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Preparation and NMR characterization of glucosamine oligomers bearing an azide function using chitosan

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ABSTRACT

In this study, a procedure to produce glucosamine oligomers with the amino functions transformed into azido groups was optimized, and HPLC purification afforded to the isolation of nine different oligosaccharides derivatives, with the reducing end transformed in alditol. These oligomers differed for the degree of polymerization and for the type of alditol at the reducing end. The first group comprehended species from di- to hexasaccharide, with all the amino functions converted into an azido group. The second and the third groups were isolated in minor yields, and were both constituted from tri- and tetrasaccharides; the difference between the two groups regarded exclusively the type of alditol found at the reducing end, which was a glucosaminitol in the first case, or a N-acetylglucosaminitol in the other. Products were fully characterized by 2D NMR spectroscopy. The azido moieties installed on these oligosaccharides can be further exploited in Cu(I) catalyzed azido-alkyne cycloaddition reactions.

1. Introduction

Chitosan is a linear polymer consisting of β-(1 → 4)-linked 2-amino-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-glucose units in varying proportions. Although present in biomass, this polysaccharide is preferentially obtained by alkaline *N*-deacetylation of chitin (homopolymer of β-(1 → 4) bound GlcNAc), a structural polysaccharide present in the exoskeleton of arthropods and in the cell-wall of many fungi (Muzzarelli, 2011; Muzzarelli, Boudrant, Meyer, Manno, DeMarchis, & Paoletti, 2012).

This biopolymer displays peculiar physiochemical and biological properties and it finds its place in a vast range of applications, in food, agriculture, medicine and drug delivery (Trombotto, Ladavière, Delolme, & Domard, 2008 and references therein). However, its insolubility at neutral pH and its high viscosity in aqueous solution restricts its application. This problem is partly circumvented nowadays making more soluble chitosan derivatives (Batista, Pinto, Gomes, & Gomes, 2006; Gomes, Gomes, Batista, Pinto, & Silva, 2008; Oliveira, Martins, Mafra, & Gomes, 2012) or working with chitosan oligomers, which display interesting antibacterial, antitumorogenic and biological properties (Lee, Nah, Kwon, Koh, Ko, & Kim, 2001; Richardson, Kolbe, & Duncan, 1999; Sato, Ishii, & Okahata, 2001).

Indeed, reduction in chitosan's molecular weight facilitates its manufacture into various biomedical devices thanks its decreased viscosity; low molecular weight chitosan has been shown to effectively complex DNA and prevent nuclease degradation in addition to improving gene expression of luciferase plasmid. Finally, lower molecular weight chitosan would likely be cleared more efficiently after intravenous injection (Knight, Shapka, & Amsden, 2007).

Indeed, the properties of chitosan or of its oligomers can be modulated varying the type of substituent on the sugar ring, so that new functional polymers are obtained (Mourya & Inamdar, 2008), decorated mostly at the primary hydroxyl function or at the amino group. Nowadays, because of the introduction of the click chemistry reactions, as the Huisgen 1,3-dipolar cycloaddition in the Cu(I)-catalyzed variant (Kolb, Finn, & Sharpless, 2001), the number of new applications involving chitosan and its suitable derivatives, is destined to increase quite rapidly.

In this frame, this work deals with preparation of oligosaccharides presenting azido functions (Fig. 1) useful for further modification according to the click chemistry approach (Kulbokaite, Ciuta, Netopilik, & Makuska, 2009) using chitosan as starting material.

This approach presents some advantages with respect to the traditional organic synthesis: the low cost of the starting material, chitosan, and the usage of water as solvent, avoiding therefore the usage of organic solvents and of protection–deprotection procedures. As shown in results, isolation of a panel of oligomers of different lengths, with the azido function installed directly on the

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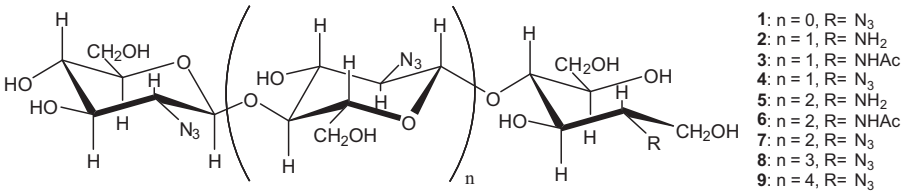

Fig. 1. Structure of the different azide glucosamine oligomers isolated via RP8 HPLC chromatography.

monosaccharide ring is made possible without the tailored synthesis of each of them.

2. Material and methods

2.1. Hydrolysis of chitosan

Chitosan (230 mg, 1.43 mmol of GlcN, Sigma–Aldrich 448869, deacetylation degree 10%, MW 612 kDa (Tsung-Lin & Tai-Horng, 2009)) was dissolved in 12.5 M aqueous HCl (2.3 mL, Fluka 96208); the solution was kept at 70 °C in a thermostatic oil-bath and after 150 min the reaction vessel was cooled at room temperature, treated with 3 volumes of ice cold acetone. Chitosan oligosaccharides were recovered by centrifugation (5000 rpm, 15 min), washed three times with cold acetone, dissolved in water; the resulting solution was neutralized adding few drops of 5 M aqueous NaOH and freeze-dried (179 mg, 1.11 mmol of GlcN, 77.9% yield).

2.2. Reduction of chito-oligosaccharide mixture

Chito-oligosaccharides (50 mg, Section 2.1.) were dissolved in 0.5 M AcOH/AcONa buffer (5.0 mL, pH 7.0) and reduced with $NaBH_4$ (3 mg) at room temperature over night; the reaction was quenched by adding with few drops of HCl 1 M and water was roto-evaporated adding MeOH.

2.3. Preparation of glucosamine oligosaccharide derivatives

Reduced glucosamine oligosaccharide mixture (50 mg, 0.310 mmol, Section 2.2) was dissolved in 10 mL of 1 M phosphate buffer together with, $CuSO_4{\cdot}5H_2O$ (0.77 mg, 0.01 equiv.), and imidazole-1-sulfonyl azide hydrochloride (78.1 mg, 1.2 equiv.; Stick reagent) (Goddard-Borger & Stick, 2007). The mixture was stirred at room temperature for 3 h, and the excess of Stick reagent was quenched with few drops of ethanol-amine. The solution was directly purified by HPLC chromatography.

2.4. HPLC purification of glucosamine oligosaccharide derivative mixture

Purification was performed on a Discovery octyl reverse phase or RP8 column (59354-U, 25 cm × 4.6 mm i.d., particle size 5 μm, pore size 180 Å) mounted on an Agilent 1100 HPLC instrument, equipped with a binary pump and an UV/Vis detector.

Chromatographic separation of the mixture was achieved working at a flow rate of 0.8 mL/min, the column was equilibrated with 5% of solvent B (0.1% TFA in MeOH) in solvent A (0.1% TFA in water), and oligosaccharides were eluted by varying solvent B after 5 min from 5% to 40% over 25 min then raising B to 100% over 15 min. The elution profile was monitored reading at 206 nm and the following yields were obtained (structures in Fig. 1): **1** (6.8 mg, yield 13.60%), **2** (1.6 mg, 3.2%), **3** (4.3 mg, 8.6%,), **4** (5.0 mg, 10%), **5** (2.8 mg, 5.6%), **6** (2.2 mg, yield 4.4%), **7** (4.8 mg, 9.6%), **8** (3.0 mg, 6.0%), **9** (3.3 mg, 6.6%).

2.5. NMR spectroscopy

^1H and ^{13}C NMR experiments were performed using a Bruker DRX-600 equipped with a cryogenic probe and spectra were calibrated using acetone (δ_H = 2.225 ppm, δ_C = 31.45 ppm) as internal standard. Commercial chitosan (5 mg) was solved in 550 μL of deuterated water (D_2O) containing 0.1% TFA. Products **1**–**9** were solved in D_2O and acetone (ca. 0.5 μL) was added with a glass capillary, as internal reference.

Two-dimensional spectra (DQ-COSY, TOCSY, TROESY, gHSQC and gHMBC) were measured using the standard Bruker software (Topspin 2.1). For the homonuclear experiment, 512 FIDs of 2048 complex data points were collected, with 24 scans per FID, a mixing time of 120 or 300 ms was applied for the TOCSY and TROESY spectra, respectively. For the HSQC and HMBC spectra, 512 FIDS of 2048 complex points were acquired with 32 scans per FID for HSQC or 50 for HMBC. Processing of Bruker data and analysis was performed with Bruker TopSpin 3 program.

3. Result and discussion

3.1. Hydrolysis of chitosan and study of DP distribution of the GlcN oligomer mixture

Chitosan was partially depolymerized with concentrated HCl producing a series of glucosamine oligomers. Strong acid hydrolysis condition was preferred to other possible approaches, as the use of nitrous acid or enzymatic treatments (Allan & Peyron, 1997; Muzzarelli, 1997) because in this case the depolymeration is accompanied from the cleavage of the *N*-acetyl group. The resulting oligomers then possess a higher number of free amino functions, with respect to that that can be observed adopting the other treatments.

Products were isolated by precipitation with cold acetone, centrifugation and freeze–drying; 78% in weight of the starting material was obtained and analyzed via ^1H NMR to determine the acetylation and the polymerization degree (Fig. S1, shown in supporting information).

^1H NMR spectrum (Fig. S1, shown in supporting information) showed different diagnostic signals: the region at approx. 2 ppm displayed the methyl signals of the *N*-acetyl groups, at 3.2 and 3.4 ppm the H-2 protons of the non acetylated GlcN were present, carbinolic protons were located in the region between 3.40 and 4.20 ppm, and the lower field region of the spectrum displayed the anomeric protons of both reducing and non reducing glucosamine units. Importantly, the group of signals at ca. 4.8 ppm corresponded to H-1 of non reducing residues while signals at 5.04, 5.40 and 5.53 corresponded to H-1 protons of the β (the first of the three) and α-reducing residues. By comparison of signal's integration of acetyl protons of GlcNAc units (0.0086) with signal's integration of H-2 proton of GlcN (0.0869 and 0.0127) it was possible to establish NH_2 free groups amount according to formula (1):

$$\%NH_{2\text{-free}} = \left(\frac{I_{\text{H-2(GlcN)}}}{I_{\text{H-2(GlcN)}} + (1/3)I_{CH_3}}\right) \times 100 = 97.2\% \quad (1)$$

Interestingly, acetylation degree for the intact chitosan was evaluated via ^{1}H NMR (data not shown) and resulted equal to 10%, indicating that the hydrolysis condition caused the depolymerization of the sample and its almost complete deacetylation. This side reaction increased the number of the free amino groups of the mixture that could be transformed later into azido functions.

Similarly, applying Eq. (2), it was also possible to establish the averaged degree of polymerization of the oligosaccharide mixture, which was 5.10.

$$\mathrm{DP} = \left(\frac{I_{\text{non reducing protons}}}{I_{\text{reducing protons}}} \right) + 1 = 5.10 \quad (2)$$

3.2. Preparation and isolation of the azido chito-oligosaccharides

The oligosaccharide mixture was reduced at the reducing anomeric center yielding to a mixture of glucosamine oligosaccharide alditols. This reaction was performed at pH = 7.0 to prevent the pH raising induced from $NaBH_4$ and the precipitation of the oligomers, which resulted in the non-complete reduction of the compounds. Among the buffer systems used, AcOH/AcONa gave better results even with respect to phosphate buffer which is usually more indicated for this pH value.

Since partial insolubility of the reduced glucosamine oligomers in the classic diazotransfer reaction conditions (Goddard-Borger & Stick, 2007), the reaction was conducted in phosphate buffer 0.5 M at pH = 7.0 with imidazol-1-sulfonyl-azide hydrochloride and without Na_2CO_3. The use of this reagent was preferred to triflyl-azide because it has similar reactivity but it is more stable, easy to handle and cheap, and it is reported to give better yields on chitosan (Kulbokaite, Ciuta, Netopilik, & Makuska, 2009).

The reaction conditions were optimized on glucosamine hydrochloride first (results not shown), and then transferred to the glucosamine oligosaccharide mixture; the reaction proceeded at room temperature and it was quenched after 3 h with few drops of ethanolamine; the mixture of products was directly purified by HPLC chromatography.

The chromatographic profile (Fig. 2) showed the occurrence of several peaks, which were purified and analyzed via ^{1}H NMR in order to identify the resulting azide oligosaccharides.

As result, the fractions eluted within the first 5 min contained mainly salts, and small amount of products related to the oligosaccharide derivatives; in some cases no signal was detected in the proton spectrum, suggesting that the sample contained NMR silent components, like an inorganic salt. This outcome was expected because the reaction mixture contained sodium acetate (from the $NaBH_4$ reduction), phosphate and copper sulfate.

3.3. Spectroscopic analysis of purified products

^{1}H NMR spectra were recorded for all the fractions collected from HPLC purification (Fig. 2) and identification of the oligosaccharides derivatives was guided from the presence of the anomeric signals of the glucosamine derivatives residues, found at ca. 4.6 ppm.

Consequently, peaks **1**–**9** were identified and divided in three groups for simplicity of discussion.

The first group comprehended oligosaccharides **1**, **4**, **7**–**9** (Fig. 3) which, differ from the other two groups, did not display any proton signal between 4.55 and 4.00 ppm.

These compounds were eluted in increasing order of length as indicated from the increasing number of anomeric protons in the low field region of the spectrum. In depth spectroscopical attribution is here reported for product **4** (Table 1, expansion of the HSQC spectrum in Fig. S2 of the supporting information), chemical shifts for the longer oligomers **8** and **9** are in the supporting information (Tables S1 and S2, expansion of the HSQC spectra in Figs. S5 and S6 of the supporting information, respectively), while structures of **1** and **7** were assigned as the fully azide derivates of disaccharide and tetrasaccharide alditols, respectively, for the similarity of their proton spectra with those of the products fully characterized (Table 2)

Product **4** (structure in Fig. 1, ^{1}H NMR in Fig. 3, expansion of the HSQC spectrum in Fig. S2 of the supporting information) presented two signals in the anomeric region and the use of 2D NMR experiments allowed the complete characterization of this oligosaccharide (Table 1); the anomeric signals at 4.64 and 4.59 ppm were labeled with a letter, **A** and **B**, respectively, and the combined use of TOCSY and COSY spectra identified the position of the signals relative to all the ring protons. H-3 and H-5 chemical shifts for each residue were also confirmed analyzing the TROESY spectrum which displayed the characteristic intra residue NOEs between H-1 and H-3 and H-1 and H-5. Carbon chemical shifts were assigned on the basis of the HSQC and HMBC spectra, of importance C-2 chemical shift of both **A** and **B** residues was found at ca. 67 ppm, which differed substantially from the chemical shift reported for glucosamine, namely 58 ppm. This finding confirmed the conversion of the chito-oligosaccharide into the azide analogue and the chemical shift variation of ca. 10 ppm was in agreement with the shift observed between methylamine (^{13}C: at 28.3 ppm) and methylazide (^{13}C: 37.9 ppm). Additionally, C-4 of residue **B** was found at 70.5 ppm, indicating that this unit was located at the non reducing end of the oligosaccharide, being not glycosylated. Differently, C-4 of unit **A** displayed a lower field value (78.9 ppm) due to the glycosylation effect (Bock & Pedersen, 1983).

With regard to the alditol moiety **C**, attribution started from the signal at 3.92 ppm which was recognized as H-2 because situated on an azide bearing carbon at 66.9 ppm. This H-2 correlated in the COSY spectrum with the geminal protons at 3.91 and 3.65 ppm, which were generated from the reduction of the glucosamine at the reducing end. No other correlations (TOCSY or COSY) were relating H-2 to the successive protons, therefore attribution of this unit was completed starting from the carbon density at 63.2 ppm, the only left hydroxyl methyl signal in the HSQC spectrum, which was assigned as C-6 of **C** unit for exclusion. Accordingly, H6s of **C** were determined, and the TOCSY spectrum of that resonating at 3.76 ppm identified two other protons, at 3.98 and 3.86 ppm: this last signal was attributed to H-4 because of the low field value (78.6 ppm) of the corresponding carbon atom, consequently, the proton at 3.98 ppm was assigned as H-5 of the alditol moiety.

Taken together, these data yielded to the complete attribution of compound **4**.

Analysis of both the TROESY and HMBC spectra confirmed the sequence of the three residues, which was **B**–**A**–**C**.

The second group of compounds comprehended **2** and **5** (structure in Fig. 1, ^{1}H NMR in Fig. 4) and a common feature of these two oligosaccharides was the occurrence of a doublet at 4.16 ppm which was instead absent in the first group. NMR analysis was focused on **2** and followed the same approach shown above for **4**; of importance, the proton at 4.16 ppm correlated with a carbon at 67.0 ppm and displayed a COSY correlation with a proton at 3.74 ppm in turn associated with a carbon at 56.2 ppm, namely a nitrogen bearing carbon; combining the HSQC and HMBC information (expansion of the HSQC spectrum in Fig. S3 of the Supporting Information), these two protons were identified as H-3 and H-2 of the alditol moiety of the oligosaccharide. The carbon chemical shift of C-2, together with the lack in the proton spectrum of a methyl group associated to an *N*-acetyl, allowed the identification of this moiety as an amino bearing alditol. Indeed product **2** was identified as a trisaccharide alditol, with the units **A** and **B** being both azido derivatives of glucosamine units and **C** a glucosamine alditol. Similarly, product **5**

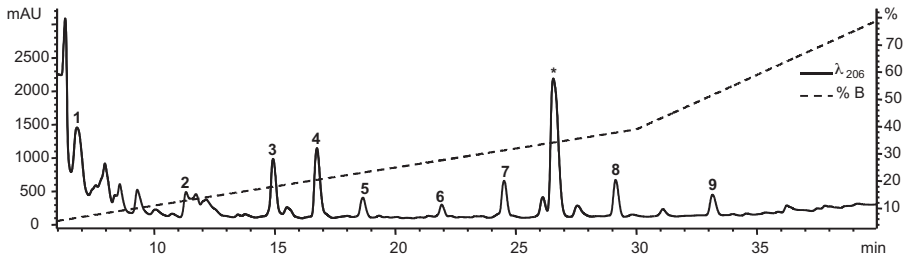

Fig. 2. Expansion of RP8-HPLC profile measured at 206 nm (solid line) of the azide glucosamine oligoalditol mixture; separation was performed with 0.1% aqueous TFA–MeOH gradient (broken line); proton spectra were measured for all peaks and the most relevant ones are labeled with a number. *: byproduct from Stick reagent.

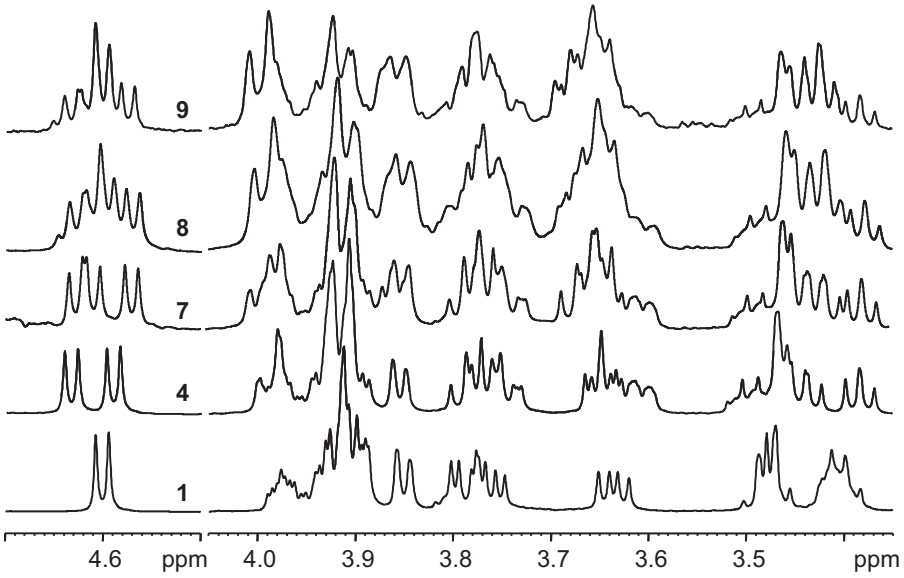

Fig. 3. (600 MHz, 298 K) ^{1}H NMR spectra of the first group of glucosamine oligosaccharide alditols derivatives. In this case, all the amino functions are converted into an azido group with the Stick reagent. Compound number follows the label on the HPLC chromatogram (Fig. 2).

Table 1
Proton (600 MHz) and carbon (150 MHz) chemical shift of **4**, measured at 305 K. **A** and **B** residues are in the pyranosidic form and β configured at the anomeric center ($^{3}J_{H1,H2}$ 8.2 Hz for both **A** and **B** residues). Spectra are calibrated with respect to internal acetone (^{1}H: 2.225 ppm, ^{13}C: 31.45 ppm). Residues sequence: **B**–**A**–**C**. Expansion of the HSQC spectrum is reported in Fig. S2 of the Supporting Information.

		1	2	3	4	5	6
A	^{1}H	4.64	3.44	3.65	3.78	3.61	3.99; 3.89
4-GlcN$_3$	^{13}C	101.9	67.0	73.9	78.9	75.9	61.0
B	^{1}H	4.59	3.39	3.51	3.47	3.47	3.91; 3.74
***t*-GlcN$_3$**	^{13}C	102.4	67.2	75.5	70.5	77.4	61.7
C	^{1}H	3.91; 3.65	3.92	3.92	3.86	3.98	3.76; 3.92
4-GlcN$_3$-alditol	^{13}C	62.4	66.9	70.4	78.6	71.9	63.2

was recognized as a tetrasaccharide with the alditol moiety having a free amino function.

The third group of oligosaccharides was composed from **3** and **6** (structure in Fig. 1, ^{1}H NMR in Fig. 5); Similarly to **4**, product **3** displayed two anomeric signals and the main difference was related to the occurrence of a multiplet at 4.27 ppm, which correlated in the HSQC spectrum with a carbon at 54.3 ppm, namely a nitrogen bearing atom (expansion of the HSQC spectrum in Fig. S4 of the Supporting Information). The complete study of the 2D NMR spectra (Table 3) together with the information of the occurrence in the

Table 2
Proton (600 MHz) and carbon (150 MHz) chemical shift of **2**, measured at 298 K. **A** and **B** residues are in the pyranosidic form and β configured at the anomeric center ($^{3}J_{H1,H2}$ 8.2 Hz for both **A** and **B** residues). Spectra are calibrated with respect to internal acetone (^{1}H: 2.225 ppm, ^{13}C: 31.45 ppm). Residues sequence: **B**–**A**–**C**. Expansion of the HSQC spectrum is reported in Fig. S3 of the Supporting Information.

		1	2	3	4	5	6
A	^{1}H	4.65	3.47	3.67	3.80	3.64	3.98; 3.91
4-GlcN$_3$	^{13}C	102.1	66.8	74.1	78.5	76.0	60.8
B	^{1}H	4.59	3.39	3.50	3.47	3.46	3.91; 3.74
***t*-GlcN$_3$**	^{13}C	102.3	67.3	78.6	70.7	77.4	61.7
C	^{1}H	3.94; 3.83	3.74	4.16	3.94	3.95	3.93; 3.80
4-GlcNH$_2$-alditol	^{13}C	60.2	56.2	67.0	79.0	71.5	63.1

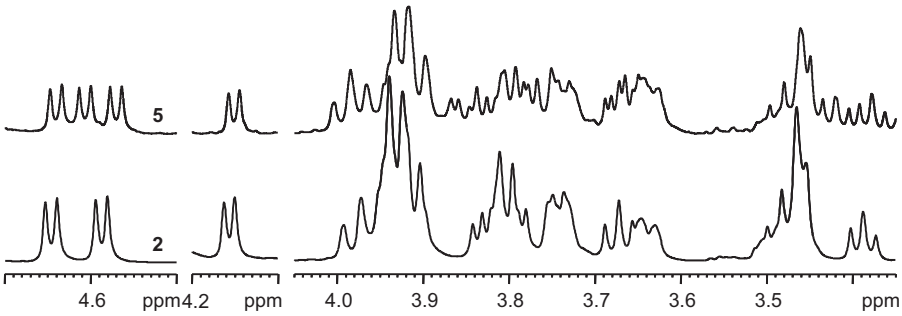

Fig. 4. (600 MHz, 298 K) ^{1}H NMR spectra of the second group of glucosamine oligosaccharide alditols. In this case, the amino function of the alditol unit at the reducing end is not converted in the corresponding azide by reaction with Stick reagent, as instead found for the other amino groups; the proton at 4.15 ppm is H-3 of the alditol unit. Compounds number follows the label on the HPLC chromatogram (Fig. 2).

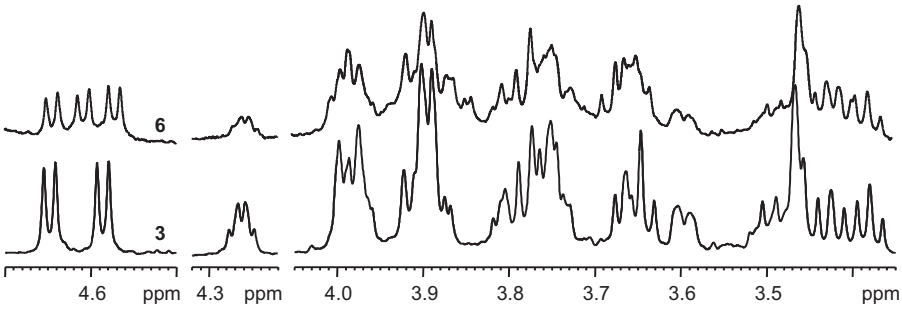

Fig. 5. (600 MHz, 298 K) 1H NMR spectra of the third group of oligosaccharide alditols derivatives. In this group, the amino function of alditol unit at the reducing end is *N*-acetylated, while all the others are transformed in azido groups; the proton at 4.26 ppm is H-2 of the alditol unit shifted for acetylation. Compounds number follows the label on the HPLC chromatogram (Fig. 2).

spectrum of an N-acetyl group, identified this product as a trisaccharide composed of two glucosamines with an azido function and a *N*-acetylglucosaminitol at the reducing end of the molecule. Similarly, product **6** was recognized as a tetrasaccharide with the alditol moiety having a *N*-acetylated amino function.

4. Discussion and conclusion

Chitosan and its low molecular weight derivatives are important for both biotechnological and medical applications.

Many of these applications exploit the occurrence of an elevated number of amino moieties, which can be modified to install different appendages, varying from chemical groups to whole organic molecules.

In this work we developed an inexpensive and rapid method to obtain pure glucosamine oligomers of different size in which the free amino groups are transformed into azide by diazo transfer reaction. In the development of this methodology, protection–deprotection reactions on the molecules were not necessary.

We focused this first investigation on small oligomers of chitosan, but modulating the chitosan hydrolysis conditions it is possible to prepare different pools of chitosan oligomers with different and increasing length.

The reaction conditions to transform the mixture of glucosamine oligosaccharide alditols into the corresponding azide compounds were optimized; for simplicity, the reducing end of the chito-oligomers was reduced to the corresponding alditol, but as alternative approach, it is possible to exploit the reactivity of the reducing glucosamine unit to introduce other chemical modification, as the derivation of the free aldehyde group with a fluorophore by means of reductive amination reaction.

Of importance, reduction and diazotransfer reaction were done consecutively, without any intermediate chromatographic purification of the sample, and finally the mixture was directly purified via HPLC chromatography, affording pure oligomers of different length.

The three groups of oligomers differed exclusively for the substitution pattern at the C-2 of the alditol moiety, with could bear an azide, an *N*-acetyl, or a free amino group (compare products **2**–**4** of Fig. 1).

With regard to the *N*-acetyl substituent, the low acetylation degree observed (<3%) after hydrolysis is due to the harsh condition used which promotes the acetyl group removal. Additionally, the

Table 3
Proton (600 MHz) and carbon (150 MHz) chemical shift of **3**, measured at 288 K. **A** and **B** residues are in the pyranosidic form and β configured at the anomeric center ($^{3}J_{H1,H2}$ 8.2 Hz for both **A** and **B** residues). Spectra are calibrated with respect to internal acetone (^{1}H: 2.225 ppm, ^{13}C: 31.45 ppm). Residues sequence: **B**–**A**–**C**. Expansion of the HSQC spectrum is reported in Fig. S4 of the supporting information.

		1	**2**	**3**	**4**	**5**	**6**
A	^{1}H	4.65	3.43	3.65	3.80	3.60	3.99; 3.90
4-GlcN$_3$	^{13}C	101.9	67.1	73.9	78.4	75.8	61.0
B	^{1}H	4.59	3.38	3.50	3.47	3.46	3.91; 3.74
***t*-GlcN$_3$**	^{13}C	102.2	67.2	75.5	70.5	77.1	61.6
C	^{1}H	3.77; 3.66	4.27	3.99	3.89	3.97	3.91; 3.77
4-GlcNAc-$_{alditol}$	^{13}C	62.8	54.3	68.9	79.1	72.1	63.2

finding of this group exclusively on the alditol moiety, and not on an internal residue, is related to the kinetic of the hydrolysis of the glucosamine glycosidic linkage (Vårum, Ottøy, & Smidsrød, 2001) which is faster for those residues bearing an *N*-acetyl substituent.

The occurrence of a free amino group was related to the not complete diazo-transfer reaction, which apparently failed only on the alditol moiety. We do not have an explanation for this experimental evidence which will be object of studies in future works.

Importantly, the spectroscopical description of this family of azido-bearing oligosaccharide derivatives, gives a valuable set of NMR chemical shift useful for the characterization of similar compounds. Finally, through the procedures developed, it is possible to obtain a family of azide chito-oligomers which are of interest in click chemistry for the synthesis of new glycoconjugates.

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We apologize to the many colleagues who have influenced our thinking through work that was not explicitly cited.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.06.010.

References

Allan, G. G., & Peyron, M. (1997). Depolymerization of chitosan by mean of nitrous acid. In A. A. Muzzarelli, & M. G. Peter (Eds.), *Chitin handbook* (pp. 153–164). Grottammare: Atec Edizioni.

Batista, M. K. S., Pinto, L. F., Gomes, C. A. R., & Gomes, P. (2006). Novel highly soluble peptide–chitosan polymers: Chemical synthesis and spectral characterization. *Carbohydrate Polymers*, *64*, 299–305.

Bock, K., & Pedersen, S. (1983). Carbon-13 nuclear magnetic resonance spectroscopy of monosaccharides. *Advances in Carbohydrate Chemistry and Biochemistry*, *41*, 27–65.

Goddard-Borger, E. D., & Stick, R. V. (2007). An efficient, inexpensive, and shelf-stable diazotransfer reagent: Imidazole-1-sulfonyl azide hydrochloride. *Organic Letters*, *9*(19), 3797–3800.

Gomes, P., Gomes, C. A. R., Batista, M. K. S., Pinto, L. F., & Silva, P. A. P. (2008). Synthesis structural characterization and properties of water-soluble *N*-(γ-propanoyl-amino acid)-chitosans. *Carbohydrate Polymers*, *71*, 54–65.

Knight, D. K., Shapka, S. N., & Amsden, B. G. (2007). Structure, depolymerization, and cytocompatibility evaluation of glycol chitosan. *Journal of Biomedical Materials Research Part A*, *83A*(3), 787–798.

Kolb, H. C., Finn, M. G., & Sharpless, K. B. (2001). Click chemistry: Diverse chemical function from a few good reactions. *Angewandte Chemie International Edition*, *40*(11), 2004–2021.

Kulbokaite, R., Ciuta, G., Netopilik, M., & Makuska, R. (2009). N-PEG'ylation of chitosan via click chemistry reactions. *Reactive and Functional Polymers*, *69*(10), 771–778.

Lee, M., Nah, J.-W., Kwon, Y., Koh, J. J., Ko, K. S., & Kim, S. W. (2001). Water-soluble and low molecular weight chitosan-based plasmid DNA delivery. *Pharmaceutical Research*, *18*(4), 427–431.

Mourya, V. K., & Inamdar, N. N. (2008). Chitosan-modifications and applications: Opportunities galore. *Reactive and Functional Polymers*, *68*, 1013–1051.

Muzzarelli, R. A. A. (1997). Depolymerization of chitins and chitosans with hemicellulase, lysozyme, papain and lipases. In R. A. A. Muzzarelli, & M. G. Peter (Eds.), *Chitin handbook* (pp. 153–164). Grottammare: Atec Edizioni.

Muzzarelli, R. A. A. (2011). Chitin nanostructures in living organism's chitin: Formation and diagenesis. *Topics in Biogeology*, *34*, 1–34.

Muzzarelli, R. A. A., Boudrant, J., Meyer, D., Manno, N., DeMarchis, M., & Paoletti, M. G. (2012). A tribute to Henri Braconnot, precursor of the carbohydrate polymers science on the chitin bicentennial. *Carbohydrate Polymers*, *87*, 995–1012.

Oliveira, J. R., Martins, M. C. L., Mafra, L., & Gomes, P. (2012). Synthesis of an *O*-alkynyl-chitosan and its chemoselective conjugation with a PEG-like amino-azide through click chemistry. *Carbohydrate Polymers*, *87*, 240–249.

Richardson, S. W., Kolbe, H. J., & Duncan, R. (1999). Potential of low molecular mass chitosan as a DNA delivery system: Biocompatibility, body distribution and ability to complex and protect DNA. *International Journal of Pharmaceutics*, *178*(2), 231–243.

Sato, T., Ishii, T., & Okahata, Y. (2001). In vitro gene delivery mediated by chitosan. Effect of pH, serum, and molecular mass of chitosan on the transfection efficiency. *Biomaterials*, *22*(15), 2075–2080.

Trombotto, S., Ladavière, C., Delolme, F., & Domard, A. (2008). Chemical preparation and structural characterization of a homogeneous series of chitin/chitosan oligomers. *Biomacromolecules*, *9*(7), 1731–1738.

Tsung-Lin, Y., & Tai-Horng, Y. (2009). The specificity of chitosan in promoting branching morphogenesis of progenitor salivary tissue. *Biochemical Biophysical Research Communications*, *381*(4), 466–470.

Vårum, K. M., Ottøy, M. H., & Smidsrød, O. (2001). Acid hydrolysis of chitosans. *Carbohydrate Polymers*, *46*(1), 89–98.