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Preparation and characterisation of oligosaccharides produced by nitrous acid depolymerisation of chitosans

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Abstract

Two chitosans with widely different chemical composition (fraction of *N*-acetylated units (F_A) < 0.001 and $F_A = 0.59$), were degraded by nitrous acid, to obtain the reactive 2,5-anhydro-D-mannose- (**M**-) unit at the new reducing end. The fully *N*-acetylated and fully *N*-deacetylated oligomers were separated by size-exclusion chromatography. Both the chemical structure and purity were studied by one- and two-dimensional ^{1}H and ^{13}C NMR methods. The fully *N*-acetylated oligomers were found to be stable, whereas the *N*-deacetylated oligomers reacted intermolecularly by a Schiff base reaction between the 2-amino group on the *N*-deacetylated units and the **M**-units, facilitating the cleavage of the glycosidic bond next to the **M**-unit and the formation of 5-hydroxymethylfurfural (HMF).

Keywords: Chitosan; Depolymerisation; Nitrous acid; ^{1}H NMR; ^{13}C NMR; Size-exclusion chromatography; MALDI-TOF MS

1. Introduction

Chitosan is a linear copolymer of β-(1→4) bound 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc, **A**-unit) and 2-amino-2-deoxy-D-glucopyranose (GlcN, **D**-unit) units produced by alkaline *N*-deacetylation of chitin (homopolymer of β-(1→4) bound GlcNAc), a structural polysaccharide in the exoskeleton of arthropods and in the cell-wall of many fungi.[1] The solution properties of chitosan are governed by the relative content of acetylated amino groups designated by the fraction of *N*-acetylated units, F_A, the distribution of **A**-units, the molecular weight, the molecular-weight distribution, the ionic strength and the pH value, as the pK_a-value of the amino-group in chitosan has been determined as 6.5.[2,3] It has previously been shown that the **A**- and **D**-units are randomly distributed along the chain in water-soluble chitosans.[4,5] Depolymerisation of chitosan by the use of nitrous acid (HONO) is a homogeneous reaction where the number of glycosidic bonds broken is roughly stoichiometric to the amount of nitrous acid used.[6,7] The mechanism involves a deamination of a **D**-unit forming 2,5-anhydro-D-mannose (**M**-units) at the new reducing end (see Scheme 1). Since the **M**-residue is unstable, the standard procedure has been to reduce to 2,5-anhydro-D-mannitol by the use of $NaBH_4$. Depolymerisation of chitosans, by use of an excess of nitrous acid and subsequent reduction, has been used previously to study the distribution of *N*-acetylated units in partially *N*-acetylated chitosan.[8]

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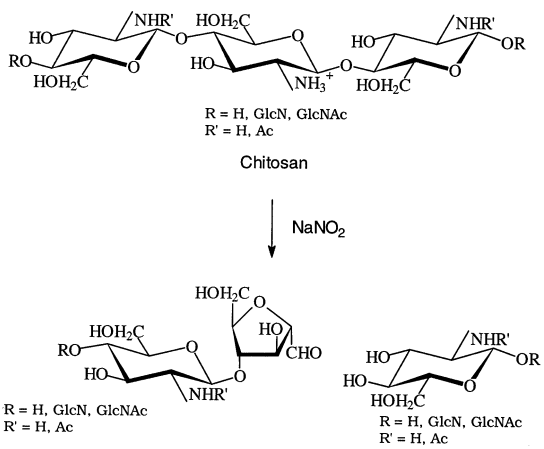


Scheme 1. The mechanism by which the nitrous acid reaction leads to chain cleavage resulting in a 2,5-anhydro-D-mannose-(**M**-) reducing end.

The scope of this study has been to prepare and characterise defined fully *N*-acetylated and *N*-deacetylated oligomers with the intact **M**-unit at the reducing end. The **M**-unit has some advantages over a normal reducing end. 2,5-Anhydro-D-mannose does not mutarotate in solution and the aldehyde group is more available for reactions (e.g., reductive amination) since it does not participate in intramolecular hemiacetals. This makes chito-oligomers with an **M**-unit as the reducing end an interesting precursor in organic synthesis of e.g., glycodendrimers.

2. Experimental

Materials.—Two chitosans with fraction of acetylation (F_A) of 0.59 and < 0.001 (as determined by ^{1}H NMR spectroscopy), where used in this study. Chitin was prepared from shrimp shells.[9] The chitosan with F_A of 0.59 was prepared by homogeneous deacetylation[10] of chitin, while the fully *N*-acetylated chitosan was prepared by further heterogeneous deacetylation of a commercial chitosan with $F_A = 0.01$. Sodium nitrite and ammonium acetate were obtained from Merck, D_2O (99.96% D atom) from Isotech Inc., DCl (11.7 M, 35%), HMF and NaOD from Sigma and Superdex 30 (prep grade) came from Pharmacia Biotech.

Preparation of fully N-*acetylated oligomers.*—Chitosan ($F_A = 0.59$, $[\eta] = 826$ mL/g, 500 mg, HCl form) was dissolved in 30 mL 2.5% v/v AcOH. Dissolved oxygen was removed by bubbling N_2 gas through the solution for 5 min. After cooling to 4 °C, a freshly prepared solution of $NaNO_2$ (1.5 mmol) was added, and the reaction was allowed to proceed for 12 h at 4 °C in darkness without stirring. The product was centrifuged (10 min, 5000 rpm) and filtered (8 μm), to remove the insoluble fractions of fully *N*-acetylated oligomers before lyophilisation.

Preparation of fully N-*deacetylated oligomers.*—A solution of chitosan ($F_A < 0.001$, $[\eta] = 283$ mL/g, 500 mg, HCl form) was prepared and degraded by HNO_2 as described for the first sample, the only exception being that the molar ratio of **D**-units to $NaNO_2$ was reduced to 0.45 (1.13 mmol $NaNO_2$). All the fully *N*-deacetylated oligomers were soluble at low pH-values.

Chromatography.—The oligomers (500 mg) were separated by gel-filtration on two 2.5 × 100 cm columns connected in series packed with Superdex 30, eluted with 0.15 M ammonium acetate at pH 4.5. A flow-rate of 0.8 mL/min was used and the entire separation took 16 h. The relative amounts of oligomers were monitored by means of an on-line refractive index (RI) detector (Shimadzu RID-6A). Fractions of 4 mL were collected and pooled to provide the purified oligomers.

NMR.—All samples were dissolved in D_2O, and transferred to 5-mm NMR tubes. The measurements were performed on a Dpx 400 Bruker Avance spectrometer. All chemical shifts were determined relative to internal TSP (sodium 3-(trimethylsilyl)-propionate-d_4 from Aldrich Chemical Co., 5 μL added from a 1% stock solution).[11] Typical conditions for the acquisition of ^{1}H NMR spectra: 400.13 MHz; 25 °C; size of spectral window, 8220 Hz; centre of the ^{1}H NMR spectra, 1880 Hz; acquisition time, 3 s; actual pulse repetition time, 4 s; number of scans, 64 and a 30° excitation pulse-angle was used; data size, 32 K. Typical conditions used for ^{13}C NMR acquisition: 100.64 MHz; 25 °C; size of spectral window, 31,850 Hz; centre of the ^{13}C NMR spectra, 12,700 Hz; acquisition time, 3 s; actual pulse

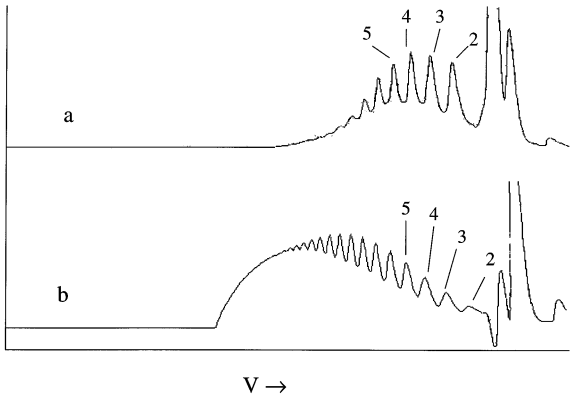


Fig. 1. Size-exclusion chromatograms of the (a) fully *N*-acetylated; and (b) fully *N*-deacetylated chito-oligomers. (Superdex 30; two 2.5 × 100 cm columns in series, 0.15 M ammonium acetate, pH 4.5, flow rate 0.8 mL/min.)

repetition time, 6 s; number of scans, 20,000; 30° excitation pulse-angle; data size, 32 K. The spin-system was completely decoupled. Conditions for acquisition of the COSY (2-D homonuclear correlation spectroscopy) spectrum:[13] memory size, 1024 (F2) × 128 K (F1); size of spectral window, 2400 (F2) × 2400 Hz (F1); centre of spectrum, 1881 Hz. Conditions for acquisition of the HETCOR (2-D heteronuclear correlation spectroscopy) spectrum:[14] memory size, 4096 (F2) × 68 K (F1); size of spectral window, 6040 (F2) × 2400 Hz (F1); centre of spectral windows, 8049 Hz for F2 and 1873 Hz for F1. Both the COSY and the HETCOR spectra were recorded in D_2O at 35 °C and pH* 5.0. The pH* (value obtained when measuring with a pH-meter, correction for isotopic effect:[12] pD = pH* + 0.4) were measured by use of a Mettler Toledo pH electrode. The pH* value was controlled in all NMR-samples.

MALDI-TOF MS.—Two samples, the *N*-acetylated hexamer (**A**–**A**–**A**–**A**–**A**–**M**) and the *N*-deacetylated tetramer (**D**–**D**–**D**–**M**) obtained from the gel-filtration where solved in deionised water (1 mg/mL) after lyophilisation. After mixing the oligomer solutions 1:4 with 2,5-dihydroxybenzoic acid (DHB) in EtOH (100mM), 3 μL from each of the two mixtures where dried on a target plate and analysed on a Bruker MALDI-TOF Reflex III mass spectrometer.

3. Results and discussion

Preparation.—The fully *N*-acetylated oligomers (**A**–[**A**]$_n$–**M**) were prepared by adding an excess of nitrous acid to a chitosan ($F_A = 0.59$) in order to convert all **D**-units to **M**-units. The fully *N*-deacetylated oligomers (**D**–[**D**]$_n$–**M**) were prepared by nitrous acid depolymerisation of a fully *N*-deacetylated chitosan ($F_A < 0.001$), where the molecular-weight distribution of the oligosaccharides can be controlled by the molar ratio of nitrous acid to **D**-units.

Separation.—The oligomers were separated by size-exclusion chromatography (SEC), and the chromatograms of the fully *N*-acetylated and fully *N*-deacetylated oligomers are given in Fig. 1. For the *N*-acetylated oligosaccharides 10–12 peaks were seen. The depolymerised chitosan contained an insoluble fraction, which were probably the higher oligosaccharides. Comparison of the reducing-end intensities (at resonance 4.14, 4.36 and 5.01 ppm) with those of the H-1 (**A**-units) signal at the glycosidic bonds (4.55–4.58 ppm) of the different *N*-acetylated fractions (Fig. 2), confirmed the separation to be according to decreasing chain-length (i.e., the longest chains were eluted first), and also the DP (degree of polymerisation) as given in Fig. 1. Similar results were seen for the *N*-deacetylated fractions.

Chemical structure of the N-*acetylated oligomers.*—The fully *N*-acetylated oligomer fractions separated by SEC were characterised using ^{1}H and ^{13}C NMR. The trimer 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2,5-anhydro-D-mannofuranose (**A**–**A**–**M**) was chosen to assign the reducing end resonances by the use of 2-D homonuclear correlation spectroscopy (COSY, see Fig. 3(a)), and 2-D heteronuclear correlated spectroscopy (HETCOR, see Fig. 3(b)). In combination with previously published spectra of fully *N*-acetylated oligosaccharides,[15] the ^{1}H and ^{13}C chemical shifts of the reducing end of the trimer (**A**–**A**–**M**) were assigned and the results are presented in Tables 1 and 2, respectively. Neither the ^{1}H nor the ^{13}C spectra

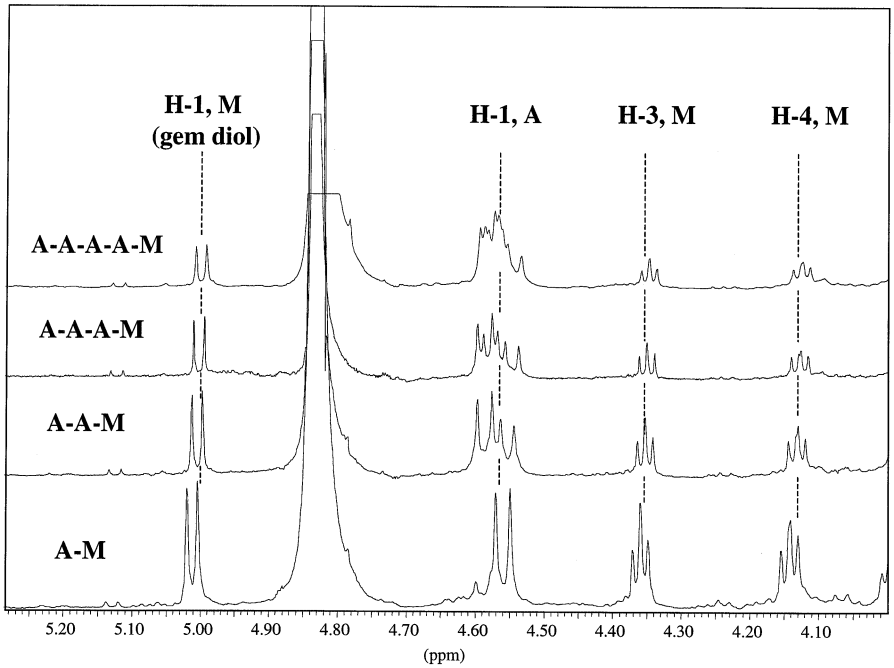


Fig. 2. ^{1}H NMR spectra of the fully *N*-acetylated oligomer fractions as they are collected from the peaks indicated in Fig. 1(a). The spectra were recorded at 400.13 MHz, 25 °C and pH* 5.0.

Table 1
^{1}H NMR (400.13 MHz) chemical shifts (ppm) and vicinal coupling constants (in Hz, given in parenthesis) for the reducing end (**M**-unit) of the *N*-acetylated trimer (**A**–**A**–**M**) in D_2O at 25 °C and pH* 5.0

H-1, gem diol	H-2	H-3	H-4	H-5	H-6a	H-6b
5.01 (6.1)	3.76 (4.5, 6.1)	4.35 (4.5, 4.5)	4.13 (4.3, 5.4)	3.98 (5.4, 9.5)	3.43 (9.5)	3.49 (9.5)

contained the resonances expected for the free aldehyde group of the **M**-residue (^{1}H: ca. 9–10 ppm and ^{13}C: ca. 180 ppm[13]). The H-1 and C-1 resonances at 5.01 and 89.5 ppm, respectively, indicated the presence of a hydrated aldehyde where a water molecule has been added to the carbonyl group (a so-called gem diol), which seems to be the only existing form of the reducing end in water. No major by-products of the nitrous acid depolymerisation reaction can be identified in the purified trimer fraction. The assignments were almost identical for the *N*-acetylated oligomers, and are not discussed further here.

Chemical structure of the N-*deacetylated oligomers.*—The chemical shifts of the **M**-residue resonances from the *N*-deacetylated trimer (**D**–**D**–**M**) were generally shifted to higher ppm-values (deshielded) relative to the same resonances from the *N*-acetylated trimer (Table 3), and the results from the former was used in combination with the published spectra of fully *N*-deacetylated oligosaccharides.[16] The ^{1}H and ^{13}C assignments of the reducing end resonances are presented in Tables 4 and 5, respectively. The H-1 proton (gem diol) of the reducing **M**-unit is sensitive to whether the neighbouring unit is a **A**- or a **D**-unit. This is also seen in the previously published spectra[4] of partially *N*-acetylated chitosan which were depolymerised using nitrous acid, where the

Table 2
^{13}C NMR (100.64 MHz) chemical shifts (ppm) for the reducing end (**M**-unit) of the *N*-acetylated trimer (**A**–**A**–**M**) in D_2O at 25 °C and pH* 5.0

C-1	C-2	C-3	C-4	C-5	C-6
91.86	88.13	79.18	88.03	85.01	61.8

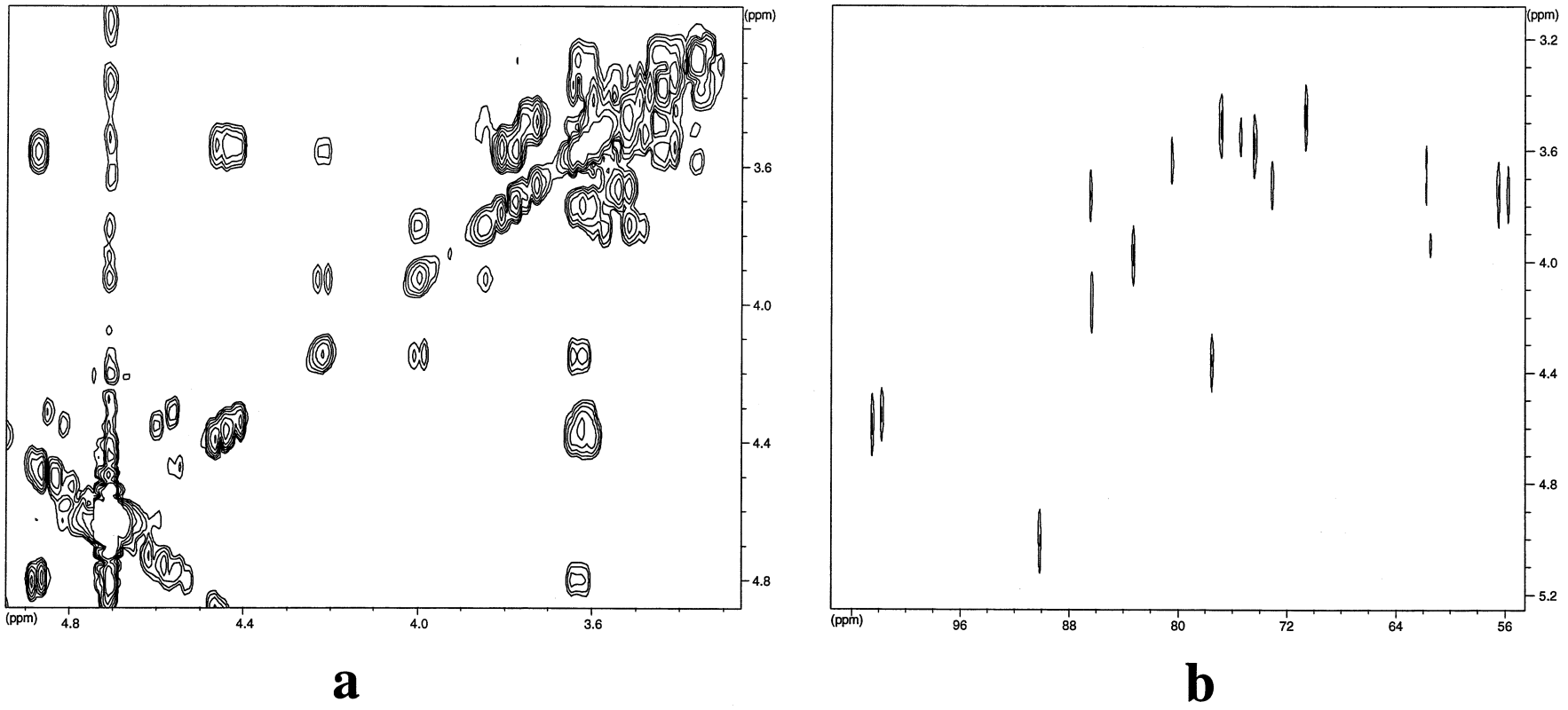


Fig. 3. The 2D NMR spectra used to elucidate the chemical shifts of the 2,5-anhydro-D-mannose reducing end of the fully *N*-acetylated trimer: (a) homonuclear correlation spectroscopy (COSY); and (b) heteronuclear correlated spectroscopy (HETCOR). The recordings were done at 400.13 MHz (^{1}H), 100.64 MHz (^{13}C), 25 °C and pH* 5.0.

Table 3
^{1}H NMR (400.13 MHz) chemical shifts (ppm), vicinal coupling constants (Hz) for the reducing end (**M**-unit) on the *N*-deacetylated trimer (**D**–**D**–**M**) in D_2O at 43 °C and pH* 5.7

H-1, free aldehyde	H-1, gem diol	H-1, Schiff base	H-2	H-3	H-4	H-5	H-6a	H-6b
9.49	5.09 (5.3)	7.93–7.97	3.84 (5.3, 5.3)	4.44 (4.4, 5.3)	4.22 (4.7, 5.3)	4.13 (5.3, 9.9)	[a] (9.9)	[a] (9.9)

[a] Due to signal overlapping, assignment were not possible for these protons which resonate in the 3.7–3.9 ppm region.

H-1 protons of the M-units are seen as two resonances at 5.01 (–**A**–**M**) and 5.09 ppm (–**D**–**M**). In contrast to the *N*-acetylated oligosaccharides, the aldehyde of the **M**-unit exists both in its hydrated (–$CH(OH)_2$) and free (–CHO) form, and was also found to be quite unstable as a result of the preparation procedure. During the lyophilisation process of the purified oligomers from the buffer (containing ammonium acetate and acetic acid), the pH value in the fractions changed from 4.5 to 8 as water and acetic acid evaporated first, leaving ammonia. The pK_a-value of the primary amino group of chitosan has been determined as 6.5,[2,3] meaning that the amino groups of the *N*-deacetylated oligomers were deprotonated during lyophilisation to give a strong nucleophile, which could react with the aldehyde of the reducing end. This produced a reversible imino bond with the release of a water molecule, and as water evaporated during the lyophilisation, the reaction was facilitated. This is a Schiff base reaction, similar to the non-enzymatic browning reactions often seen in foods (Maillard browning), where side chain amino groups of proteins react with mono- and oligosaccharides producing melanoidin pigments.[17,18] The occurrence of the imino proton (–C*H*=N–) is clearly seen in the ^{1}H NMR spectrum for the *N*-deacetylated trimer (**D**–**D**–**M**) shown in Fig. 4(a), with a characteristic chemical shift at 8.1 ppm.[19,20] When the pH* was reduced from 5.7 to 4.0, the spectrum changed drastically, as shown in Fig. 4(b). The intensity of the imino protons had almost disappeared, and was replaced by the resonances characteristic of 5-hydroxymethylfurfural (HMF), as indicated in Fig. 4(b) and Table 5. These were identical to the resonances and coupling constants obtained when a ^{1}H NMR spectrum of pure (commercial) HMF was acquired. In addition, the resonances and coupling constants of a normal, reducing end of a *N*-deacetylated dimer (**D**–**D**) were identified in the spectrum (see Fig. 4(b) and Table 5) when compared to earlier work.[16,21] HMF is a common by-product of Maillard browning, produced by two eliminations of water followed by chain cleavage. According to earlier studies,[22] the rate-limiting step in the formation of HMF, is the first β elimination leading to 3-deoxy-hexulose. When the *N*-acetylated trimer (**A**–**A**–**M**) was subjected to strongly acidic conditions (pH* 1), only negligible amounts of HMF (ca. 1%) were detected by ^{1}H NMR. Gottschalk showed in 1952, that while fructose under mild acidic conditions (2 M acetic acid) was converted to HMF (63%) in the presence of phenylalanine, only negligible amounts of HMF could be detected without the presence of the amino acid.[23] Therefore it seems likely

Table 4
^{13}C NMR (100.64 MHz) chemical shifts (ppm) for the reducing end (**M**-unit) of the *N*-deacetylated trimer (**D**–**D**–**M**) in D_2O at 25 °C and pH* 5.7

C-1, gem diol	C-2	C-3	C-4	C-5	C-6
89.8	86.5	77.0	85.6	82.6	63.1

Table 5
^{1}H NMR (400.13 MHz) chemical shifts (ppm) and vicinal coupling constants (Hz) confirming the presence of dimer $(GlcN)_2$ (**D**–**D**) and 5-hydroxymethylfurfural (HMF) in the *N*-deacetylated trimer (**D**–**D**–**M**) fraction, after adjusting to pH* 4.0 (43 °C, D_2O)

HMF	H-1	H-3	H-4	H-6
	9.50	7.52 (3.8)	6.66 (3.8)	4.68
$(GlcN)_2$	H-1α	H-1β		
	5.44 (3.2)	4.92 (8.5)		

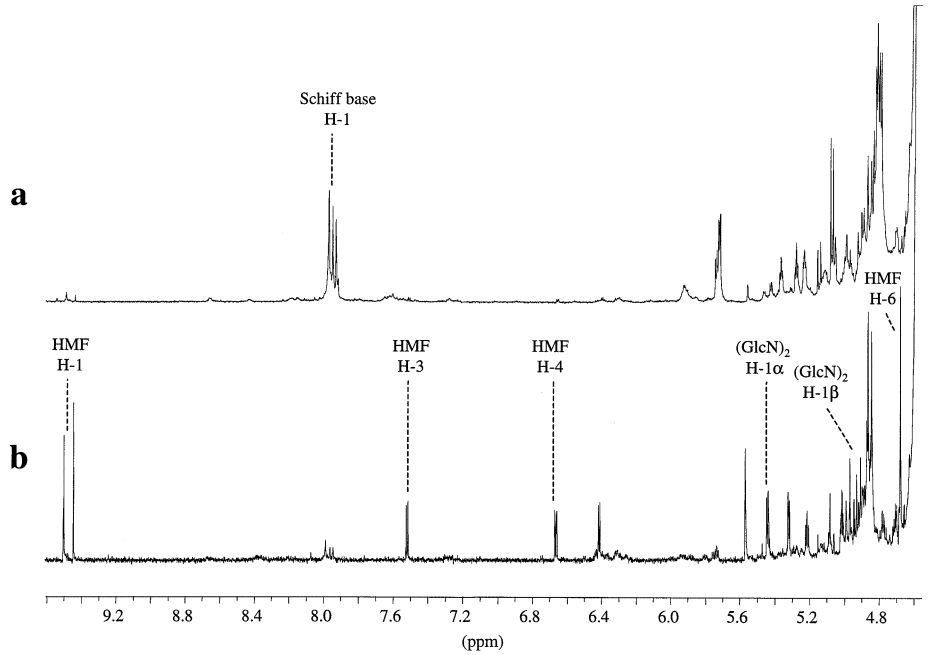
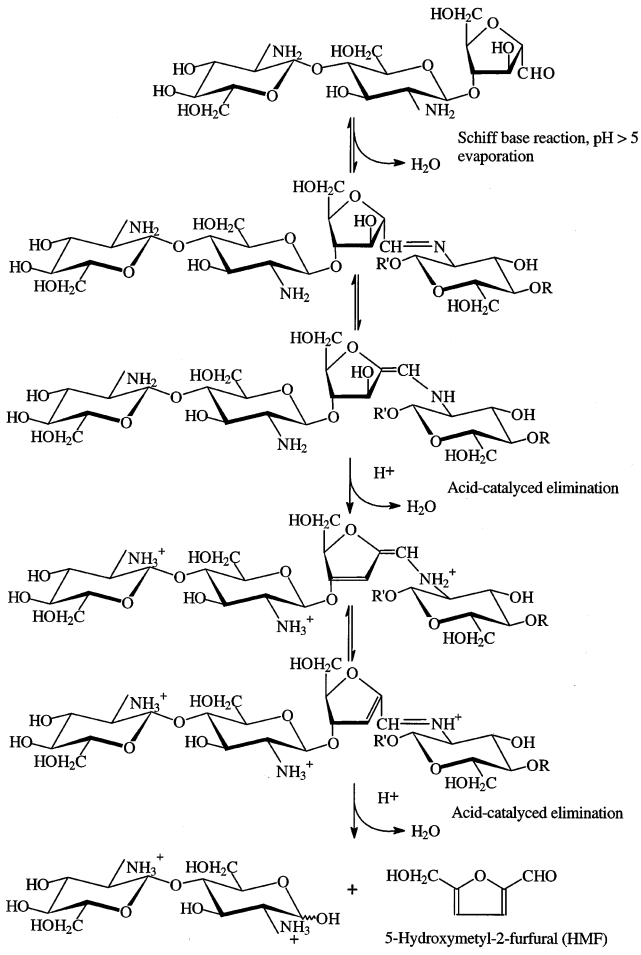


Fig. 4. ^{1}H NMR spectrum (400.13 MHz, 43 °C in D_2O) of the *N*-deacetylated trimer (**D**–**D**–**M**) after lyophilisation: (a) at pH* 5.7. The intensities of the imino protons (Schiff base) are indicated in the spectrum (8.1 ppm). (b) At pH* 4.0 (adjusted with 0.1 M DCl). The resonances of HMF and the reducing end of the GlcN-dimer (**D**–**D**) are indicated.

to conclude that the formation of a Schiff base facilitated the elimination reactions leading to the formation of HMF and the subsequent cleavage of the glycosidic bond (Scheme 2) in a similar manner as for β-carbonyl groups (β elimination).[23] The formation of Schiff bases could only occur during the final lyophilisation step, where the pH increased to well above the pK_a-value of the amino group of the **D**-unit (6.5). During all the prior steps the oligomers were kept at acidic conditions (pH was never above 4.5), and the amino group will only exist in the protonated form, i.e., as a poor nucleophile. It is only as a nucleophile (deprotonated) that the amino group can react with the carbonyl group to form a Schiff base. When the pH value is reduced to well below the pK_a-value of the amino group, existing imino nitrogens are protonated and the divalent bonds are either dissociated reversibly to the precursors, or an elimination reaction may occur to yield the 3-deoxy derivative of 2,5-anhydro-D-mannose. After this rate-determining step, HMF is formed directly, or via its Schiff-base derivative, produced by elimination of the GlcN-disaccharide (**D**–**D**). If the *N*-deacetylated oligomers are to be isolated in their pure form, they have to be separated and dried in a system where the pH value can be kept well below the pK_a-value of the 2-amino group. As an additional confirmation of the chemical structure of the separated oligomers, the *N*-acetylated hexamer (**A**–**A**–**A**–**A**–**A**–**M**) and *N*-deacetylated tetramer (**D**–**D**–**D**–**M**) were analysed by matrix-assisted laser desorption/ionisation time-of-flight mass spectroscopy (MALDI-TOF MS). For the *N*-acetylated hexamer (**A**–**A**–**A**–**A**–**A**–**M**), a major peak was obtained at m/z 1199.38 which was interpreted as the hydrated $[M + Na]^+$-ion of the oligomer. For the *N*-deacetylated tetramer (**D**–**D**–**D**–**M**), the two main peaks were the $[M + Na]^+$-ions of the *N*-deacetylated trimer (**D**–**D**–**D**) and the tetramer (**D**–**D**–**D**–**M**) at m/z 507.17 and 668.18, respectively. These mass/charge ratios (m/z) are in agreement with the NMR study.

4. Conclusion

Fully *N*-acetylated low-molecular weight chito-oligomers with hydrated aldehyde reducing-ends were produced by nitrous acid depolymerisation of a partially *N*-acetylated



Scheme 2. Proposed mechanism of the Schiff base reaction (trimer, **D**–**D**–**M**) facilitating the elimination of water leading to the formation of HMF and chain cleavage.

chitosan. Their stability and reactivity makes them well suited as synthetic precursors. It was possible to produce fully *N*-deacetylated oligomers by nitrous acid depolymerisation of fully *N*-deacetylated chitosan. However, the reducing end aldehyde groups and the 2-amino groups of the **D**-units reacted to produce Schiff-bases under neutral/basic conditions with subsequent elimination reactions and formation of by-products upon acidification, and are therefore less suited for use in synthesis.

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