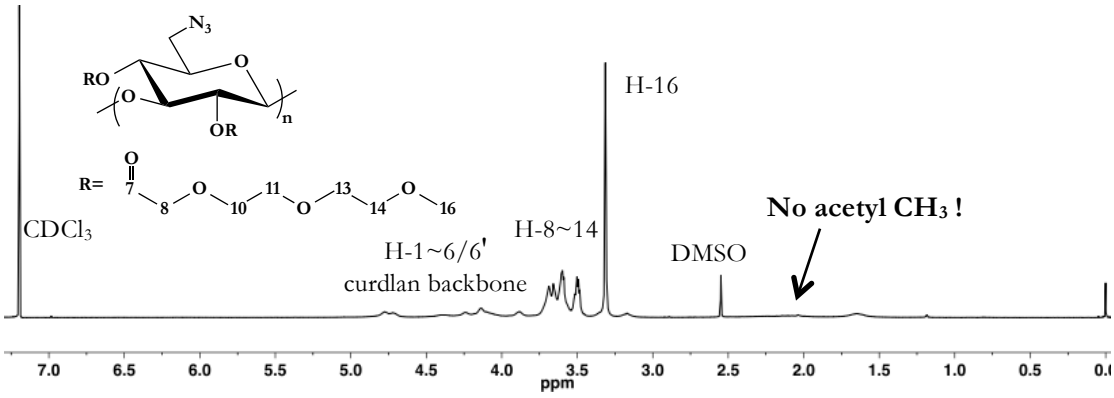


Fig. S4.3. [1]H NMR spectra of peracetylated 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoyl-curdlan.

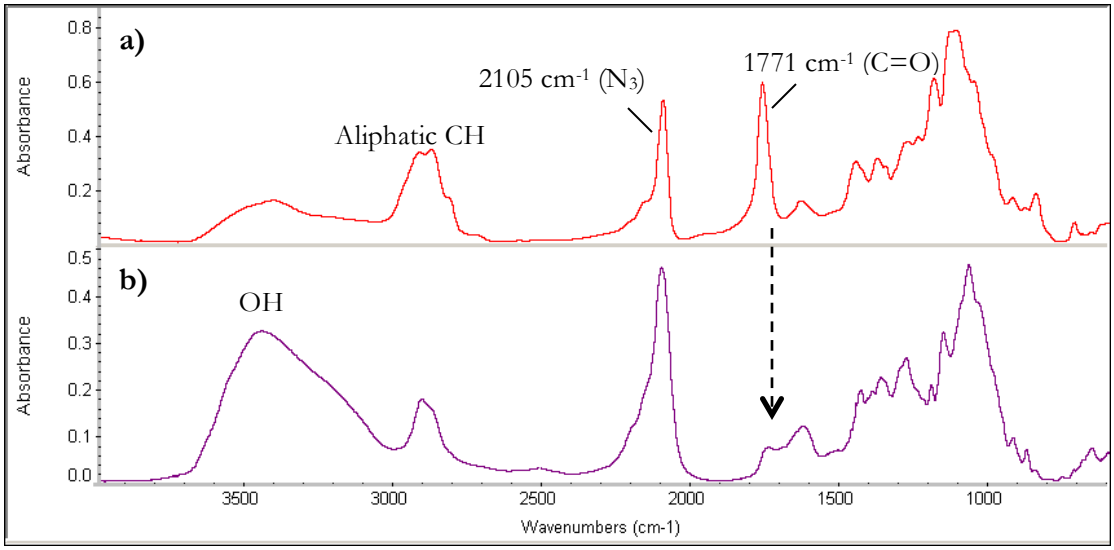


Fig. S4.4. FTIR spectra of **a)** 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoyl-curdlan and **b)** its residue after saponification and back-titration.

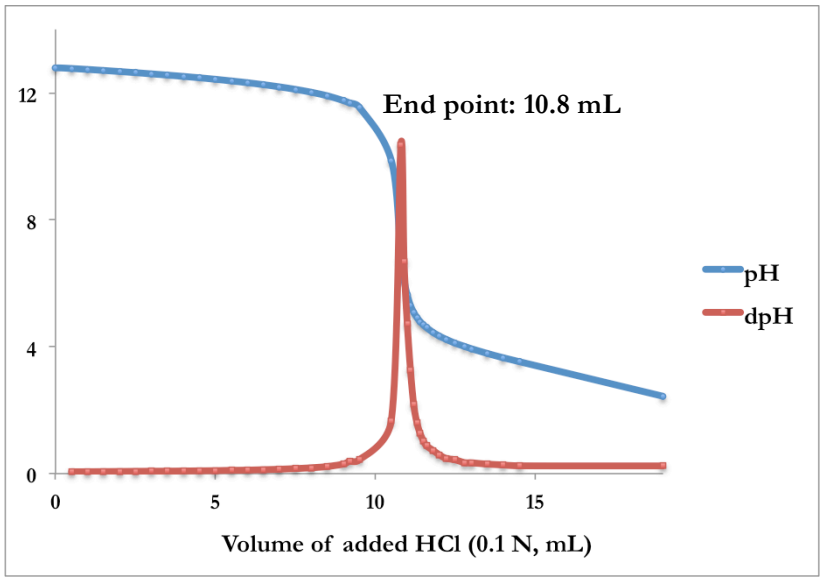


Fig. S4.5. Titration curves of 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoyl-curdlan after saponification at 25 °C for 24 h: pH or dpH change vs. volume of added HCl (0.1 N, mL).

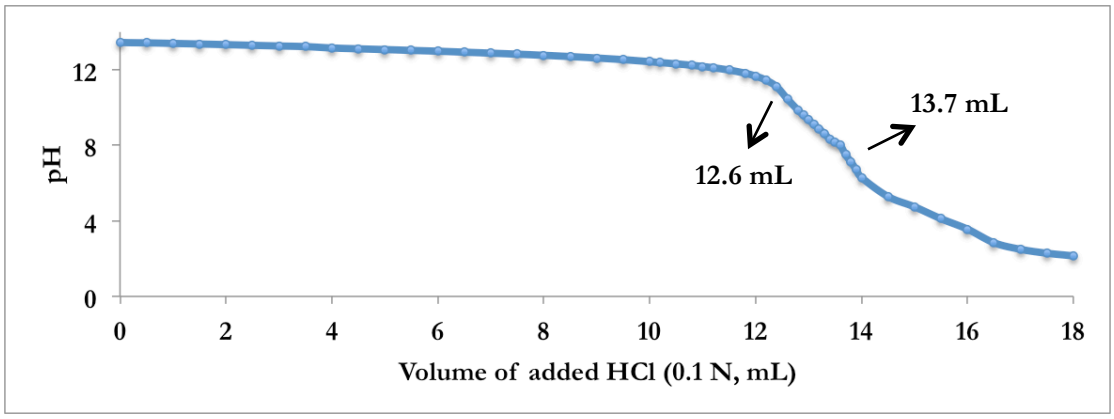


Fig. S4.6. Titration curve of the reductive product from 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoyl-curdlan by sodium borohydride after saponification at 25 °C for 24 h: pH change vs. volume of added HCl (0.1 N, mL).

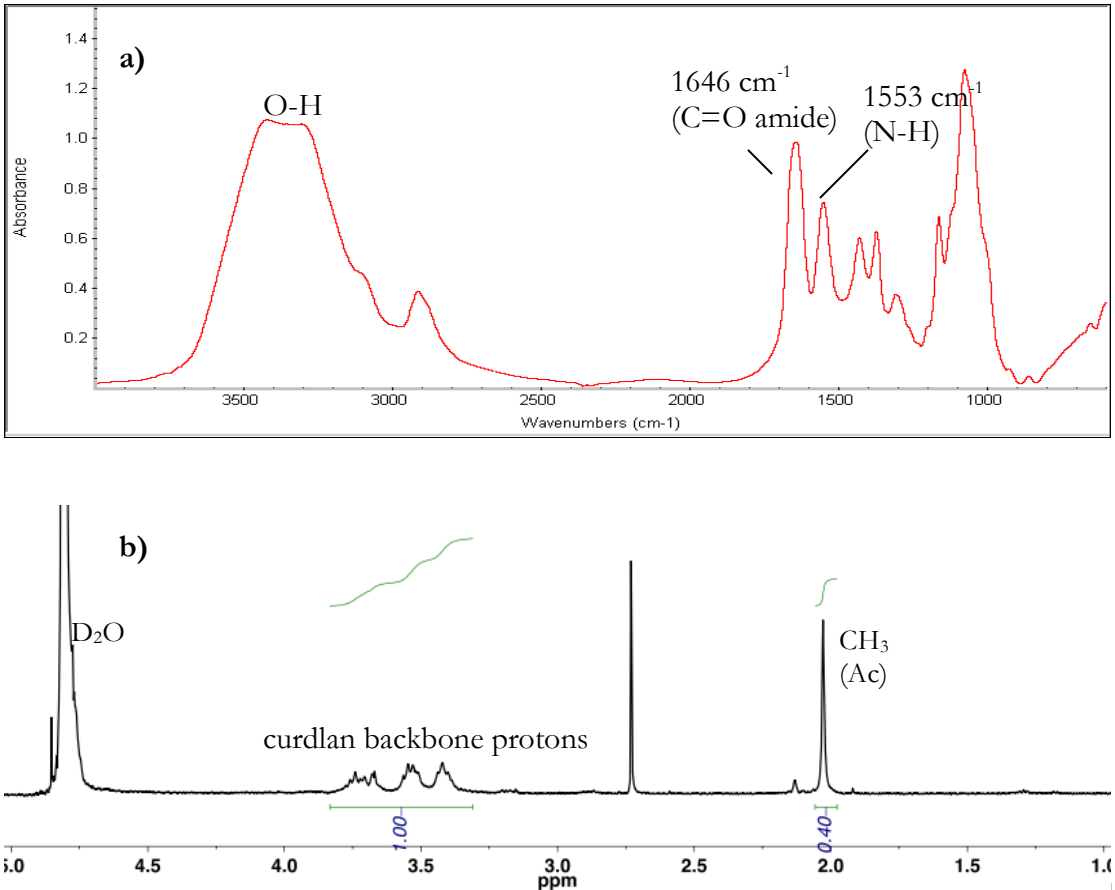


Fig. S4.7. Sodium borohydride reduction of 6-azido-6-deoxy-2,4-acetyl-curdlan to produce 6-acetamido-6-deoxycurdlan: **a)** FTIR spectrum with amide carbonyl absorption at 1646 cm^{-1} and N-H stretch at 1553 cm^{-1} and **b)** ^{1}H NMR spectrum with single peak around 2.0 ppm with DS_{Ac}~0.93.

4.7 Acknowledgements

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4.8 References

1. (a) Hassani, L. N.; Hendra, F.; Bouchemal, K., Auto-associative amphiphilic polysaccharides as drug delivery systems. *Drug Discovery Today* **2012,** *17* (11–12), 608-614; (b) Henni-Silhadi, W.; Deyme, M.; Boissonnade, M.-M.; Appel, M.; Le Cerf, D.; Picton, L.;

Rosilio, V., Enhancement of the Solubility and Efficacy of Poorly Water-Soluble Drugs by Hydrophobically-Modified Polysaccharide Derivatives. *Pharmaceutical Research* **2007,** *24* (12), 2317-2326; (c) Liesiene, J.; Matulioniene, J., Application of water-soluble diethylaminoethylcellulose in oral drug delivery systems. *Reactive and Functional Polymers* **2004,** *59* (2), 185-191.

2. (a) Roemhild, K.; Wiegand, C.; Hipler, U.-C.; Heinze, T., Novel Bioactive Amino-Functionalized Cellulose Nanofibers. *Macromol. Rapid Commun.* **2013,** *34* (22), 1767-1771; (b) Lee, J. W.; Yeomans, W. G.; Allen, A. L.; Gross, R. A.; Kaplan, D. L., Microbial production of water-soluble non curdlan type exopolymer-B with controlled composition by agrobacterium sp. *Biotechnol. Lett.* **1997,** *19* (12), 1217-1221.

3. Ravi Kumar, M. N. V., A review of chitin and chitosan applications. *Reactive and Functional Polymers* **2000,** *46* (1), 1-27.

4. Yeh, T.-H.; Hsu, L.-W.; Tseng, M. T.; Lee, P.-L.; Sonjae, K.; Ho, Y.-C.; Sung, H.-W., Mechanism and consequence of chitosan-mediated reversible epithelial tight junction opening. *Biomaterials* **2011,** *32* (26), 6164-6173.

5. Sadeghi, A. M. M.; Dorkoosh, F. A.; Avadi, M. R.; Weinhold, M.; Bayat, A.; Delie, F.; Gurny, R.; Larijani, B.; Rafiee-Tehrani, M.; Junginger, H. E., Permeation enhancer effect of chitosan and chitosan derivatives: Comparison of formulations as soluble polymers and nanoparticulate systems on insulin absorption in Caco-2 cells. *European Journal of Pharmaceutics and Biopharmaceutics* **2008,** *70* (1), 270-278.

6. Hajeb, P.; Selamat, J., A Contemporary Review of Seafood Allergy. *Clinic Rev Allerg Immunol* **2012,** *42* (3), 365-385.

7. Ranaldi, G.; Marigliano, I.; Vespignani, I.; Perozzi, G.; Sambuy, Y., The effect of chitosan and other polycations on tight junction permeability in the human intestinal Caco-2 cell line. *The Journal of Nutritional Biochemistry* **2002,** *13* (3), 157-167.

8. Fox, S. C.; Edgar, K. J., Staudinger Reduction Chemistry of Cellulose: Synthesis of Selectively O-Acylated 6-Amino-6-deoxy-cellulose. *Biomacromolecules* **2012,** *13* (4), 992-1001.

9. Zhang, R.; Edgar, K. J., Synthesis of curdlan derivatives regioselectively modified at C-6: O-(N)-Acylated 6-amino-6-deoxycurdlan. *Carbohydr. Polym.* **2014,** *105* (0), 161-168.

10. Cimecioglu, A. L.; Ball, D. H.; Kaplan, D. L.; Huang, S. H., Preparation of Amylose Derivatives Selectively Modified at C-6. 6-Amino-6-deoxyamylose. *Macromolecules* **1994,** *27* (11), 2917-2922.

11. Pereira, J. M.; Edgar, K. J., Regioselective synthesis of 6-amino- and 6-amido-6-deoxypullulans. *Cellulose (Dordrecht, Neth.)* **2014,** *21* (4), 2379-2396.

12. Furuhata, K.; Koganei, K.; Chang, H.-S.; Aoki, N.; Sakamoto, M., Dissolution of cellulose in lithium bromide-organic solvent systems and homogeneous bromination of cellulose with N-bromosuccinimide triphenylphosphine in lithium bromide - N,N-dimethylacetamide. *Carbohydr. Res.* **1992,** *230*, 165-177.

13. Fox, S. C.; Edgar, K., Synthesis of regioselectively brominated cellulose esters and 6-cyano-6-deoxycellulose esters. *Cellulose* **2011,** *18* (5), 1305-1314.

14. (a) Furuhata, K.; Chang, H. S.; Aoki, N.; Sakamoto, M., Chlorination of cellulose with N-chlorosuccinimide-triphenylphosphine under homogeneous conditions in lithium chloride-N,N-dimethylacetamide. *Carbohydr. Res.* **1992,** *230* (1), 151-64; (b) Furuhata, K.; Koganei, K.; Chang, H. S.; Aoki, N.; Sakamoto, M., Dissolution of cellulose in lithium bromide-organic solvent systems and homogeneous bromination of cellulose with N-bromosuccinimide-triphenylphosphine in lithium bromide-N,N-dimethylacetamide. *Carbohydr. Res.* **1992,** *230* (1), 165-77.

15. Fox, S. C.; Li, B.; Xu, D.; Edgar, K. J., Regioselective Esterification and Etherification of Cellulose - A Review. *Biomacromolecules* **2011,** *12*, 1956-1972.

16. (a) Boger, J.; Corcoran, R. J.; Lehn, J. M., Cyclodextrin chemistry. Selective modification of all primary hydroxyl groups of α- and β-cyclodextrins. *Helv. Chim. Acta* **1978,** *61* (6), 2190-218; (b) Cimecioglu, A. L.; Ball, D. H.; Huang, S. H.; Kaplan, D. L., A Direct Regioselective Route to 6-Azido-6-deoxy Polysaccharides under Mild and Homogeneous Conditions. *Macromolecules* **1997,** *30* (1), 155-156.

17. Matsui, Y.; Ishikawa, J.; Kamitakahara, H.; Takano, T.; Nakatsubo, F., Facile synthesis of 6-amino-6-deoxycellulose. *Carbohydrate Research* **2005,** *340* (7), 1403-1406.

18. Brown, M. S.; Rapoport, H., The Reduction of Esters with Sodium Borohydride1. *The Journal of Organic Chemistry* **1963,** *28* (11), 3261-3263.

19. (a) Baumann, H.; Liu, C.; Faust, V., Regioselectively Modified Cellulose and Chitosan Derivatives for Mono- and Multilayer Surface Coatings of Hemocompatible Biomaterials. *Cellulose* **2003,** *10* (1), 65-74; (b) Liu, C.; Baumann, H., Exclusive and complete introduction of amino groups and their N-sulfo and N-carboxymethyl groups into the 6-position of cellulose without the use of protecting groups. *Carbohydrate Research* **2002,** *337* (14), 1297-1307.

20. (a) Harada, T.; Harada, A., Curdlan and succinoglycan. In *Polysaccharides in Medicinal Applications*, Dumitriu, S., Ed. Dekker: New York, 1996; pp 21-57; (b) Zhang, R.; Edgar, K. J., Properties, Chemistry, and Applications of the Bioactive Polysaccharide Curdlan. *Biomacromolecules* **2014,** *15* (4), 1079-1096.

21. Miwa, M.; Nakao, Y.; Nara, K., Food Applications of Curdlan. In *Food Hydrocolloids*, Nishinari, K.; Doi, E., Eds. Springer US: 1993; pp 119-124.

22. (a) Jin, Y.; Zhang, H.; Yin, Y.; Nishinari, K., Comparison of curdlan and its carboxymethylated derivative by means of Rheology, DSC, and AFM. *Carbohydrate Research* **2006,** *341* (1), 90-99; (b) Na, K.; Park, K.-H.; Kim, S. W.; Bae, Y. H., Self-assembled hydrogel nanoparticles from curdlan derivatives: characterization, anti-cancer drug release and interaction with a hepatoma cell line (HepG2). *Journal of Controlled Release* **2000,** *69* (2), 225-236; (c) Popescu, I.; Pelin, I. M.; Butnaru, M.; Fundueanu, G.; Suflet, D. M., Phosphorylated curdlan microgels. Preparation, characterization, and in vitro drug release studies. *Carbohydrate Polymers* **2013,** *94* (2), 889-898.

23. (a) Heinze, T.; Schaller, J., New water soluble cellulose esters synthesized by an effective acylation procedure. *Macromolecular Chemistry and Physics* **2000,** *201* (12), 1214-1218; (b) Dorn, S.; Pfeifer, A.; Schlufter, K.; Heinze, T., Synthesis of water-soluble cellulose esters applying carboxylic acid imidazolides. *Polym. Bull.* **2010,** *64* (9), 845-854.

24. Zhou, Q.; Zhang, L.; Okamura, H.; Minoda, M.; Miyamoto, T., Synthesis and properties of O-2-[2-(2-methoxyethoxy)ethoxy]acetyl cellulose. *Journal of Polymer Science Part A: Polymer Chemistry* **2001,** *39* (3), 376-382.

25. Liu, H.; Edgar, K. J., Synthesis and characterization of neutral and anionic cellulosic amphiphiles. *Carbohydr. Polym.* **2014,** *113* (0), 480-489.

26. Edgar, K. J.; Arnold, K. M.; Blount, W. W.; Lawniczak, J. E.; Lowman, D. W., Synthesis and Properties of Cellulose Acetoacetates. *Macromolecules* **1995,** *28* (12), 4122-4128.

27. (a) Freire, C. S. R.; Silvestre, A. J. D.; Pascoal Neto, C.; Rocha, R. M. A., An Efficient Method for Determination of the Degree of Substitution of Cellulose Esters of Long Chain Aliphatic Acids. *Cellulose* **2005,** *12* (5), 449-458; (b) Malm, C. J.; Genung, L. B.; Williams, J. R. F.; Pile, M. A., Analysis of Cellulose Derivatives Total Acyl in Cellulose Organic Esters by Saponification in Solution. *Industrial & Engineering Chemistry Analytical Edition* **1944,** *16* (8), 501-504; (c) Stojanović, Ž.; Jeremić, K.; Jovanović, S.; Lechner, M. D., A Comparison of Some Methods for the Determination of the Degree of Substitution of Carboxymethyl

Starch. *Starch - Stärke* **2005,** *57* (2), 79-83; (d) Tihlarik, K.; Pasteka, M., Estimation of functional groups and degree of substitution in carboxymethylstarch. *Chem. Pap.* **1987,** *41* (5), 703-8.

28. Scriven, E. F. V.; Turnbull, K., Azides: their preparation and synthetic uses. *Chemical Reviews* **1988,** *88* (2), 297-368.

29. (a) Cao, P.; Raleigh, D. P., Ester to Amide Switch Peptides Provide a Simple Method for Preparing Monomeric Islet Amyloid Polypeptide under Physiologically Relevant Conditions and Facilitate Investigations of Amyloid Formation. *J. Am. Chem. Soc.* **2010,** *132* (12), 4052-4053; (b) Sohma, Y.; Hayashi, Y.; Skwarczynski, M.; Hamada, Y.; Sasaki, M.; Kimura, T.; Kiso, Y., O/N intramolecular acyl migration reaction in the development of prodrugs and the synthesis of difficult sequence-containing bioactive peptides. *Peptide Science* **2004,** *76* (4), 344-356.

30. Sproviero, J. F., Stereospecificity of the O→ N acyl migration in 2, 3, 4, 6-tetra- *O*-benzoyl-β-D-mannopyranosylamine. *Carbohydr. Res.* **1973,** *26* (2), 357-363.

31. Pereira, J. M.; Edgar, K. J., Regioselective synthesis of 6-amino-and 6-amido-6-deoxypullulans. *Cellulose*, 1-18.

Chapter 5. Regioselective Synthesis of Cationic 6-Deoxy-6-(*N*,*N*,*N*-trialkylammonio)curdlan Derivatives

Zhang, R.; Edgar, K. J. *Carbohydrate Polymers* **2016**, *136*, 474-484. Used with permission of Elsevier, 2016.

5.1 Abstract

Curdlan, a bioactive β-1,3-glucan, is of intense interest for pharmaceutical and biomedical applications. Cationic derivatives of curdlan and other polysaccharides are especially attractive for their potential to interact in controlled fashion with proteins, among many other possible applications, but relatively few methods exist for their synthesis. Herein we report a regioselective method for preparation of cationic, water-soluble 6-(*N*,*N*,*N*-trialkylammonio)-6-deoxycurdlan salts by reaction of 6-bromo-6-deoxycurdlan and its 2,4-*O*-diesters with trialkylamines. Dimethyl sulfoxide was identified as the optimal solvent for this nucleophilic displacement to produce cationic curdlan derivatives (80 °C, 24 h), providing maximum degree of triethylammonium substitution (DS) of 0.89, exclusively at the C-6 position. 6-Bromo-6-deoxycurdlan was also reacted with heterocyclic amines such as pyridine and imidazole, providing ammonium-substituted curdlan derivatives with substantial DS (0.66 and 0.86, respectively). The new combination of regioselective Furuhata bromination and bromine displacement under optimized conditions with tertiary amines provides access to quaternized curdlan derivatives that possess high, permanent positive charge and are readily water-soluble, properties that indicate potential application promise including for mucoadhesion, permeation enhancement, and delivery of genes and anionic drugs. Regioselectivity and DS of those curdlan ammonium derivatives were quantified by means of ^{1}H NMR, ^{13}C NMR and FTIR spectroscopic methods and by elemental analysis.

5.2 Introduction

Cationic polysaccharides are materials of interest for a number of applications, ranging from industrial (e.g., flocculants[1] and anti-bacterial coatings[2]) to biomedical (e.g., vehicles for gene delivery[3] and for delivery of anionic drugs[4]). Despite the potential utility of such materials, there are relatively few synthetic approaches to cationic polysaccharides, especially to those that carry a permanent positive charge (and thus are charged even at neutral pH). Amine

substitution of polysaccharides, for example by displacement of a 6-tosylate or 6-halide, is known and is a route to 6-amino-6-deoxypolysaccharides;[5] it is less clear whether this would be a useful route to permanently charged polysaccharides. Substituents bearing pendent charged groups can be attached by well-known chemistries such as etherification[6] or "click" chemistry[7]. Alternatively, existing amino groups (i.e., the 2-amino-2-deoxy moiety in chitosan) may be peralkylated[8] to create permanent charge. Only a few studies describe the seemingly straightforward approach of displacement of a polysaccharide-appended leaving group by a trialkylamine nucleophile, including by displacement of the tosyl group from *p*-toluenesulfonyl cellulose with various nucleophiles, such as n-butylamine, pyridine[9], triethylamine, *N*,*N*-dimethyl-1,3-diaminopropane and 2,4,6-tris-(*N*,*N*-dimethyl-aminomethyl)phenol[10]. 6-Bromo-6-deoxy derivatives have special appeal as substrates for nucleophilic displacement (S_N) reactions, since bromide is a good leaving and activating group and the bromination reaction can be so highly regioselective.[11]

Cationic polysaccharides are of particular interest for their influence on the tight junctions between the enterocytes that line the small intestine and constitute the barrier between the intestinal lumen and the bloodstream. Macromolecular drugs such as proteins are not passively absorbed through the enterocytes (transcellular permeation) due to their abundance of hydrogen bond donors and acceptors[12], and relatively high molar mass values. Efficient oral delivery of protein therapeutics is thus difficult or impossible at present. Researchers have explored the possibility that absorption of these hydrophilic macromolecules may be facilitated by temporary opening of the tight junctions between the enterocytes, thereby clearing a pathway for the large proteins[13] (termed paracellular absorption). A number of compounds, referred to as "paracellular permeability enhancers (PPE)[14]" have been evaluated to increase paracellular transport between the enterocytes.[15]

Cationic chitosan derivatives have been of particular interest for this purpose; the mechanism by which they effect tight junction opening is not fully understood but is believed to involve electrostatic interaction with the proteins that bridge the tight junctions.[16] Molecules that effect opening of tight junctions must do so only fleetingly, so as not to seriously impair their protective function.[17] Paracellular permeation enhancement by chitosan was shown to be pH-dependent, since the free amino groups of chitosan are protonated at

low pH, increasing charge as well as water solubility. Peralkylation of free chitosan amines affords trimethyl chitosan (TMC), which has been heavily explored by Junginger and others as a permanently charged polymer for tight junction opening.[18] However, chitosan derivatives may not be ideal for such purposes; for example the precursor chitin is extracted from crustacean shell composites that contain large amounts of shellfish proteins, which may provoke severe immune responses in susceptible individuals[19]. Alkaline hydrolysis of chitin to chitosan also requires severe conditions not amenable to control or selectivity. Most importantly, semisynthetic chitosan is not natural, and there is no *a priori* reason to assume that it is the ideal aminopolysaccharide for interacting with tight junction proteins to enhance paracellular transport; more extensive structure-property studies are needed.

Curdlan (β-1,3-glucan) is a bacterial, neutral, linear homopolysaccharide produced by *Alcaligenes Faecalis* var. *myxogenes* strain 10C3.[20] Curdlan and its derivatives have shown potential in biomedical and pharmaceutical applications as biological response modifiers (antitumor, anti-infective, anti-inflammatory and anti-coagulant) to enhance immune responses.[21] Curdlan's water insolubility, attributed to the presence of extensive inter-/intra-molecular hydrogen bonds, has limited its biological application. "Click" chemistry has been applied by Shinkai group[7] to incorporate quaternary ammonium moieties into the curdlan backbone; the resultant cationic derivatives demonstrated excellent binding properties with polymeric guest molecules in water. Suflet *et al.*[22] recently reported the synthesis of cationic water-soluble curdlan ammonium with DS(ammonium) up to 0.15 by etherification with two types of ω-trialkylammonium substituents. The viscosity behavior of these cationic curdlan derivatives and their interaction with anionic species have been investigated *vs.* DS and nature of the quaternary substituent, and ionic strength of the aqueous solution. Our lab is interested in synthesis of 6-amino-6-deoxycurdlans with complete control of regiochemistry and amine substitution; we have previously reported synthesis of two water-soluble aminocurdlans, 6-amino-6-deoxy- and 6-trioxadecanamido-6-deoxycurdlan with high regioselectivity by chemoselective azide reduction using sodium borohydride.[23] We hypothesize that permanently charged 6-deoxy-6-trialkylammoniocurdlan derivatives can be prepared with essentially perfect regioselectivity by direct displacement reactions of 6-halo-6-deoxycurdlans with appropriate trialkylamines and heterocyclic amines. We further hypothesize that these structurally well-defined derivatives, analogs and in some cases

isomers of peralkylated chitosans, may be useful for biomedical applications like tight junction opening, without the drawback of potentially harmful contamination.

Herein we report our attempts to achieve such selective displacements of 6-halides from curdlan derivatives in order to prepare permanently cationic, regioselectively substituted 6-deoxy-(*N*,*N*,*N*-trialkylammonio)- curdlan salts, cationic analogs of trialkyl chitosans, with an eye towards water solubility enhancement and to enable later exploration of the structure-property relationship with regard to tight junction opening.

5.3 Experimental

5.3.1 Materials

Curdlan (DP ~ 500) was obtained from Wako Chemicals and dried under vacuum at 40 °C overnight prior to use. Lithium bromide (LiBr, laboratory grade, Fisher) was dried under vacuum at 125 °C. *N*-Bromosuccinimide (NBS, 99%, Acros) was recrystallized from boiling water and dried for two days under reduced pressure over anhydrous calcium chloride. *N, N*-Dimethylacetamide (DMAc, reagent grade, Fisher), dimethyl sulfoxide (DMSO, HPLC grade, Fisher) and *N, N*-dimethylformamide (DMF, HPLC grade, Fisher) were stored over 4 Å molecular sieves. 4-Dimethylaminopyridine (DMAP, Acros), triphenylphosphine (Ph_3P, 99%, Acros), pyridine (Pyr, anhydrous, 99%, AcroSeal®), triethylamine (TEA, 99%, Acros), tributylamine (TBA, 99%, Acros), diethylamine (DEA, reagent grade, Fisher), 1-methylimidazole (MeIMID, 99+%, Sigma-Aldrich), *N*, *N*-dimethylpropionamide (DMPr, >98%, TCI), sodium iodine (Fisher), acetic anhydride (Ac_2O, 99+%, Sigma-Aldrich), ethanol (HPLC grade, Fisher) and regenerated cellulose dialysis tubing (MW 3500, Fisher) were used as received.

5.3.2 Measurements

^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance II 500MHz spectrometer in DMSO-d_6 or D_2O at room temperature or 50 °C, employing 32 and 15,000 scans, respectively. Infrared spectroscopic analyses of samples as pressed KBr pellets were performed on a Thermo Electron Nicolet 8700 instrument with 64 scans and 4 cm^{-1} resolution. Elemental analyses (EA) to determine carbon, nitrogen and bromine contents were performed by Micro Analysis Inc. using a Perkin Elmer 2400 II analyzer. Carbon and

nitrogen contents were determined by flask combustion followed by ion chromatography and bromine content was measured with a thermal conductivity detector.

5.3.3 Methods

5.3.3.1 Dissolution of curdlan in DMAc/LiBr

Dissolution of curdlan in DMAc/LiBr was performed by a previously reported procedure[24]. A mixture of dried curdlan (4.00 g, 24.7 mmol AGU) and DMAc (150 mL) was kept at 165 °C for 26 min with vigorous stirring under nitrogen. LiBr (36.00 g, 42.4 mmol) was added, and the mixture was stirred at 170°C for 8 min. DMAc (40 mL) was distilled off to facilitate water removal. The slurry was allowed to cool to room temperature while being stirred continuously overnight, during which time dissolution occurred to form a transparent solution.

5.3.3.2 Regioselective bromination of curdlan

Ph_3P (25.96 g, 4 eq per AGU) and NBS (17.58 g, 4 eq per AGU) were separately dissolved in 50 mL of dry DMAc each. The Ph_3P solution was added dropwise via a liquid addition funnel to a standard curdlan (4.00 g curdlan) solution in DMAc/LiBr, followed by the addition of the NBS solution in similar fashion. The reaction solution was then heated at 70 °C for 1 h. The mixture was added slowly to 1 L of a 50:50 mixture of methanol and deionized water and then filtered to recover the precipitate. The isolated product was re-dissolved in DMSO and re-precipitated in ethanol twice. The sample was dried under vacuum at 40 °C overnight to yield 6-bromo-6-deoxycurdlan. ^{13}C NMR (DMSO-d_6; **Fig. S5.1c**): δ 103.2 (C-1), 84.9 (C-3), 74.4 (C-5), 73.6 (C-2), 70.1 (C-4), 34.6 (C-6-Br). Yield: 86%.

5.3.3.3 Peracetylation of 6-bromo-6-deoxycurdlan was performed by a previously reported procedure[24]

Briefly, dry 6-bromo-6-deoxycurdlan (1.00 g, 4.44 mmol), 4-dimethylaminopyridine (DMAP, 20 mg), pyridine (3.6 mL, 10 eq per AGU), and acetic anhydride (8.4 mL, 20 eq per AGU) were combined under N_2. The resulting solution was heated to 80 °C and held 24 h, then cooled and added slowly to 200 mL deionized water. The crude product was collected by filtration, and then re-dissolved in $CHCl_3$ (10 mL). This solution was added slowly with rapid stirring to EtOH (200 mL). The precipitate was isolated by filtration, washed with EtOH

and then water, then dried under vacuum (40 °C) overnight to yield 6-bromo-6-deoxy-2,4-di-*O*-acetyl-curdlan. ^{13}C NMR (DMSO-d_6; **Fig. S5.1a**): δ 169.3 (C=O), 100.0 (C-1), 78.6 (C-3), 72.8 (C-5), 72.1 (C-2), 70.4 (C-4), 32.8 (C-6-Br), 21.2 (CH_3-acetate). Yield: 91%.

5.3.3.4 Synthesis of 6-(*N*,*N*,*N*-trialkylammonio)/pyridinio/(1-methylimidazolio)-6-deoxycurdlan bromide

Dry 6-bromo-6-deoxycurdlan (0.5 g) was dissolved in 25 mL of DMSO in a 100 mL flask. Triethylamine (6.2 mL, 20 eq per AGU), pyridine (3.6 mL, 20 eq per AGU) or 1-methylimidazole (3.5 mL, 20 eq per AGU) was added dropwise. In the case of tributylamine (10.6 mL, 20 eq per AGU), it was mixed with 15 mL of DMPr, and then the mixture was added into the flask. The mixture was heated to 80 °C and stirred at that temperature for 24 h. The solution was cooled to room temperature and transferred to 3500 g/mol MWCO dialysis tubing (prewet with water) that was placed in a beaker containing 400 mL of ethanol. The mixture was dialyzed for at least three days and the ethanol was replaced daily. The dialysis tubing was then transferred to 1 L of deionized water for another three days. For water-insoluble products, the resulting precipitate was filtered out and dried under vacuum at 40°C overnight. For water-soluble products, the solution was then freeze-dried.

6-(*N*,*N*,*N*-Triethylammonio)-6-deoxycurdlan bromide: ^{1}H NMR (DMSO-d_6; **Fig. 5.4b**): δ 5.0-3.0 (curdlan backbone protons & *N*-$\underline{CH_2}$-CH_3), 1.31 (*N*-CH_2-$\underline{CH_3}$); ^{13}C NMR (DMSO-d_6; **Fig. 5.3**): δ 103.0 (C-1), 102.3 (C-1'), 84.7 (C-3), 83.6 (C-3'), 74.2 (C-5), 73.8 (C-5'), 73.4 (C-2), 73.1 (C-2'), 70.1 (C-4), 69.6 (C-4'), 57.9 (C-6-*N*-triethyl), 54.3 (*N*-$\underline{CH_2}$-CH_3), 33.2 (C-6-Br), 7.2 (*N*-CH_2-$\underline{CH_3}$). Yield: 81%; DS_{Et3N} = 0.58 (no NaI) or 0.89 (NaI added). The DS value of triethylammonium group was determined according to **Eq. (5.1)** by ^{1}H NMR. Elemental analysis: %C 45.33, %H 6.93, %N 3.52 (Theoretical (DS 1.0) %C 38.62, %H 6.43, %N 3.75); $DS_{Et3N,\,EA}$ = 0.91 (NaI added).

$$DS_{Et3N} = \frac{7}{\frac{9\times I_{(curdlan\ backbone+CH_2)}}{I_{CH_3}} - 6} \qquad \textbf{(Eq. 5.1)}$$

6-(*N*,*N*,*N*-tributylammonio)-6-deoxycurdlan bromide: ^{1}H NMR (DMSO-d_6; **Fig. S5.4**): δ 5.0-3.0 (curdlan backbone protons & *N*-$\underline{CH_2}$-CH_2-CH_2-CH_3), 1.67 (*N*-CH_2-$\underline{CH_2}$-CH_2-CH_3),

1.38 (*N*-CH_2-CH_2-$\underline{CH_2}$-CH_3), 0.99 (*N*-CH_2-CH_2-CH_2-$\underline{CH_3}$). Yield: 75%; DS_{Bu3N} = 0.04 (no NaI) or 0.35 (NaI added). The DS value of tributylammonium group was determined according to **Eq. (5.2)** by ^{1}H NMR.

$$DS_{Bu3N} = \frac{7}{\frac{6\times I_{(curdlan\ backbone+CH_2)}}{I_{CH_2}} - 6} \qquad (\textbf{Eq. 5.2})$$

6-Deoxy-6-pyridinio-curdlan bromide: ^{1}H NMR (DMSO-d_6; **Fig. 5.6a**): δ 9.1 (*N*-$\underline{CH}$-CH-CH), 8.6 (*N*-CH-CH-$\underline{CH}$), 8.2 (*N*-CH-$\underline{CH}$-CH), 5.3-2.9 (curdlan backbone protons). Yield: 87%; DS_{Pyr} = 0.66. The DS value of the pyridinium group was determined according to **Eq. (5.3)** by ^{1}H NMR. Elemental analysis: %C 42.26, %H 4.89, %N 3.44, %Br 17.51 (Theoretical (DS 1.0) %C 43.42, %H 4.60, %N 4.61, %Br 26.31); $DS_{Pyr,\ EA}$ = 0.64.

$$DS_{Pyr} = \frac{7\times I_{CH-ring}}{5\times I_{curdlan\ backbone}} \qquad (\textbf{Eq. 5.3})$$

6-(1-Methylimidazolio)-6-deoxycurdlan bromide: ^{1}H NMR (DMSO-d_6; **Fig. 5.6b**): δ 9.10 (*N*-$\underline{CH}$-N-CH_3), 7.82 (*N*-CH-$\underline{CH}$-N-CH_3), 7.78 (*N*-$\underline{CH}$-CH-N-CH_3), 4.9-2.9 (curdlan backbone protons & N-$\underline{CH_3}$). Yield: 89%; DS_{MeIMID} = 0.86. The DS value of methylimidazolium group was determined according to **Eq. (5.4)** by ^{1}H NMR. Elemental analysis: %C 40.58, %H 5.44, %N 8.74, %Br 14.94 (Theoretical (DS 1.0) %C 39.08, %H 4.88, %N 9.12, %Br 26.06); $DS_{MeIMID,\ EA}$ = 0.87.

$$DS_{MeIMID} = \frac{7}{\frac{3\times I_{(curdlan\ backbone+CH_3)}}{I_{CH-ring}} - 3} \qquad (\textbf{Eq. 5.4})$$

5.3.3.5 Synthesis of 6-(*N*,*N*,*N*-triethylammonio)-6-deoxy-2,4-di-*O*-acetyl-curdlan bromide

Dry 6-bromo-6-deoxy-2,4-di-*O*-acetyl-curdlan (0.5 g) was dissolved in 25 mL of DMSO in a 100 mL flask. Triethylamine (TEA, 100 eq per AGU) was added dropwise. The mixture was heated to 80 °C and stirred at that temperature for 24 h. The homogeneous solution was cooled to room temperature and transferred to 3500 g/mol MWCO dialysis tubing (prewet with water) that was placed in a beaker containing 400 mL of ethanol. The mixture was dialyzed for at least three days and the ethanol was replaced once daily. The resulting precipitate was filtered out and dried under vacuum at 40 °C overnight. ^{1}H NMR (DMSO-d_6;

Fig. 5.1): δ 5.3-3.0 (curdlan backbone protons & *N*-$\underline{CH_2}$-CH_3), 1.9-2.3 (CH_3-acetate), 1.5-0.9 (*N*-CH_2-$\underline{CH_3}$). Yield: 85%; DS_{Et3N} = 0.27.

5.3.3.6 Synthesis of 6-(*N*,*N*-diethylamino)-6-deoxy-(2,4-di-*O*-acetyl-)curdlan

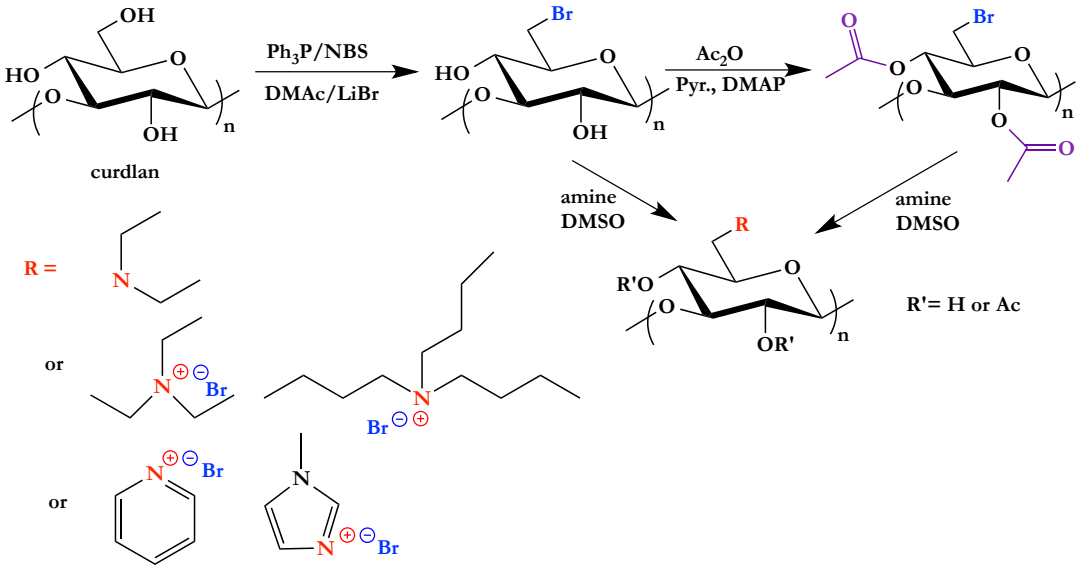
Dry 6-bromo-6-deoxy-(2,4-di-*O*-acetyl-)curdlan (0.5 g) was dissolved in 25 mL of DMSO in a 100 mL flask. Diethylamine (20 eq per AGU) was added dropwise. The mixture was heated to 50 °C and stirred at that temperature for 24 h. The homogeneous solution was cooled to room temperature and transferred to 3500 g/mol MWCO dialysis tubing (prewet with water) that was placed in a beaker containing 400 mL of ethanol. The mixture was dialyzed for at least two days and ethanol was replaced once daily. The dialysis tubing was then transferred in 1 L of deionized water for another two days. The resulting precipitate was collected by filtration and dried under vacuum at 40 °C overnight. 6-(*N*,*N*-Diethylamino)-6-deoxycurdlan: ^{1}H NMR (DMSO-d_6; **Fig. 5.5b**): δ 5.0-3.0 (curdlan backbone protons & *N*-$\underline{CH_2}$-CH_3), 1.20 (*N*-CH_2-$\underline{CH_3}$); ^{13}C NMR (DMSO-d_6; **Fig. 5.5c**): δ 103.1 (C-1), 84.7 (C-3), 74.0 (C-5), 71.0 (C-2), 69.9 (C-4), 52.2 (C-6-*N*-diethyl), 48.3 (*N*-$\underline{CH_2}$-CH_3), 9.2 (*N*-CH_2-$\underline{CH_3}$). Yield: 90%; DS_{Et2N} = 0.98. 6-(*N*,*N*-Diethylamino)-6-deoxy-2,4-di-*O*-acetyl-curdlan: ^{1}H NMR (DMSO-d_6; **Fig. S5.4**): δ 5.0-3.0 (curdlan backbone protons & *N*-$\underline{CH_2}$-CH_3), 1.8-2.3 (CH_3-acetate), 1.23 (*N*-CH_2-$\underline{CH_3}$). Yield: 80%; DS_{Et2N} = 0.64. The DS value of diethylamino group was determined according to **Eq. (5.5)** by ^{1}H NMR.

$$DS_{Et2N} = \frac{7}{\frac{6\times I_{(curdlan\ backbone+CH_2)}}{I_{CH_3}} - 4} \qquad (\textbf{Eq. 5.5})$$

5.4. Results and discussion

As we have previously reported[24], curdlan can be brominated with complete regioselectivity at C-6 to DS_{Br} up to 1.0 using triphenylphosphine and *N*-bromosuccinimide in the DMAc/LiBr solvent system, and the resulting 6-bromo-6-deoxycurdlan can be easily esterified by carboxylic anhydrides to produce 2,4-*O*-diester derivatives readily soluble in a range of organic solvents. During the bromination of curdlan, reaction time, temperature and reagent molar ratio can be adjusted to obtain 6-brominated curdlan derivatives with different DS_{Br} values. Conversion of these bromo curdlans to primary 6-amines is readily accomplished by azide displacement, followed by azide reduction. Peralkylation of these

primary amines would be one route to *N*,*N*,*N*-trialkylammonium derivatives, but we felt that this was unnecessarily indirect and could be plagued by issues of N/O alkylation selectivity. Therefore we pursued the possibility of direct nucleophilic displacement of the 6-halide by trialkylamines and heterocyclic amines (**Scheme 5.1**).



Scheme 5.1. Synthetic scheme for 6-(trialkylammonio/imidazolio/pyridinio/dialkylamino)-6-deoxy-(2,4-di-*O*-acetyl)-curdlan bromide.

5.4.1 6-(*N*,*N*,*N*-Triethylammonio)-6-deoxy-2,4-di-*O*-acetyl-curdlan bromide

Peracetylation of 6-bromo-6-deoxycurdlan (DS_{Br} 0.97) with acetic anhydride in pyridine was utilized to afford readily organic solvent-soluble 2,4-di-*O*-acetyl curdlan ester substrates, whose identity and regioselectivity of acetyl group substitution was established by NMR spectroscopy using methods described by us previously[24]. Diagnostic features of the ^{13}C NMR spectrum (**Fig. S5.1a**) included the brominated C-6 resonance at δ 33 ppm, the acetyl carbonyls resonance at δ 169.3 ppm, and one for the acetyl methyl carbons at δ 21.2 ppm. The DS_{Ac} was calculated as 2.03 by integration of the ^{1}H NMR spectrum (**Fig. S5.1b**).

TEA was used to explore initial reaction conditions with this esterified 6-bromo curdlan in several solvents including DMAc, DMF, DMSO, and by using TEA as both solvent and reactant. Product structures were confirmed by ^{1}H NMR spectroscopy (**Fig. 5.1**), in which the ammonium methyl resonances appeared around δ 1.24 ppm, and the ammonium

methylene resonances (~3.4 ppm) overlapped with those of curdlan backbone protons. **Table 5.1** summarizes the DS values of ammonium products obtained in different solvents. With 20 equiv. of triethylamine, **entry 3** using DMSO as solvent afforded the highest DS_{Et3N} of 0.12, far lower than the theoretical maximum degree of substitution (ca. 1.0). By increasing the molar ratio of TEA to 100 equiv., the DS_{Et3N} reached 0.27 (**entry 5**). We also explored using TEA as both reagent and solvent to provide maximum TEA concentration and maximize ammonium substitution. However, whatever amount of TEA was used as solvent (20 vs. 100 equiv.), the product DS_{Et3N} was less than 0.1 (**entry 4** and **6**). The relative failure of the reactions where TEA is both solvent and reactant is likely due at least in part to the heterogeneous nature of the reaction mixture, due to insufficient solubility of the starting curdlan derivative in TEA. We concluded that DMSO was the optimal solvent among the polar aprotic solvents tested for maximizing ammonium DS of 6-(*N*,*N*,*N*-triethylammonio)-6-deoxy-2,4-di-*O*-acetyl-curdlan bromide.

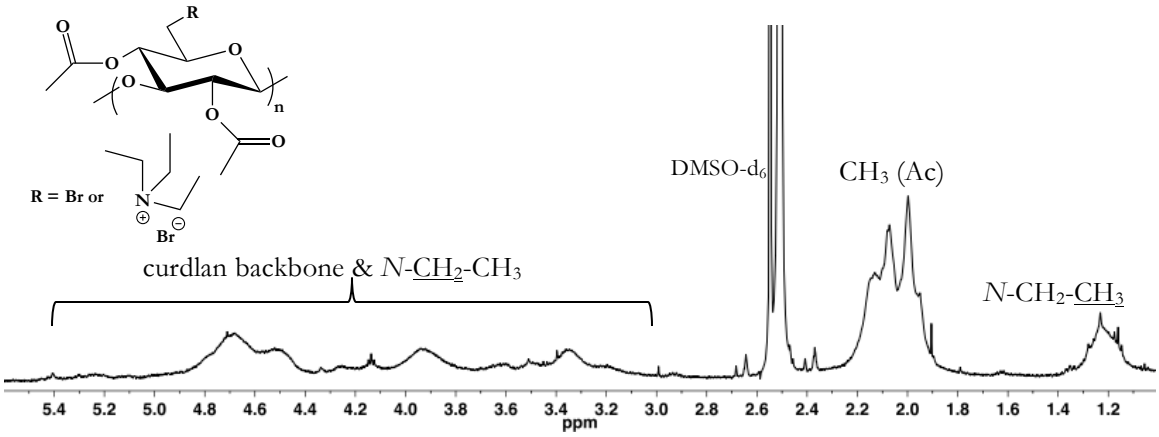


Fig. 5.1. ^{1}H NMR spectrum of 6-(*N*,*N*,*N*-triethylammonio)-6-deoxy-2,4-di-*O*-acetyl-curdlan bromide (DS_{Br}~0.97, 100 equiv. TEA/AGU, 80 °C, 24 h, in DMSO): DS_{Et3N} = 0.27.

We hypothesized that the increasing density of positive charge along the curdlan chain as the reaction proceeds leads to increasing, energetically costly electrostatic repulsion, and that this is responsible for the rather low DS_{Et3N} values obtained. To test this hypothesis, we synthesized two 6-bromo-6-deoxycurdlan esters in which the C-6 bromination was incomplete, by adjusting the stoichiometry (from 4 equiv. of Ph_3P and NBS at 70 °C for 1 h to 2 equiv. of reagents at 70 °C for 0.5 h (DS_{Br} = 0.30) or 1 h (DS_{Br} = 0.85)). The DS_{Br} values of the obtained partially 6-brominated curdlans were calculated from proton NMR spectra

of the corresponding peracetylated products (**Fig. S5.2**). By integrations of **Fig. S5.3**, DS_{Et3N} of **entry 7** and **8** were 0.10 and 0.13, still less than the potential maximum values (0.85 and 0.30, respectively). However, the percent bromide substitution of the derivative with the lowest DS_{Br} (0.30, **entry 8**) reached 43%, much higher than those observed for DS_{Br} of 0.85 (**entry 5**, 27%) or 0.97 (**entry 7**, 12%), supporting the hypothesis that charge accumulation limits the achievable DS_{Br}. To further explore this hypothesis, we treated esterified 6-bromo-6-deoxycurdlan (DS_{Br}=0.97) with 20 equiv. of diethylamine at 50 °C for 24 h to provide non-charged 6-(*N*,*N*-diethylamino)-2,4-di-*O*-acetyl-curdlan. DS_{Et2N} was determined as 0.64 by integration of **Fig. S5.4**, almost five times higher than that achieved in preparation of the corresponding triethylammonium product under equivalent conditions, providing additional support for the hypothesis that the developing charge density retards nucleophilic displacement by trialkylamines to form new cationic moieties.

Table 5.1.

Substitution achieved (DS_{Et3N}) vs. conditions for 6-(*N*,*N*,*N*-triethylammonio)-6-deoxy-2,4-di-*O*-acetyl-curdlan bromide.[a]

Entry	DS_{Br}	TEA (eq/AGU)	Solvent	DS_{Et3N}	PS[c] (%)	Water solubility[d]
1	0.97	20	DMAc	0.06	6	–
2			DMF	0.11	11	–
3			DMSO	0.12	12	–
4			TEA	0.08[b]	8	–
5		100	DMSO	0.27	27	–
6			TEA	0.08[b]	8	–
7	0.85	100	DMSO	0.10	12	–
8	0.30			0.13	43	–

[a] 6-Bromo-6-deoxy-2,4-*O*-acetylcurdlan starting material, all reactions 80 °C, 24 h.

[b] Heterogeneous reactions in TEA as solvent, homogeneous reactions in other solvents.

[c] $\text{Percent substitution (PS)} = \frac{\text{DS}_{\text{ammonium}}}{\text{DS}_{\text{Br}}}; \text{PS}(\%)_{\text{max}} = 100\%.$

[d] + = soluble; − = insoluble.

5.4.2 Synthesis of 6-(*N*,*N*,*N*-triethylammonio)-6-deoxycurdlan bromide

The acetylated, partially substituted 6-(*N*,*N*,*N*-triethylammonio)-6-deoxycurdlan derivatives described in the last section have poor aqueous solubility. This is likely due to the low DS_{Et3N} values, as well as the hydrophobicity of the acetyl esters. The poor aqueous solubility would be limiting with regard to testing and usage in potential applications, whether in tight junction opening, flocculant, or most other envisioned applications. Therefore, we pursued the synthesis of 6-(*N*,*N*,*N*-triethylammonio)-6-deoxycurdlan derivatives with free hydroxyls at the C-2/4 positions, in order to enhance water solubility. 6-Bromo-6-deoxycurdlan was treated with 100 equiv. of triethylamine in several polar aprotic solvents; DMAc, DMF, or DMSO, at 80 °C for 24 h. All reaction mixtures were homogeneous throughout the reaction time. Product chemical structures were confirmed by FTIR (**Fig. 5.2**) and ^{13}C NMR spectroscopy (**Fig. 5.3**). Several infrared spectral changes provided information about the nature of the product. The strong peak near 540 cm^{-1} (**Fig. 5.2a**) assigned to the C-Br stretching absorption was greatly reduced in the ammonium product (**Fig. 5.2b**), indicating successful displacement by TEA. A new peak appeared at high wave number (1635 cm^{-1}) which was assigned to the C_6-*N* absorption of the quaternary ammonium salt.[25] In the ^{13}C NMR spectrum (**Fig. 5.3**) the curdlan C-6 carbon substituted with triethylammonium resonated at δ 58 ppm, shifted significantly downfield from the C-6-Br starting material, though residual bromodeoxy carbon was still observed (δ 33 ppm). Quaternary amine substitution was further confirmed by the triethylammonium carbon resonances at δ 54.3 ppm and δ 7.2 ppm, from the CH_2 and CH_3 carbons of the *N,N,N*-triethylammonium groups, respectively. Due to the presence of multiple C-6 substituents (Br vs. $N^+(CH_2CH_3)_3Br^-$), multiple curdlan backbone carbon resonances were observed in the range of δ 105-70 ppm.

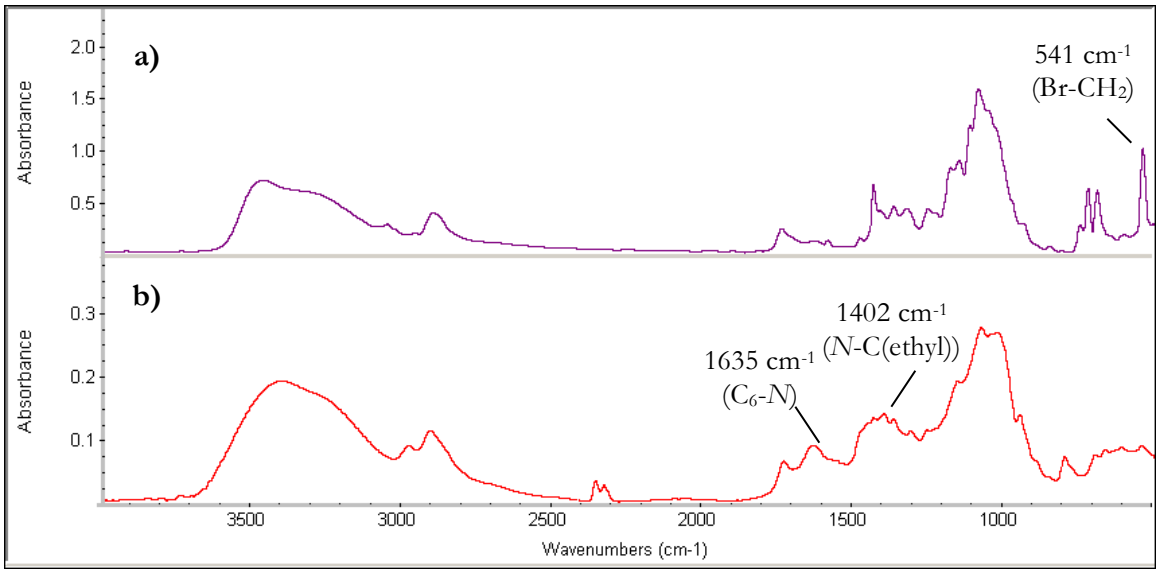


Fig. 5.2. FTIR spectra of **(a)** 6-bromo-6-deoxycurdlan (DS_{Br} 0.97) and **(b)** 6-(*N*,*N*,*N*-triethylammonio)-6-deoxycurdlan bromide (20 equiv. TEA/AGU, 80 °C, 24 h, DMSO).

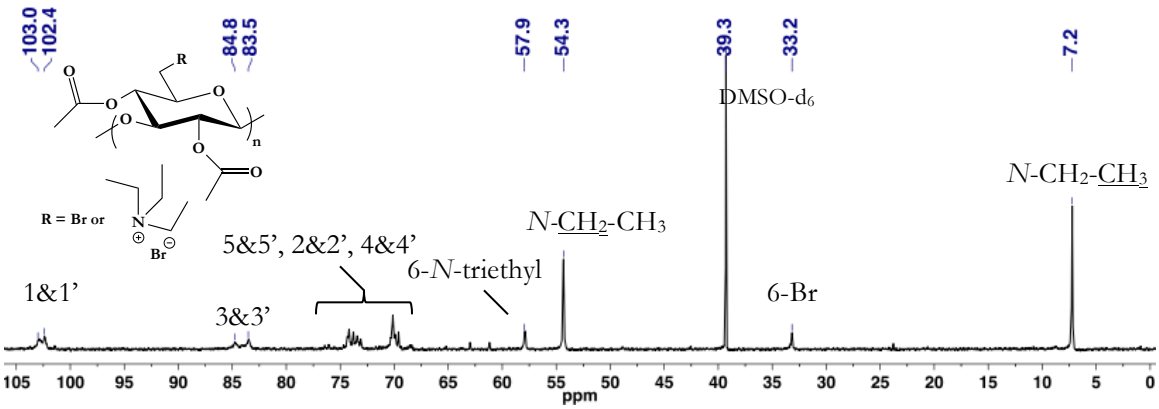


Fig. 5.3. ^{13}C NMR spectrum of 6-(*N*,*N*,*N*-triethylammonio)-6-deoxycurdlan bromide from brominated curdlan (DS_{Br} 0.97, 20 equiv. TEA/AGU, 80 °C, 24 h, DMSO).

Calculated DS_{Et3N} values in each solvent were based on ^{1}H NMR spectral integration (**Fig. 5.4**, **Fig. S5.5**) as summarized in **Table 5.2**. We can easily differentiate the low DS product (**Fig. 5.4a**) from the high DS product (**Fig. 5.4b**) in the range of δ 5.0-3.0 ppm of the spectra. There was a signal around δ 3.4 ppm corresponding to the *N*-$\underline{CH_2}$-CH_3 in **Fig. 5.4b** (DS_{Et3N} 0.58), which was difficult to identify in **Fig. 5.4a** (DS_{Et3N} 0.08). Negligible differences were observed among spectra of TEA-substituted products prepared in DMAc (**Fig. 5.4a**), DMF (**Fig. S5.5a**) and TEA (**Fig. S5.5b**). From **Table 2**, **entry 1-4** (compare with **Table**

5.1, **entry 1-6**) DMSO was the most useful solvent for the production of curdlan 6-ammonium salts with the highest DS_{Et3N}. In order to figure out the optimal molar ratio of triethylamine and the peak value of DS_{Et3N}, we varied the added amount of TEA (20, 50, 100 equiv. per AGU) as well as the reaction time (24 h vs. 48 h). Interestingly, there was no significant difference in DS_{Et3N} among the products of **entries 3** and **5-7**, demonstrating that the highest DS 0.58 could be achieved with at the lowest TEA/AGU (20 equiv. per AGU, 80 °C, 24 h). Since iodide is superior to bromide as a leaving group, we added sodium iodide (5 equiv./AGU) during the reaction of 6-bromo-6-deoxycurdlan with triethylamine under the same reaction conditions, in order to generate the 6-iodo product *in situ* in hopes of capture by TEA as it was generated, potentially affording higher DS_{Et3N}.[26] Based on the ^{1}H NMR spectrum (**Fig. S5.8a**) and **Eq. (5.1)**, it is clear that this tactic succeeded. The product has DS_{Et3N} 0.89, much higher than the maximum DS_{Et3N} previously achieved (0.58); no apparent residual bromodeoxy carbon was observed (δ 33 ppm, **Fig. S5.8b**). Finkelstein displacement[27] of bromide by iodide in a separate step would of course be an alternative approach. Importantly, these 6-ammonio-deoxycurdlan products, prepared in DMSO with $DS_{Et3N} > 0.5$ are readily soluble in water, while those with DS ~0.1 have poor water solubility. Those water-soluble products are promising candidates for studies in tight junction opening, drug delivery and other biomedical areas.

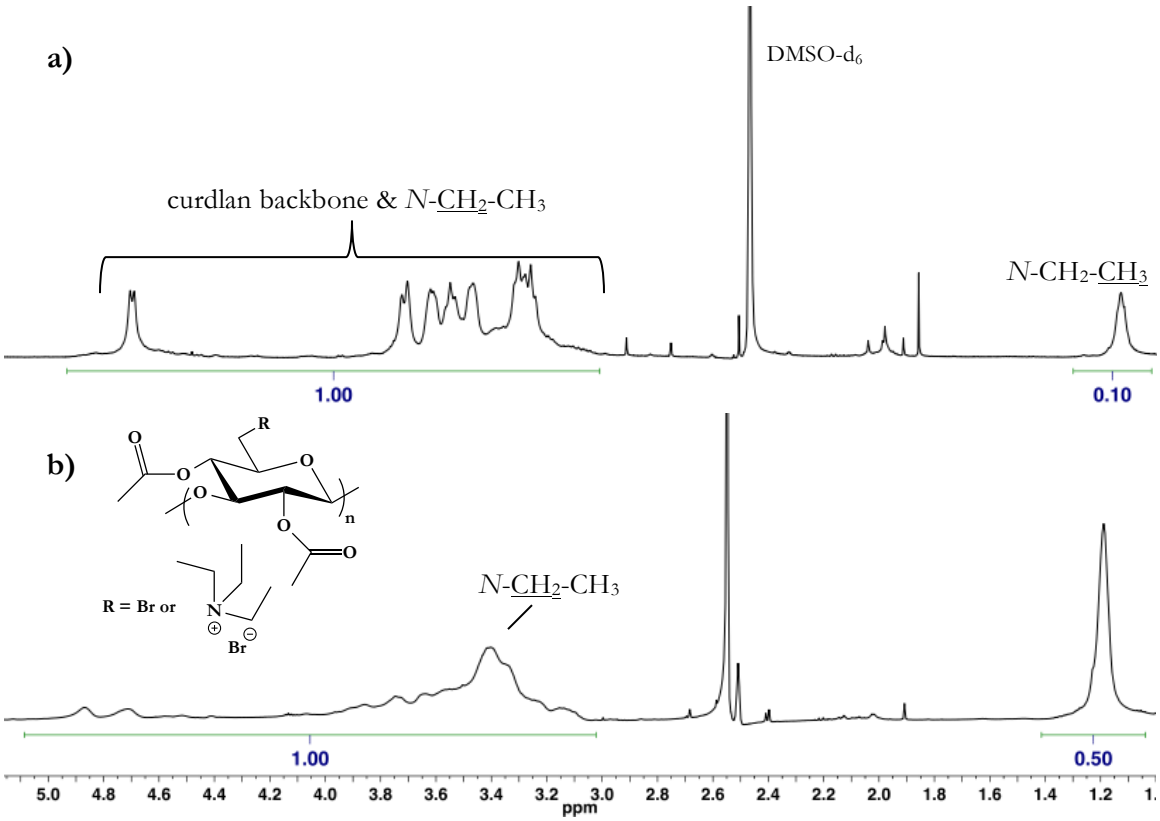


Fig. 5.4. ^{1}H NMR spectra of 6-(*N*,*N*,*N*-triethylammonio)-6-deoxycurdlan bromide (DS_{Br} 0.97, 80 °C, 24 h): **(a)** 100 equiv. TEA/AGU, DMAc, DS_{Et3N} = 0.08; **(b)** 20 equiv. TEA/AGU, DMSO, DS_{Et3N} = 0.58.

Table 5.2.

DS_{Et3N} values of 6-(*N*,*N*,*N*-triethylammonio)-6-deoxycurdlan bromide.[a]

Entry	DS_{Br}	TEA (eq/AGU)	Solvent	DS_{Et3N}	Water solubility[c]
1	0.97	100	DMAc	0.08	–
2			DMF	0.11	–
3			DMSO	0.53	+
4			TEA	0.10[b]	–
5		100	DMSO	0.57	+
6		50		0.57	+
7		20		0.58	+
8		20	DMSO	0.89	+

			(NaI)[d]	(0.91)[e]	

[a] 6-Bromo-6-deoxycurdlan starting material, all reactions 80 °C, 24 h except **entry 5** (80 °C, 48 h).

[b] Heterogeneous reactions in TEA, homogeneous reactions in other solvents.

[c] + = soluble; − = insoluble.

[d] NaI (5 equiv./AGU) added.

[e] DS determined by elemental analysis.

We explored treatment of 6-bromo-6-deoxycurdlan with 20 equiv. of diethylamine to investigate whether the absence of accumulating charge would increase the substitution of amine in this case as well. The FTIR spectrum (**Fig. 5.5a**) showed a new peak around 1643 cm^{-1} representing the C_6-*N* absorption of the DEA substituent. In the ^{13}C NMR spectrum (**Fig. 5.5c**), the resonances at δ 48.3 and 9.2 ppm were assigned to the methylene and methyl carbons of DEA. Additionally, distinct, single peaks for C-1 to C-5 of the curdlan backbone were present between δ 70 and 103 ppm. The strong upfield chemical shift for C-6 upon substitution by DEA to δ 52 ppm is diagnostic for the postulated amination. No peak was observed at δ 34 ppm where the original C-6-Br would resonate, indicating essentially complete conversion. **Fig. 5.5b** demonstrated an apparent signal at δ 1.2 ppm corresponding to the *N*-CH_2-$\underline{CH_3}$ as well as a signal at δ 3.2 ppm assigned to the *N*-$\underline{CH_2}$-CH_3. By integration of this proton spectrum, DS_{Et2N} was determined as 0.98 using **Eq. (5.5)**, far higher than the maximum DS_{Et3N} 0.58 achieved by reaction with TEA under comparable conditions, further indicating the strong influence of accumulating charge on the outcome of displacements that create permanent *N*-centered positive charge appended to C-6.

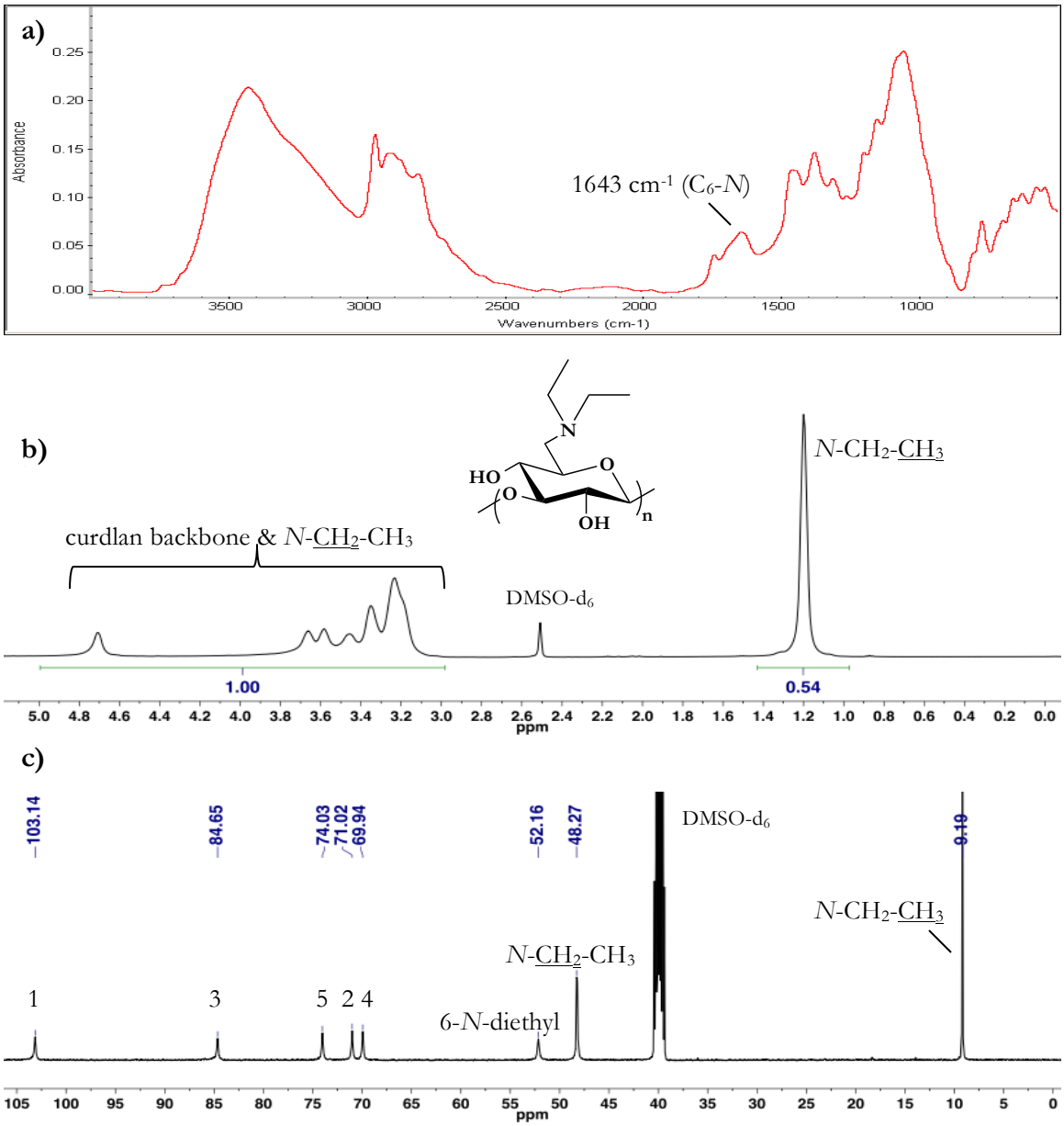


Fig. 5.5. 6-(*N*, *N*-diethylamino)-6-deoxycurdlan (20 equiv. DEA/AGU, DMSO, 50 °C, 24h, DS_{Et2N} = 0.98): **(a)** FTIR, **(b)** ^{1}H NMR and **(c)** ^{13}C NMR spectra.

Seeking to understand the impact of steric bulk of the amine nucleophile, we carried out the reaction of 6-bromo-6-deoxycurdlan with tributylamine (20 equiv., 80 °C, 24h). Curdlan backbone peaks in the range of δ 3.0-5.0 ppm of the product ^{1}H NMR spectrum (**Fig. S5.6a**) were similar to those of low DS_{Et3N} products (**Fig. 5.4**, **Fig. S5.5**), indicating relatively low TBA substitution. Peaks at δ 1.67 (*N*-CH_2-$\underline{CH_2}$-CH_2-CH_3), 1.38 (*N*-CH_2-CH_2-$\underline{CH_2}$-CH_3) and 0.99 (*N*-CH_2-CH_2-CH_2-$\underline{CH_3}$) were assigned to the alkyl protons of the tributylammonium

substituent, while the DS_{Bu3N} was calculated as 0.04 by **Eq. (5.2)**. The extremely low DS was probably due to the immiscibility of TBA and available solvent for bromo curdlan (DMSO, DMAc, DMF). Alternatively, we employed DMSO/DMPr co-solvents to overcome the issue of TBA immiscibility; using these co-solvents, DS_{Bu3N} increased from 0.04 to 0.16 (**Fig. S5.6b**). Adding NaI (5 equiv./AGU) further enhanced tributylammonium substitution, thereby reaching DS_{Bu3N} 0.35 (**Fig. S5.6c**); the resultant product was partially soluble in water. Compared with the higher DS of 6-ammonium substituent obtainable by reaction with TEA under similar conditions, the relatively lower DS_{Bu3N} could result in part from the greater steric bulk of the TBA moiety.

5.4.3 Synthesis of 6-pyridinio/(1-methylimidazolio)-6-deoxycurdlan bromide

It was of interest to study nucleophilic halide displacement in 6-bromo-6-deoxycurdlan by heterocyclic amines such as pyridine and 1-methylimidazole, since we expected that the sp^2 hybridized nitrogen atoms would be more nucleophilic[9, 28], displacement could be more efficient, and the products would be of significant interest. These displacement reactions were carried out under favorable conditions determined for triethylamine (20 equiv./AGU, 80 °C, 24h, in DMSO), though in this case we did not employ added NaI. Identity and DS of the resulting 6-pyridinium/(1-methylimidazolium)-6-deoxycurdlan bromide were established by FTIR and NMR spectroscopy. FTIR analysis (**Fig. S5.7**) showed C_6-*N* absorptions of the quaternary ammonium salt around 1600 cm^{-1}, and significantly reduced C-Br absorption around 540 cm^{-1}, indicating high conversion and DS_{Pyr}. In the pyridinium-substituted derivative ^1H NMR spectrum (**Fig. 5.6a**), signals at δ 9.1, 8.7 and 8.2 ppm were assigned to five CH protons in the pyridinium ring, confirming the incorporation of the pyridinium moiety. To further confirm its regioselectivity, we carried out a heteronuclear multibond correlation NMR experiment (HMBC, **Fig. 5.7**), in which the cross peak between C-6 and H-7 of the pyridinium ring was diagnostic of the position of substitution at C-6 however no other correlation peaks observed between C-2/4 and H-7. Similarly, in **Fig. 5.6b** are observed three resonances at δ 9.1, 7.8 and 7.7 ppm corresponding with the three protons of the imidazolium ring. There was a sharp single peak at δ 3.9 ppm that was attributed to the CH_3 group of MeIMID, supporting imidazole displacement. Compared with the maximum DS_{Et3N} achieved (0.58) for the TEA products in the absence of NaI, the

higher DS_{Pyr} 0.66 (0.64 by EA) and DS_{MeIMID} 0.86 (0.87 by EA) can be attributed to the less sterically hindered and more nucleophilic sp^2 nitrogens of pyridine and 1-methylimidazole. Elemental analysis of these derivatives indicated DS_{Br} 0.29 and 0.09, respectively, for the pyridinium and imidazolium products after S_N2 displacement. Therefore the combined DS values of cationic substituent and bromide were 0.93 and 0.96 as determined by **Eqs.** (**5.6**) and (**5.7**), respectively. As the starting 6-bromo derivative had DS_{Br} 0.97, this allows us to place an upper limit of approximately 4% loss of bromide due to any adventitious hydrolysis. Importantly, these two ammoniocurdlan derivatives are water-soluble due to the influence of the high DS of positively charged nitrogen substituents.

Pyridinium product: $DS_{substituent}$ =0.29(Br) + 0.64(pyridinium)=0.93 (**Eq. 5.6**)

Imidazolium product: $DS_{substituent}$ =0.09(Br) + 0.87(imidazolium)=0.96 (**Eq. 5.7**)

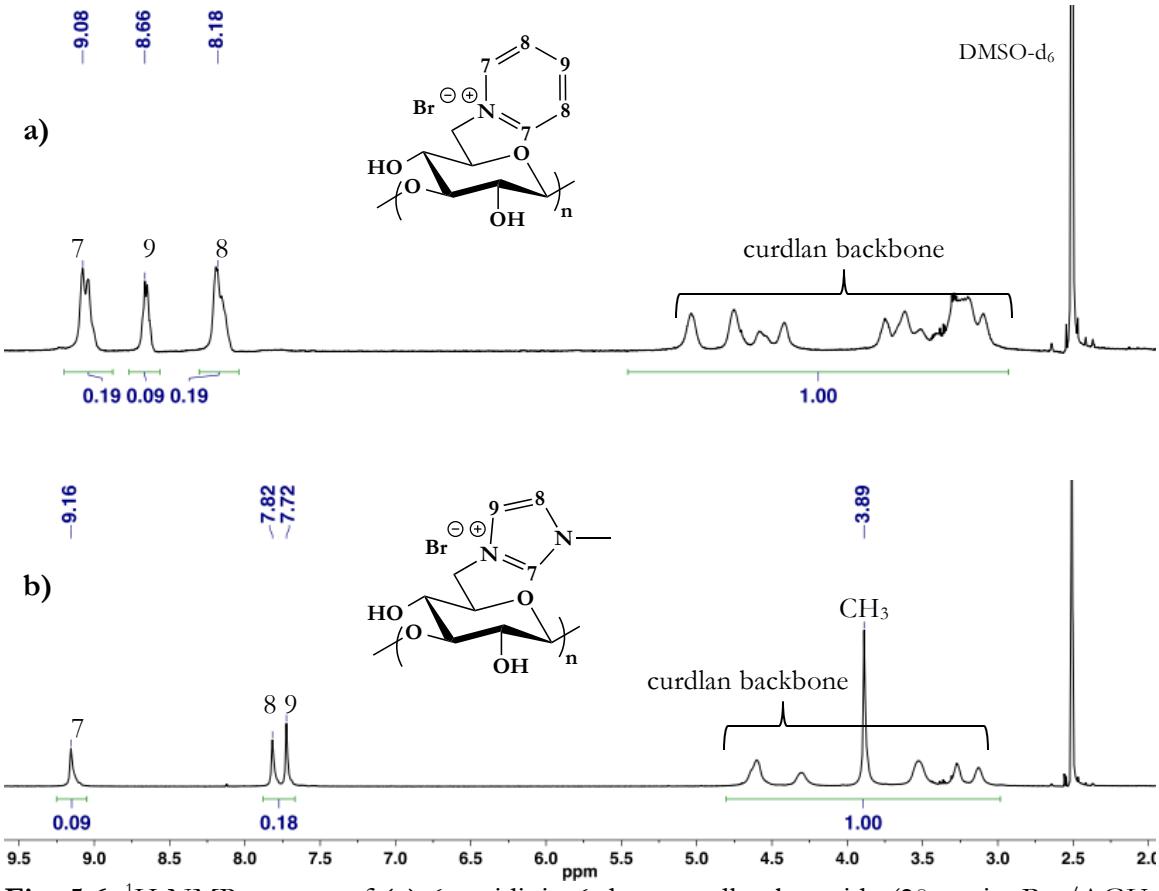


Fig. 5.6. ^{1}H NMR spectra of **(a)** 6-pyridinio-6-deoxycurdlan bromide (20 equiv. Pyr/AGU, 80 °C, 24 h, DMSO, DS_{Pyr} = 0.66) and **(b)** 6-(1-methyl-imidazolio)-6-deoxycurdlan bromide (20 equiv. MeIMID/AGU, 80 °C, 24 h, DMSO, DS_{MeIMID} = 0.86).

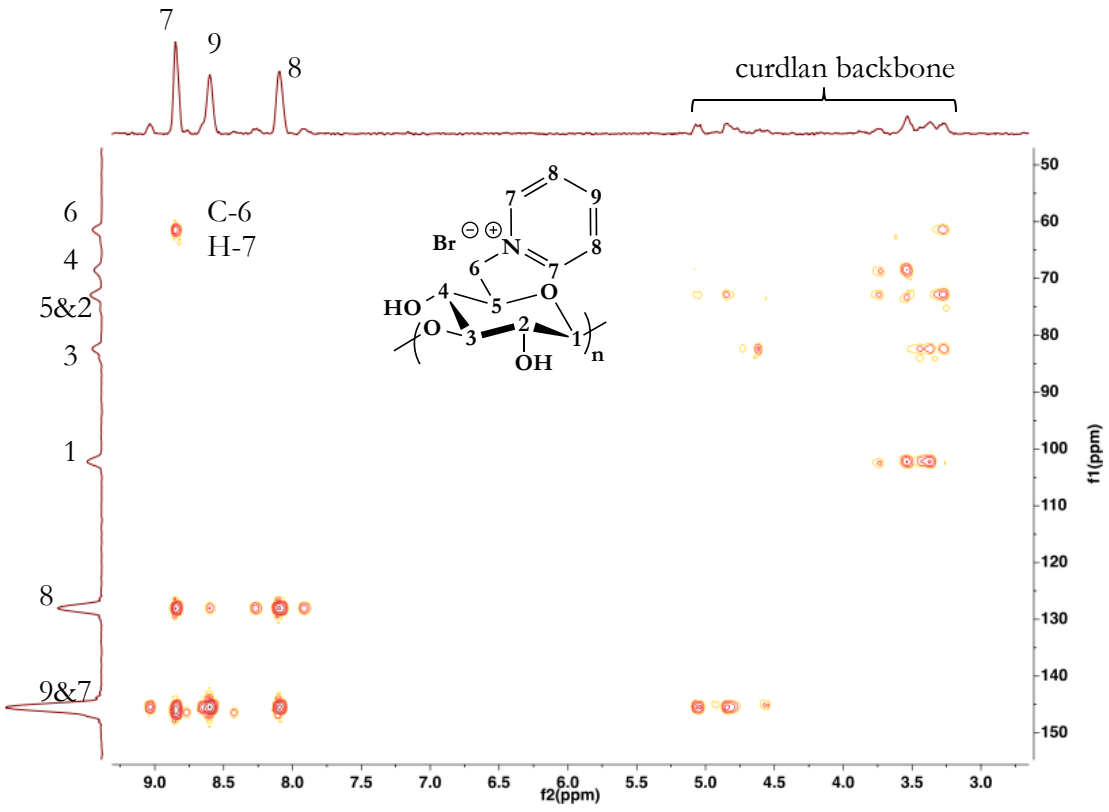


Fig. 5.7. HMBC NMR (in D_2O) spectrum of 6-pyridinio-6-deoxycurdlan bromide (20 equiv. Pyr/AGU, 80 °C, 24 h, DMSO, DS_{Pyr}=0.66).

5.5 Conclusions

These results extend the utility of 6-bromo-6-deoxycurdlan intermediates, providing access to derivatives containing quaternary ammonium substituents at C-6. At the same time, we have identified roadblocks to this direct displacement approach. In particular, we provide substantial evidence to support the hypothesis that developing charge on the curdlan molecule as displacement of the 6-halo substituents proceeds at some point reduces the rate of additional substitution reactions that would create additional positive charge on the molecule, making complete displacement at C-6 difficult to achieve. We further demonstrate useful synthetic tactics for attacking this issue and achieving relatively high DS(ammonium) at C-6; these include use of the polar, aprotic solvent DMSO, use of excess tertiary or heterocyclic amine, and *in situ* formation of more labile 6-iodo-6-deoxy intermediates by reaction with added sodium iodide. We also show that displacement can be carried out on curdlan derivatives that are acylated at the 2- and 4-hydroxyls, but that displacement is more

efficient when these hydroxyls are not acylated. Overall we have succeeded in creating useful approaches for synthesis of water-soluble curdlan analogs of quaternized chitosan derivatives.

The derivatives available by this chemistry containing very significant levels of permanent positive charge will be of great interest for studies ongoing in our laboratories, such as for promotion of absorption of small polar molecules as well as macromolecular (peptide and protein) drugs across the mucosal epithelia, with the hypothetical mechanism of tight junction opening. Access to this family of water-soluble, regioselectively substituted quaternized curdlan derivatives will permit investigations of structure-property relationships, and comparison with quaternized chitosan derivatives with regard to the influence of the type of glucosidic linkage (1,3- vs. 1,4-) as well the position of the positively charged substituent (C-6 vs. C-2). These aspects as well as biomedical properties and applications of these derivatives will be topics of further study in our laboratory. They may also be of future interest for formation of polyelectrolyte complexes, for example complexes with poly(nucleic acids) for gene delivery applications, and as carriers for small molecule anionic drugs. We fully expect that this chemistry will prove useful for synthesis of charged derivatives of other 6-hydroxy polysaccharides and are exploring this possibility as well.

5.6 Supplemental material

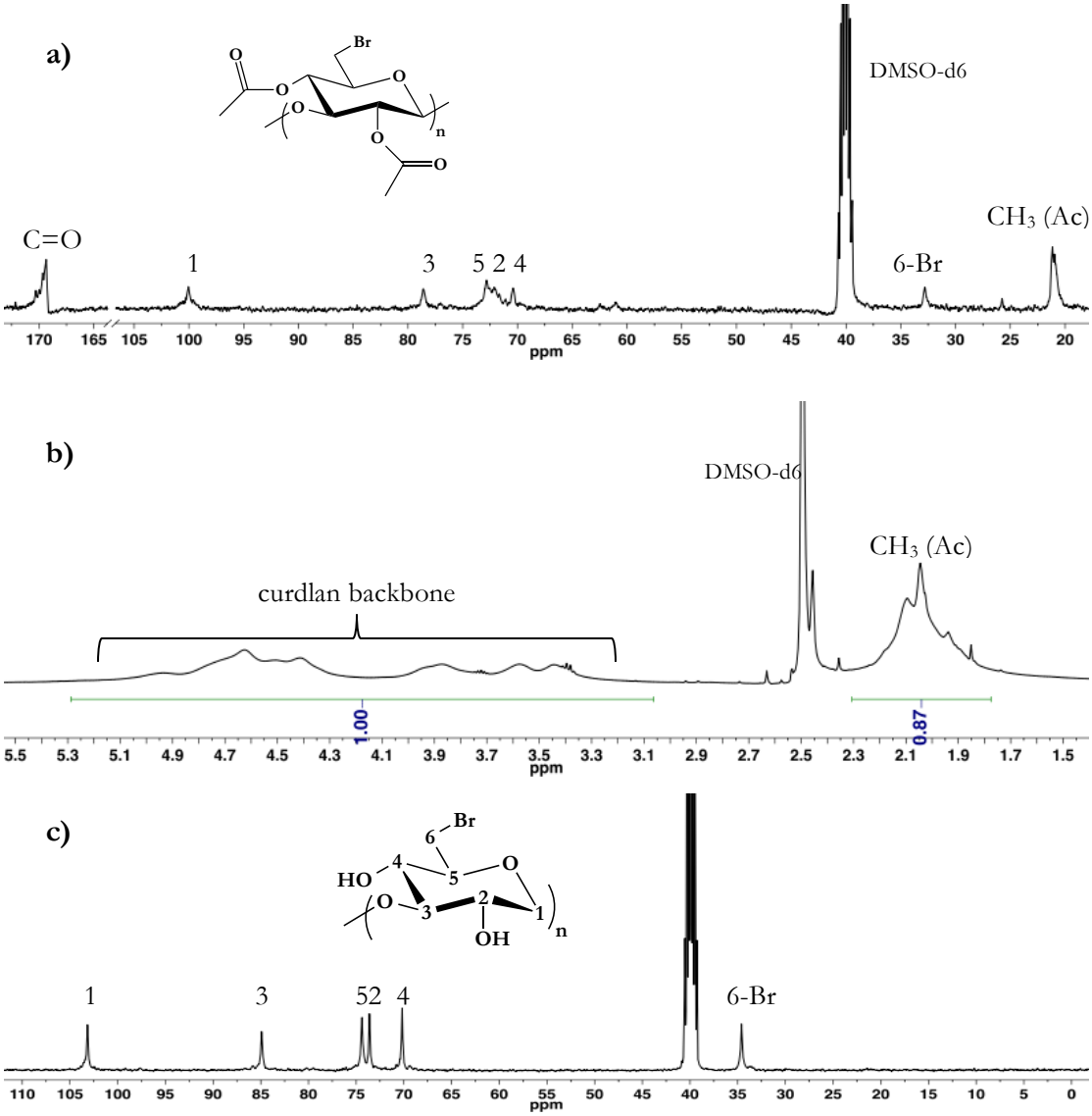


Fig. S5.1. ^{13}C NMR spectra of **(a)** 6-bromo-6-deoxy-2,4-acetyl-curdlan and **(c)** 6-bromo-6-deoxycurdlan; **(b)** ^{1}H NMR spectrum of 6-bromo-6-deoxy-2,4-acetyl-curdlan.

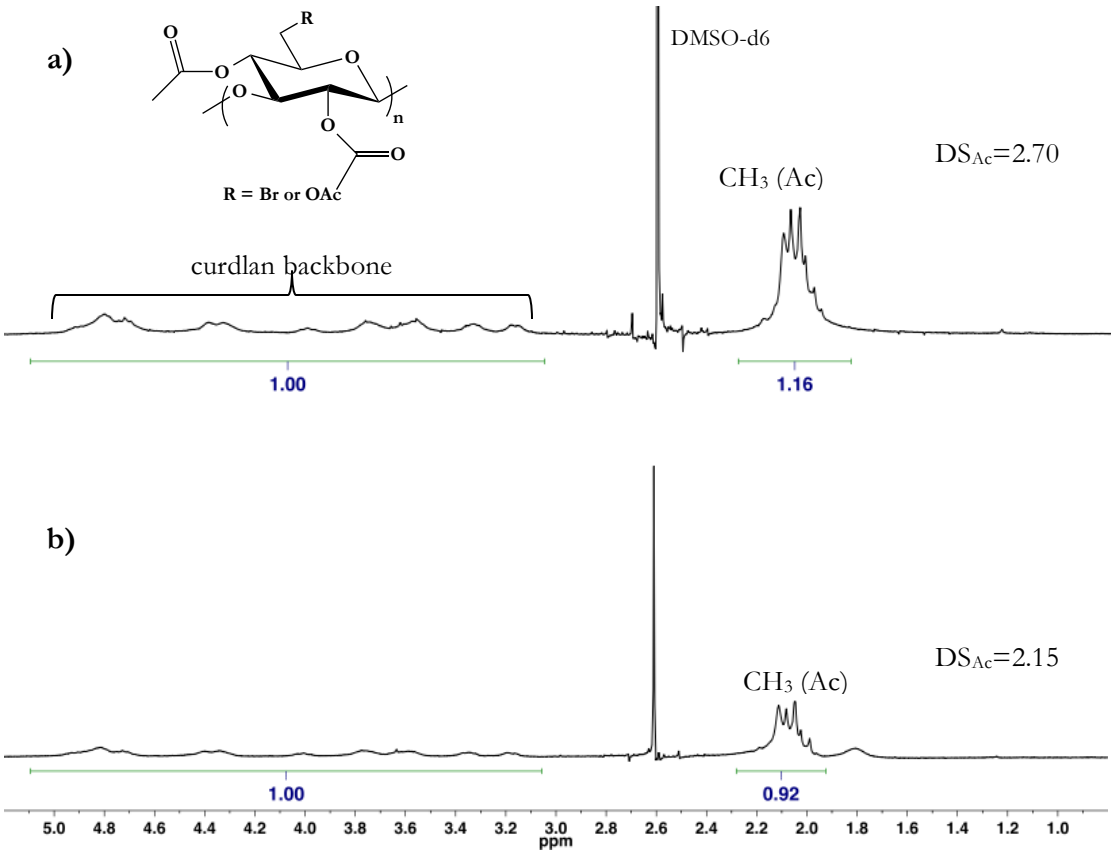


Fig. S5.2. ^{1}H NMR spectra of bromo curdlans with incomplete bromination (2 equiv./AGU Ph_3P and NBS at 70 °C): **(a)** DS_{Br}=0.30 (0.5 h); **(b)** DS_{Br}=0.85 (1 h).

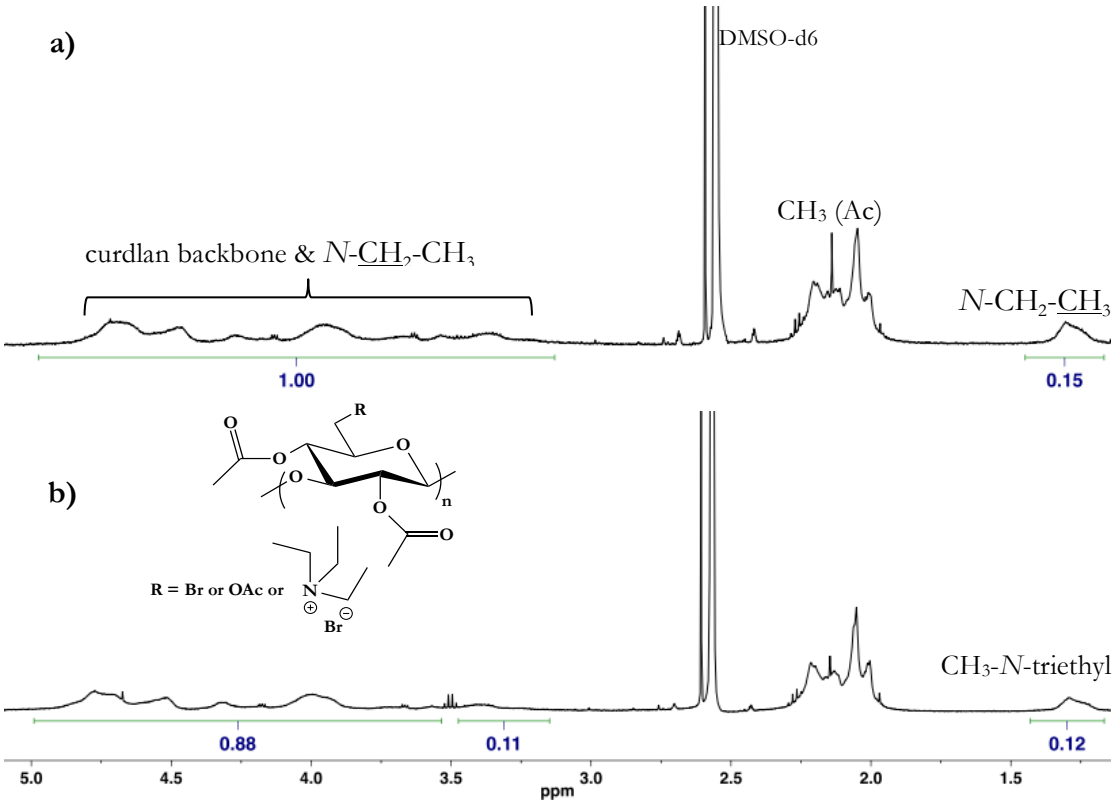


Fig. S5.3. [1]H NMR spectra of 6-(*N*,*N*,*N*-triethylammonio)-6-deoxy-2,4-di-*O*-acetyl-curdlan bromide from partially brominated curdlans (100 equiv. TEA/AGU, 80 °C, 24 h, in DMSO) : **(a)** DS_{Et3N}=0.13 from DS_{Br} 0.30; **(b)** DS_{Et3N}=0.10 from DS_{Br} 0.85.

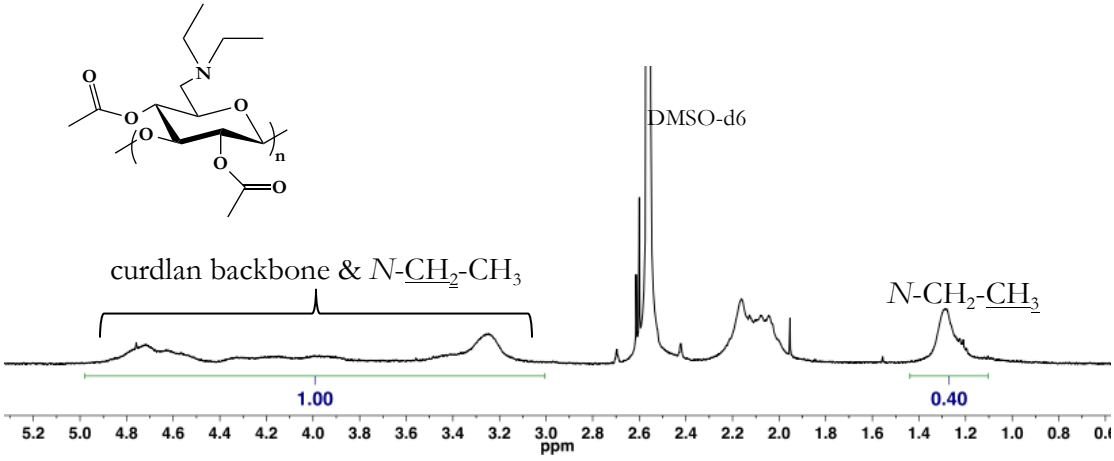


Fig. S5.4. [1]H NMR spectra of 6-(*N*,*N*-diethylamino)-6-deoxy-2,4-di-*O*-acetyl-curdlan (20 equiv. DEA/AGU at 50 °C, 24 h, DS_{Et2N}=0.64.

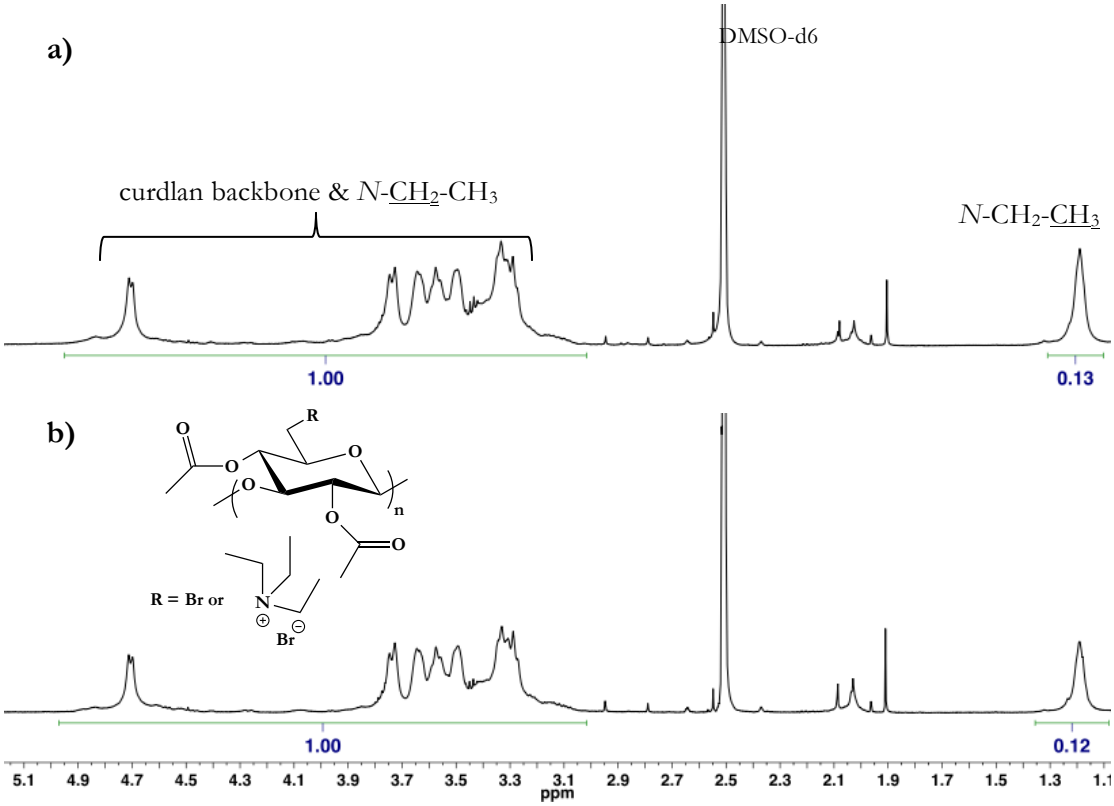


Fig. S5.5. ^{1}H NMR spectra of 6-(*N*,*N*,*N*-triethylammonio)-6-deoxycurdlan bromide from brominated curdlan (DS_{Br} 0.97, 100 equiv. TEA/AGU, 80 °C, 24 h): **(a)** in DMF, DS_{Et3N}=0.11; **(b)** in TEA, DS_{Et3N}=0.10.

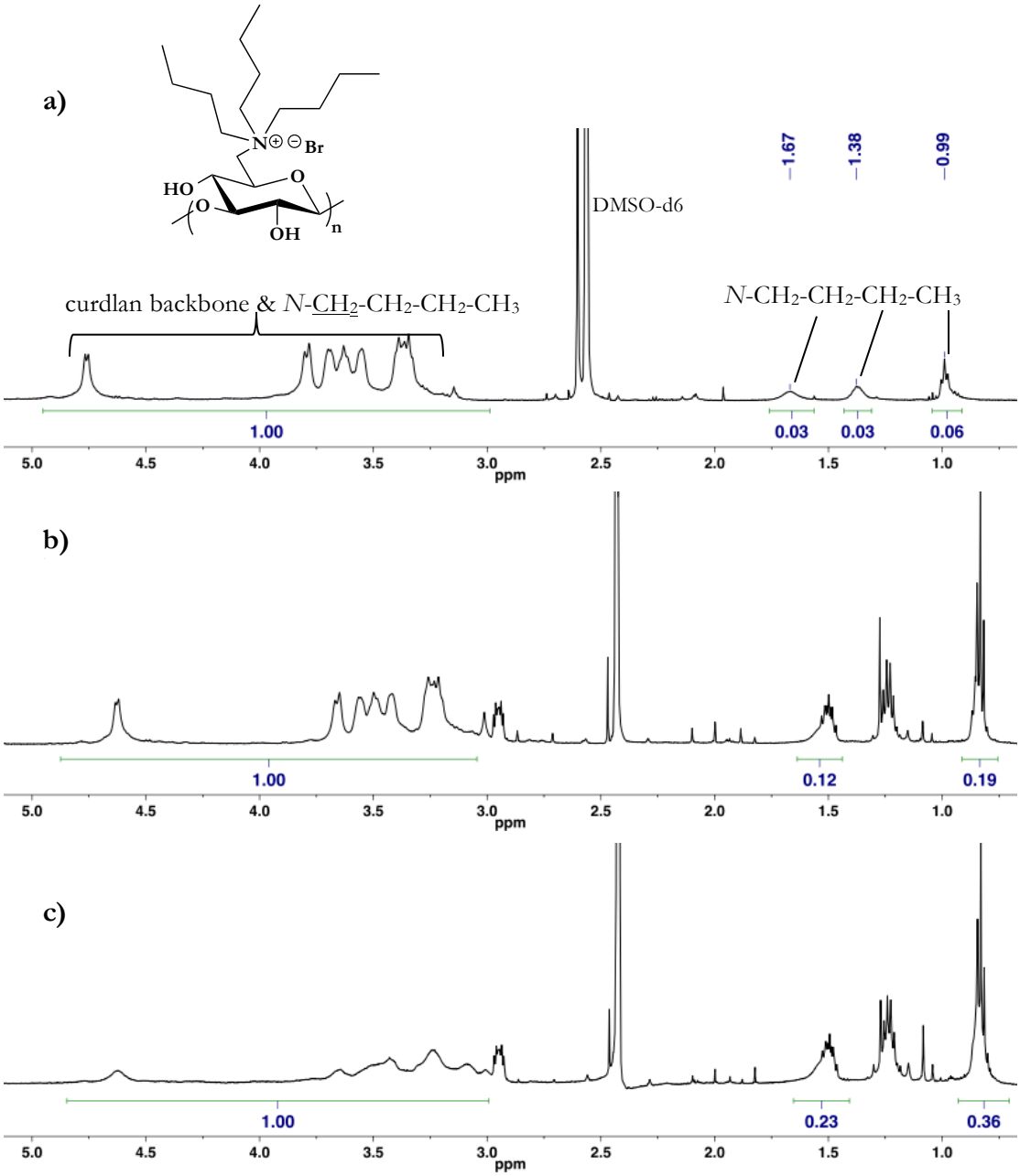


Fig. S5.6. ^{1}H NMR spectra of 6-(*N*,*N*,*N*-tributylammonio)-6-deoxycurdlan bromide: **(a)** 20 equiv. TBA/AGU, 80 °C, 24 h, in DMSO, DS_{Bu3N}=0.04; **(b)** 20 equiv./AGU TBA in DMPr, 80 °C, 24 h, in DMSO, DS_{Bu3N}=0.16; **(c)** 20 equiv. TBA/AGU in DMPr, 5 equiv. NaI/AGU, 80 °C, 24 h, in DMSO, DS_{Bu3N}=0.35.

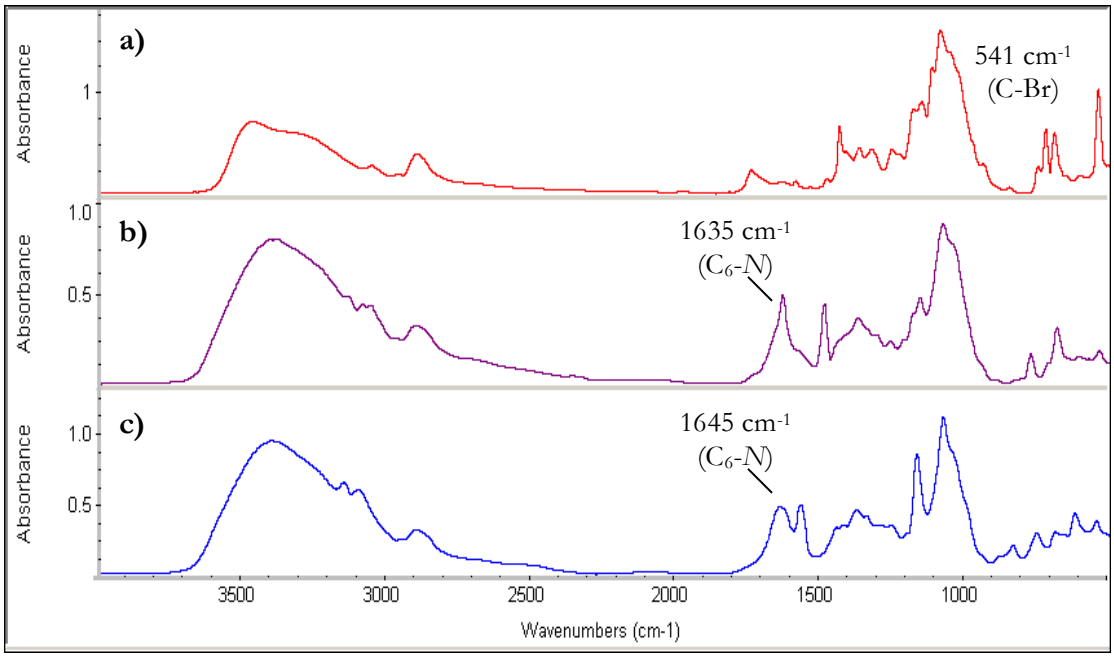


Fig. S5.7. FTIR spectra of **(a)** 6-bromo-6-deoxycurdlan; **(b)** 6-pyridinio-6-deoxycurdlan bromide (20 equiv. Pyr/AGU, 80 °C, 24 h, DMSO, DS_{Pry}=0.66); **(c)** 6-(1-methyl-imidazolio)-6-deoxycurdlan bromide (20 equiv. MeIMID/AGU, 80 °C, 24 h, DMSO, DS_{MeIMID}=0.86).

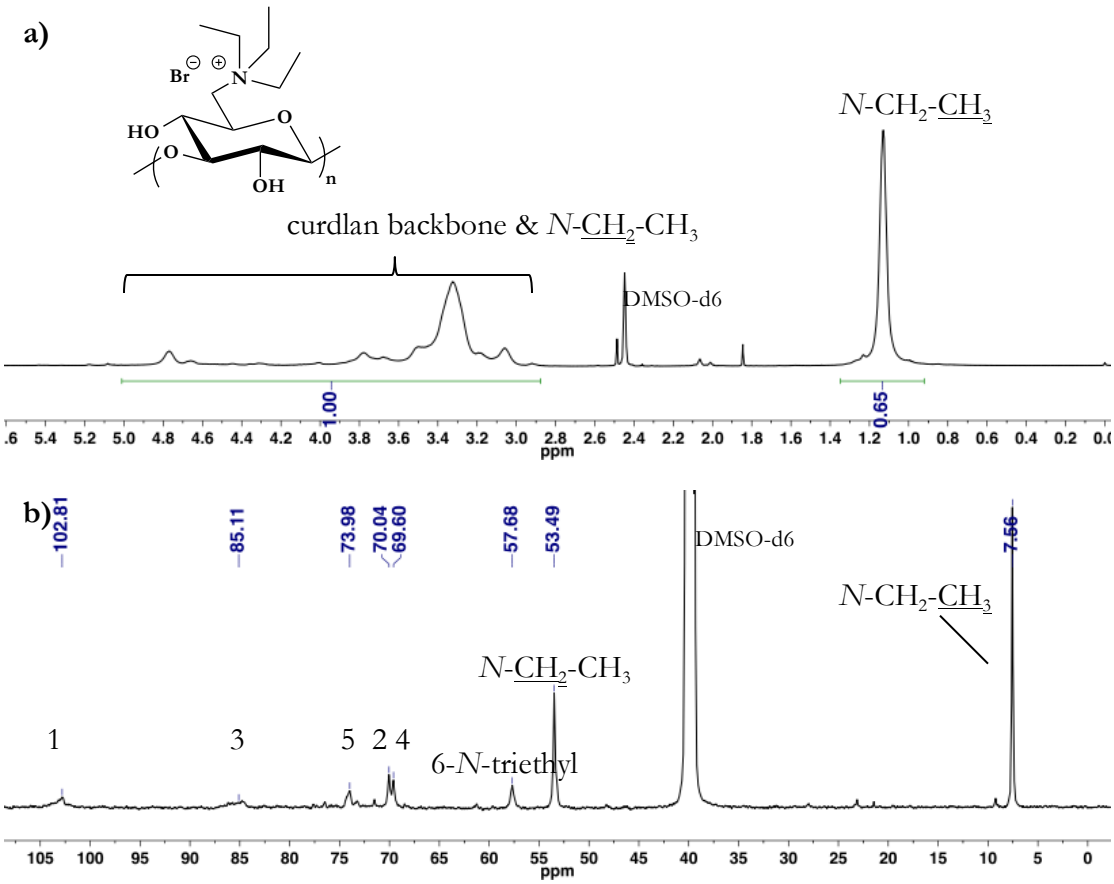


Fig. S5.8. 6-(*N*,*N*-Triethylammonio)-6-deoxycurdlan (20 equiv. TEA/AGU, 5 equiv. NaI/AGU, DMSO, 80 °C, 24h, DS_{Et3N} = 0.89): **(a)** ^{1}H NMR and **(b)** ^{13}C NMR spectra.

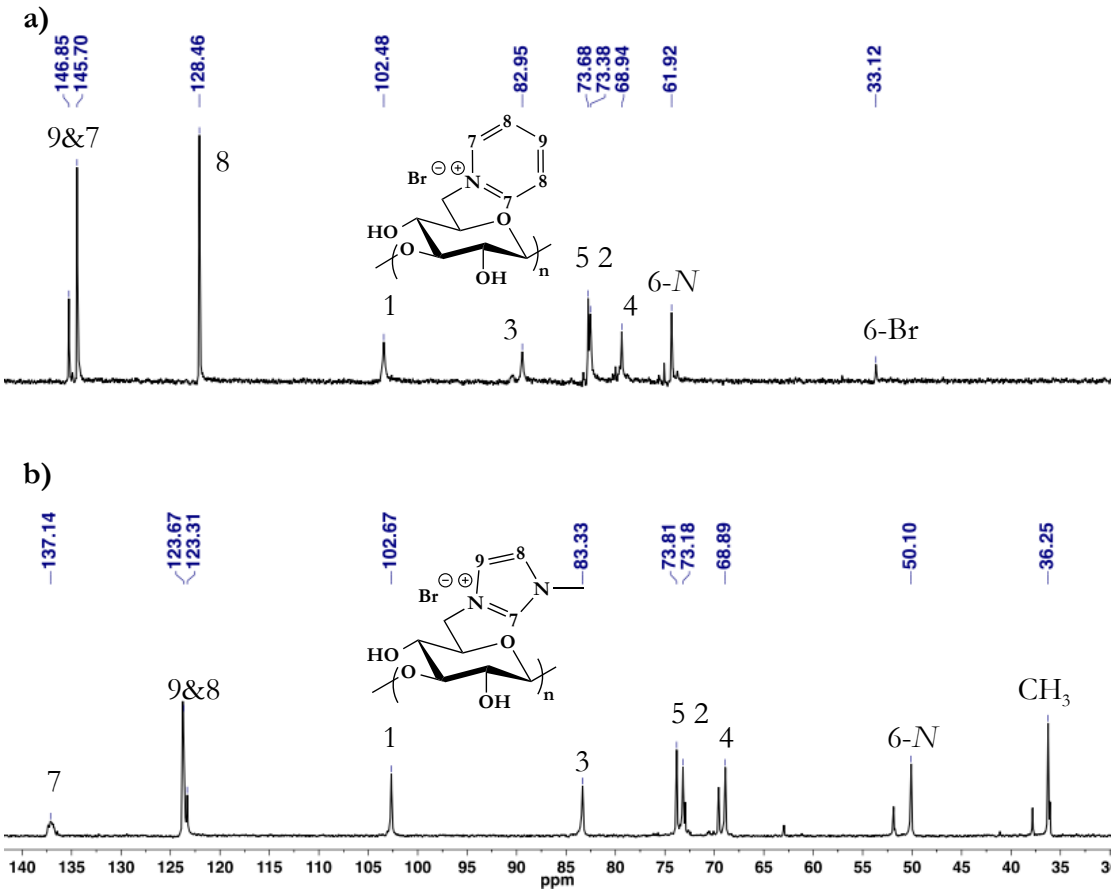


Fig. S5.9. ^{13}C NMR spectra of **(a)** 6-pyridinio-6-deoxycurdlan bromide (20 equiv. Pyr/AGU, 80 °C, 24 h, DMSO, DS_{Pyr}=0.66) and **(b)** 6-(1-methyl-imidazolio)-6-deoxycurdlan bromide (20 equiv. MeIMID/AGU, 80 °C, 24 h, DMSO, DS_{MeIMID}=0.86).

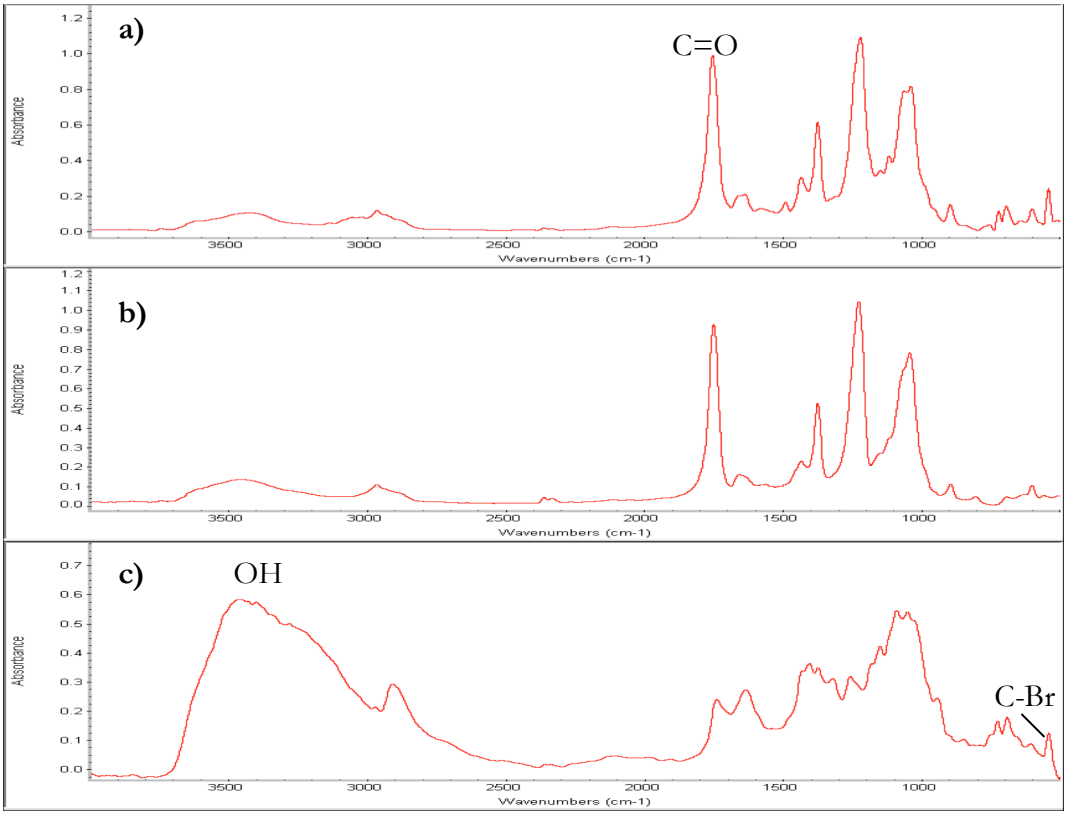


Fig. S5.10. FTIR spectra of **(a)** 6-bromo-6-deoxy-2,4-acetyl-curdlan (DS_{Br}=0.97), **(b)** 6-bromo-6-deoxy-2,4,6-acetyl-curdlan (DS_{Br}=0.30) and **(c)** 6-bromo-6-deoxycurdlan (DS_{Br}=0.97).

5.7 Acknowledgements

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5.8 References

1. Pal, S.; Mal, D.; Singh, R. P., Cationic starch: an effective flocculating agent. *Carbohydr. Polym.* **2005,** *59* (4), 417-423.

2. Dilamian, M.; Montazer, M.; Masoumi, J., Antimicrobial electrospun membranes of chitosan/poly(ethylene oxide) incorporating poly(hexamethylene biguanide) hydrochloride. *Carbohydr. Polym.* **2013,** *94* (1), 364-371.

3. (a) Mai, K.; Zhang, S.; Liang, B.; Gao, C.; Du, W.; Zhang, L.-M., Water soluble cationic dextran derivatives containing poly(amidoamine) dendrons for efficient gene delivery. *Carbohydr. Polym.* **2015**, Ahead of Print; (b) Ikeda, M.; Minari, J.; Shimada, N.; Numata, M.; Sakurai, K.; Shinkai, S., Complex formation between cationic [small beta]-1,3-glucan and hetero-sequence oligodeoxynucleotide and its delivery into macrophage-like cells to induce cytokine secretion. *Organic & Biomolecular Chemistry* **2007,** *5* (14), 2219-2224.

4. (a) Bernkop-Schnurch, A.; Dunnhaupt, S., Chitosan-based drug delivery systems. *Eur J Pharm Biopharm* **2012,** *81* (3), 463-9; (b) Tamaru, S.-i.; Tokunaga, D.; Hori, K.; Matsuda, S.; Shinkai, S., Giant amino acids designed on the polysaccharide scaffold and their protein-like structural interconversion. *Organic & Biomolecular Chemistry* **2014,** *12* (5), 815-822.

5. (a) Fox, S. C.; Edgar, K. J., Staudinger Reduction Chemistry of Cellulose: Synthesis of Selectively O-Acylated 6-Amino-6-deoxy-cellulose. *Biomacromolecules* **2012,** *13* (4), 992-1001; (b) Heinze, T.; Koschella, A.; Brackhagen, M.; Engelhardt, J.; Nachtkamp, K., Studies on non-natural deoxyammonium cellulose. *Macromol. Symp.* **2006,** *244* (New Cellulose Products and Composites), 74-82; (c) Liu, C.; Baumann, H., New 6-butylamino-6-deoxycellulose and 6-deoxy-6-pyridiniumcellulose derivatives with highest regioselectivity and completeness of reaction. *Carbohydr. Res.* **2005,** *340* (14), 2229-2235; (d) Matsui, Y.; Ishikawa, J.; Kamitakahara, H.; Takano, T.; Nakatsubo, F., Facile synthesis of 6-amino-6-deoxycellulose. *Carbohydrate Research* **2005,** *340* (7), 1403-1406.

6. Song, Y.; Sun, Y.; Zhang, X.; Zhou, J.; Zhang, L., Homogeneous Quaternization of Cellulose in NaOH/Urea Aqueous Solutions as Gene Carriers. *Biomacromolecules* **2008,** *9* (8), 2259-2264.

7. Ikeda, M.; Hasegawa, T.; Numata, M.; Sugikawa, K.; Sakurai, K.; Fujiki, M.; Shinkai, S., Instantaneous Inclusion of a Polynucleotide and Hydrophobic Guest Molecules into a Helical Core of Cationic β-1,3-Glucan Polysaccharide. *Journal of the American Chemical Society* **2007,** *129* (13), 3979-3988.

8. Kotze, A. F.; Thanou, M. M.; Lueen, H. L.; De Boer, A. G.; Verhoef, J. C.; Junginger, H. E., Enhancement of paracellular drug transport with highly quaternized N-trimethyl

chitosan chloride in neutral environments: in vitro evaluation in intestinal epithelial cells (Caco-2). *J. Pharm. Sci.* **1999,** *88* (2), 253-257.

9. Liu, C.; Baumann, H., New 6-butylamino-6-deoxycellulose and 6-deoxy-6-pyridiniumcellulose derivatives with highest regioselectivity and completeness of reaction. *Carbohydrate Research* **2005,** *340* (14), 2229-2235.

10. Koschella, A.; Heinze, T., Novel Regioselectively 6-Functionalized Cationic Cellulose Polyelectrolytes Prepared via Cellulose Sulfonates. *Macromolecular Bioscience* **2001,** *1* (5), 178-184.

11. Furuhata, K.; Koganei, K.; Chang, H. S.; Aoki, N.; Sakamoto, M., Dissolution of cellulose in lithium bromide-organic solvent systems and homogeneous bromination of cellulose with N-bromosuccinimide-triphenylphosphine in lithium bromide-N,N-dimethylacetamide. *Carbohydr. Res.* **1992,** *230* (1), 165-77.

12. Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J., Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Delivery Rev.* **2001,** *46* (1-3), 3-26.

13. Pan, Y.; Li, Y.-j.; Zhao, H.-y.; Zheng, J.-m.; Xu, H.; Wei, G.; Hao, J.-s.; Cui, F.-d., Bioadhesive polysaccharide in protein delivery system: chitosan nanoparticles improve the intestinal absorption of insulin in vivo. *Int. J. Pharm.* **2002,** *249* (1-2), 139-147.

14. Ward, P. D.; Tippin, T. K.; Thakker, D. R., Enhancing paracellular permeability by modulating epithelial tight junctions. *Pharm. Sci. Technol. Today* **2000,** *3* (10), 346-358.

15. Sadeghi, A. M. M.; Dorkoosh, F. A.; Avadi, M. R.; Weinhold, M.; Bayat, A.; Delie, F.; Gurny, R.; Larijani, B.; Rafiee-Tehrani, M.; Junginger, H. E., Permeation enhancer effect of chitosan and chitosan derivatives: Comparison of formulations as soluble polymers and nanoparticulate systems on insulin absorption in Caco-2 cells. *Eur. J. Pharm. Biopharm.* **2008,** *70* (1), 270-278.

16. Hsu, L.-W.; Lee, P.-L.; Chen, C.-T.; Mi, F.-L.; Juang, J.-H.; Hwang, S.-M.; Ho, Y.-C.; Sung, H.-W., Elucidating the signaling mechanism of an epithelial tight-junction opening induced by chitosan. *Biomaterials* **2012,** *33* (26), 6254-6263.

17. Deli, M. A., Potential use of tight junction modulators to reversibly open membranous barriers and improve drug delivery. *Biochim. Biophys. Acta, Biomembr.* **2009,** *1788* (4), 892-910.

18. Thanou, M.; Verhoef, J. C.; Junginger, H. E., Chitosan and its derivatives as intestinal absorption enhancers. *Bull. Tech. Gattefosse* **2001,** *94*, 87-96.

19. Hajeb, P.; Selamat, J., A Contemporary Review of Seafood Allergy. *Clinic Rev Allerg Immunol* **2012,** *42* (3), 365-385.

20. (a) Zhang, R.; Edgar, K. J., Properties, Chemistry, and Applications of the Bioactive Polysaccharide Curdlan. *Biomacromolecules* **2014,** *15* (4), 1079-1096; (b) Harada, T.; Harada, A., Curdlan and succinoglycan. In *Polysaccharides in Medicinal Applications*, Dumitriu, S., Ed. Dekker: New York, 1996; pp 21-57; (c) Vandamme, E. J.; De Baets, S.; Steinbuechel, A.; Editors, *Biopolymers, Volume 5: Polysaccharides I, Polysaccharides from Prokaryotes*. Wiley-VCH Verlag GmbH: 2002; p 532 pp.

21. (a) Bohn, J. A.; BeMiller, J. N., (1→3)- β -D-Glucans as biological response modifiers: a review of structure-functional activity relationships. *Carbohydr. Polym.* **1995,** *28* (1), 3-14; (b) Stone, B. A.; Clarke, A. E., *Chemistry and biology of (1→3)-β-D-glucans*. La Trobe University: Melbourne, 1992; (c) Yotsuzuka, F., Curdlan. *Food Sci. Technol. (N. Y., NY, U. S.)* **2001,** *113* (Handbook of Dietary Fiber), 737-757.

22. Suflet, D. M.; Popescu, I.; Pelin, I. M.; Nicolescu, A.; Hitruc, G., Cationic curdlan: Synthesis, characterization and application of quaternary ammonium salts of curdlan. *Carbohydrate Polymers* **2015,** *123*, 396-405.

23. Zhang, R.; Edgar, K. J., Water-soluble aminocurdlan derivatives by chemoselective azide reduction using NaBH4. *Carbohydrate Polymers* **2015,** *122* (0), 84-92.

24. Zhang, R.; Edgar, K. J., Synthesis of curdlan derivatives regioselectively modified at C-6: O-(N)-Acylated 6-amino-6-deoxycurdlan. *Carbohydrate Polymers* **2014,** *105* (0), 161-168.

25. de Britto, D.; Assis, O. B. G., A novel method for obtaining a quaternary salt of chitosan. *Carbohydrate Polymers* **2007,** *69* (2), 305-310.

26. McCortney, B. A.; Jacobson, B. M.; Vreeke, M.; Lewis, E. S., Methyl transfers. 14. Nucleophilic catalysis of nucleophilic substitution. *Journal of the American Chemical Society* **1990,** *112* (9), 3554-3559.

27. Finkelstein, H., Preparation of Organic Iodides from the Corresponding Bromides and Chlorides. *Ber. Dtsch. Chem. Ges.* **1910,** *43*, 1528-32.

28. (a) Henderson, W. A.; Schultz, C. J., The Nucleophilicity of Amines. *The Journal of Organic Chemistry* **1962,** *27* (12), 4643-4646; (b) Phan, T. B.; Breugst, M.; Mayr, H., Towards a

General Scale of Nucleophilicity? *Angewandte Chemie International Edition* **2006,** *45* (23), 3869-3874.

Chapter 6. Studies on Subsequent Reactions of Staudinger Ylide: Reductive Amination and Peralkylation of Aminocurdlan

6.1 Abstract

Staudinger-related reactions between azides and phosphines have been widely applied to organic and polymer chemistry due to their chemoselectivity, high efficiency, and mild reaction conditions. Application to polysaccharides has largely focused on hydrolysis or acylation of the intermediate Staudinger ylide. Curdlan, a natural, nontoxic, biocompatible and bioactive β-1,3-glucan, has remarkable potential in biomedical and pharmaceutical applications. Herein we report three methods for preparation of regioselectively aminated curdlan derivatives via a Staudinger ylide. 6-Azido-6-deoxy-(2,4-di-*O*-acyl-)curdlan was first treated with triphenylphosphine to generate the highly nucleophilic iminophosphorane intermediate available for subsequent syntheses of: i) 6-imino curdlans by reaction with aromatic aldehydes (PhCHO, *p*-NO_2PhCHO, *p*-ClPhCHO or 2-PyrCHO), ii) 6-monoalkylamino curdlans by reductive amination using these aldehydes and sodium cyanoborohydride, and iii) 6-dialkylamino-/tri-alkylammoniocurdlans by reacting with methyl iodide. In order to understand how to carry them out most efficiently, the scope of those amination reactions has been investigated as well, including the influence of key process parameters: solvent, temperature, time, and molar ratios of added reagents. Regioselectivity and DS of those aminated curdlan derivatives were quantified by means of 1D (^{1}H, ^{13}C) and 2D NMR spectroscopic methods.

6.2 Introduction

Numerous products derived from natural polysaccharides play prominent roles in various industrial and biomedical applications. Considerable attention has been devoted to the study of chemically modified polysaccharide derivatives with specific properties. Additionally, understanding the fundamental relationship between polysaccharide structure and properties has prompted the development of new chemical approaches for the selective modification of native polysaccharides. Curdlan, a native extracellular polysaccharide, stands out from the family due to its valuable rheological properties and inherent bioactivity. The simple homopolymeric, unbranched and uncharged structure of the (1,3)-β-D-glucan curdlan

simplifies a range of chemical modifications, such as esterification[1], carboxymethylation[2], phosphorylation[3], sulfation[4] and TEMPO oxidization[5]. Our laboratory has developed a series of regioselective modifications of curdlan to synthesize 6-deoxy-6-(bromo/azido/amino/amido/ammonium) derivatives[6] that are promising candidates for biomedical and pharmaceutical applications. We have observed that the iminophosphorane intermediate generated during Staudinger reduction of 6-azido-6-deoxycurdlan to produce 6-amino-6-deoxycurdlan is highly nucleophilic, in accord with the previous work of Bertozzi[7] and others. This reactivity raises the question of whether the iminophosphorane could be harnessed to carry out additional, useful subsequent reactions. 6-Azido-6-deoxycurdlan is an efficient ylide precursor that upon treatment with triphenylphosphine at ambient temperature forms a phosphazide, which in turn loses nitrogen gas to form the desired iminophosphorane.

Iminophosphoranes are organic compounds of general composition $R_3P{=}NR$ that possess a highly polarized P=N bond and are best described as resonance hybrids of the two extreme forms A and B (**Fig. 6.1**)[8]. Staudinger reaction is by far the most widely used method to synthesize iminophosphoranes, in which a phosphine (R_3P:) reacts with an organic azide. Although Staudinger and Meyers[9] prepared the first aza-Wittig reagent $Ph_3P{=}NPh$ in 1919, the chemistry of iminophosphoranes was not heavily explored until three decades later. Since then, aza-Wittig reactions between iminophosphoranes and aldehydes have become a powerful tool in small molecule organic synthetic strategies due to the absence of catalysts, mild reaction conditions, and relatively high yield. Small molecule iminophosphoranes can also react with other carbonyl compounds, such as ketones, esters, thioesters, amides, and anhydrides, to afford an excellent method to construct C=N double bonds.[10] Our previous papers have reported application of iminophosphorane intermediates, generated by Staudinger reduction of azides, to the synthesis of *O*-acylated 6-amido-6-deoxy-cellulose[11], -curdlan[6a], and -pullulan[12] derivatives using *in situ* reaction of the ylide with water, or with excess carboxylic anhydride.

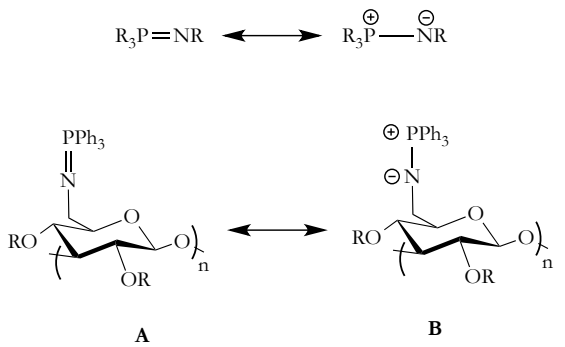
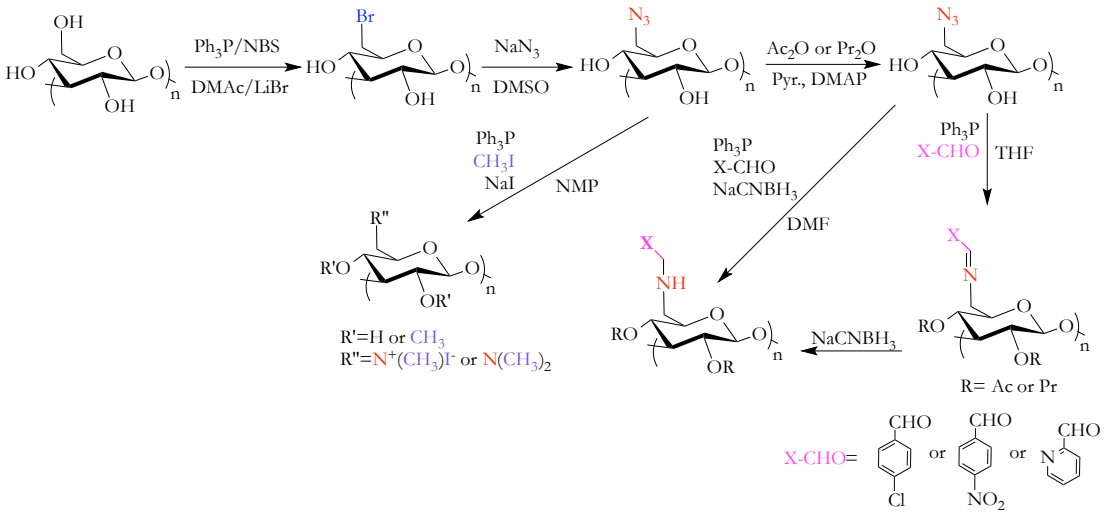


Fig. 6.1. The two forms of a general iminophosphorane and a specific curdlan iminophosphorane generated during Staudinger reaction

The resultant imines can be further reduced by borohydride to produce amines, known as indirect reductive amination. Sodium borohydride is a highly selective reducing agent for aldehydes, ketones and imines. Previous investigators have confirmed the successful reduction of chitosan Schiff base (imine) by $NaBH_4$ in methanol; they concluded that it was $NaBH_4$ but not the Schiff base that should be completely soluble in the chosen solvent for the reduction to be effective.[13] However, it is also known that $NaBH_4$ can reduce ester groups to primary alcohols in the presence of excess reagent, which may be undesirable for ester containing products. Instead, sodium cyanoborohydride with its strongly electron withdrawing cyano group is a milder and more selective reductant than sodium borohydride.[14] In the direct reductive amination process, a carbonyl compound, most commonly an aldehyde or a ketone, will condense with an amine to form an intermediate imine, in the presence of a reducing agent.[15] Since the imine formation and reduction occur sequentially in one pot and the iminium ion needs to be reduced much faster than the carbonyl group, the selectivity of $NaCNBH_3$ makes it a preferred reducing agent. There appear to be no previous reports of employing the one-pot reductive amination between an aldehyde and a polysaccharide iminophosphorane intermediate formed by Staudinger reaction; this could be due to concerns about achieving the necessary reactivity and selectivity in a somewhat rigid, sterically encumbered polysaccharide derivative. We hypothesize that the curdlan-based 6-iminophosphorane intermediate generated during the Staudinger reduction may be an active nucleophile to undergo the one-pot reductive amination with aldehydes in the presence of a reductant. Herein we report our attempts to

prepare a family of 6-monoalkyl curdlans by reacting 6-azido-6-deoxy-2,4-di-*O*-acyl-curdlan with Ph_3P and aldehydes (**Scheme 6.1**).

In addition, inspired by the peralkylation of chitosan to produce *N*-trimethyl chitosan chloride (TMC) with excellent absorption enhancing effect across mucosal epithelia, we are interested in investigating the possibility of preparing water-soluble trialkylaminocurdlans by peralkylation of the Staudinger ylide. Peralkylated chitosan is able to open up tight junctions, which seal the paracellular pathways, thereby facilitating the paracellular diffusion of peptide drugs.[16] The charge density, determined by the degree of quaternization, plays an important role on its paracellular permeability and mucoadhesive properties.[17] Herein we synthesized a series of 6-di/tri-alkylamino curdlans by reacting 6-azido-6-deoxy-curdlan with alkyl iodide in the presence of Ph_3P (**Scheme 6.1**). The influence of key process parameters including reaction temperature, time, and molar ratio of the added alkyl iodide have been investigated to tailor the charge density of final peralkylated aminocurdlan products.



Scheme 6.1. Synthetic scheme for 6-aminated curdlan derivatives via Staudinger ylide.

6.3 Experimental

6.3.1 Materials

Curdlan (-$C_6H_{10}O_5$- as anhydroglucose unit (AGU), DP ~ 500) was obtained from Wako Chemicals and dried under vacuum at 40 °C overnight prior to use. Lithium bromide (LiBr, laboratory grade, Fisher) was dried under vacuum at 125 °C. *N*-Bromosuccinimide (NBS,

99%, Acros) was recrystallized from boiling water and dried for two days under reduced pressure over anhydrous calcium chloride. *N,N*-Dimethylacetamide (DMAc, reagent grade, Fisher) and *N*,*N*-dimethylformamide (DMF, HPLC grade, Fisher) were stored over 4 Å molecular sieves. Tetrahydrofuran (THF, 99.8%, extra dry, stabilized, AcroSeal®), *N*-methyl-2-pyrrolidone (NMP, 99.5%, extra dry, AcroSeal®), pyridine (Pyr, anhydrous, 99%, AcroSeal®), benzaldehyde (PhCHO, purified by redistillation, ≥ 99.5%, Aldrich), 4-nitrobenzaldehyde (NO_2PhCHO, 98%, Aldrich), 4-chlorobenzaldehyde (ClPhCHO, 98%, Aldrich), 2-pyridinecarboxaldehyde (PyrCHO, 99%, Aldrich), 4-dimethylaminopyridine (DMAP, Acros), triphenylphosphine (Ph_3P, 99%, Acros), sodium azide (NaN_3, 99%, Alfa Aesar), sodium iodine (NaI, Fisher), methyl iodide (CH_3I, stabilized, 99%, Acros), sodium hydroxide (NaOH, reagent grade, 97%, Sigma-Aldrich), sodium cyanoborohydride ($NaCNBH_3$, reagent grade, 95%, Aldrich), acetic anhydride (Ac_2O, 99+%, Sigma-Aldrich), propionic anhydride (Pr_2O, 97% Sigma-Aldrich), n-butyric anhydride (Bu_2O, 98%, Acros), ethanol (HPLC grade, Fisher), molecular sieves (4 Å, Fisher) and regenerated cellulose dialysis tubing (MW 3500, Fisher) were used as received.

6.3.2 Measurements

^{1}H, ^{13}C and HSQC NMR spectra were obtained on a Bruker Avance II 500MHz spectrometer in $CDCl_3$, DMSO-d_6, DMF-d_7, or D_2O at room temperature or 50 °C, employing 32, 15,000 and 19,000 scans, respectively.

6.3.3 Methods

6.3.3.1 Dissolution and bromination of curdlan in DMAc/LiBr[6a]

Dissolution of dried curdlan (4.00 g, 24.7 mmol AGU) in DMAc (110 mL) and LiBr (36.00 g, 42.4 mmol) was performed by a previously reported procedure.[6a] Ph_3P (25.96 g, 4 eq per AGU) and NBS (17.58 g, 4 eq per AGU) in dry DMAc (50 mL) each were added dropwise, sequentially, to the curdlan solution. The reaction solution was then heated at 70 °C for 1 h. The mixture was added slowly to 1 L of a 50:50 mixture of methanol and deionized water and then filtered to recover the precipitate. The isolated sample was washed with ethanol twice and then dried under vacuum (40 °C) overnight to yield 6-bromo-6-deoxycurdlan. ^{13}C NMR (DMSO-d_6): δ 103.2 (C-1), 84.9 (C-3), 74.4 (C-5), 73.6 (C-2), 70.1 (C-4), 34.6 (C-6-Br). Yield: 86%.

6.3.3.2 Azide displacement of 6-bromo-6-deoxycurdlan (performed by a previously reported procedure)[6a]

Briefly, dry 6-bromo-6-deoxycurdlan (1.00 g, 4.44 mmol) was dissolved in DMSO (25 mL). Then NaN_3 (1.44 g, 5 eq per AGU) was added to the solution. The resulting mixture was heated at 80 °C for 24 h under nitrogen. The product was isolated by pouring into 300 mL of deionized water and collected by filtration. The precipitate was re-dissolved in acetone and re-precipitated in deionized water followed by filtration. The sample was dried under vacuum (40 °C) overnight to yield 6-azido-6-deoxycurdlan. ^{13}C NMR (DMSO-d_6): δ 103.4 (C-1), 84.9 (C-3), 74.9 (C-5), 73.9 (C-2), 69.4 (C-4), 51.7 (C-6-N_3). Yield: 92%.

6.3.3.3 General procedure for peracylation of 6-azido-6-deoxycurdlan[6a]

Dry 6-azido-6-deoxycurdlan (1.00 g, 5.35 mmol), 4-dimethylaminopyridine (DMAP, 20 mg), pyridine (3.6 mL, 10 eq per AGU), and 20 eq per AGU of carboxylic anhydride (Ac_2O, 10.1 mL; Pr_2O, 13.8 mL) were combined. The mixture was stirred at 80 °C for 24 h, then cooled and added slowly to 200 mL deionized water to recover the precipitate which was re-dissolved in chloroform and re-precipitated in ethanol followed by filtration. The products were washed with ethanol and water several times and then dried under vacuum (40 °C) overnight to yield 6-azido-6-deoxy-2,4-di-*O*-acyl-curdlan. ^1H NMR ($CDCl_3$): δ 4.7 (H-5), 4.6 (H-5), 4.3 (H-1), 3.7 (H-6), 3.5 (H-6'), 3.3 (H-3), 3.1 (H-2), 2.2-1.9 (CH_3-acetate), 2.6-2.0 (CH_2-propionate), 1.3-1.0 (CH_3-propionate).

6.3.3.4 General procedure for 6-imino-6-deoxy-2,4-di-*O*-acetyl-curdlan synthesis

Dry 6-azido-6-deoxy-2,4-di-*O*-acetyl-curdlan (0.25 g, 0.92 mmol) was dissolved in 15 mL of THF or DMAc in a 50 mL flask with molecular sieves. Then Ph_3P (2 eq per AGU) and 30 eq per AGU of aldehyde (PhCHO (2.94 g), NO_2PhCHO (4.18 g), ClPhCHO (3.88 g), or PyrCHO (2.96 g) were added to the flask. The reaction was run under nitrogen at room temperature for 24 h. The solution was transferred to 3,500 g/mol MWCO dialysis tubing that was then placed in a large beaker containing ethanol. After three to five days of dialysis,

the precipitate formed within the tubing was isolated by filtration and then dried under vacuum (40 °C) overnight to yield 6-imino-6-deoxy-2,4-di-*O*-acyl-curdlan.

6.3.3.5 Borohydride reduction of 6-imino-6-deoxy-2,4-di-*O*-acetyl-curdlan

Dry 6-benzimino-6-deoxy-2,4-di-*O*-acetyl-curdlan (0.1 g, 0.30 mmol) was dissolved in 10 mL of THF in a 50 mL flask. Then 10 eq per AGU of $NaCNBH_3$ (0.19 g, 3.0 mmol) was added to the flask. The reaction was run at ambient temperature (ca. X° C) for 24 h. The mixture was added to 100 mL of ethanol. The precipitate was isolated by filtration and washed with ethanol, then dried under vacuum (40 °C) overnight.

6.3.3.6 One-pot reductive amination of 6-azido-6-deoxy-2,4-di-*O*-acyl-curdlan via Staudinger ylide

Dry 6-azido-6-deoxy-2,4-di-*O*-acetyl-curdlan (0.25 g, 0.92 mmol) was dissolved in 15 mL of DMF in a 50 mL flask containing molecular sieves (2 g, 4 Å). Then Ph_3P (2 eq per AGU), PhCHO (2.82 mL, 30 eq per AGU), and $NaCNBH_3$ (0.23 g, 10 eq per AGU) were added to the flask. The reaction was run under nitrogen at ambient temperature for 24 h. The solution was transferred to 3,500 g/mol MWCO dialysis tubing and dialyzed against ethanol for at least three days and the ethanol was replaced daily. The precipitate formed within the tubing was isolated by filtration and then dried under vacuum (40 °C) overnight.

6.3.3.7 Peralkylation of 6-amino-6-deoxycurdlan via Staudinger ylide

<u>One-step synthesis:</u>

Dry 6-azido-6-deoxycurdlan (0.1 g, 0.54 mmol) was dissolved in 5 mL of NMP in a 25 mL flask. Ph_3P (0.28 g, 2 eq per AGU), NaI (0.2 g), aqueous NaOH solution (15%, 0.08 mL, 4 eq per AGU) and CH_3I (0.51 mL, 15 eq per AGU) were added. The mixture kept stirring at 60 °C for 8 h. The solution was transferred to 3,500 g/mol MWCO dialysis tubing that was then placed in a large beaker containing ethanol. After one day of dialysis, the yellow precipitate formed within the tubing was isolated by filtration and then dried under vacuum (40 °C) overnight.

<u>Two-step synthesis:</u>

Dry 6-azido-6-deoxycurdlan (0.1 g, 0.54 mmol) was dissolved in 5 mL of NMP in a 25 mL flask. Ph_3P (0.28 g, 2 eq per AGU) was then added. The solution was stirred at room temperature for 7 h. NaI (0.2 g), aqueous NaOH solution (15%, 0.08 mL, 4 eq per AGU) and CH_3I (0.17 mL, 5 eq per AGU) were added. The mixture was stirred at room temperature for another 24 h. The solution was transferred to 3,500 g/mol MWCO dialysis tubing that was then placed in a large beaker containing ethanol. After one day of dialysis, the yellow precipitate that formed within the tubing was isolated by filtration and then dried under vacuum (40 °C) overnight.

6.4 Results and discussion

6.4.1 Synthesis of 6-benzimino-6-deoxy-2,4-di-*O*-acyl-curdlan

Our lab has reported a series of C-6 modifications of curdlan[6a, 6b, 18] to produce primary and tertiary amines, 6-ammonio derivatives, and 6-amides, starting from 6-bromo- or 6-azido-6-deoxycurdlan, with high overall regioselectivity and with DS at C-6 that frequently approached 1.0. Esterification of 6-azido-6-deoxycurdlan can be easily carried out by reaction with various carboxylic (acetic/propionic) anhydrides to produce 2,4-*O*-diester derivatives readily soluble in a range of organic solvents, in which the azide moiety serves effectively as a protected amine. Proton NMR spectroscopy was useful for characterizing these peracylated derivatives, 6-azido-6-deoxy-2,4-di-*O*-acetyl/propionyl-curdlan (DS_{ester} =2.0) (**Fig. S6.1**), which serve as important starting materials for the current work as well. We found that the resultant 6-azido-6-deoxy-2,4-*O*-acetyl-curdlan could be treated with triphenylphosphine and benzaldehyde to synthesize 6-benzimino-6-deoxy-2,4-acetyl-curdlan, almost certainly via the iminophosphorane ylide intermediate. We attempted to optimize the reaction conditions with respect to solvent (DMAc *vs.* THF), temperature (RT *vs.* 50 °C), reaction time (24 h *vs.* 36 h) and added benzaldehyde molar ratios (20 *vs.* 30 equiv. per AGU). As can be seen from **Table 6.1 entries 1** and **2**, reaction in THF gave slightly higher DS_{imine} than that in DMAc. By extending the reaction time for another 12 h, DS_{imine} decreased 0.16 (**entry 3** *vs.* **1**) probably due to hydrolysis of the initially formed imine. Increasing either the excess of PhCHO (**entry 4**) or the reaction temperature (**entry 5**) afforded slightly increased DS (DS_{imine} 0.92), which approached full conversion. Due to the potential for degradation and instability of imino derivatives at higher temperatures, we applied the reaction conditions of **entry 4** for the following imine syntheses.

Table 6.1.

Substitution achieved (DS_{imine}) *vs.* conditions for 6-benzimino-6-deoxy-2,4-di-*O*-acetyl-curdlan.

Entry	PhCHO (eq/AGU)	Temp. (°C)	Time (h)	Solvent	DS_{imine}
1	20	RT	24	THF	0.89
2	20	RT	24	DMAc	0.83
3	20	RT	36	THF	0.73
4	30	RT	24	THF	0.91
5	20	50	24	THF	0.91

^{13}C NMR was useful for characterizing the 6-benzimino-6-deoxy-2,4-di-*O*-acetyl-curdlan product (**Fig. 6.2b**); resonances in the range of δ 126-137 ppm were assigned to the aromatic carbons of the phenyl ring. Additionally, signals at δ 62 and 162 ppm were assigned to the $\underline{\mathbf{C}}_6$-N and N=$\underline{\mathbf{C}}_7$ respectively. Diagnostic features of the proton NMR spectrum (**Fig. 6.2a**) included the aromatic proton resonances at δ 7.5 ppm ($H_{10,11,12}$) and δ 7.7 ppm ($H_{9,13}$) as well as the imino proton signal at δ 8.2 ppm (H_7) from the $\underline{\mathbf{H}}$-C_7=N moiety. DS_{imine} values were calculated by integration of protons $H_{7\text{-}13}$ in comparison with curdlan backbone protons ($H_{1\text{-}6/6'}$) and are summarized in **Table 6.1**. Our previous work indicated that the Staudinger reaction (Ph_3P) to reduce the curdlan azide to amine is sufficiently mild to preserve ester bonds against reduction,[6a] which was confirmed by the presence of acetyl groups around δ 2.0 ppm, integration of which indicated full 2, 4-*O*-substitution (DS_{Ac} 2.0). However, there were some small peaks evident close to the imino aromatic signals as well as the apparent aldehyde proton signal at δ 10.0 ppm and the carbonyl carbon signal at δ 192.0 ppm, attributed to the residual benzaldehyde impurity; this can of course be the result of imide hydrolysis rather than or in addition to failure to separate excess benzaldehyde from the product by dialysis. In order to increase the degree of substitution and avoid the accompanying hydrolysis by external water/moisture, we repeated the reactions in the presence of molecular sieves, to capture any adventitious water. When molecular sieves were

so used, benzaldehyde signals were absent in the products, as evidenced by the ^{1}H NMR spectra (**Fig. 6.3**) with complete substitution DS_{imine} 1.0.

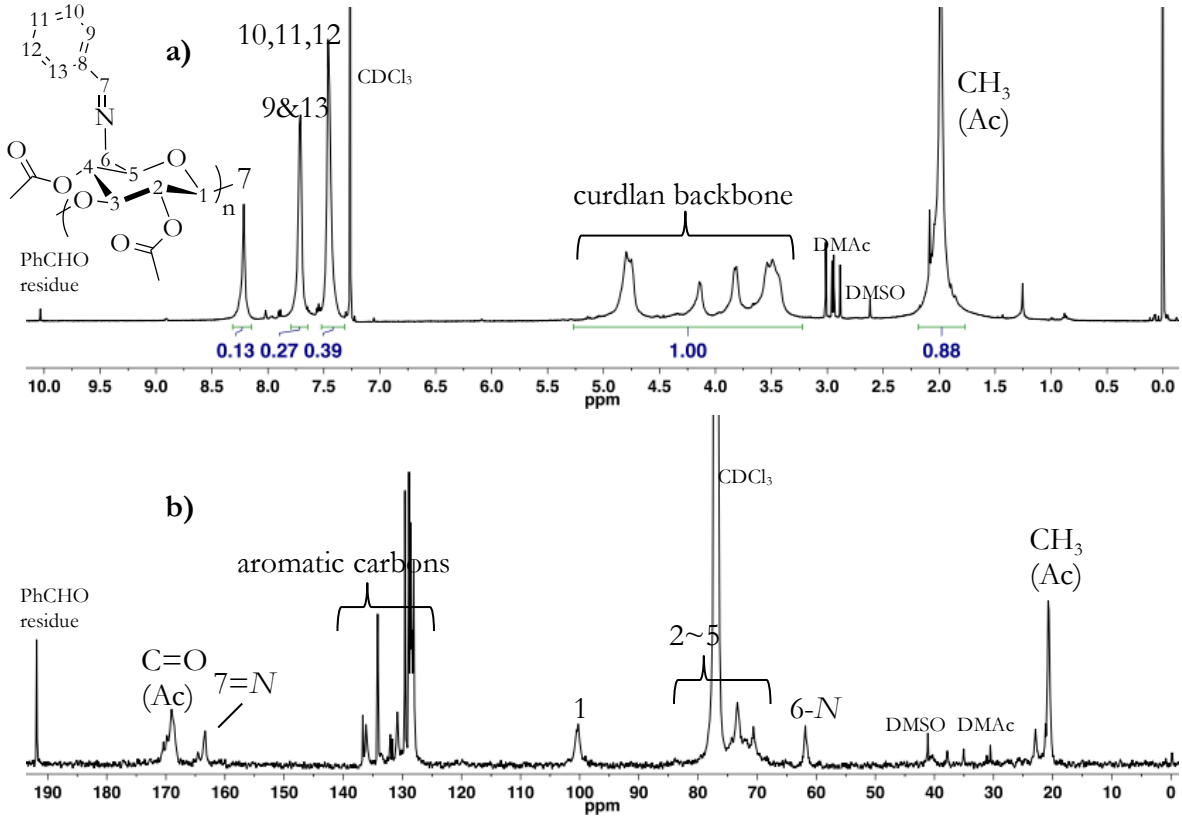


Fig. 6.2. 6-Benzimino-6-deoxy-2,4-di-*O*-acetyl-curdlan (30 equiv. PhCHO/AGU, THF, RT, 24 h, DS_{imine} = 0.91): **(a)** ^{1}H NMR and **(b)** ^{13}C NMR spectra.

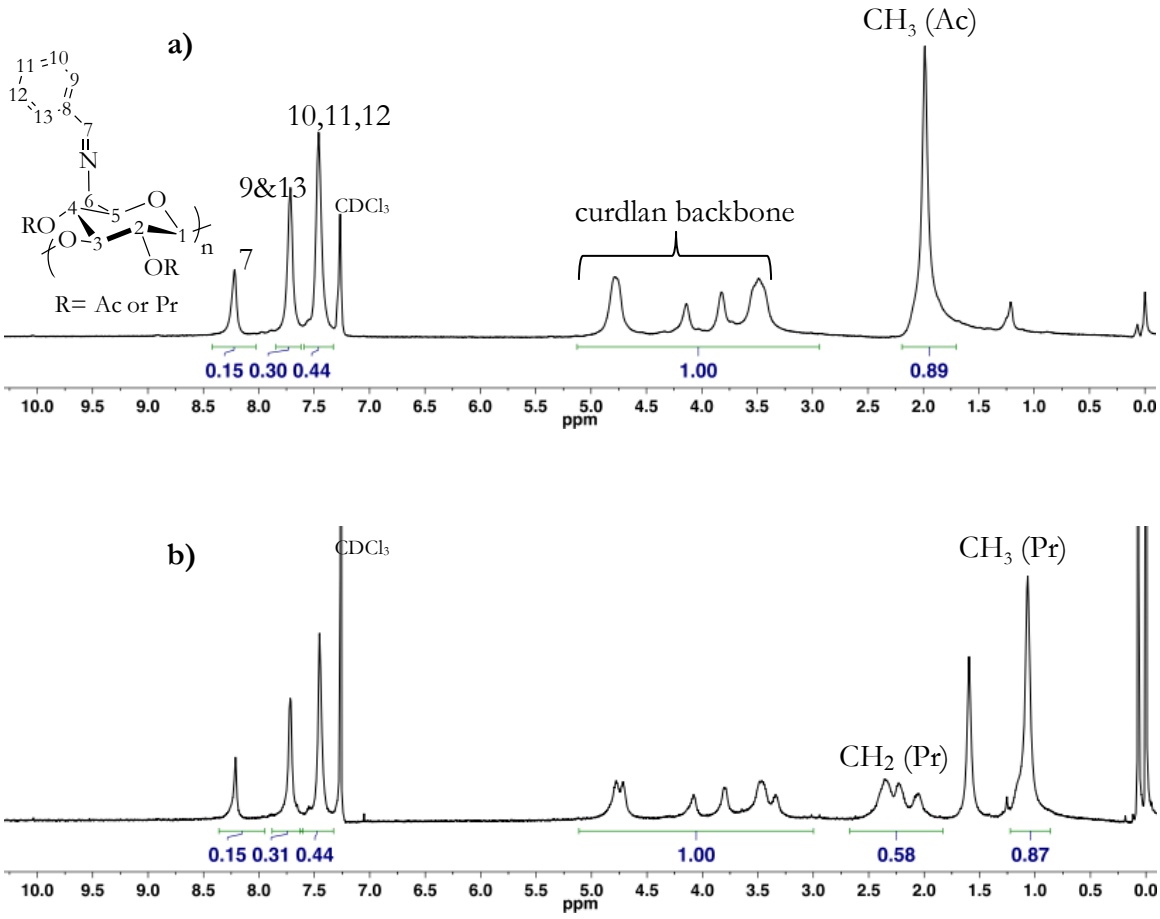
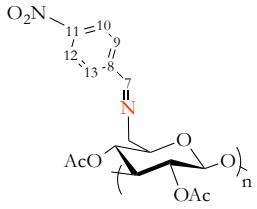
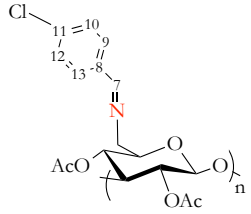
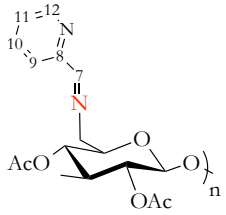


Fig. 6.3. ^{1}H NMR spectra of **(a)** 6-benzimino-6-deoxy-2,4-di-*O*-acetyl-curdlan and **(b)** 6-benzimino-6-deoxy-2,4-di-*O*-propionyl-curdlan (30 equiv. PhCHO/AGU, THF, RT, 24 h, molecular sieves, DS_{imine} = 1.0).

To broaden the applicability of this 6-deoxy-6-iminocurdlan synthetic method, three other aldehydes including NO_2PhCHO, ClPhCHO and PyrCHO were reacted with 6-azido-6-deoxycurdlan under the same conditions (**Scheme 6.1**). Analogous imino substituted products were obtained, which could also be well-characterized by NMR spectroscopy (**Fig. S6.2**). In each case, resonances of curdlan backbone protons fell within δ 5.0-3.0 ppm while aromatic ring and imine moiety protons in the range of δ 8.7-7.4 ppm. **Table 6.2** summarizes the chemical structure, DS_{imine}, product yield and chemical shifts assignments of each imino analogue. The degree of substitution of each imino product was determined as $DS_{ClPh\text{-}imine}$ 0.63, $DS_{Pyr\text{-}imine}$ 0.76 and $DS_{NO2Ph\text{-}imine}$ 0.77, respectively. The incomplete displacement might be due to the lower miscibility of those aldehydes and the solvent THF.

Table 6.2

^{1}H NMR chemical shift assignments for aromatic ring and imine protons, DS_{imine} values and yields of 6-imino-6-deoxycurdlans.

Imine structure	Aldehyde used	^{1}H NMR assignment		DS_{imine}	Yield (%)
		proton	d (ppm)		
	NO_2PhCHO	10,12	8.3	0.77	81
		7,9,13	7.9		
	ClPhCHO	7	8.1	0.63	87
		9,13	7.6		
		10,12	7.4		
	PyrCHO	12	8.6	0.76	84
		7,9	8.3-8.2		
		10,11	7.7-7.9		

6.4.2 Borohydride reduction of 6-benzimino-6-deoxy-2,4-di-*O*-acyl-curdlan

As explained earlier, sodium cyanoborohydride is more selective than $NaBH_4$ for reduction of imines in the presence of ester groups. We treated 6-benzimino-6-deoxy-2,4-di-*O*-acety-curdlan (DS_{imine} 1.0) with different molar ratios of $NaCNBH_3$ to perform the imine to amine reduction. By integration of **Fig. S6.3**, we can easily obtain the DS_{amine} 0.18 (**Table 6.3, entry 1)**, far lower than the starting DS_{imine} 1.0. The resultant DS went up to 0.38 (**entry 2**) as the reaction time increased from 5 h to 24 h, while raising the added reductant amount did not remarkably improve the DS_{amine}, which leveled off at around 0.4.

Table 6.3.

Substitution achieved (DS_{amine}) *vs.* conditions by borohydride reduction of isolated 6-benzimino-6-deoxy-2,4-di-*O*-acetyl-curdlan.

Entry	$NaCNBH_3$ (eq/AGU)	Temp. (°C)	Time (h)	Solvent	DS_{amine}
1	2	RT	5	THF	0.18
2			24		0.38
3	5				0.41
4	10				0.38
5	20				0.42

6.4.3 One-pot reductive amination via Staudinger ylide

The traditional direct reductive amination involves reaction of a carbonyl compound with an amine to form an intermediate imine, which is not isolated but rather is reduced directly in situ by a selective reductant like $NaBH_3CN$. We attempted to emulate the one-pot method in order to avoid isolation of the hydrolytically sensitive imine, and thus improve overall selectivity and efficiency. Our one-pot reductive amination started with a Staudinger iminophosphorane generated from the corresponding azide, reacting with excess aldehyde in the presence of sodium cyanoborohydride, in which all reagents (6-azido-6-deoxy-2,4-*O*-acety-curdlan, Ph_3P, PhCHO, $NaCNBH_3$) were added at the very beginning in various solvent systems. As can be seen from **Table 6.4 entry 1-3**, solvent comparison at 10 equiv. of $NaCNBH_3$ per AGU afforded a maximum DS_{amine} of 0.56 (**Fig. S6.4**) in DMF solvent. In an attempt to further improve DS, we used even larger excess of $NaCNBH_3$ (40 equiv., **entry 4**); this higher excess afforded a satisfying DS_{amine} = 0.89. **Fig. 6.4** clearly showed the broad aromatic protons in the range of δ 7.5-7.3 ppm with disappearance of the imino proton at δ 8.2 ppm, indicating high conversion to 6-benzamino curdlan.

Table 6.4.

Substitution achieved (DS_{amine}) *vs.* conditions for one-pot reductive amination.

Entry[a]	$NaCNBH_3$ (eq/AGU)	Solvent	DS_{amine}
1	10	THF	0.27
2		NMP	0.31
3		DMF	0.56
4	40	THF	0.89

[a]6-Azido-6-deoxy-2,4-*O*-acetyl-curdlan starting material except **entry 4** (6-azido-6-deoxycurdlan), all reactions with Ph_3P (2 eq/AGU), PhCHO (30 eq/AGU), RT, 24 h.

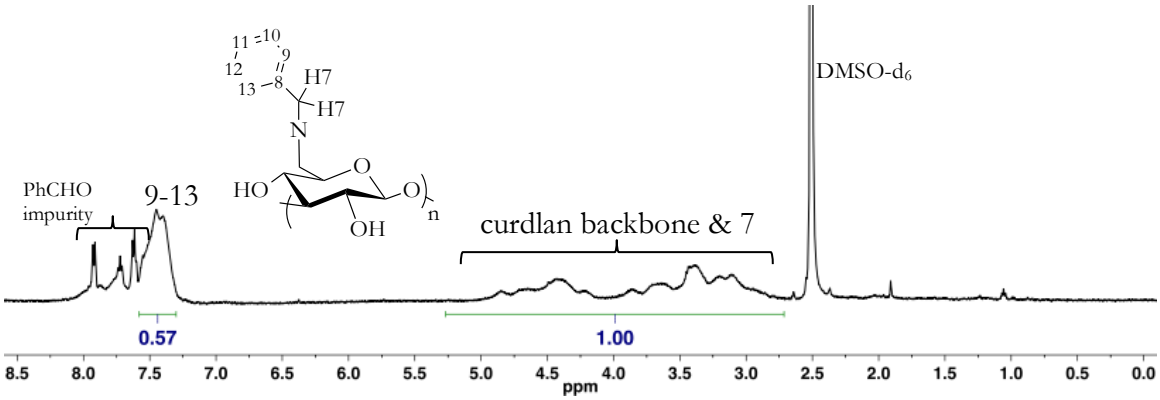


Fig. 6.4. ^{1}H NMR spectrum of 6-benzylamino-6-deoxycurdlan (**Table 6.3**, **entry 4**).

6.4.4 Peralkylation of aminocurdlan

As elaborated previously,[6c] a series of cationic curdlan derivatives with pendent charged ammonium group were synthesized by displacement of the bromide group in the starting 6-bromo-6-deoxycurdlan with a trialkylamine nucleophile. We felt that an alternative approach could be by peralkylation of the reactive iminophosphorane ylide available from 6-azido-6-deoxycurdlan; in any case we wished to investigate the reactivity of these highly nucleophilic ylides towards alkylation by alkyl halides. 6-Azido-6-deoxycurdlan was first treated with triphenylphosphine to generate the iminophosphorane ylide, that was then further reacted with methyl iodide. By changing the equivalents of added CH_3I per AGU and the reaction time of each step, we were capable to control the degree of substitution resulting in mono-, di- and tri-methylated curdlan derivatives. We initially attempted to perform a one-step synthetic approach by adding triphenylphosphine, sodium iodide and excess methyl iodide all together at the beginning (**Table 6.5**, **entry 6**). The product was water-soluble and was

analyzed by NMR spectroscopy in deuterium oxide (D_2O). In parallel with the results of previous studies on chitosan trimethylation,[17a, 19] the ^{13}C NMR spectrum (**Fig. 6.5**) showed that the curdlan C-6 carbon substituted with trimethyl ammonio group ($N^+(CH_3)_3$) or dimethyl amino group ($N(CH_3)_2$) resonated at δ 67.6 and 61.9 ppm respectively, shifted significantly downfield from the azide substituted C-6 resonance (δ 51 ppm) of the starting material, though residual azide-bearing carbon was still observed. Quaternary and tertiary amine substitutions were further confirmed by the trimethyl and dimethyl carbon resonances at δ 54.9 and δ 48.6 ppm respectively. Due to the presence of multiple C-6 substituents ($N^+(CH_3)_3$ *vs.* $N(CH_3)_2$ *vs.* N_3), broad curdlan backbone carbon resonances were observed in the range of δ 105-68 ppm.

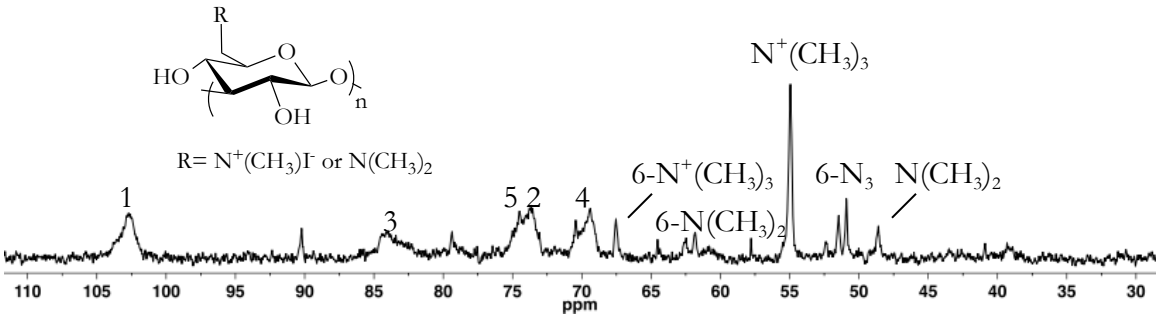


Fig. 6.5. ^{13}C NMR spectrum of 6-*N*-trimethyl curdlan iodide prepared from 6-azido-6-deoxycurdlan (2 equiv. Ph_3P/AGU, 15 equiv. CH_3I/AGU, NMP, 60 °C, 8 h).

Table 6.5.

Substitution achieved *vs.* conditions for aminocurdlan peralkylation with one-step or two-step synthetic method.

Entry	i) + Ph_3P		ii) + NaI & CH_3I			DS	
	Temp. (°C)	Time (h)	Temp. (°C)	Time (h)	CH_3I (eq/AGU)	$N(CH_3)_2$	$N^+(CH_3)_3$
1	RT	4	RT	15	1	0.55	0.07
2		7		24		0.63	0.11
3		7		24	5	0.19	0.66
4		14	60	8	1	0.48	0.43

5		4		15		0.47	0.42
6	One-step: + Ph_3P, NaI, CH_3I (15 eq/AGU) 60 °C, 8 h					--	

The two-step approach of aminocurdlan peralkylation involved the addition of Ph_3P to first generate the iminophosphorane ylide followed by adding CH_3I. We varied the reaction temperature and time for each step as well as the added methyl iodide amount (**Table 6.5**, **entry 1-5**) to investigate their effects on producing trimethyl and dimethyl amino curdlan derivatives. From the proton NMR spectrum of **entry 3**, the apparent peak at δ 3.1 ppm (**Fig. 6.6**) was assigned to the trimethyl ammonio group. To further confirm the peak assignment, we carried out a Heteronuclear Single-Quantum Correlation NMR experiment (HSQC, **Fig. 6.7**), in which the cross peak between the *N*-trimethyl carbon at δ 55 ppm and a proton signal at δ 3.1 ppm was diagnostic of the assignment of *N*-trimethyl protons.

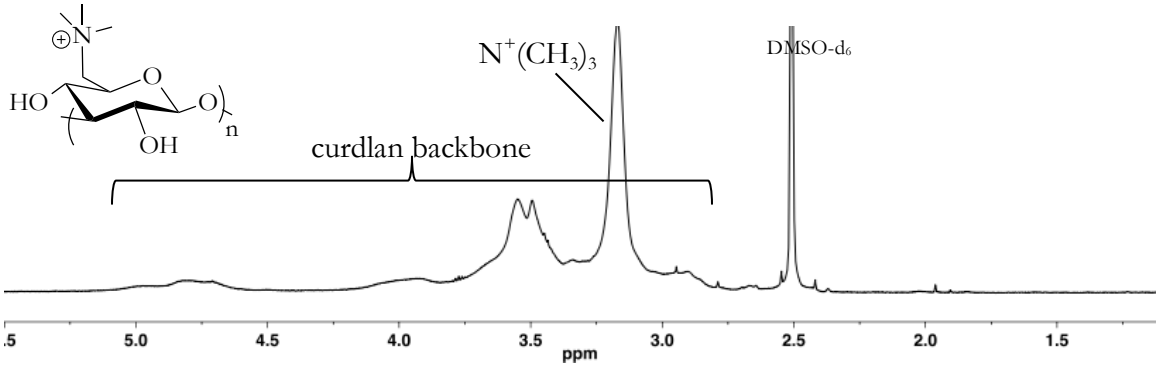


Fig. 6.6. ^{1}H NMR (in DMSO-d_6) spectrum of 6-*N*-trimethyl curdlan iodide prepared from 6-azido-6-deoxycurdlan in NMP (**Table 6.4**, **entry 3**).

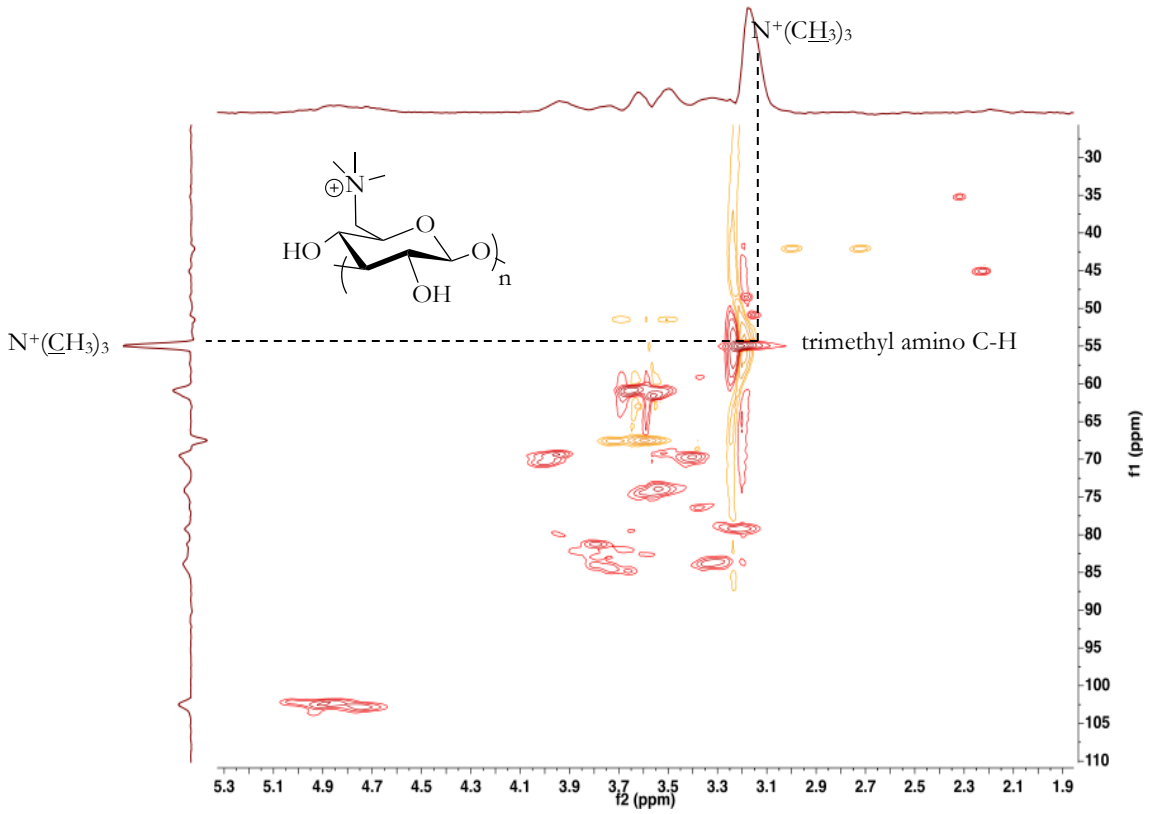


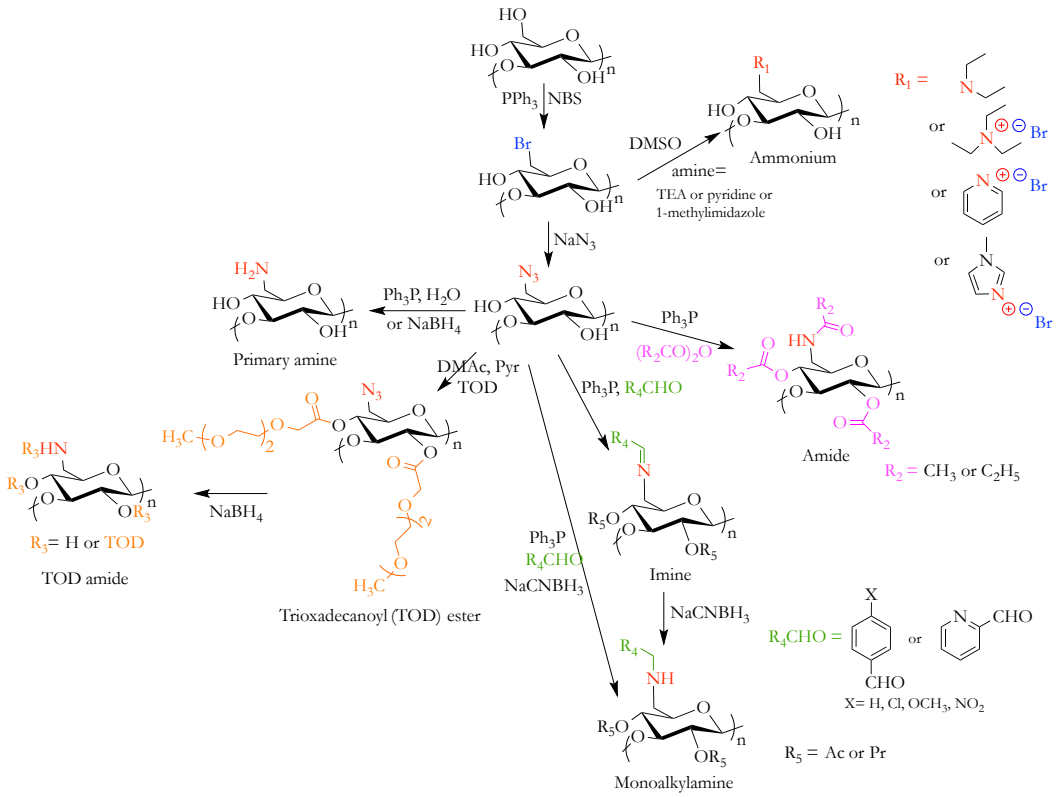
Fig. 6.7. HSQC NMR (in D_2O) spectrum of 6-deoxy-6*N*-trimethylammoniocurdlan iodide prepared from 6-azido-6-deoxycurdlan in NMP (**Table 6.4**, **entry 3**).

Fig. S6.5 shows the stacked ^{1}H NMR spectra of **Table 6.5 entries 1-5** prepared under different reaction conditions, in which the trimethyl and dimethyl resonances appeared around δ 3.1 and 3.3 ppm respectively, partially overlapping with those of curdlan backbone protons (δ 3.0-5.0 ppm). With 1 equiv. of methyl iodide, **entry 5** preformed at 60 °C afforded higher $DS_{N+(CH3)3}$ than **entry 1** at ambient temperature. By increasing the molar ratio of CH_3I to 5 equiv., the $DS_{N+(CH3)3}$ reached 0.66 (**entry 3**) while the $DS_{N(CH3)2}$ was lower than **entry 2** due to higher conversion of trialkylamine to tetraalkyl ammonium. We also explored varying the time for each step (**entry 4** *vs.* **5**), however this had little impact on DS values, indicating that the shorter reaction times were quite adequate. Importantly, all of the ammoniocurdlan derivatives so synthesized, throughout this di-/trimethyl ratio ($DS_{N(CH3)2}$/ $DS_{N+(CH3)3}$) range, are readily soluble in water.

6.5 Conclusions

We have investigated two nucleophilic reactions of the ylide (iminophosphorane intermediate) obtained by Staudinger reduction of 6-azido-6-deoxycurdlans, for the synthesis of 6-aminated curdlans, specifically reductive amination and alkylation. 6-Azido-6-deoxy-(2,4-di-*O*-acetyl-)curdlan was first exposed to Ph_3P in an appropriate solvent to create the highly nucleophilic iminophosphorane. By adding aldehydes in the anhydrous environment, a series of 6-imino curdlans with different functionalities at C-6 were synthesized with DS_{imine} up to 1.0. The resultant imino derivatives were further reduced by borohydride to produce the corresponding 6-monoalkylamino-6-deoxycurdlans. Alternatively, a one-pot reductive amination method was created by adding Ph_3P, aldehyde together with the reducing agent without the isolation of imino intermediate to achieve the same aminocurdlans, thus providing a new strategy for regioselective incorporation of a range of monoalkylamine functional pendants at C-6 of curdlan. In addition, as an alternative to trimethylchitosan polymers as promising paracellular permeability enhancers in tight junction opening for oral delivery of protein drugs, we prepared a range of 6-di/tri-alkylamino curdlans by alkylation of the Staudinger ylide with excess alkyl iodide. In the two-step route, by selecting the correct reaction temperature and molar excess of methyl iodide, we can control the $DS_{dialkylamine}$ *vs.* $DS_{trialkylamine}$. This class of water-soluble curdlan derivatives having tunable charge density can also be further modified with hydrophobic or ionic moieties for potential pharmaceutical use.

So far we have developed the synthetic strategies for a family of regioselectively aminated curdlan derivatives including 6-imino-, 6-amido-, 6-primary/secondary/tertiary-amino and 6-quaternary ammonium curdlans starting from 6-bromo/azido-6-deoxycurdlan (**Scheme. 6.2**) The S_N2 substitution reaction to synthesize curdlan derivatives brominated at C-6 affords perfect regioselectivity and has served as a gateway reaction to incorporate a range of pendent functionalities with desired properties. The comprehensive syntheses open doors to a wide variety of potentially useful aminated polymers in biomedical and pharmaceutical fields. We can expect further exploitation of this sort of chemistry as well to involve other chemical modifications on curdlan, taking advantage of its very high regioselectivity.



Scheme. 6.2. Comprehensive syntheses of regioselectively aminated curdlan derivatives at C-6.

6.6 Supplemental material

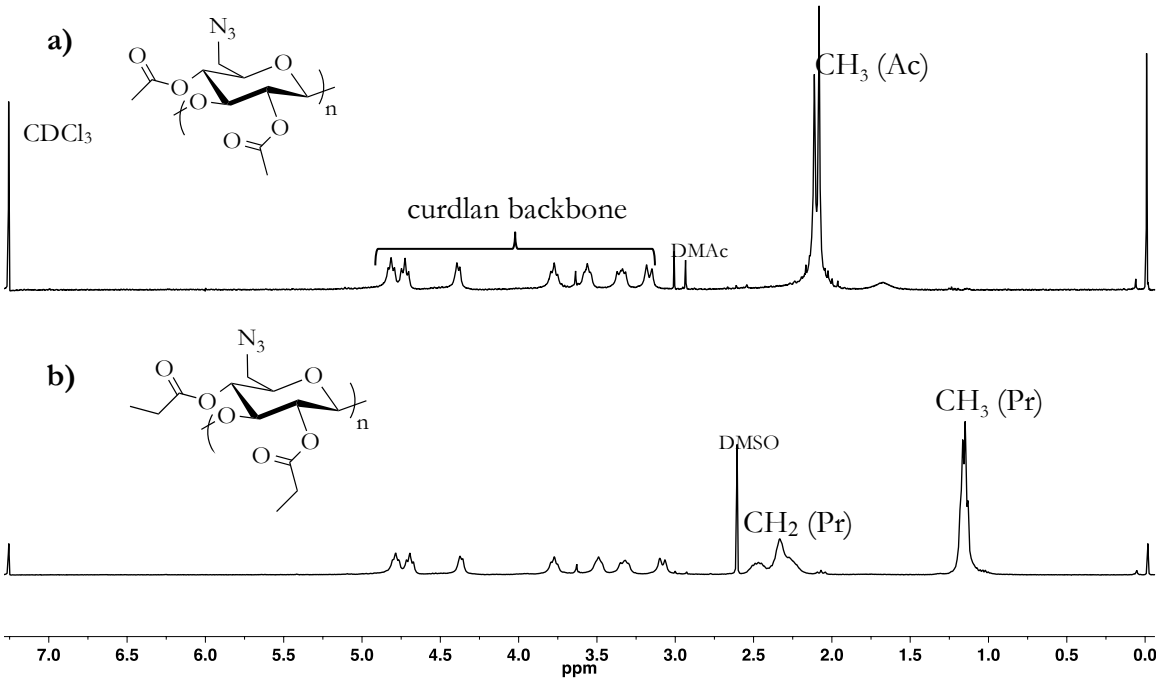


Fig. S6.1. ^{1}H NMR spectra (DS_{ester} =2.0) of: **(a)** 6-azido-6-deoxy-2,4-di-*O*-acetyl-curdlan and **(b)** 6-azido-6-deoxy-2,4-di-*O*-propionyl-curdlan.

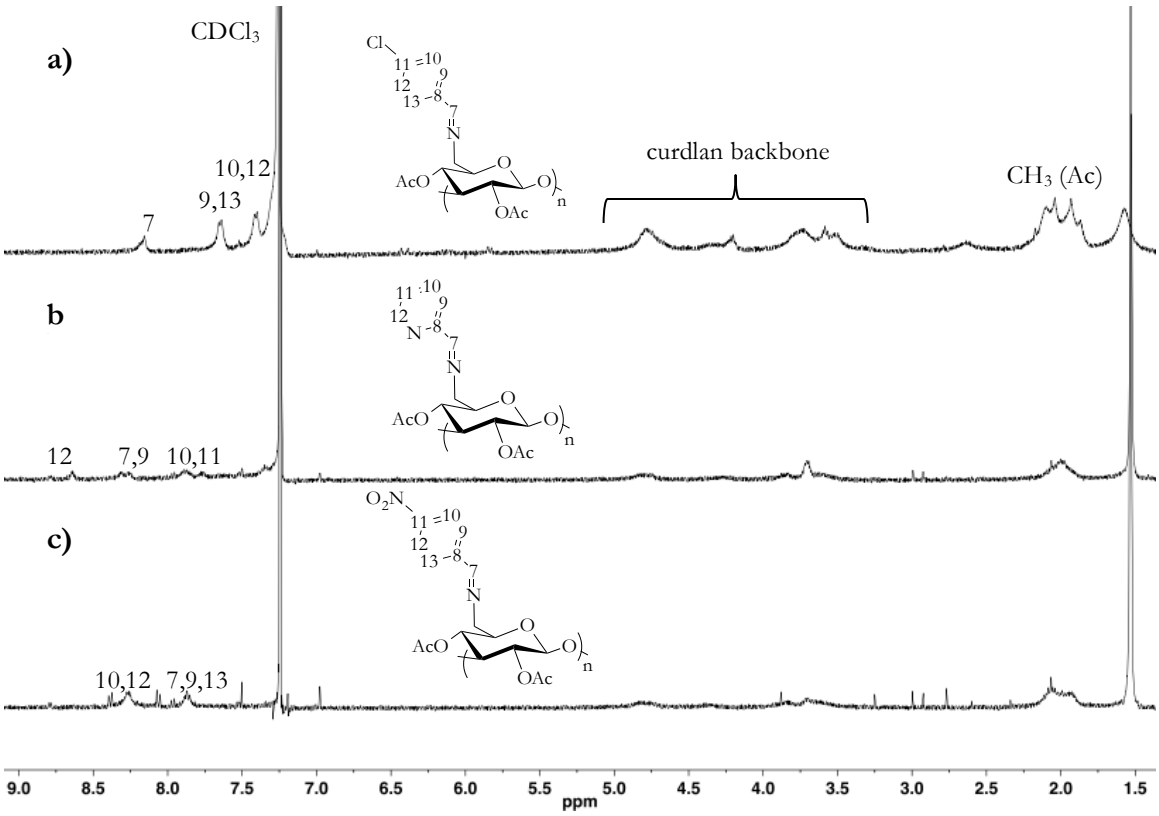


Fig. S6.2. ^{1}H NMR spectra of **(a)** 6-chlorobenzimino-6-deoxy-2,4-di-*O*-acetyl-curdlan and **(b)** 6- pyridinecarboximino-6-deoxy-2,4-di-*O*-propionyl-curdlan and **(c)** 6-nitrobenzimino-6-deoxy-2,4-di-*O*-acetyl-curdlan (30 equiv. aldehyde/AGU, THF, RT, 24 h, molecular sieves).

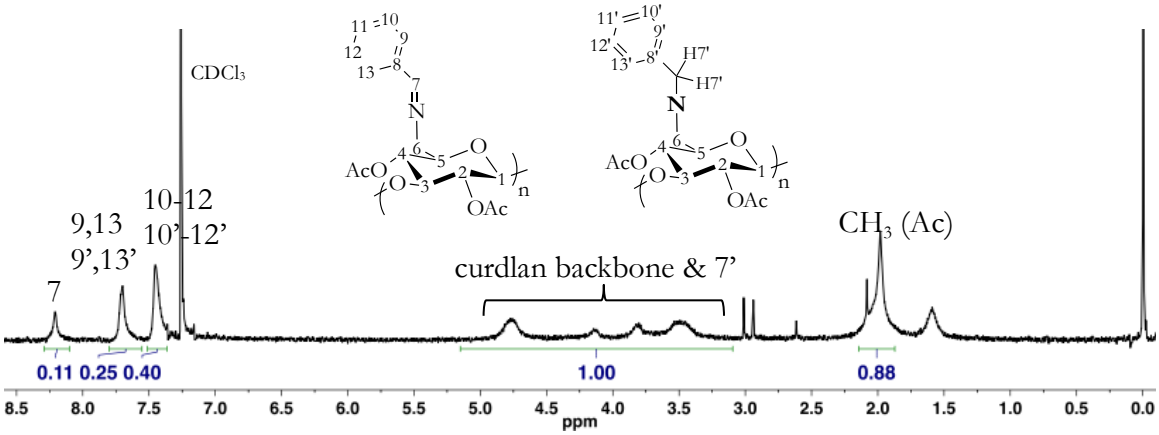


Fig. S6.3. ^{1}H NMR (in $CDCl_3$) spectrum of 6-benzamino-6-deoxy-2,4-di-*O*-acetyl-curdlan reduced by $NaCNBH_3$ (**Table 6.2, entry 1**).

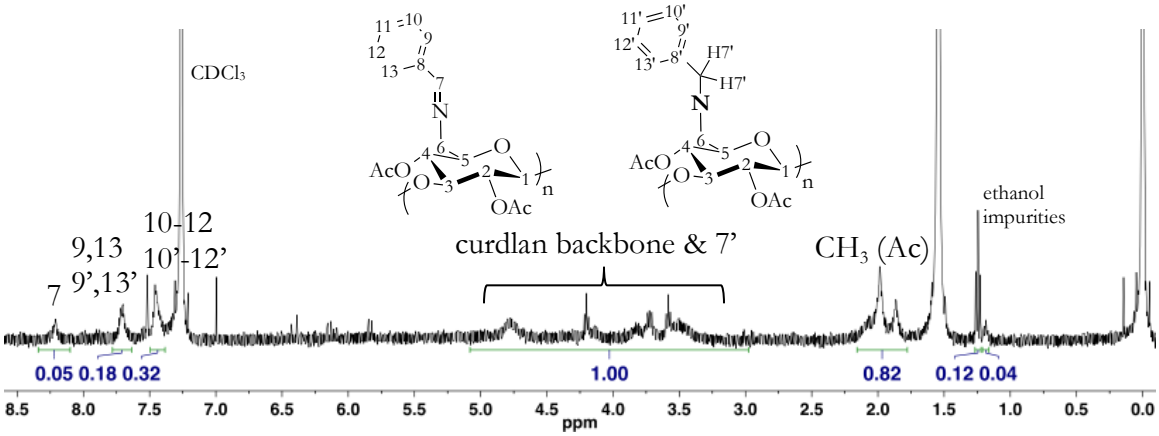


Fig. S6.4. ^{1}H NMR (in DMSO-d$_6$) spectrum of 6-benzamino-6-deoxy-2,4-di-*O*-acetyl-curdlan prepared by one-pot method (**Table 6.3**, **entry 3**).

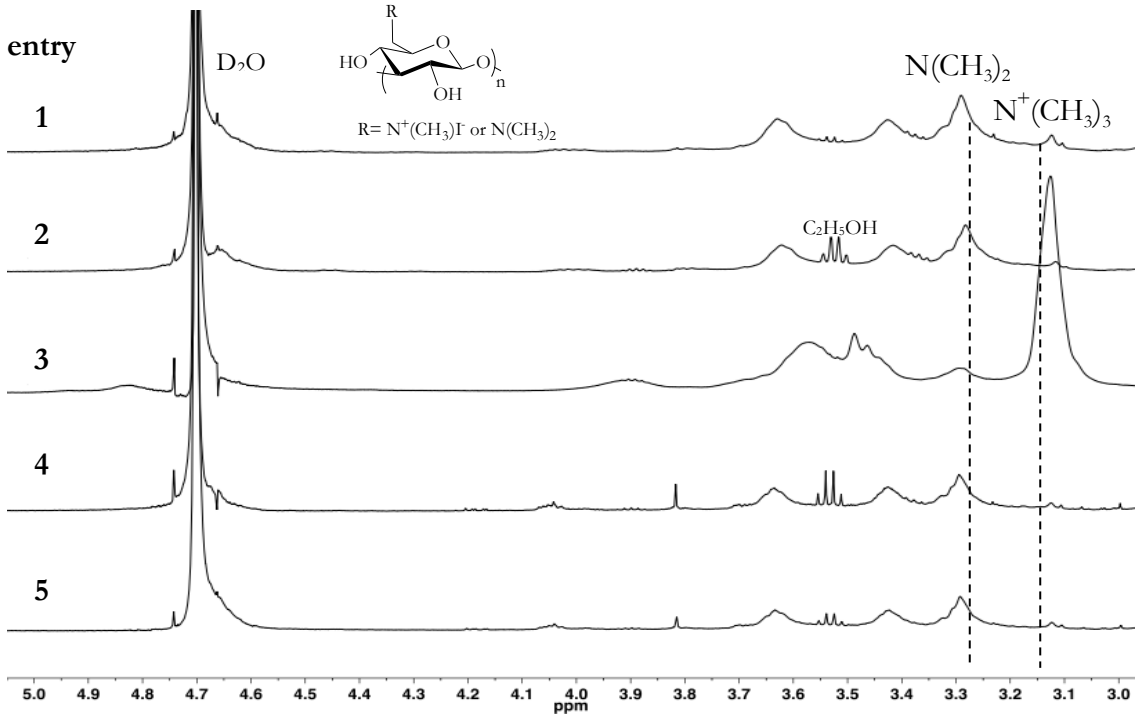


Fig. S6.5. ^{1}H NMR (in D$_2$O) spectra of peralkylated 6-aminocurdlans prepared from 6-azido-6-deoxycurdlan in NMP (**Table 6.4**, **entry 1-5**).

6.7 Acknowledgements

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Biomaterials (SBIO) at Virginia Tech for their financial, facilities and educational support. We thank the USDA for partial support of this work through grant No. 2011-67009-20090.

6.8 References

1. Marubayashi, H.; Yukinaka, K.; Enomoto-Rogers, Y.; Takemura, A.; Iwata, T., Curdlan ester derivatives: Synthesis, structure, and properties. *Carbohydrate Polymers* **2014,** *103*, 427-433.

2. Jin, Y.; Zhang, H.; Yin, Y.; Nishinari, K., Comparison of curdlan and its carboxymethylated derivative by means of Rheology, DSC, and AFM. *Carbohydrate Research* **2006,** *341* (1), 90-99.

3. Suflet, D. M.; Nicolescu, A.; Popescu, I.; Chitanu, G. C., Phosphorylated polysaccharides. 3. Synthesis of phosphorylated curdlan and its polyelectrolyte behaviour compared with other phosphorylated polysaccharides. *Carbohydr. Polym.* **2011,** *84*, 1176-1181.

4. (a) Yoshida, T.; Hatanaka, K.; Uryu, T.; Kaneko, Y.; Suzuki, E.; Miyano, H.; Mimura, T.; Yoshida, O.; Yamamoto, N., Synthesis and structural analysis of curdlan sulfate with a potent inhibitory effect in vitro of AIDS virus infection. *Macromolecules* **1990,** *23*, 3717-2; (b) Osawa, Z.; Morota, T.; Hatanaka, K.; Akaike, T.; Matsuzaki, K.; Nakashima, H.; Yamamoto, N.; Suzuki, E.; Miyano, H.; et, a., Synthesis of sulfated derivatives of curdlan and their anti-HIV activity. *Carbohydr. Polym.* **1993,** *21*, 283-8.

5. (a) Tamura, N.; Hirota, M.; Saito, T.; Isogai, A., Oxidation of curdlan and other polysaccharides by 4-acetamide-TEMPO/NaClO/NaClO2 under acid conditions. *Carbohydrate Polymers* **2010,** *81* (3), 592-598; (b) Delattre, C.; Rios, L.; Laroche, C.; Le, N. H. T.; Lecerf, D.; Picton, L.; Berthon, J. Y.; Michaud, P., Production and characterization of new families of polyglucuronic acids from TEMPO-NaOCl oxidation of curdlan. *Int. J. Biol. Macromol.* **2009,** *45*, 458-462.

6. (a) Zhang, R.; Edgar, K. J., Synthesis of curdlan derivatives regioselectively modified at C-6: O-(N)-Acylated 6-amino-6-deoxycurdlan. *Carbohydrate Polymers* **2014,** *105* (0), 161-168; (b) Zhang, R.; Edgar, K. J., Water-soluble aminocurdlan derivatives by chemoselective azide reduction using NaBH4. *Carbohydrate Polymers* **2015,** *122* (0), 84-92; (c) Zhang, R.; Liu, S.; Edgar, K. J., Regioselective synthesis of cationic 6-deoxy-6-(N,N,N-trialkylammonio)curdlan derivatives. *Carbohydrate Polymers* **2016,** *136*, 474-484.

7. (a) Saxon, E.; Armstrong, J. I.; Bertozzi, C. R., A "Traceless" Staudinger Ligation for the Chemoselective Synthesis of Amide Bonds. *Organic Letters* **2000,** *2* (14), 2141-2143; (b) Saxon, E.; Bertozzi, C. R., Cell Surface Engineering by a Modified Staudinger Reaction. *Science* **2000,** *287* (5460), 2007-2010.

8. García-Álvarez, J.; García-Garrido, S. E.; Cadierno, V., Iminophosphorane–phosphines: Versatile ligands for homogeneous catalysis. *Journal of Organometallic Chemistry* **2014,** *751*, 792-808.

9. Staudinger, H.; Meyer, J., New organic compounds of phosphorus. II. Phosphazines. *Helv. Chim. Acta* **1919,** *2*, 619-35.

10. Palacios, F.; Alonso, C.; Aparicio, D.; Rubiales, G.; de los Santos, J. M., The aza-Wittig reaction. An efficient tool for the construction of carbon-nitrogen double bonds. *Tetrahedron* **2007,** *63* (3), 523-575.

11. Fox, S. C.; Edgar, K. J., Staudinger Reduction Chemistry of Cellulose: Synthesis of Selectively O-Acylated 6-Amino-6-deoxy-cellulose. *Biomacromolecules* **2012,** *13* (4), 992-1001.

12. Pereira, J.; Edgar, K., Regioselective synthesis of 6-amino- and 6-amido-6-deoxypullulans. *Cellulose* **2014,** *21* (4), 2379-2396.

13. Billman, J. H.; Diesing, A. C., Reduction of Schiff Bases with Sodium Borohydride. *The Journal of Organic Chemistry* **1957,** *22* (9), 1068-1070.

14. Lane, C. F., Sodium cyanoborohydride, a highly selective reducing agent for organic functional groups. *Synthesis* **1975,** (3), 135-46.

15. Borch, R. F.; Bernstein, M. D.; Durst, H. D., Cyanohydridoborate anion as a selective reducing agent. *J. Amer. Chem. Soc.* **1971,** *93* (12), 2897-904.

16. (a) de Britto, D.; Assis, O. B. G., A novel method for obtaining a quaternary salt of chitosan. *Carbohydrate Polymers* **2007,** *69* (2), 305-310; (b) Kotzé, A. F.; Lueßen, H. L.; de Leeuw, B. J.; de Boer, B. G.; Coos Verhoef, J.; Junginger, H. E., Comparison of the effect of different chitosan salts and N-trimethyl chitosan chloride on the permeability of intestinal epithelial cells (Caco-2). *Journal of Controlled Release* **1998,** *51* (1), 35-46; (c) Kotze, A. F.; Thanou, M. M.; Lueen, H. L.; De Boer, A. G.; Verhoef, J. C.; Junginger, H. E., Enhancement of paracellular drug transport with highly quaternized N-trimethyl chitosan chloride in neutral environments: in vitro evaluation in intestinal epithelial cells (Caco-2). *J. Pharm. Sci.* **1999,** *88* (2), 253-257.

17. (a) Polnok, A.; Borchard, G.; Verhoef, J. C.; Sarisuta, N.; Junginger, H. E., Influence of methylation process on the degree of quaternization of N-trimethyl chitosan chloride. *European Journal of Pharmaceutics and Biopharmaceutics* **2004,** *57* (1), 77-83; (b) Sieval, A. B.; Thanou, M.; Kotze, A. F.; Verhoef, J. C.; Brussee, J.; Junginger, H. E., Preparation and NMR characterization of highly substituted N-trimethyl chitosan chloride. *Carbohydr. Polym.* **1998,** *36* (2/3), 157-165; (c) Snyman, D.; Hamman, J. H.; Kotze, J. S.; Rollings, J. E.; Kotze, A. F., The relationship between the absolute molecular weight and the degree of quaternisation of N-trimethyl chitosan chloride. *Carbohydr. Polym.* **2002,** *50* (2), 145-150.

18. Zhang, R.; Liu, S.; Edgar, K. J., Regioselective synthesis of cationic 6-deoxy-6-(N, N, N-trialkylammonio)curdlan derivatives. *Carbohydrate Polymers*.

19. (a) Sieval, A. B.; Thanou, M.; Kotze´, A. F.; Verhoef, J. C.; Brussee, J.; Junginger, H. E., Preparation and NMR characterization of highly substitutedN-trimethyl chitosan chloride. *Carbohydrate Polymers* **1998,** *36* (2–3), 157-165; (b) Snyman, D.; Hamman, J. H.; Kotze, J. S.; Rollings, J. E.; Kotzé, A. F., The relationship between the absolute molecular weight and the degree of quaternisation of N-trimethyl chitosan chloride. *Carbohydrate Polymers* **2002,** *50* (2), 145-150.

Chapter 7. Glycan Ester Deacylation by TBAOH or TBAF: Regioselectivity vs. Polysaccharide Structure

Zhang, R.; Zheng, X.; Kuang, J.; Edgar, K. J. *Carbohydrate Polymers* **2014**, *113,* 159-165. Used with permission of Elsevier, 2014.

7.1 Abstract

Tetrabutylammonium fluoride (TBAF) and tetrabutylammonium hydroxide (TBAOH) have been found to mediate regioselective deacylation of cellulose esters, with unexpected selectivity for removal of acyl groups at the more hindered secondary O-2/3 positions. This simple, efficient, one-step process triggers our investigation of TBAF/TBAOH deacylation on other glycan (amylose, curdlan, dextran, pullulan and glucomannan) esters to examine the generality of this reaction and the impact of glucan and glucomannan structure on deacylation regioselectivity. Remarkably regioselective O-2/3 deacylation was observed with amylose triesters, while moderately regioselective O-2/4 deacylation of curdlan triester occurred. No regioselectivity was observed with dextran triester, as predicted due to the lack of primary OH groups. We observed deacylation of pullulan and glucomannan triesters, but due to the complexity of the partially substituted products have not yet been able to determine the deacylation regioselectivity for these glycan esters.

7.2 Introduction

Polysaccharides are ubiquitous biopolymers with a wide variety of structures, properties and functions. Esterification of polysaccharides has been a reliable and useful modification strategy, providing ready access to a broad variety of bio-based materials with desirable properties.[1] Though polysaccharide esterification has been known for over a century, it remains very challenging to control the position of acylation of polysaccharides. Regioselective substitution of polysaccharides is difficult to perform due to the slight reactivity differences among different positions and the generally poor organic solubility of polysaccharides. The most powerful approach for regioselective polysaccharide derivative synthesis has thus far been the protection/deprotection approach.[2] However, this method is

practically limited to preparation of lab scale samples due to the multiple steps involved, low overall yields, expensive reagents, and the potential for inadequate reactivity of some of the protected intermediates. Those issues motivate us to seek out an efficient, broadly applicable procedure for synthesis of regioselectively substituted polysaccharide derivatives.

In the 1990s researchers investigated the deacylation of fully substituted cellulose esters by aliphatic amines in order to synthesize regioselectively substituted derivatives but the best regioselectivity (O-6 vs. O-2/3) obtained was only around ~60%.[3] Recently Xu and Edgar[4] have discovered that tetrabutylammonium fluoride (TBAF) smoothly deacylates cellulose esters, with unexpected regioselectivity, and a remarkable predominance of deacylation at the more hindered secondary positions O-2 and O-3. This simple one-step process produces cellulose-6-*O*-esters with high regioselectivity, which can also be easily modified into cellulose-2,3-*O*-A-6-*O*-B-triesters. The mechanism of this unexpected reaction has been investigated by methods including kinetic isotope effect (KIE) studies[5]. Deacylation at the secondary O-2/3 positions occurs by an E1cB mechanism involving a ketene intermediate, while deacylation at the primary O-6 occurs by a different mechanism, general base catalysis involving a tetrahedral intermediate. A further investigation of reaction scope explored the influences of factors including solvent, temperature and water content upon deacylation regioselectivity and provided results consistent with the mechanistic proposals above[6]. Reactions of cellulose esters (acetate, propionate, butyrate, hexanoate and benzoate) with TBAF in DMSO at 50 °C for 18 h provided the most efficient conditions for regioselective deacylation at O-2/3. Reaction with the strongly basic tetrabutylammonium hydroxide (TBAOH) has been shown to afford similarly regioselective deacylation of cellulose esters[7], but with the advantages vs. TBAF of milder reaction conditions, less expensive reagent, and the potential for easier recycling by simple ion exchange. We have hypothesized that chelation between the tetrabutylammonium cation and the ester oxygen atoms of the vicinal 2,3-acyl groups is responsible for the observed regioselectivity of TBA-catalyzed deacylation.

We felt that investigating the possible extension of this chemistry to esters of other glucans and glucomannans might be of interest, not only because of the potential value of a general method for regioselective synthesis of derivatives of these polysaccharides. By studying deacylation of these other polysaccharide esters, we also hoped to gain further insight into

the source of the unusual regioselectivity of these deacylation reactions. For example, deacylation of esters of amylose (a component of plant starch[8]) would give information about the influence of anomeric stereochemistry upon deacylation selectivity, since amylose differs from cellulose nearly exclusively in that it has α-1→4 rather than β-1→4 linkages. On the other hand, deacylation of esters of unbranched dextran would provide an example in which there were no primary alcohol esters present, due to the α-1→6 backbone linkages of dextran; dextran is a bacterial polysaccharide that is often branched in nature, but in some cases, including the material used here, is unbranched. Dextran is highly water-soluble and finds wide utility in injectable formulations for human use.[9] What would be the regioselectivity of deacylation in the absence of primary alcohol esters? Esters of pullulan, a bacterial polysaccharide useful in buccal delivery formulations and food applications[10], would provide an internal comparison of results from deacylation of monosaccharides containing α-1→4 vs. α-1→6 linkages, since pullulan has a maltotriose (glucose trimer with internal α-1→4 linkages) repeat unit connected by α-1→6 linkages.

The absence of a vicinal diol structure in curdlan, due to its β-1→3 linkages, would test our previously expressed hypothesis that complexation of the tetralkylammonium ion with the 2,3-vicinal diester of cellulose esters is a primary driving force for the observed selectivity of that reaction for deacylation of the 2,3-esters. Curdlan is a glucan which is also a bacterial polysaccharide, produced by the bacterium *Alcaligenes faecalis var.* myxogenes, and is water-insoluble[11]. Curdlan is used in food applications based on its excellent thermal-dependent gelation properties, ability to mimic fat/meat mouth-feel, and ability to modify rheological properties of sauces and dairy products.[12] Finally, glucomannan esters would provide interesting information as well, since the 2,3-diol in mannose has the *cis* orientation, vs. the *trans* orientation in cellulose, amylose, dextran and pullulan. Glucomannan consists of β-1,4-linked D-glucose and D-mannose; in the sample we employed, the glucose:mannose ratio was 1.6 : 1.[13] Water-soluble glucomannan is frequently obtained from plant seeds, finds utility as a natural gum in foods, and is also used in films, coatings, and drug delivery systems.[14] We anticipated that deacylation of glucomannan esters would provide understanding of the potential influence of 2,3-diester orientation upon the proposed cation coordination mechanism.

We report herein our results with regard to extent and regioselectivity of deacylation of each of these triesters with TBAF or TBAOH, and the implications of those results for the generality and mechanism of these methods for synthesis of regioselectively substituted glucan and glucomannan esters.

7.3 Experimental

7.3.1 Materials

Amylose from potato (DP~13,000, Sigma Co.), unbranched dextran (DP~56-68, Sigma Co.) and curdlan (DP~6173, Wako Chemicals) were purchased and dried under vacuum at 40 °C overnight prior to use. Pullulan (DP~1235) was purchased from Hayashibara Company (Okayama, Japan) and was dried under vacuum at 120 °C overnight prior to use. Konjac glucomannan (KGM, DP~1475) (Propol® A) was kindly provided by Shimizu Chemical Company (Hiroshima, Japan). Tetrabutylammonium fluoride (TBAF, approximately as trihydrate), tetrabutylammonium hydroxide (TBAOH 40 wt% in water), 4-dimethylaminopyridine (DMAP), lithium chloride (LiCl), pyridine (anhydrous, 99%, AcroSeal®), tetrahydrofuran (THF, 99.8%, extra dry, AcroSeal®), acetic anhydride (Ac_2O, 99+%) and propionic anhydride (Pr_2O, 97%) were purchased from Acros Organics and used as received. *N, N*-Dimethylacetamide (DMAc, reagent grade, Fisher) and dimethyl sulfoxide (DMSO, HPLC grade, Fisher) were stored over 4 Å molecular sieves. Iodine (I_2, Fisher), methanol (HPLC grade, Fisher), ethanol (HPLC grade, Fisher) and sodium thiosulfate (anhydrous, J.B.Baker Inc.) were used as received.

7.3.2 Measurements

^1H, ^{13}C, HMBC, and COSY NMR spectra were obtained on a Bruker Avance II 500MHz spectrometer in $CDCl_3$ or DMSO-d_6 at room temperature or 50 °C, with number of scans of 32, 15,000, 19,200 and 9,400 respectively. Total and partial DS (degree of substitution) values of glycan ester products after peracylation were determined by calculating the ratios of acetyl or propionyl proton integrals to those of the backbone hydrogens.[4, 15]

7.3.3 Preparation of glycan triesters

7.3.3.1 Preparation of amylose and curdlan triesters

Dissolution of amylose or curdlan in DMAc/LiCl was adapted from a method previously reported for cellulose dissolution[16]. A mixture of dried amylose or curdlan (1 g, 6.2 mmol anhydroglucose unit (AGU)) and DMAc (37.5 mL) was kept at 150 °C for 26 min with vigorous stirring under nitrogen. Anhydrous LiCl (1.88 g) was added, and the mixture was stirred at 165 °C for 8 min. DMAc (10 mL) was distilled off to facilitate water removal. The slurry was cooled to room temperature while being stirred overnight, during which time dissolution occurred.

Synthesis of amylose or curdlan triesters was adapted from a previously published procedure[4-6]. Amylose tripropionate (ATP) or curdlan triacetate (CTA) was prepared by adding DMAP (20 mg), pyridine (5.8 mL, 10 eq per AGU) and propionic anhydride (7.9 mL, 10 eq per AGU) or acetic anhydride (5.8 mL, 10 eq per AGU) to the amylose or curdlan solution. The solution was kept at 80 °C for 24 h and then added slowly to 500 mL deionized water under vigorous stirring. The crude product was isolated by filtration, and then re-dissolved in 10 mL chloroform. This solution was added slowly with rapid stirring to 50 mL of ethanol. After filtration and washing with ethanol and water several times, the sample was dried under vacuum at 40 °C overnight. DS_{Pr}(ATP) = 3.0 and DS_{Ac}(CTA) = 3.0 by ^{1}H NMR ($CDCl_3$): ATP: δ 0.86-1.26 (CH_3-propionate), δ 2.02-2.54 (CH_2-propionate), δ 3.70-5.52 (amylose backbone); CTA: δ 1.84-2.19 (CH_3-acetate), δ 3.49-5.07 (curdlan backbone). Yields: ATP 80%, CTA 89%.

7.3.3.2 Preparation of dextran triester

Synthesis of dextran triacetate (DTA) was performed by a previously published procedure[17]. Iodine (0.5 g) was added to a three-necked round bottom flask. Acetic anhydride (5.8 mL, 10 eq per AGU) was added and the reaction mixture was kept under stirring for 15 min. Pre-dried dextran (1.0 g, 6.2 mmol) was added and the resulting mixture was refluxed at 50 °C for 3 h under nitrogen. The excess of iodine (catalyst) was removed by adding a saturated aqueous sodium thiosulfate solution to the reaction mixture. The thus formed precipitate was filtered off, washed twice with methanol and cold water, and then dried under vacuum

at 40 °C overnight. DS_{Ac}(DTA) = 3.0 by ^{1}H NMR ($CDCl_3$): δ 1.85-2.18 (CH_3-acetate), δ 3.39-5.65 (dextran backbone). Yield: 92%.

7.3.3.3 Preparation of pullulan triester

Synthesis of pullulan triacetate (PTA) was performed by a previously published procedure[18]. Pullulan (2 g, hydroxyl group 37 mmol) was dissolved in DMAc (50 mL). Pyridine (8.8 mL, 10 eq per mol anhydroglucose unit (AGU) of pullulan,) was added to the solution, followed by addition of acetyl chloride (10 mL, 10 eq per mol AGU of pullulan). The mixture was stirred at 60 °C for 20 h, and then the solution was added slowly to ethanol (500 mL) under vigorous stirring. The formed precipitate was filtered and washed with ethanol several times, and dried under vacuum at 40 °C overnight. DS_{Ac}(PTA) = 3.0 by ^{1}H NMR (DMSO-d_6): δ 1.80-2.20 (CH_3-acetate), δ 3.42-5.67 (pullulan backbone). Yield: 82%.

7.3.3.4 Preparation of glucomannan triester

Synthesis of glucomannan triacetate (GTA) was performed as previously described.[19] Briefly, glucomannan (0.5 g) was pretreated by dissolving in water (50 mL) and freeze-drying. A pre-mixed solution of acetic acid (20 mL) and trifluoroacetic anhydride (TFAA, 20 mL), which had been stirred at 50 °C for 20 min, was immediately added to the freeze-dried glucomannan. The reaction solution was stirred at 50 °C for 1 h under nitrogen. After cooling to room temperature, the solution was slowly added to ethanol (500 mL). The crude product was collected by filtration, re-dissolved in chloroform (10 mL), and re-precipitated into ethanol for further purification. The sample was dried under vacuum at 40 °C overnight. DS_{Ac}(GTA) = 3.0 by ^{1}H NMR ($CDCl_3$): δ 1.86-2.10 (CH_3-acetate), δ 3.36-5.32 (glucomannan backbone). Yield: 84%.

7.3.4 General procedures for deacylation of glycan triesters

7.3.4.1 Tetrabutylammonium fluoride deacylation of glycan triesters

To a solution of glycan triester in DMSO or THF (40 mL per g of esters) was added TBAF trihydrate (4 eq per mol glucopyranosyl group), and the reaction solution was kept at 50 °C for 24 h. For CTA, PTA and GTA, the solution was added slowly to water (250 mL). For ATP, the solution was transferred to 3,500 g/mol MWCO dialysis tubing that was then

placed in a large beaker containing water for 3 days. For DTA, the solution was added slowly to methanol (250 mL). The thus formed precipitate was collected by filtration, washed several times with water or methanol, and dried under vacuum at 40 °C overnight. The samples were further perpropionylated or peracetylated (details below) for DS determination.

7.3.4.2 Tetrabutylammonium hydroxide deacylation of glycan triesters

To a solution of glycan triester in DMSO or THF (40 mL per g of ester) was added TBAOH (2 eq per mol glucopyranosyl group), and the reaction solution was kept at room temperature for 1 h or at 50 °C for 24 h. For CTA, PTA and GTA, the solution was added slowly to water (250 mL). For ATP, the solution was transferred to 3,500 g/mol MWCO dialysis tubing that was then placed in a large beaker containing water for 3 days. For DTA, the solution was added slowly to methanol (250 mL). The thus formed precipitate was collected by filtration, washed several times with water or methanol, and dried under vacuum at 40 °C overnight. The samples were further perpropionylated or peracetylated (details below) for DS determination.

7.3.5 General procedure for peracylation of deacylated glycan esters

Deacylated products were peracetylated or perpropionylated for easier NMR analysis, according to previously published procedures.[4, 15] DMAP (15 mg) and acetic anhydride (4 mL) or propionic anhydride (4 mL) were added to the solution of deacylated glycan esters (0.3 g) in pyridine (4 mL). After stirring at 80 °C for 24 h, the crude product was obtained by precipitation into water or ethanol (200 mL) and washed several times by water or ethanol. Re-dissolving the crude product in chloroform and re-precipitating it into ethanol further purified the product. The sample was dried under vacuum at 40 °C overnight for analysis. The total and partial DS values for the peracetylated or perpropionylated products were determined according to **Eqs** (**7.1**) and (**7.2**) by ^{1}H NMR.

$$DS_{ester} = 3 - \frac{7I_{H,acetyl\ or\ propionyl}}{3I_{H,AGU}}; I = integral \quad \textbf{(Eq. 7.1)}$$

$$DS_{ester(n)} = 1 - \frac{7I_{H,acetyl\ or\ propionyl\ (n)}}{3I_{H,AGU}}; I = integral, n = Position\ 2, 3, 4\ or\ 6 \quad \textbf{(Eq. 7.2)}$$

7.4. Results and discussion

7.4.1 Deacylation of amylose tripropionate (ATP)

Due to the poor solubility of amylose triacetate, ATP was chosen as the deacylation substrate. Conditions chosen were those that had worked well with cellulose esters (4 equiv. TBAF, THF solvent, 50 °C, 24 h)[4]. The product amylose propionate had DS_{Pr} of approximately 1.03, as calculated by ^{1}H NMR peak integration (**Fig. 7.1**). Two strong resonances for the acetyl methyl groups appeared at δ 1.9 – 2.1. To further confirm the positions of ATP deacylation and calculate the partial DS of each position, we carried out a heteronuclear multibond correlation NMR experiment (HMBC), in which the cross peaks between ester carbonyls and the nearest ring hydrogens of the AGU (three-bond correlation) are diagnostic of the position of substitution. Two clear correlation peaks were observed between the two diastereotopic H-6 resonances at 4.45 and 4.19 ppm and the propionate carbonyl ^{13}C resonance at 174 ppm (**Fig. 7.2**). The calculated partial $DS_{Ac(2)}$=0.97 and $DS_{Ac(3)}$=0.98 supported nearly perfectly regioselective deacylation of ATP at O-2/3 (98% regioselectivity, **Table 7.1**, **entry 1**).

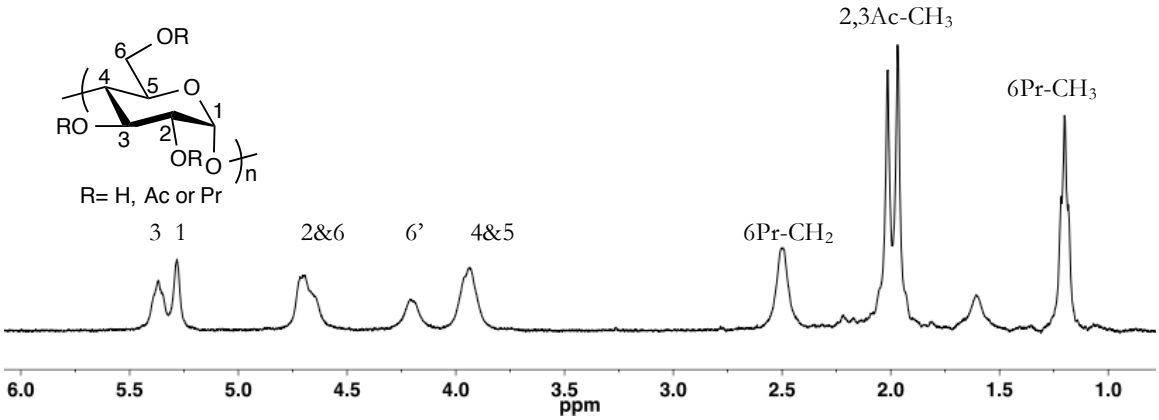


Fig. 7.1. ^{1}H NMR spectrum of the product of amylose tripropionate deacylation by TBAF (24 h, THF, 50 °C), after peracetylation.

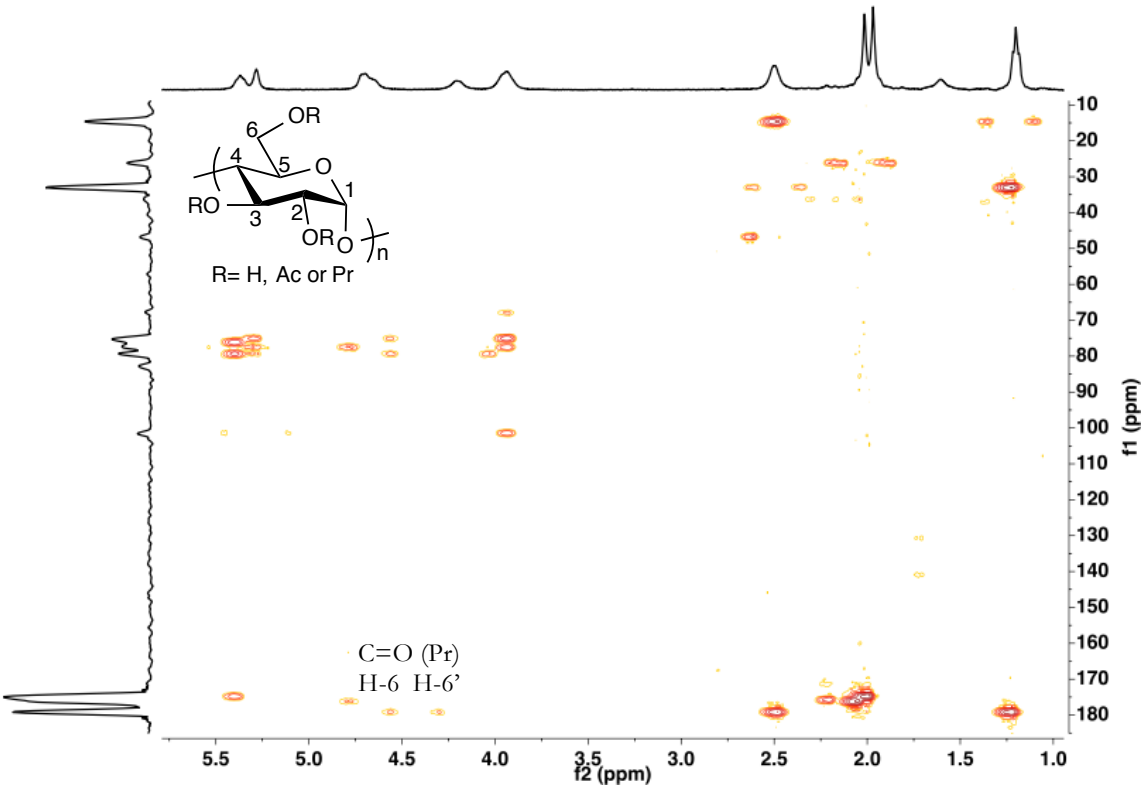


Fig. 7.2. HMBC NMR spectrum of the product of amylose tripropionate deacylation by TBAF (24 h, THF, 50 °C), after peracetylation.

Reactions of ATP with TBAOH were performed to study the impact on regioselectivity of varying reaction time, temperature and reagent molar ratios (**Table 7.1**). Treatments of ATP with 1 eq/AGU TBAOH (**entry 2**, **3**, **4**) provided incomplete deacylation of propionyl groups at O-2/3. Treatments of ATP with 2 eq/AGU TBAOH (**entry 5**, **6**, **7**) gave highly regioselective deacylation, affording a product with residual propionate DS_{Pr} 0.92-0.99. Under the best conditions TBAOH deacylation was also nearly perfectly regioselective (99% regioselectivity). The ^{1}H NMR spectra showed the absence of propionate esters at O-2/3, similar to the results with TBAF.

Table 7.1.

Results of TBAF/TBAOH deacylation of ATP in THF.

Entry	TBAF/ TBAOH (eq/AGU)[a]	Temp. (°C)	Time (h)	$DS_{Pr(6)}$	$DS_{Pr(2+3)}$	$DS_{Pr(total)}$	PR[b]

0	0	--	--	1.00	2.00	3.00	--
1	4	50	24	0.98	0.05	1.03	98%
2	1	50	24	0.98	0.72	1.70	97%
3	1	50	1	0.98	0.88	1.86	96%
4	1	RT	1	0.99	1.03	2.02	98%
5	2	50	24	0.99	0.03	1.02	99%
6	2	50	1	0.95	0.02	0.97	95%
7	2	RT	1	0.92	0.07	0.99	92%

[a]Entry 1: TBAF Entry 2~7: TBAOH

[b]Percent regioselectivity (PR)= $\frac{DS_{2+3(S)}-DS_{2+3(P)}}{DS_{total(S)}-DS_{total(P)}} - \frac{DS_{6(S)}-DS_{6(P)}}{DS_{total(S)}-DS_{total(P)}}$;

S=starting amylose tripropionate, P=product.

7.4.2 Deacylation of curdlan triacetate (CTA)

Curdlan triacetate (DS_{Ac} 3.0) was subjected to deacylation with TBAF in THF (4 equiv, 50 °C, 24 h). The precedent of preference for TBAF deacylation of cellulose and amylose esters at the secondary positions might lead one to expect selective TBAF deacylation of curdlan triacetate at the O-2/4 positions; however, the absence of the vicinal diester functionality in curdlan triacetate (*vs.* the presence of the vicinal 2,3-diacetate groups in cellulose and amylose triacetates) might be expected to reduce regioselectivity if cation coordination by the vicinal diester were, as we have hypothesized, the driving force of regioselectivity. The product of TBAF deacylation of curdlan triacetate, upon perpropionylation, displayed peaks around δ 2.1 – 2.6 and δ 0.9 – 1.3 in the proton NMR spectrum (**Fig. 7.3**). We assigned these resonances to propionyl methylene and methyl groups, indirectly indicating the expected acetate deacylation with DS_{Ac} of 1.79. HMBC was performed to assign the positions of substitution and thus reveal deacylation regioselectivity. The HMBC spectrum showed a clear correlation peak between the H-2/4 resonances at 4.7 and the propionate carbonyl ^{13}C resonance at 172 ppm (**Fig. 7.4**). Due to the overlapping of acetyl protons at different positions, it was difficult to calculate the partial DS values of acetyl residues at O-2/4/6 after deacylation. Interestingly, the ^{1}H NMR spectrum of the unperpropionylated product after TBAF treatment (**Fig. S7.3**) demonstrated a well-separated acetyl peak for each position even though this particular sample contained a small amount of tetrabutylammonium salt

contaminant. Partial DS values were calculated as $DS_{Ac(2)}$=0.50, $DS_{Ac(4)}$=0.51 and $DS_{Ac(6)}$=0.78 with a percent regioselectivity (PR) around 64%.

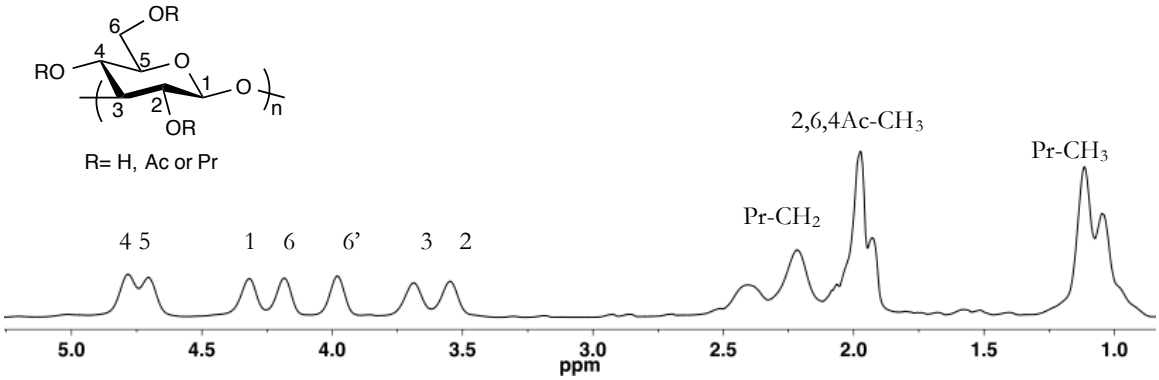


Fig. 7.3. ^{1}H NMR spectrum of the product of curdlan triacetate deacylation by TBAF (24 h, THF, 50 °C), after perpropionylation.

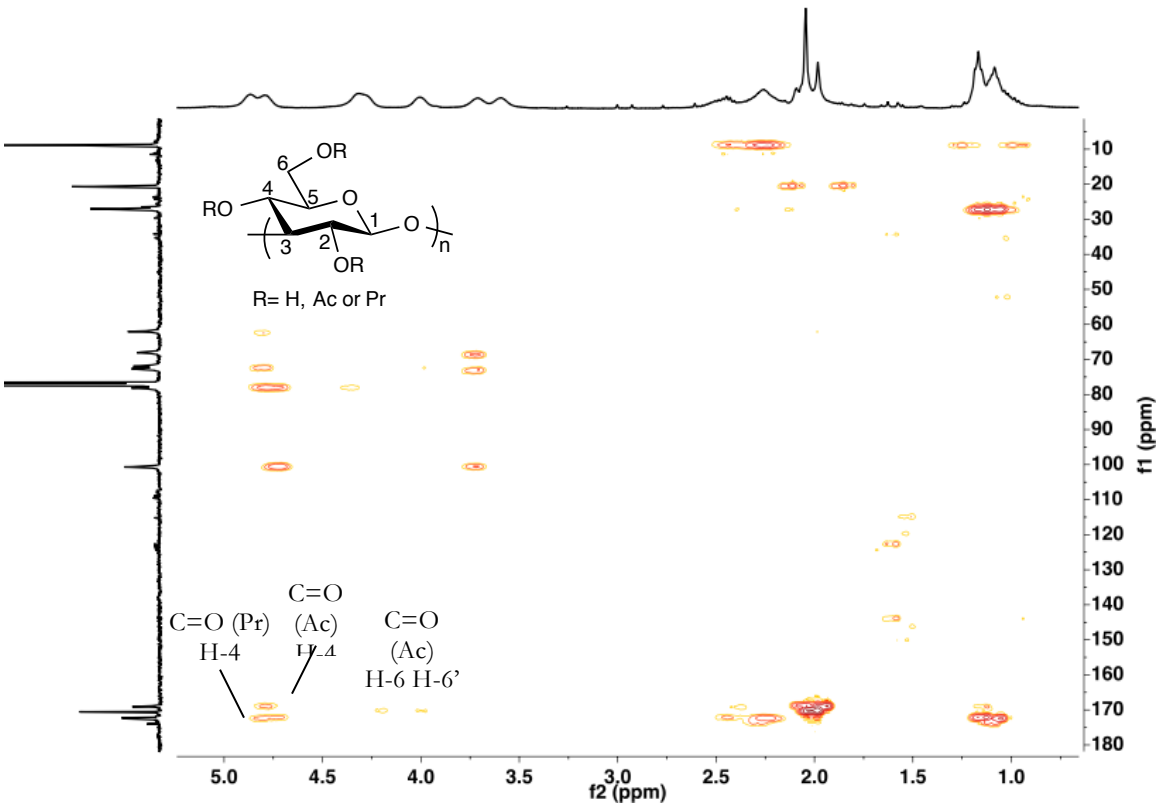


Fig. 7.4. HMBC NMR spectrum of the product of curdlan triacetate deacylation by TBAF (24 h, THF, 50 °C), after perpropionylation.

Reaction of CTA with 2 equiv. TBAOH at room temperature for 1 h provided similar deacylation extent and regioselectivity to that afforded at longer time and higher temperature (50 °C for 24 h), indicating that deacylation is rapid even under mild conditions. Three separate peaks around δ 1.97-2.13 (**Fig. 7.5**) arise from the residual acetyl methyl groups at O-2/4/6 with $DS_{total(Ac)}$ of 1.19. Partial DS_{Ac} values for each position were determined by the ratio of the partial acetyl resonances (O-2 δ 2.09-2.13, O-6 δ 2.03-2.08, O-4 δ 1.97-2.01) to the integrals of the curdlan backbone protons. The resultant curdlan acetate with $DS_{Ac(6)}$=0.44, $DS_{Ac(2)}$=0.28, and $DS_{Ac(4)}$=0.30 indicated slight preference for deacylation at O-2/4 over O-6, with almost equal levels of deacylation occurring at the two secondary positions (O-2 and O-4). This is consistent with the hypothesis that coordination of the cation by vicinal ester groups is necessary for maximum selectivity for secondary alcohol ester deacylation.

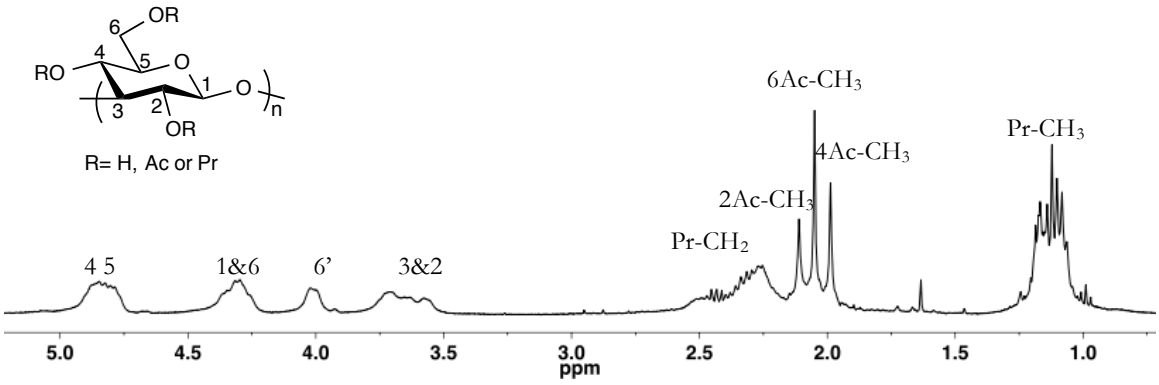


Fig. 7.5. ^{1}H NMR spectrum of the product of curdlan triacetate deacylation by TBAOH (1 h, DMSO, RT), after perpropionylation.

7.4.3 Deacylation of dextran triacetate (DTA)

TBAF deacylation of the triacetate ester of unbranched dextran (DTA, DS_{Ac} 3.0) was carried out under the same conditions as for amylose tripropionate and curdlan triacetate (4 equiv. TBAF, THF solvent, 50 °C for 24 h). The resulting dextran acetate had DS_{Ac} 2.05 (**Fig. 7.6**). HMBC analysis combined with ^{1}H NMR integration again provided information about regioselectivity. Due to the overlapping resonances of the O-2/4 acetyl groups, we were not able to ascertain partial DS values for every individual position, but were able to determine that $DS_{Ac(2+4)}$ = 1.28 and $DS_{Ac(3)}$ = 0.61. This is consistent with the interpretation that

deacylation of the acetyl groups at O-2 and O-4 proceeded to the same extent as that observed at O-3, since $DS_{Ac(2+4)}$ is almost twice $DS_{Ac(3)}$. These results support our hypothesis that reaction rates at all three ester groups of unbranched dextran triacetate should be similar, since all three are esters of secondary alcohols, and since vicinal diester relationships exist between both the O-2 and O-3 positions, and the O-3 and O-4 positions.

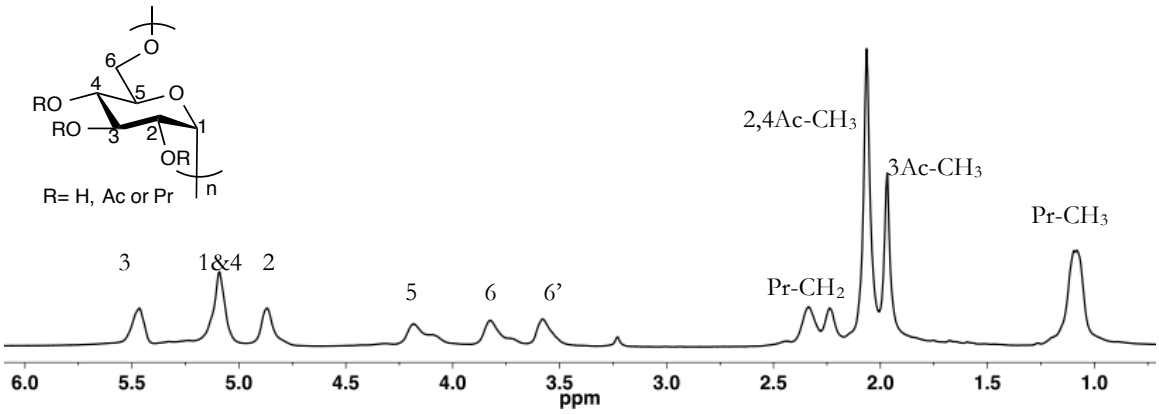


Fig. 7.6. ^{1}H NMR spectrum of the product of dextran triacetate deacylation by TBAF (24 h, THF, 50 °C), after perpropionylation.

We carried out deacylation treatments in which dextran triacetate was exposed to TBAOH, examining the effect of TBAOH/DTA stoichiometry (**Table 7.2**). After 1 h reaction of DTA with 1 equiv. TBAOH in THF (entry 2), the DS acetate was reduced from 3.0 to 1.93 with $DS_{Ac(2+4)}$=1.28 and $DS_{Ac(3)}$=0.65, similar results as for TBAF deacylation. Using 2 equiv. TBAOH, the residual DS_{Ac} at O-2/4 and O-3 decreased to the half of above values, with $DS_{Ac(2+4)}$ again approximately twice $DS_{Ac(3)}$, indicating that TBAOH deacylation also led to very similar levels of deacylation of each secondary alcohol acetate. On the other hand, 3 equiv. TBAOH led to complete deacylation of DTA, as expected. TBAOH deacylation of dextran triacetate is rapid, quantitative, and appears not to be regioselective, consistent with our predictions based on mechanistic understanding and experience with the reaction.

Table 7.2.

Results of TBAOH deacylation of DTA.[a]

Entry	TBAOH (eq/AGU)	$DS_{Ac(2+4)}$	$DS_{Ac(3)}$	$DS_{Ac(total)}$

1	0	2.00	1.00	3.00
2	1	1.28	0.65	1.93
3	2	0.63	0.32	0.95
4	3	0	0	0

[a] Starting DTA DS 3.0, solvent THF, room temperature, time 1 h.

7.4.4 Deacylation of pullulan triacetate (PTA)

Deacylation of PTA was carried out under the standard TBAF conditions (4 equiv. TBAF, DMSO, 50 °C, 24h). This afforded pullulan acetate with DS_{Ac} 1.36. **Fig. 7.7** shows the ^{1}H NMR spectrum of the PTA deacylation product after perpropionylation. Unfortunately, the acetyl proton resonances for the different positions overlap severely, so it was difficult to completely assign the acetyl protons. Reaction of PTA with 2 equiv. TBAOH for 24 h at 50 °C gave the resulting pullulan acetate with DS_{Ac} of 1.21; however in this case also it was difficult to determine regioselectivity by ^{1}H NMR due to excessive signal overlap.

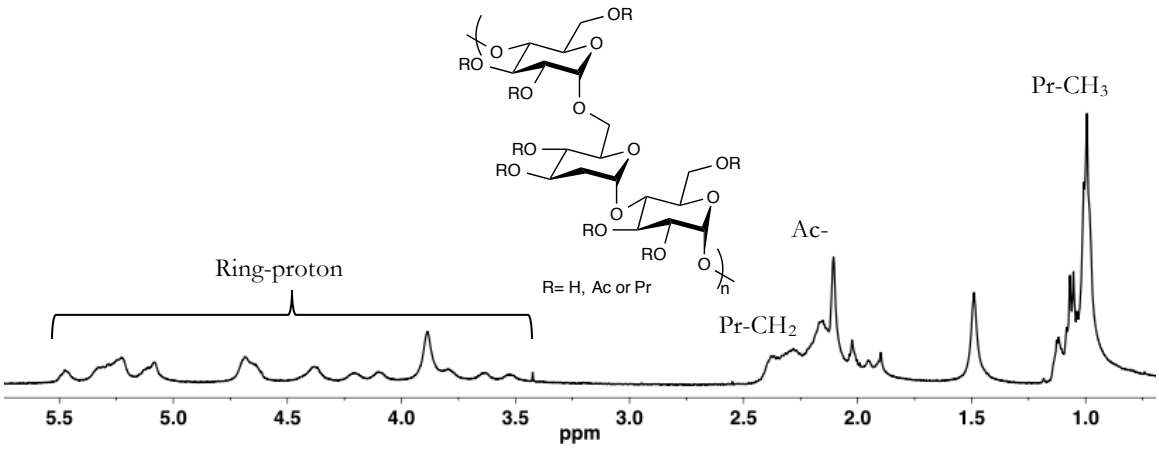


Fig. 7.7. ^{1}H NMR spectrum of the product of pullulan triacetate deacylation by TBAF (24 h, DMSO, 50 °C), after perpropionylation.

7.4.5 Deacylation of glucomannan triacetate (GTA)

Reaction of GTA with 4 equiv. TBAF for 24 h at 50 °C in DMSO provided successful deacylation, affording glucomannan acetate with DS_{Ac} of 1.10 (**Fig. S7.4**). Even though the backbone protons of glucomannan acetate have been completely assigned by a previously published paper[19], it was difficult to determine regioselectivity due to significant signal

overlap in these partially substituted glucomannan ester products. The signals at 2.04 - 2.14 ppm were assigned to C6 acetates of glucose and mannose, C2 acetates of mannose, and C3 acetates of glucose. The resonances between 1.90 - 2.01 ppm were assigned to C2 acetates of glucose and C3 acetates of mannose. Reaction of GTA with 2 equiv. TBAOH for 24 h at 50 °C in DMSO also gave the expected deacylation providing glucomannan acetate with DS_{Ac} of 1.00; positional assignment again was precluded by excessive signal overlap.

7.5. Conclusions

Inspired by discovery of efficient one-step synthetic methods to prepare highly regioselectively substituted cellulose-6-*O*-esters by tetrabutylammonium fluoride- or tetrabutylammonium hydroxide-promoted deacylation, we investigated the effectiveness of these reactions for deacylation of five other polysaccharide triesters, selected for their importance and for the mechanistic and selectivity insights they might provide. Deacylation of amylose tripropionate removed nearly all propionyl groups at the more hindered secondary O-2/3 positions as seen with cellulose esters, showing that the switch from β to α anomeric stereochemistry did not impair regioselectivity. This method for making amylose 6-esters provides nearly perfect regioselectivity and should be useful for the design and synthesis of new regioselectively substituted amylose ester materials. Deacylation of curdlan triacetate was only slightly regioselective, favoring deacylation of the secondary O-2/4 esters over the primary O-6 ester; we attribute this lower regioselectivity to the absence of a vicinal diacetate moiety to provide complexation of the tetrabutylammonium cation and thereby localization of the reactive anion. In deacylation of dextran triacetate, all three positions, O-2/3/6, are esters of secondary alcohols (and constitute two vicinally oriented pairs) and therefore as predicted appeared to undergo the same degree of deacylation without regioselectivity. The complex structures of partially acylated pullulan and glucomannan polysaccharides prevented complete positional resonance assignments in the corresponding NMR spectra, so we were not able to determine the regiochemistry of these deacylations, but did observe the expected total percent deacylations. TBAF did successfully deacylate each of these glycan esters, to approximately the same extent as with cellulose esters under similar conditions. In each case, we also observed that treatment with TBAOH resulted in

quantitative removal of a number of equivalents of acyl groups equal to the TBAOH equivalents used.

All of these results are consistent with our previous mechanistic observations and proposals that deacylation at secondary positions differs from that at the primary position, and that regioselectivity is driven by complexation of the tetraalkylammonium cation by vicinal diester groups. Among those five glycan triesters, only amylose tripropionate showed excellent regioselectivity of TBAF and TBAOH deacylations, which is likely due to the fact that it is the only glycan ester examined that has both a vicinal 2,3-ester functionality and a 6-O primary alcohol ester (and which provided product spectra where resonance separation permitted full positional selectivity analysis). Taken together, the results of these explorations of TBAF- and TBAOH-catalyzed deacylations of polysaccharide esters provide confidence about the generality of these reactions in polysaccharide chemistry, and create considerable predictive power to help us understand when we can expect regioselective deacylation, and what that regioselectivity might be, given the structure of the particular polysaccharide ester.

7.6 Supplemental material

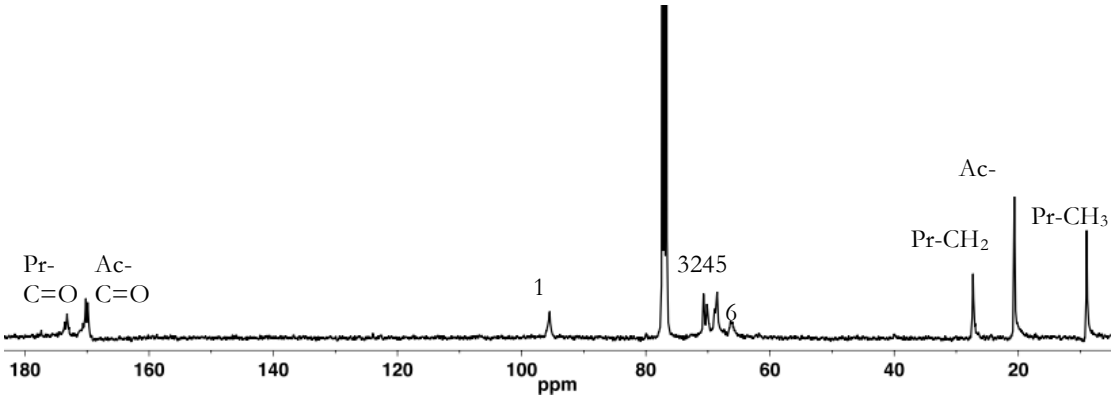


Fig. S7.1. ^{13}C NMR spectrum of the product of dextran triacetate deacylation by TBAF (24 h, THF, 50 °C), after perpropionylation.

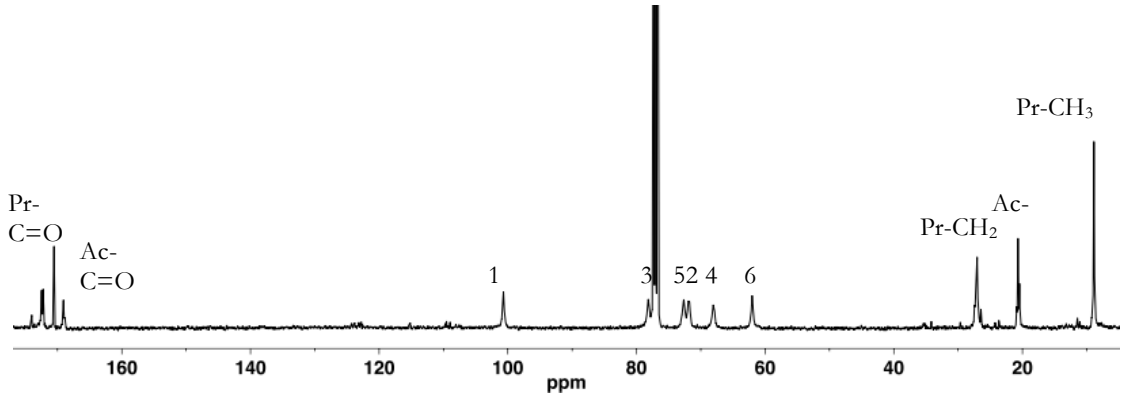


Fig. S7.2. ^{13}C NMR spectrum of the product of curdlan triacetate deacylation by TBAF (24 h, THF, 50 °C), after perpropionylation.

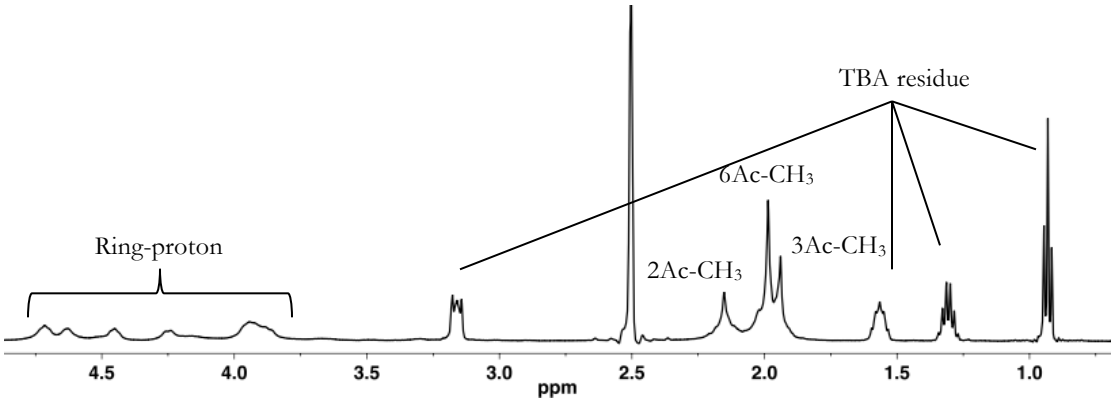


Fig. S7.3. ^{1}H NMR spectrum of the product of curdlan triacetate deacylation by TBAF (24 h, THF, 50 °C).

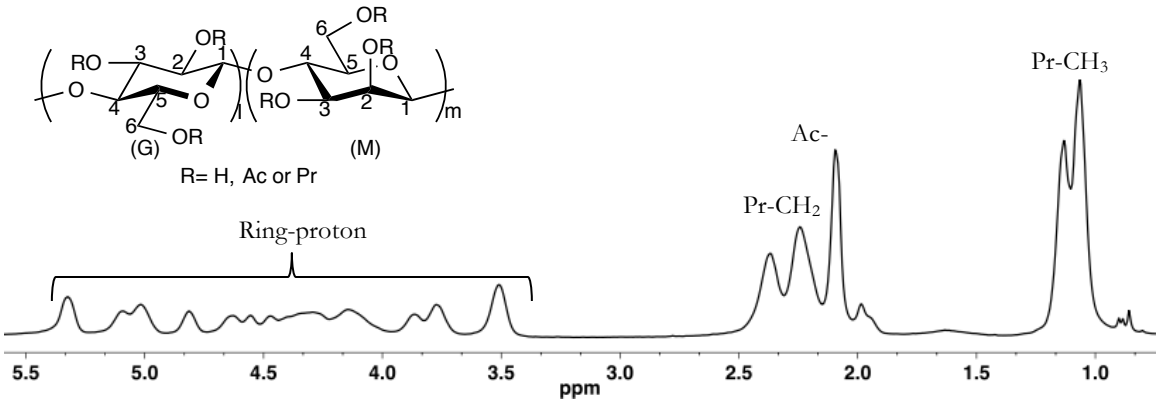


Fig. S7.4. ^{1}H NMR spectrum of the product of glucomannan triacetate deacylation by TBAF (24 h, DMSO, 50 °C), after perpropionylation.

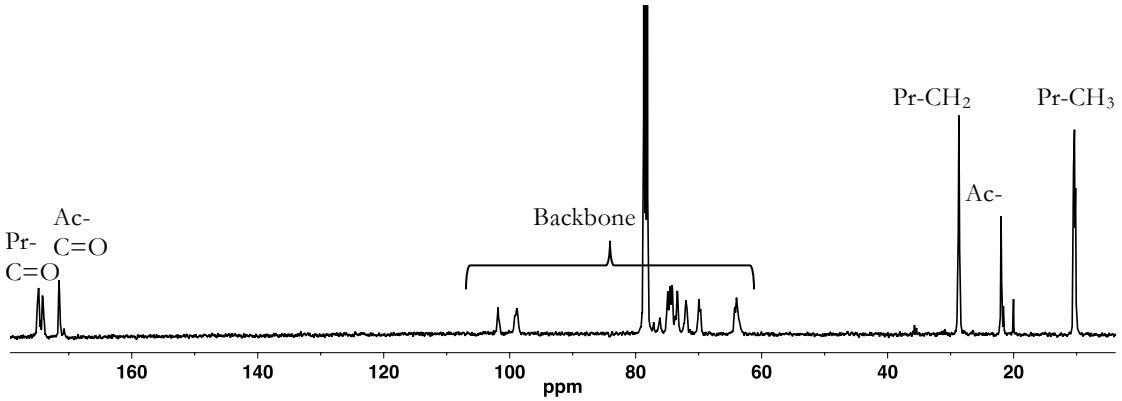


Fig. S7.5. ^{13}C NMR spectrum of the product of glucomannan triacetate deacylation by TBAF (24 h, DMSO, 50 °C), after perpropionylation.

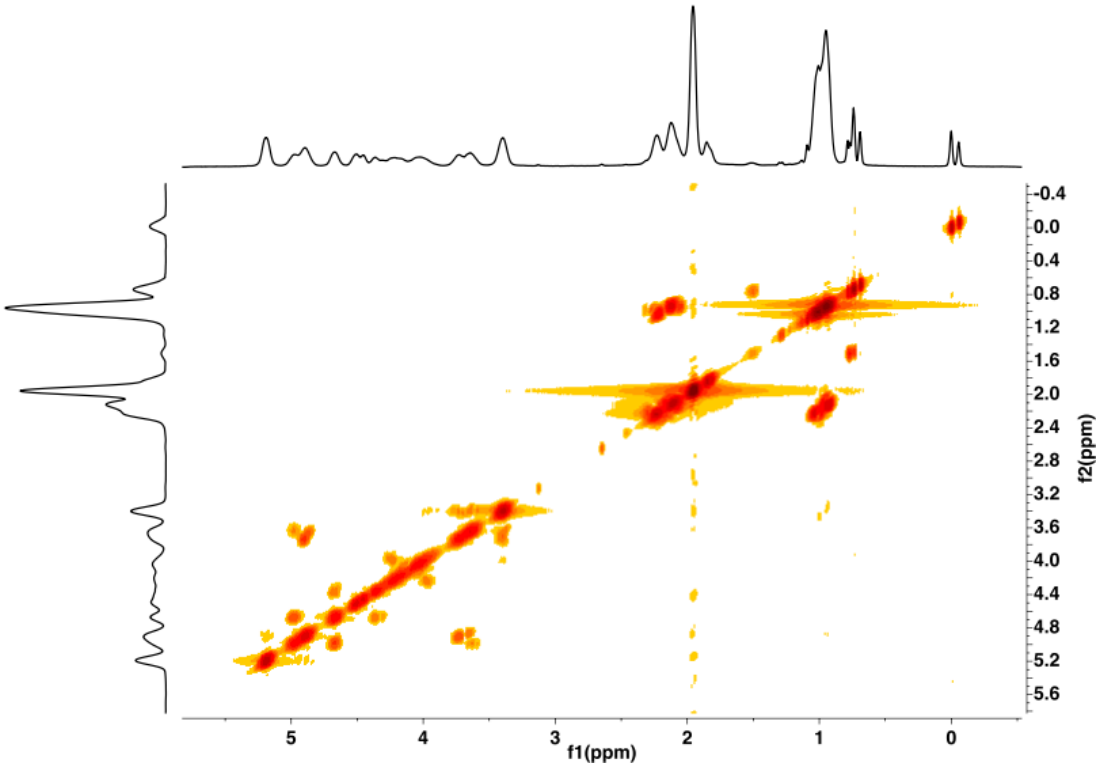


Fig. S7.6. COSY NMR spectrum of the product of glucomannan triacetate deacylation by TBAF (24 h, DMSO, 50 °C), after perpropionylation.

7.7 Acknowledgements

We thank the Institute for Critical Technologies and Applied Science (ICTAS) and Macromolecules and Interfaces Institute (MII) at Virginia Tech for their financial, facilities and educational support. We thank the USDA through grant number 2011-67009-20090.

7.8 References

1. Heinze, T.; Liebert, T.; Koschella, A., Introduction and Objectives. In *Esterification of Polysaccharides*, Springer Berlin Heidelberg: 2006; pp 1-3.

2. Fox, S. C.; Li, B.; Xu, D.; Edgar, K. J., Regioselective Esterification and Etherification of Cellulose: A Review. *Biomacromolecules* **2011,** *12* (6), 1956-1972.

3. (a) Philipp, B.; Wagenknecht, W.; Wagenknecht, M.; Nehis, I.; Klemm, D.; Stein, A.; Heinze, T.; Heinze, U.; Helbig, K.; et, a., Regioselective esterification and etherification of cellulose and cellulose derivatives. Part 1. Problems and description of the reaction systems. *Papier (Darmstadt)* **1995,** *49* (1), 3-7; (b) Wagenknecht, W., Regioselectively substituted cellulose derivatives by modification of commercial cellulose acetates. *Papier (Darmstadt)* **1996,** *50* (12), 712-720.

4. Xu, D.; Edgar, K. J., TBAF and Cellulose Esters: Unexpected Deacylation with Unexpected Regioselectivity. *Biomacromolecules* **2012,** *13* (2), 299-303.

5. Zheng, X.; Gandour, R. D.; Edgar, K. J., Probing the Mechanism of TBAF-Catalyzed Deacylation of Cellulose Esters. *Biomacromolecules* **2013,** *14* (5), 1388-1394.

6. Zheng, X.; Gandour, R. D.; Edgar, K. J., TBAF-catalyzed deacylation of cellulose esters: Reaction scope and influence of reaction parameters. *Carbohydrate Polymers* **2013,** *98* (1), 692-698.

7. Zheng, X.; Gandour, R. D.; Edgar, K. J., Remarkably regioselective deacylation of cellulose esters using tetraalkylammonium salts of the strongly basic hydroxide ion. *Carbohydrate Polymers* **2014,** *111* (0), 25-32.

8. Fuentes-Zaragoza, E.; Riquelme-Navarrete, M. J.; Sánchez-Zapata, E.; Pérez-Álvarez, J. A., Resistant starch as functional ingredient: A review. *Food Research International* **2010,** *43* (4), 931-942.

9. (a) Åberg, M.; Hedner, U.; Bergentz, S.-E., The Antithrombotic effect of dextran. *Scandinavian Journal of Haematology* **1979,** *23* (S34), 61-68; (b) Maia, J.; Ribeiro, M. P.; Ventura, C.; Carvalho, R. A.; Correia, I. J.; Gil, M. H., Ocular injectable formulation assessment for oxidized dextran-based hydrogels. *Acta Biomaterialia* **2009,** *5* (6), 1948-1955.

10. (a) Hosseinkhani, H.; Aoyama, T.; Ogawa, O.; Tabata, Y., Liver targeting of plasmid DNA by pullulan conjugation based on metal coordination. *Journal of Controlled Release* **2002,**

83 (2), 287-302; (b) Leathers, T. D., Biotechnological production and applications of pullulan. *Applied Microbiology and Biotechnology* **2003,** *62* (5-6), 468-473.

11. Zhang, R.; Edgar, K. J., Properties, Chemistry, and Applications of the Bioactive Polysaccharide Curdlan. *Biomacromolecules* **2014,** *15* (4), 1079-1096.

12. McIntosh, M.; Stone, B. A.; Stanisich, V. A., Curdlan and other bacterial (1→3)-β-d-glucans. *Appl Microbiol Biotechnol* **2005,** *68* (2), 163-173.

13. Katsuraya, K.; Okuyama, K.; Hatanaka, K.; Oshima, R.; Sato, T.; Matsuzaki, K., Constitution of konjac glucomannan: chemical analysis and 13C NMR spectroscopy. *Carbohydrate Polymers* **2003,** *53* (2), 183-189.

14. Yu, H.; Huang, A.; Xiao, C., Characteristics of konjac glucomannan and poly(acrylic acid) blend films for controlled drug release. *J. Appl. Polym. Sci.* **2006,** *100* (2), 1561-1570.

15. Liebert, T.; Hussain, M. A.; Heinze, T., Structure Determination of Cellulose Esters via Subsequent Functionalization and NMR Spectroscopy. *Macromolecular Symposia* **2005,** *223* (1), 79-92.

16. (a) Edgar, K. J.; Arnold, K. M.; Blount, W. W.; Lawniczak, J. E.; Lowman, D. W., Synthesis and properties of cellulose acetoacetates. *Macromolecules* **1995,** *28* (12), 4122-4128; (b) Zhang, R.; Edgar, K. J., Synthesis of curdlan derivatives regioselectively modified at C-6: O-(N)-Acylated 6-amino-6-deoxycurdlan. *Carbohydrate Polymers* **2014,** *105* (0), 161-168.

17. Hussain, M. A.; Shahwar, D.; Tahir, M. N.; Sher, M.; Hassan, M. N.; Afzal, Z., An efficient acetylation of dextran using in situ activated acetic anhydride with iodine. *J. Serb. Chem. Soc.* **2010,** *75* (2), 165-173.

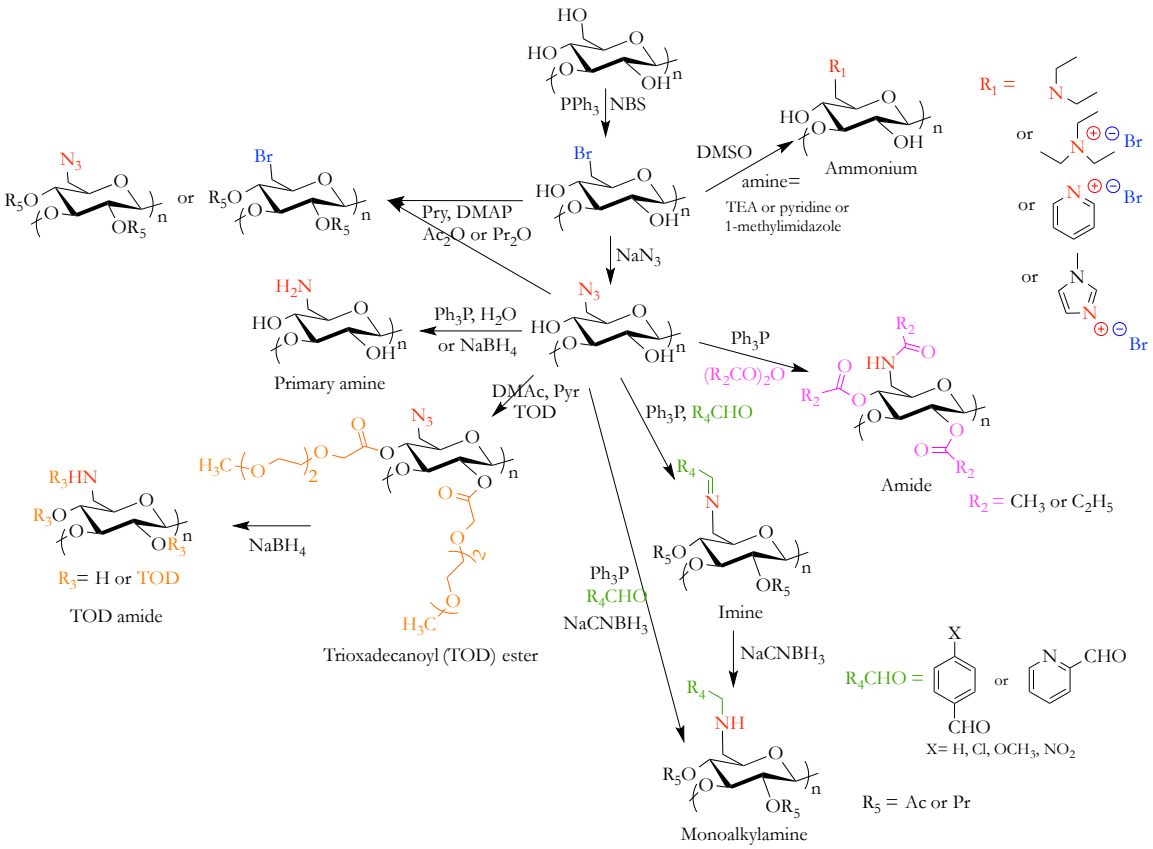
18. Teramoto, N.; Shibata, M., Synthesis and properties of pullulan acetate. Thermal properties, biodegradability, and a semi-clear gel formation in organic solvents. *Carbohydrate Polymers* **2006,** *63* (4), 476-481.

.19. Enomoto-Rogers, Y.; Ohmomo, Y.; Iwata, T., Syntheses and characterization of konjac glucomannan acetate and their thermal and mechanical properties. *Carbohydrate Polymers* **2013,** *92* (2), 1827-1834.

Chapter 8 Summary and Future Work

Aminated polysaccharides have demonstrated critical natural functions in the biomedical field, including interactions with proteins, encapsulation of drugs, and formulation with genes or other biological compounds. The natural polysaccharide chitin and its partially *N*-deacetylated derivative chitosan are the most common biopolymers with significant benefits for these applications, which however have some inevitable drawbacks, for example, the residual protein impurities, sequence control difficulty and lower efficiency in specific cases. Curdlan is a natural-based, nontoxic, biocompatible and bioactive β-1,3-glucan. Even though extremely challenging, regioselective functionalization of curdlan is of great importance for us to produce a family of promising aminated candidates on biomedical and pharmaceutical applications.

My doctoral research has focused on the development of the comprehensive family of regioselectively aminated curdlan derivatives including 6-imino-, 6-amido-, 6-primary/secondary/tertiary amino-, and 6-quaternary ammonium curdlans starting from 6-bromo/azido-6-deoxycurdlan (**Scheme 8.1**). We described extensive studies on synthesis and characterization of those curdlan derivatives.



Scheme 8.1. Comprehensive syntheses of regioselectively substituted curdlan derivatives

8.1 Synthesis of curdlan derivatives regioselectively modified at C-6: *O*-(*N*)-Acylated 6-amino-6-deoxycurdlan

Regioselective bromination of curdlan with NBS and Ph_3P in DMAc/LiBr at C-6 position has been demonstrated to synthesize 6-bromo-6-deoxycurdlan with high DS_{Br}. Kinetic studies of the subsequent nucleophilic azide displacement by sodium azide in DMSO proved the completion of producing 6-azido-6-deoxycurdlan within 1 h with no spectroscopic evidence of side reactions. We have developed multiple synthetic routes to converse the azide to amine or amides, either appending the same acyl group to form both esters and amides at C-2/4/6 positions, or attaching dissimilar *N*- and *O*-acyl groups to form curdlan 6-amide (A) and 2,4-diester (B) regioselectively substituted polymers. Staudinger reaction by Ph_3P has been employed to selectively reduce the azide to amine in the presence of esters, however the resultant 6-amino-6-deoxy-(2,4-di-*O*-acyl)curdlan appeared to have poor solubility in both water and organic solvents. In order to obtain solubility and thereby processability, we presented the syntheses of a series of *O*-acylated 6-amido-6-(2,4-di-*O*-

acyl)deoxycurdlan using Staudinger reduction followed by *in situ* reaction with excess carboxylic anhydride without isolating the 6-amino intermediate. We found that amide products have much improved solubility *vs.* 6-amino-6-deoxycurdlan or its *O*-esters. The synthetic methods open doors to a wide variety of potentially useful aminocurdlan and amidocurdlan polymers.

8.2 Water-soluble aminocurdlan derivatives by chemoselective azide reduction using $NaBH_4$

Two water-soluble aminocurdlan derivatives were synthesized as intriguing target molecules for study of their interactions with the proteins that form tight junctions between enterocytes. After initial C-6 bromination of curdlan and azide displacement of the bromide, the hydrophilic, uncharged oligo(ethylene oxide) 3,6,9-trioxodecanoate (TOD) esters were appended to the 2- and 4-OH groups of 6-azido-6-deoxycurdlan by simple acylation. Upon $NaBH_4$ reduction of the 6-azide to the 6-amine, acyl group migration occurred, along with concomitant reduction of residual ester groups, providing the *N*-TOD amide, which is also water-soluble. Alternatively, direct borohydride reduction of the parent 6-azidocurdlan afforded 6-amino-6-deoxycurdlan that was also water-soluble, as opposed to the water insolubility observed for the Staudinger reduction product of the same azide. We hypothesized that the residual arylphosphorus impurities (Ph_3P, $Ph_3P{=}O$) or the presence of residual phosphorus ylide moieties at C-6 were responsible for the poor solubility of the Staudinger product. Access to these families of water-soluble, regioselectively substituted curdlan derivatives should permit investigations of structure-property relationships for a variety of biomedical applications.

8.3 Regioselective synthesis of cationic 6-deoxy-6-(*N*,*N*,*N*-trialkylammonio) curdlan derivatives

A family of cationic water-soluble 6-(*N*,*N*,*N*-trialkylammonio)-6-deoxycurdlan salts were prepared by reaction of 6-bromo-6-deoxycurdlan and its 2,4-*O*-diesters with trialkylamines or aromatic amines. Substantial evidence was provided to support the hypothesis that the accumulation of charges on the curdlan backbone as nucleophilic displacement of the 6-halo substituents proceeds at some point reduces the rate of additional substitution, making it

difficult to achieve complete displacement at C-6. By using the polar aprotic solvent DMSO (80 °C, 24 h), excess tertiary or heterocyclic amine (20 equiv. TEA/pyridine/1-methyl imidazole per AGU), and *in situ* formation of more labile 6-iodo-6-deoxy intermediates by addition of sodium iodide, we have achieved relatively high DS(ammonium) at C-6 (DS_{TEA} 0.89, DS_{Pyr} 0.66 and DS_{MeIMID} 0.86). Elemental analysis and NMR spectroscopic methods used to determine DS values gave results in quite good agreement, within 0.02 of one another. In the future, we will investigate the structure-property relationships of these regioselectively quaternized curdlan derivatives compared with quaternized chitosan derivatives with regard to the type of glucosidic linkage (1,3- *vs.* 1,4-) as well the position of the positively charged substituent (C-6 *vs.* C-2).

8.4 Studies on subsequent reactions of Staudinger ylide: Reductive amination and peralkylation of aminocurdlan

The triphenylphosphonium ylide intermediate generated during Staudinger reduction is highly nucleophilic, making it a candidate to carry out additional subsequent reactions. 6-Azido-6-deoxy-2,4-di-*O*-acyl-curdlan was treated with Ph_3P and aromatic aldehydes (PhCHO, *p*-NO_2PhCHO, *p*-ClPhCHO, *p*-CH_3OPhCHO and 2-PyrCHO) to synthesize a family of 6-imino curdlans with DS_{imine} up to 1.0. The resultant imino derivatives were further reduced by borohydride to produce the corresponding 6-monoalkylamino-6-deoxycurdlans. Alternatively, a one-pot reductive amination method was performed by adding Ph_3P, aldehyde together with the reducing agent without the isolation of imino product to achieve the same aminocurdlans, which provided a new strategy for incorporation of a range of monoalkylamine functional pendants to the aminocurdlan backbone.

A series of 6-di/tri-alkylamino curdlans was prepared by peralkylation of the Staudinger ylide intermediate resulting from reduction of 6-azidocurdlan, with alkyl iodides. We compared the effects on substitution of using different reaction temperature, time and molar ratios of added alkyl iodide. This class of water-soluble curdlan derivatives having tunable charge density can be further modified with hydrophobic or ionic moieties for potential pharmaceutical use.

8.5 Glycan ester deacylation by TBAOH or TBAF: Regioselectivity vs. polysaccharide structure

TBAF and TBAOH have been found to efficiently mediate regioselective deacylation of cellulose esters, with unexpected selectivity for removal of acyl groups at the more hindered secondary O-2/3 positions. We carried out experiments in which five glycan (amylose, curdlan, dextran, pullulan and glucomannan) esters were exposed to TBAF or TBAOH, examining the effectiveness of this reaction and the impact of glucan and glucomannan structure on deacylation regioselectivity.

Deacylation of amylose triesters removed almost all ester groups at the more hindered secondary O-2/3 positions as seen with cellulose esters with percent regioselectivity (PR) over 95%, indicating no impairment of the switch from β to α anomeric stereochemistry. For curdlan triesters, deacylation favored the secondary O-2/4 esters over the primary O-6 ester with slight regioselectivity (PR 64%). This lower (than for cellulose or amylose) regioselectivity was attributed to the absence of a vicinal diacyl moiety to provide complexation of the TBA cation and thereby localization of the reactive anion. Since all three positions (O-2/3/6) of dextran triacetate are esters of secondary alcohols and constitute two vicinally oriented pairs, deacylation appeared to occur to the same degree at each position, without regioselectivity. We were not able to determine the deacylation regioselectivity of pullulan and glucomannan esters due to their complicated structures, but did observe the expected total percent deacylations. We also observed that TBAOH deacylation led to quantitative removal of a number of equivalents of acyl groups equal to the added TBAOH equivalents. All of these results are consistent with our previous mechanistic hypothesis that deacylation at secondary positions differs from that at the primary position, and that regioselectivity is driven by complexation of the TBA cation by vicinal diester groups.

8.6 Proposed future work

Novel aminated curdlan derivatives with valuable properties as described above are targeted for biomedical and pharmaceutical applications, however many issues still have to be addressed so that these polymers may be used as potent drug/gene delivery agents. 6-Amino-6-deoxycurdlan produced by Staudinger reduction had residual arylphosphorus

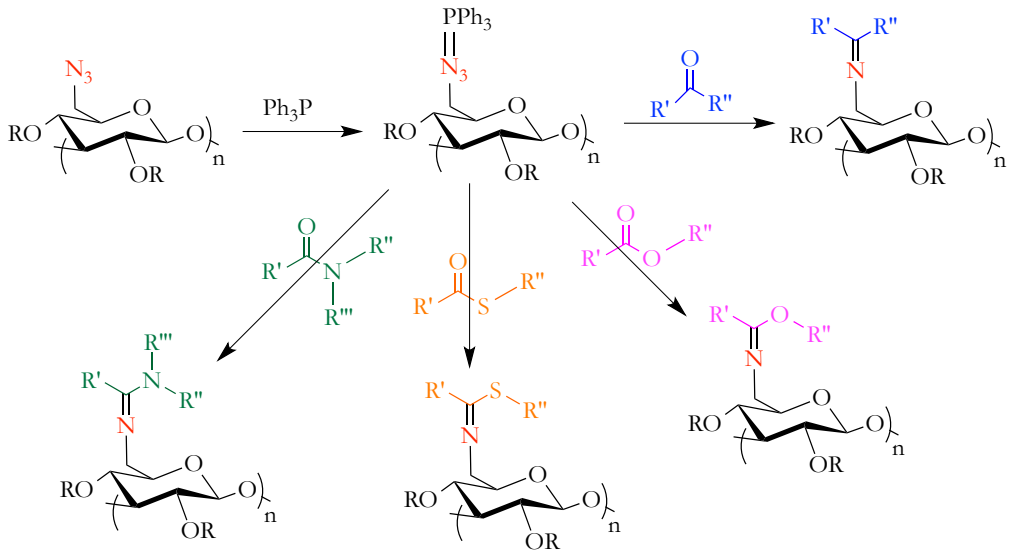
impurities (Ph_3P, $Ph_3P=O$) that were extremely difficult to remove. Trimethyl phosphine (PMe_3) has been used as an efficient reductant in carbohydrate chemistry to reduce the azide to amine, which permitted very clean separation of product from P-containing residual reagent and P-O byproduct.[1] The reduction was carried out in THF in the presence of NaOH to maintain the optimum pH 10 to drive the hydrolysis to completion. It is worthwhile to try PMe_3 in the reduction of 6-azido-6-deoxycurdlan while we need optimize pH and duration for ester containing products (6-azido-6-deoxy-2,4-di-*O*-acyl-curdlan).

The synthesis of the new type of water-soluble aminated curdlan 3,6,9-trioxodecanoate (TOD) opens interesting new possibilities for the development of other orally compatible polymer amphiphile candidates. 3,6-Dioxaheptanoate (DOH) is another promising hydrophilic ester that can be introduced to curdlan backbone so as to enhance its aqueous solubility. By adjusting the added reagent amount, we can prepare a series of water-soluble curdlan esters with different DS values. Hydrophobic ethers (methyl, ethyl, propyl), esters (acetate, propionate, butyrate, benzoylate) and pH-responsive carboxyl groups (phthalate, succinate, adipate) can be further attached to tune the properties of the polymer to accommodate drug compounds having a wide range of physiochemical properties.

Cationic 6-(*N*,*N*,*N*-trialkylammonio)-6-deoxycurdlan salts, analogs of trimethyl chitosan, are promising candidates to interact with proteins that form the tight junctions between enterocytes. Though we have achieved curdlan ammonium derivatives with high $DS_{ammonium}$ (>0.85), still there were bromides present after reaction. Thiolated polymers have been proved to improve mucoadhesive properties and permeation-enhancing properties in tight junction opening area.[2] Therefore the nucleophilic substitution reaction of those curdlan ammonium derivatives with thiols would not only eliminate the residual bromides but also enhance the paracellular transportation efficiency.

Some preliminary results showed that the iminophosphorane could be a versatile intermediate. Other than the reductive amination reaction of treating 6-azido curdlan with aldehydes to synthesize 6-imino- and 6-monoalkylamino curdlans, we will also be looking for other subsequent possibilities. Following the mechanism of aza-Wittig reaction, the generated Staudinger ylide may be capable to react with some other carbonyl compounds

such as ketones, esters, thioesters and amides,[3] which will afford a wide range of acyclic and heterocyclic functionalities on curdlan backbone (**Scheme 8.2**).



Scheme 8.2. Subsequent reactions of iminophosphorane intermediate

8.7 References

1. (a) Noti, C.; de Paz, J. L.; Polito, L.; Seeberger, P. H., Preparation and use of microarrays containing synthetic heparin oligosaccharides for the rapid analysis of heparin-protein interactions. *Chem. - Eur. J.* **2006,** *12* (34), 8664-8686; (b) Zong, C.; Venot, A.; Dhamale, O.; Boons, G.-J., Fluorous Supported Modular Synthesis of Heparan Sulfate Oligosaccharides. *Organic letters* **2013,** *15* (2), 342-345.

2. (a) Bhalekar, M. R.; Bargaje, R. V.; Upadhaya, P. G.; Madgulkar, A. R.; Kshirsagar, S. J., Formulation of mucoadhesive gastric retentive drug delivery using thiolated xyloglucan. *Carbohydrate Polymers* **2016,** *136*, 537-542; (b) Kast, C. E.; Bernkop-Schnürch, A., Thiolated polymers — thiomers: development and in vitro evaluation of chitosan–thioglycolic acid conjugates. *Biomaterials* **2001,** *22* (17), 2345-2352.

3. Palacios, F.; Alonso, C.; Aparicio, D.; Rubiales, G.; de los Santos, J. M., The aza-Wittig reaction: an efficient tool for the construction of carbon–nitrogen double bonds. *Tetrahedron* **2007,** *63* (3), 523-575.