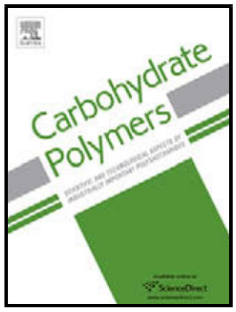

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Water-soluble aminocurdlan derivatives by chemoselective azide reduction using $NaBH_4$

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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.

Keywords: aminocurdlan; amidocurdlan; regioselectivity; sodium borohydride; water-soluble

Highlights

- Highly regioselective bromination and azide displacement of curdlan at C-6
- Chemoselective reduction of azidocurdlan to amine by sodium borohydride
- Two water-soluble 6-aminocurdlans starting from 6-bromo-6-deoxycurdlan
- Pathway to regiochemically defined 6-amino- and 6-amido-6-deoxycurdlan derivatives

1. 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 (Hassani, Hendra & Bouchemal, 2012; Henni-Silhadi et al., 2007; Liesiene & Matulioniene, 2004), and for use as antimicrobial agents (Lee, Yeomans, Allen, Gross & Kaplan, 1997; Roemhild, Wiegand, Hipler & Heinze, 2013). 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 (Ravi Kumar, 2000). 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 (Yeh et al., 2011). The protonated amine groups of chitosan drive the necessary interactions with the tight junction proteins; permanently charged *N*-peralkylated derivatives are even more effective (Sadeghi et al., 2008). 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 (Hajeb & Selamat, 2012). Ranaldi *et al.* (Ranaldi, Marigliano, Vespignani, Perozzi & Sambuy, 2002) 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.

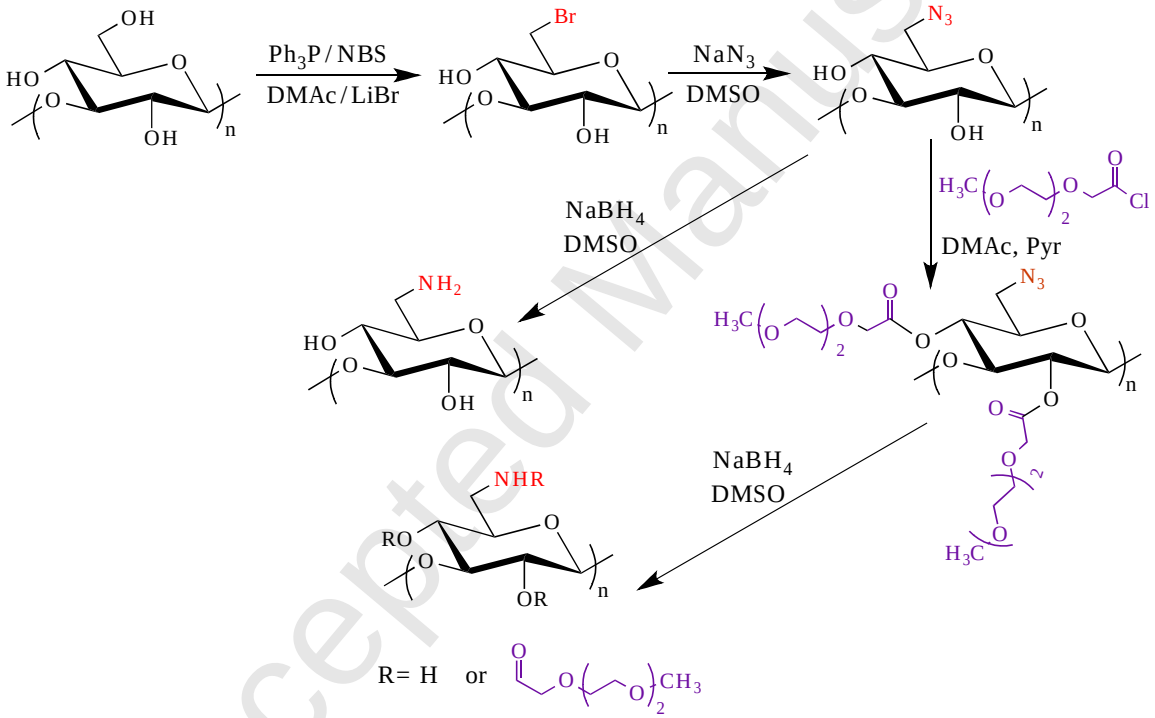
Recently researchers have explored amination of other polysaccharides including cellulose (Fox & Edgar, 2012), curdlan (Zhang & Edgar, 2014b), amylose (Cimecioglu, Ball, Kaplan & Huang, 1994), and pullulan (Pereira & Edgar, 2014) in an attempt to understand structure-property relationships, and ultimately 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)) (Furuhata, Koganei, Chang, Aoki & Sakamoto, 1992a). This chemistry involves a double S_N2 mechanism (Fox & Edgar, 2011) that provides essentially perfect C-6 selectivity for polysaccharides (Furuhata, Chang, Aoki & Sakamoto, 1992; Furuhata, Koganei, Chang, Aoki & Sakamoto, 1992b) that possess a C-6 primary alcohol; this is perhaps the most regioselective reaction known in polysaccharide chemistry (Fox, Li, Xu & Edgar, 2011). 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 (Boger, Corcoran & Lehn, 1978; Cimecioglu, Ball, Huang & Kaplan, 1997; Cimecioglu, Ball, Kaplan & Huang, 1994; Fox & Edgar, 2012) 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.* (Matsui, Ishikawa, Kamitakahara, Takano & Nakatsubo, 2005) 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 (Brown & Rapoport, 1963). Rapoport observed (Brown & Rapoport, 1963) “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.* (Baumann, Liu & Faust, 2003; Liu & Baumann, 2002) described a clever approach to completely regioselectively substituted 6-amino-6-deoxycellulose with DS_{C-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 (Harada & Harada, 1996; Zhang & Edgar, 2014a). Its propensity to undergo thermal gelation has proven valuable in foods (Miwa, Nakao & Nara, 1993), including as a thickener, fat substitute, food additive, and other functions (Jin, Zhang, Yin & Nishinari, 2006; Na, Park, Kim & Bae, 2000; Popescu, Pelin, Butnaru, Fundueanu & Suflet, 2013). 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 (Zhang & Edgar, 2014b).

The Heinze (Dorn, Pfeifer, Schlufter & Heinze, 2010; Heinze & Schaller, 2000), Zhang (Zhou, Zhang, Okamura, Minoda & Miyamoto, 2001), and Edgar (Liu & Edgar, 2014) 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 1). We also describe unexpected benefits from borohydride as opposed to Staudinger reduction of the 6-azido-6-deoxy polysaccharides.



Scheme 1. Synthetic scheme for 6-amino-6-deoxycurdlans.

2. Experimental

2.1 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.

2.2 Measurements

2.2.1 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.

2.2.2 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:

$$DS_{TOD\,(azido)} = \frac{\Delta V \times 0.1 \times (187 + 160 DS_{TOD})}{1000\ m} \Rightarrow DS_{TOD(azido)} = \frac{18.7}{1000\frac{m}{\Delta V} - 16} \quad \text{Eq. (1)}$$

or

$$DS_{TOD\,(amino)} = \frac{\Delta V \times 0.1 \times (161 + 160 DS_{TOD})}{1000\ m} \Rightarrow DS_{TOD(amino)} = \frac{16.1}{1000\frac{m}{\Delta V} - 16} \quad \text{Eq. (2)}$$

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.

2.2.3 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.

2.3 Methods

2.3.1 Dissolution of curdlan in DMAc/LiBr (referred to in examples as “standard cellulose solution in DMAc/LiCl” (Edgar, Arnold, Blount, Lawniczak & Lowman, 1995))

Dissolution of curdlan in DMAc/LiBr was performed by a procedure reported in our previous paper (Zhang & Edgar, 2014b). 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.

2.3.2 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%.

2.3.3 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 %.

2.3.4 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.

2.3.5 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

281 then freeze-dried to yield 6-trioxadecanamido-6-deoxycurdlan. ^{13}C NMR (D_2O): δ 173.3
282 (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),
283 Yield: 79 %.
284
285 2.3.6 Synthesis of 6-amino-6-deoxycurdlan using sodium borohydride
286 Dry 6-azido-6-deoxycurdlan (0.2 g) was dissolved in 20 mL of DMSO in a 100 mL flask.
287 Then $NaBH_4$ (0.81 g, 20 eq per AGU) was added to the flask and dissolved. The solution
288 was heated to 100°C and stirred for 24 h. It was cooled to 0°C, then HCl (1M) was added
289 dropwise to the mixture at 0°C in an ice bath until gas generation ceased. The mixture was
290 neutralized using saturated $NaHCO_3$ solution and then transferred to 3500 g/mol MWCO
291 dialysis tubing. After 3 days of dialysis, the colorless solution was then freeze-dried to yield
292 6-amino-6-deoxycurdlan. ^{13}C NMR (D_2O): 6-amino-6-deoxycurdlan: δ 105.4 (C-1), 86.1 (C-
293 3), 77.4 (C-5), 76.4 (C-2), 72.4 (C-4), 44.1 (C-6-NH_2), Yield: 71%.
294
295 2.3.7 Synthesis of 6-acetamido-6-deoxycurdlan by sodium borohydride reduction of 6-azido-
296 6-deoxy-2,4-di-*O*-acetylcurdlan
297 Under nitrogen, dry 6-azido-6-deoxycurdlan (0.5 g, 2.67 mmol) and 4-
298 dimethylaminopyridine (DMAP, 20 mg) were weighed into a 50 mL round-bottom flask.
299 Pyridine (2.15 mL, 10 eq per AGU) and acetic anhydride (2.73 mL, 10 eq per AGU) were
300 then added dropwise to the solution. The mixture was reacted at 80°C for 24h under
301 nitrogen. The homogeneous mixture was then added slowly to 200 mL deionized water. The
302 crude product was collected by filtration, and then re-dissolved in 10 mL chloroform. This
303 solution was added slowly with rapid stirring to 200 mL of ethanol. After filtration and
304 washing with ethanol and water several times, the sample was dried under vacuum at 40°C
305 overnight to yield 6-azido-6-deoxy-2,4-di-*O*-acetyl-curdlan. ^{13}C NMR (DMSO-d_6): δ 169.6
306 (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-
307 Ac), Yield: 97%.
308
309 Dry 6-azido-6-deoxy-2,4-di-*O*-acetylcurdlan (0.2 g, 0.74 mmol) was dissolved in 20 mL of
310 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%.

3. Results and discussion

As we have previously reported (Zhang & Edgar, 2014b), 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 (Cimecioglu, Ball, Kaplan & Huang, 1994; Fox & Edgar, 2012; Zhang & Edgar, 2014b). 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.

342

343 3.1 Synthesis of 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoyl-curdlan

344 Acylation of 6-azido-6-deoxycurdlan with the appropriate acid chloride seemed like a
345 promising approach to the 2,4-di-*O*-trioxodecanoyl esters; therefore TODCl (Fig. S2) was
346 synthesized by reaction of TODA with thionyl chloride just prior to its use, then was reacted
347 with 6-azido-6-deoxycurdlan in DMAc in the presence of pyridine catalyst.

348

349 Identity and DS of the resulting 6-azido-6-deoxy-2,4-*O*-bis(trioxadecanoyl)curdlan were
350 established by FTIR and NMR spectroscopy, peracetylation, and saponification followed by
351 back-titration. FTIR analysis showed that, in comparison with the 6-azido starting material
352 (Fig. 5a), a characteristic ester carbonyl absorption at 1771 cm^{-1} was present (Fig. 1a),
353 indicating successful TOD ester introduction. Proton NMR is normally very useful for DS
354 determination of polysaccharide derivatives, but peaks for the trioxadecanoate methylene
355 groups at around 3.3-4.1 ppm overlap with the curdlan backbone proton resonances (3.7-4.8
356 ppm, Fig. 2) preventing DS_{TOD} calculation by this method (unsurprisingly since all are
357 protons attached to carbons that also bear an electron-withdrawing oxygen atom). The ^{13}C
358 NMR spectrum (Fig. 3a) contained a TOD carbonyl signal at δ 169.1 ppm and a single C-1
359 signal at δ 100.1 ppm. Resonances assigned to the carbons of the TOD moiety and the
360 curdlan C-2, 3, 4, 5 carbons are visible at $\delta = 59.0$ (C-16) and $\delta = 65.7 – 73.8$ (C-2 through 5,
361 C-8 through 14), with similar issues of signal overlap as observed in the ^{1}H NMR. Therefore,
362 we treated the putative 6-azido-6-deoxy-2,4-*O*-bis(trioxadecanoyl)curdlan with acetic
363 anhydride in pyridine to yield products in which the proton NMR should show acetyl methyl
364 signals at 1.9-2.1 ppm if $DS_{TOD} < 2$. However, no apparent acetyl methyl resonances were
365 observed (Fig. S3), indicating complete TOD esterification (DS_{TOD}~2) at C-2/4 within the
366 ^{1}H NMR detection limits.

367

368 Alternatively, we employed saponification of 6-azido curdlan trioxadecanoate with aqueous
369 NaOH and back-titration of the excess base with HCl solution to determine DS_{TOD}. This
370 approach has long been used for DS determination of polysaccharide esters (Freire, Silvestre,
371 Pascoal Neto & Rocha, 2005; Malm, Genung, Williams & Pile, 1944; Stojanović, Jeremić,
372 Jovanović & Lechner, 2005; Tihlarik & Pasteka, 1987). Complete trioxadecanoate hydrolysis

under mild conditions (0.1 N aq NaOH, 25 °C, 24 h) can be confirmed by FTIR (Fig. S4b), 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. S5). The peak at 10.8 mL indicated the equilibrium point that was used to calculate the DS_{TOD} = 1.89 by Eq. (1).

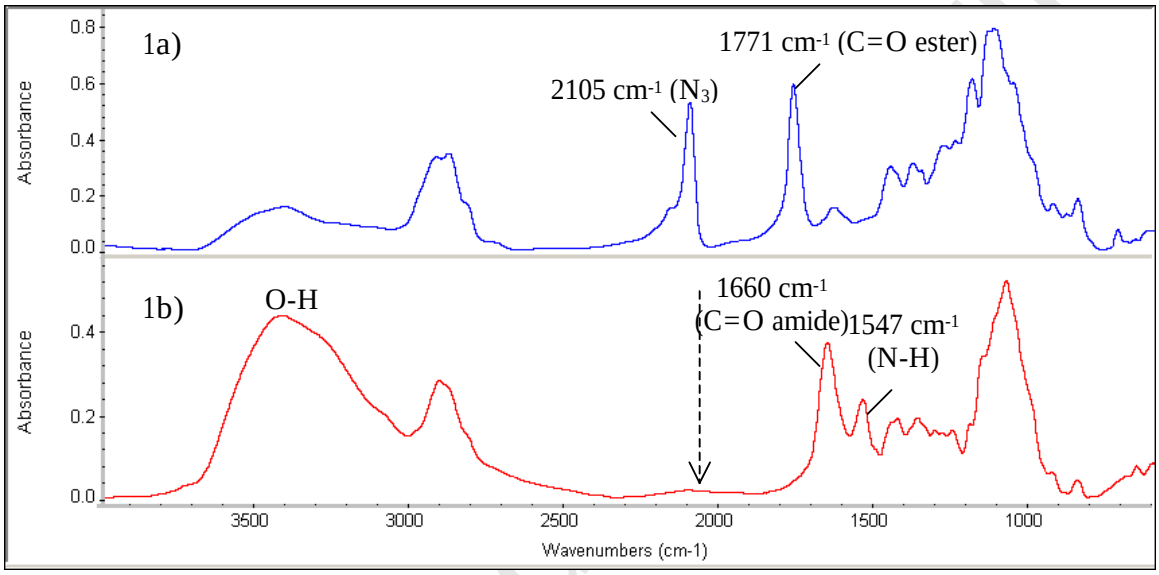


Figure 1. FTIR spectra of 1a) 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoyl-curdlan and 1b) 6-trioxadecanamido-6-deoxycurdlan synthesized by $NaBH_4$ reduction.

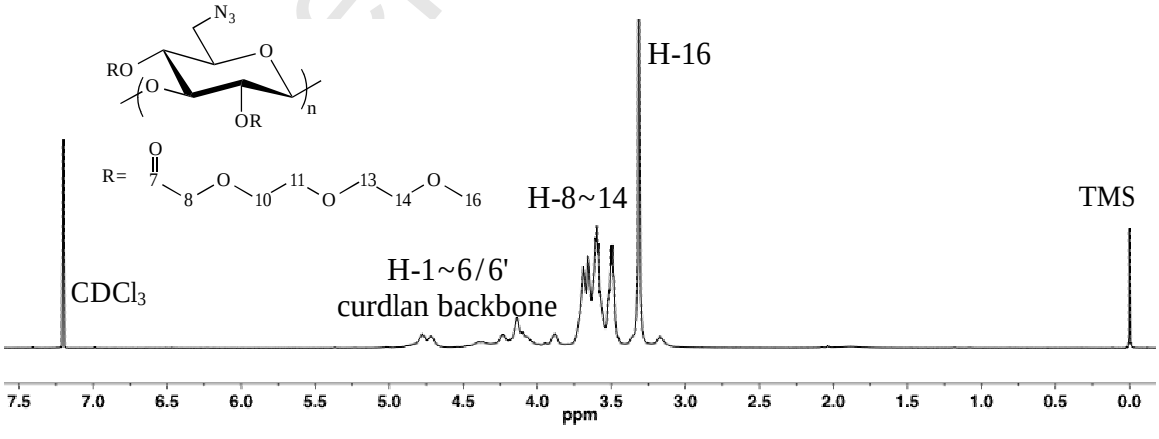


Figure 2. ^{1}H NMR spectrum of 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoylcurdlan.

3.2 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 (Scriven & Turnbull, 1988), 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. 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. 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 (Pereira & Edgar, 2014; Zhang & Edgar, 2014b). 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. 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 (Cao & Raleigh, 2010; Sohma et al., 2004) and even in carbohydrate chemistry (Sproviero, 1973). 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

418 intermolecularly. The excess sodium borohydride reductively cleaved the remaining TOD
419 esters at C-2/4, affording the observed amide product. Such acyl group migrations from O
420 to N have been observed by us before, for 6-amino-6-deoxycellulose 2,3-*O*-esters (Fox &
421 Edgar, 2012), and for the analogous derivatives of pullulan (Pereira & Edgar). However, in
422 those cases Staudinger reduction was used to reduce azide to amine, so that acyl migration
423 occurred from ester oxygen to a negatively charged nitrogen of the Staudinger ylide
424 intermediate. In order to confirm this hypothesis, we subjected 6-azido-6-deoxy-2,4-acetyl-
425 curdlan to the same $NaBH_4$ reduction conditions (since the acetyl methyl resonates upfield
426 of the curdlan backbone hydrogens and is readily observed and quantified). IR and NMR
427 analyses (Fig. S7) of this reduction product show acetyl migration to form the amide and
428 reductive cleavage of the residual *O*-acetate esters, in exactly the same fashion observed for
429 the TOD esters. It is interesting to note that the 6-acetamido-6-deoxycurdlan product, an
430 isomer of chitin (which is insoluble in water and simple organic solvents), is soluble in water.
431 This sharp difference in properties is worthy of further exploration.
432
433 In order to quantify residual trioxadecanoate following borohydride reduction of the TOD
434 ester, the product was saponified with aqueous NaOH (0.1 N) followed by back-titration
435 using HCl (0.1 N). Two inflection points were evident in the titration curve (Fig. S6); the
436 first one for titration of excess NaOH, and the second for titration of the weakly basic 6-
437 NH_2 groups. Based on the titrant consumed at the first inflection point (12.6 mL), DS_{TOD}
438 was calculated to be 1.09 by Eq. (2). Combined with previous spectrometric evidence,
439 almost all ester groups had been reduced to hydroxyls during azide reduction (compare with
440 DS_{TOD} of 1.89 in the starting 6-azido curdlan trioxadecanoate) and a new trioxadecanamide
441 was formed at C-6.

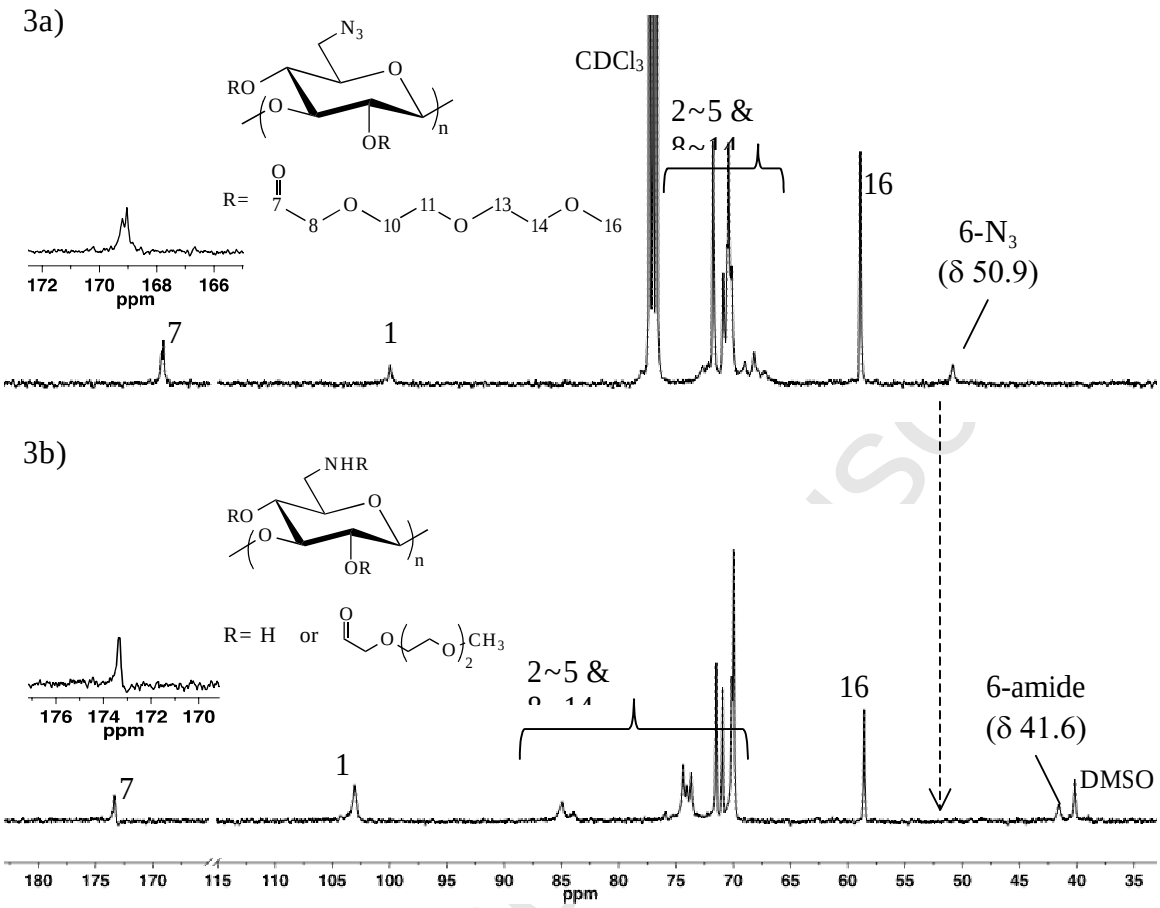


Figure 3. ^{13}C NMR spectra of 3a) 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoyl-curdlan and 3b) 6-amino-6-deoxy-2,4-di-*O*-trioxadecanoyl-curdlan.

3.3 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. 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. S1b), 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. S1a). 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. 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 5 shows the FTIR spectra of 6-azido- and 6-amino-6-deoxycurdlan. The strong azide absorptions at 2107 cm^{-1} (Fig. 5a) present in the starting material are virtually completely eliminated in the borohydride reduction product (6-amino-6-deoxycurdlan, Fig. 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.

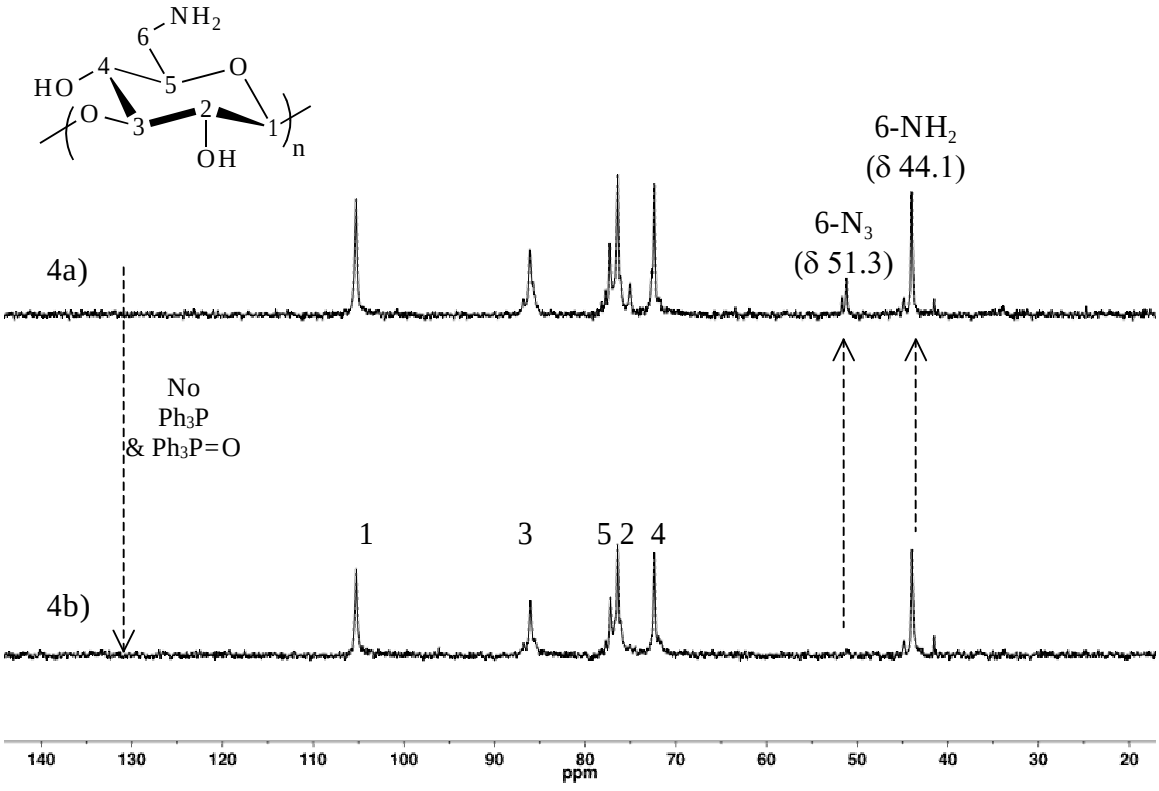


Figure 4. ^{13}C NMR spectra of 6-amino-6-deoxycurdlan: 4a) 60°C, 24 h and 4b) 100°C, 24 h.

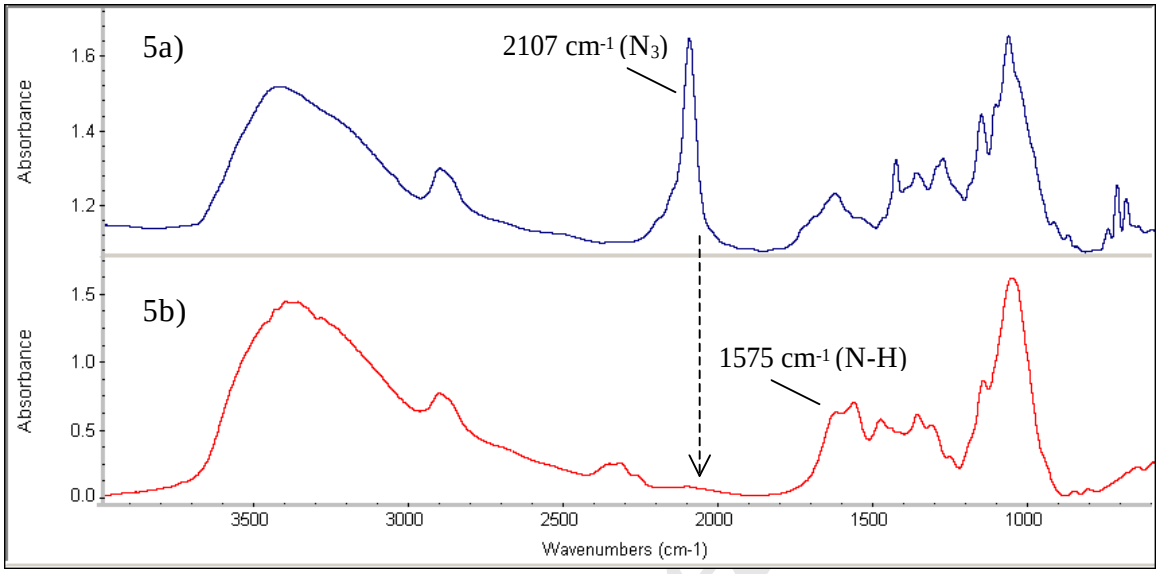


Figure **5**. FTIR spectra of 5a) 6-azido-6-deoxycurdlan and 5b) 6-amino-6-deoxycurdlan synthesized by $NaBH_4$ reduction.

4. 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.

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Supporting Information ^{13}C NMR spectra of 6-amino-6-deoxycurdlans prepared under these conditions; 80°C, 24 h and 60°C, 48 h. ^{1}H NMR spectra of 3,6,9-trioxadecanoic acid (TODA), 3,6,9-trioxadecanoyl chloride (TODCl) and peracetylated 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoyl-curdlan. FTIR spectra of 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoyl-curdlan and its residue after saponification. FTIR and ^{1}H NMR spectra of 6-acetamido-6-deoxycurdlan by sodium borohydride reduction of 6-azido-6-deoxy-2,4-acetyl-curdlan. Titration curves of 6-azido-6-deoxy-2,4-di-*O*-trioxadecanoyl-curdlan after saponification at 25°C for 24 h: pH and dpH change vs. volume of added HCl (0.1 N, mL). Titration curve of the product of reduction of 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).

References

Baumann, H., Liu, C., & Faust, V. (2003). Regioselectively Modified Cellulose and Chitosan Derivatives for Mono- and Multilayer Surface Coatings of Hemocompatible Biomaterials. *Cellulose, 10*, 65-74.

Boger, J., Corcoran, R. J., & Lehn, J. M. (1978). Cyclodextrin chemistry. Selective modification of all primary hydroxyl groups of ⟨- and ®-cyclodextrins. *Helv. Chim. Acta, 61*, 2190-2218.

Brown, M. S., & Rapoport, H. (1963). The Reduction of Esters with Sodium Borohydride. *J. Org. Chem., 28*, 3261-3263.

Cao, P., & Raleigh, D. P. (2010). 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., 132*, 4052-4053.

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

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

Davis, M. E., Pun, S. H., Bellocq, N. C., Reineke, T. M., Popielarski, S. R., Mishra, S., & Heidel, J. D. (2004). Self-assembling nucleic acid delivery vehicles via linear, water-soluble, cyclodextrin-containing polymers. *Curr. Med. Chem., 11*, 179-197.

Dorn, S., Pfeifer, A., Schlufter, K., & Heinze, T. (2010). Synthesis of water-soluble cellulose esters applying carboxylic acid imidazolides. *Polym. Bull., 64*, 845-854.

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

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

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

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

Freire, C. S. R., Silvestre, A. J. D., Pascoal Neto, C., & Rocha, R. M. A. (2005). An Efficient Method for Determination of the Degree of Substitution of Cellulose Esters of Long Chain Aliphatic Acids. *Cellulose, 12*, 449-458.

Furuhata, K., Chang, H. S., Aoki, N., & Sakamoto, M. (1992). Chlorination of cellulose with N-chlorosuccinimide-triphenylphosphine under homogeneous conditions in lithium chloride-N,N-dimethylacetamide. *Carbohydr. Res., 230*, 151-164.

Furuhata, K., Koganei, K., Chang, H.-S., Aoki, N., & Sakamoto, M. (1992a). 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., 230*, 165-177.

Furuhata, K., Koganei, K., Chang, H. S., Aoki, N., & Sakamoto, M. (1992b). 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., 230*, 165-177.

Hajeb, P., & Selamat, J. (2012). A Contemporary Review of Seafood Allergy. *Clin. Rev. Allerg. Immu., 42*, 365-385.

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

Hassani, L. N., Hendra, F., & Bouchemal, K. (2012). Auto-associative amphiphilic polysaccharides as drug delivery systems. *Drug Discov. Today, 17*, 608-614.

Heinze, T., & Schaller, J. (2000). New water soluble cellulose esters synthesized by an effective acylation procedure. *Macromol. Chem. Phys., 201*, 1214-1218.

Henni-Silhadi, W., Deyme, M., Boissonnade, M.-M., Appel, M., Le Cerf, D., Picton, L., & Rosilio, V. (2007). Enhancement of the Solubility and Efficacy of Poorly Water-Soluble Drugs by Hydrophobically-Modified Polysaccharide Derivatives. *Pharm. Res., 24*, 2317-2326.

Jagodzinski, P. P., Wiaderkiewicz, R., Kurzawski, G., Kloczewiak, M., Nakashima, H., Hyjek, E., Yamamoto, N., Uryu, T., Kaneko, Y., Posner, M.R., Kozbor, D. (1994). Mechanism of the inhibitory effect of curdlan sulfate on HIV-1 infection in vitro. *Virology, 202*, 735-745.

Jin, Y., Zhang, H., Yin, Y., & Nishinari, K. (2006). Comparison of curdlan and its carboxymethylated derivative by means of Rheology, DSC, and AFM. *Carbohydr. Res., 341*, 90-99.

Kato, Y., Onishi, H., & Machida, Y. (2005). Contribution of chitosan and its derivatives to cancer chemotherapy. *In Vivo, 19*, 301-310.

Lee, J. W., Yeomans, W. G., Allen, A. L., Gross, R. A., & Kaplan, D. L. (1997). Microbial production of water-soluble non curdlan type exopolymer-B with controlled composition by agrobacterium sp. *Biotechnol. Lett., 19*, 1217-1221.

Lee, M., Nah, J.-W., Kwon, Y., Koh, J., Ko, K., & Kim, S. (2001). Water-Soluble and Low Molecular Weight Chitosan-Based Plasmid DNA Delivery. *Pharm. Res., 18*, 427-431.

Lehtovaara, B. C., Verma, M. S., & Gu, F. X. (2012). Synthesis of curdlan-graft-poly(ethylene glycol) and formulation of doxorubicin-loaded core-shell nanoparticles. *J. Bioact. Compat. Polym., 27*, 3-17.

Liesiene, J., & Matulioniene, J. (2004). Application of water-soluble diethylaminoethylcellulose in oral drug delivery systems. *React. Funct. Polym., 59*, 185-191.

Liu, C., & Baumann, H. (2002). 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. *Carbohydr. Res., 337*, 1297-1307.

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

Liu, X.-p., Zhou, S.-t., Li, X.-y., Chen, X.-c., Zhao, X., Qian, Z.-y., Zhou, L.-n., Li, Z.-y., Wang, Y.-m., Zhong, Q., Yi, T., Li, Z.-y., He, X., & Wei, Y.-q. (2010). Anti-tumor activity of N-trimethyl chitosan-encapsulated camptothecin in a mouse melanoma model. *J. Exp. Clin. Cancer Res., 29*, 76.

Malm, C. J., Genung, L. B., Williams, J. R. F., & Pile, M. A. (1944). Analysis of Cellulose Derivatives Total Acyl in Cellulose Organic Esters by Saponification in Solution. *Ind. Eng. Chem. Anal. Ed., 16*, 501-504.

Matsui, Y., Ishikawa, J., Kamitakahara, H., Takano, T., & Nakatsubo, F. (2005). Facile synthesis of 6-amino-6-deoxycellulose. *Carbohydr. Res., 340*, 1403-1406.

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

Na, K., Park, K.-H., Kim, S. W., & Bae, Y. H. (2000). Self-assembled hydrogel nanoparticles from curdlan derivatives: characterization, anti-cancer drug release and interaction with a hepatoma cell line (HepG2). *J. Contr. Rel., 69*, 225-236.

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

Popescu, I., Pelin, I. M., Butnaru, M., Fundueanu, G., & Suflet, D. M. (2013). Phosphorylated curdlan microgels. Preparation, characterization, and in vitro drug release studies. *Carbohydr. Polym., 94*, 889-898.

Ranaldi, G., Marigliano, I., Vespignani, I., Perozzi, G., & Sambuy, Y. (2002). The effect of chitosan and other polycations on tight junction permeability in the human intestinal Caco-2 cell line. *J. Nutr. Biochem., 13*, 157-167.

Ravi Kumar, M. N. V. (2000). A review of chitin and chitosan applications. *React. Funct. Polym., 46*, 1-27.

Rege, P. R., & Block, L. H. (1999). Chitosan processing: influence of process parameters during acidic and alkaline hydrolysis and effect of the processing sequence on the resultant chitosan's properties. *Carbohydr. Res., 321*, 235-245.

Roemhild, K., Wiegand, C., Hipler, U.-C., & Heinze, T. (2013). Novel Bioactive Amino-Functionalized Cellulose Nanofibers. *Macromol. Rapid Commun., 34*, 1767-1771.

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. (2008). 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., 70*, 270-278.

Scriven, E. F. V., & Turnbull, K. (1988). Azides: their preparation and synthetic uses. *Chem. Rev., 88*, 297-368.

Shi, B., Zhang, H., Shen, Z., Bi, J., & Dai, S. (2013). Developing a chitosan supported imidazole Schiff-base for high-efficiency gene delivery. *Polym. Chem., 4*, 840-850.

Sohma, Y., Hayashi, Y., Skwarczynski, M., Hamada, Y., Sasaki, M., Kimura, T., & Kiso, Y. (2004). O-N intramolecular acyl migration reaction in the development of prodrugs and the synthesis of difficult sequence-containing bioactive peptides. *Peptide Sci., 76*, 344-356.

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

Stojanović, Ž., Jeremić, K., Jovanović, S., & Lechner, M. D. (2005). A Comparison of Some Methods for the Determination of the Degree of Substitution of Carboxymethyl Starch. *Starch, 57*, 79-83.

Tihlarik, K., & Pasteka, M. (1987). Estimation of functional groups and degree of substitution in carboxymethylstarch. *Chem. Pap., 41*, 703-708.

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

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

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

Zhou, Q., Zhang, L., Okamura, H., Minoda, M., & Miyamoto, T. (2001). Synthesis and properties of O-2-[2-(2-methoxyethoxy)ethoxy]acetyl cellulose. *J. Polym. Sci. Part A: Polym. Chem., 39*, 376-382.