Carbohydrate Polymers 98 (2013) 770–777

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Access to tetra-*N*-acetyl-chitopentaose by chemical *N*-acetylation of glucosamine pentamer

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ARTICLE INFO

Article history:
Received 19 March 2013
Received in revised form 28 June 2013
Accepted 30 June 2013
Available online 3 July 2013

Keywords:
Chitopentaose
Chitooligosaccharide
Chitosan oligomer
Acetylation
MALDI-TOF mass spectrometry

ABSTRACT

Nowadays, the easy access of tetra-*N*-acetyl-chitopentaose and its counterparts is highly interesting since such chemical compounds are precursors of biological signal molecules with a strong agro-economic impact. The chemical synthesis of tetra-*N*-acetyl-chitopentaose by controlled *N*-acetylation of the glucosamine pentamer hydrochloride under mild conditions is described herein. A systematic study on the influence of the different parameters involved in this reaction, such as the solvent, the acetylating agent, and the base used for the deprotonation of ammonium groups of the starting material was carried out. The characterization of final reaction products by HPLC and MALDI-TOF mass spectrometry showed that each of these parameters affects differently the acetylation reaction. Whereas the solvent plays an important role in the *N*- or *O*-acetylation selectivity, the acetylating agent and the base were found to influence both the degree of *N*-acetylation and the distribution of the partially *N*-acetylated derivatives in the product mixtures. Based on these results, optimized reaction conditions have been established allowing tetra-*N*-acetyl-chitopentaose to be synthesized in a one-pot deprotonation/*N*-acetylation of the glucosamine pentamer hydrochloride in a moderate yield (ca 30%).

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1. Introduction

Chitosan is a random linear polysaccharide of D-glucosamine (GlcN) and *N*-acetyl-D-glucosamine (GlcNAc) units linked by β-(1→4) glycosidic bonds. Although present in some fungi (*Mucoraceae*), chitosan is commonly obtained by chemical or enzymatic *N*-deacetylation of chitin, the second most abundant naturally occurring polymer produced industrially from shells of crustaceans and squid pens (Aranaz, Harris, & Heras, 2010; Kurita, 2006; Pacheco et al., 2011). Thanks to its physicochemical and biological properties such as non-toxicity, bio-compatibility and biodegradability, chitosan has found a wide range of potential applications in the fields of medicine, gene delivery, cosmetics, agriculture, water treatments, food packaging and biotechnological industries (Dash, Chiellini, Ottenbrite, & Chiellini, 2011; Domard & Domard, 1994; El Hadrami, Adam, El Hadrami, & Daayf, 2010; Kumar, 2000; Rinaudo, 2006).

Chitooligosaccharides (COS) are homo- or hetero-oligomers of GlcN and/or GlcNAc. They are also defined as chitosan oligomers with an average molecular weight up to ~4000 g/mol, and possess several physicochemical advantages compared to chitosan (Mourya, Inamdar, & Choudhari, 2011). Indeed, their use can overcome the well-known limitations of chitosan related to its low solubility, its high viscosity, or its tendency to form aggregates in aqueous media. Moreover, COS exhibit numerous biological properties such as antibacterial, antifungal, antitumor activities, immuno-enhancing effects on animal health, as well as an anti HIV-1 activity (Aam et al., 2010; Kim & Rajapakse, 2005; Xia, Liu, Zhang, & Chen, 2011). Several studies have shown that these bioactivities seem to depend on the structural parameters of COS such as their degree of *N*-acetylation (DA), their degree of polymerization (DP), and/or their pattern of *N*-acetylation (PA) (Aam et al., 2010; Wendt dos Santos, El Gueddari, Trombotto, & Moerschbacher, 2008; Xia et al., 2011). In this context, the design of well-defined COS is of a great interest in order to understand the relationship between their biological activities and their corresponding structures. Conventionally, COS can be produced from depolymerization of chitin or chitosan using chemical, enzymatic, and physical methods leading to complex COS mixtures with different DP and DA distributions (Aam et al., 2010; Mourya et al., 2011; Trombotto, Ladavière,

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http://dx.doi.org/10.1016/j.carbpol.2013.06.078

Delolme, & Domard, 2008). COS can also be prepared by total chemical syntheses from D-glucosamine hydrochloride by means of multiple protection/deprotection strategies leading to a perfect control of the three previously mentioned structural parameters (Aly, Ibrahim, El Sahry, & Schmidt, 2001; Barroca-Aubry, Pernet-Poil-Chevrier, Domard, & Trombotto, 2010; Kuyama et al., 1993; Vijayakrishnan, Issaree, & Corilo, 2011). However, most of these methods are limited to the elaboration of homo-oligomers of GlcNAc or GlcN, and only a very few of them deals with the preparation of hetero-COS.

Herein, we report the straight synthesis of well-defined hetero-COS structures composed of 4 GlcNAc units and only 1 GlcN unit. Indeed, these structures are considered as very interesting precursors for the synthesis of important biological signal molecules (Nod and myc factors) involved in natural processes (D'Haeze & Holsters, 2002; Maillet et al., 2011; Samain, Drouillard, Heyraud, Driguez, & Geremia, 1997). The preparation of this tetra-*N*-acetyl-chitopentaose $(GlcNAc)_4(GlcN)_1$ was carried out under mild conditions by a chemical *N*-acetylation of the commercially available chitopentaose hydrochloride $(GlcN, HCl)_5$. To this end, different *N*-acetylation reaction parameters influencing the degree of *N*-acetylation and the distribution of the partially acetylated compounds were studied, and are described in this paper.

2. Materials and methods

2.1. Materials

Chitopentaose hydrochloride ($(GlcN, HCl)_5$, assay ≥98%) was purchased from Seikagaku. Acetic anhydride (Ac_2O, assay ≥99%) and triethylamine (TEA, assay ≥99%) were purchased from Fluka. Acetyl chloride (AcCl, assay ≥98%), acetonitrile (assay ≥99%), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, assay ≥ 99%), *N*,*N*-diisopropylethylamine (DIPEA, assay ≥99%), Dowex 50WX8 resin, pentafluorophenyl acetate (PFPA, assay ≥99%), pyridine (Py, assay ≥99%), and trimethylamine (TMA, assay ≥99%) were purchased from Sigma–Aldrich. All reactants were used directly without any further purification.

2.2. Typical procedure for N-acetylation of chitopentaose hydrochloride

10 mg (8.9 μmol) of chitopentaose hydrochloride $(GlcN, HCl)_5$ were dissolved in 0.4 mL of water/methanol 10/90 (v/v). 5.5 μL of TEA (39.9 μmol, 4.5 molar equiv./chitopentaose) and 11.3 μL of acetic anhydride (120.1 μmol, 13.5 molar equiv./chitopentaose) were successively added and the mixture was stirred at room temperature. After 5 h, the reaction was stopped by addition of water, then the reaction mixture was separated by adsorption on a Dowex 50WX8 ion exchange resin followed by successive desorptions: (i) with pure water for the recovery of per-*N*-acetyl-chitopentaose, and then (ii) with dilute ammonia solution (7% v/v in water) for the recovery of partially *N*-acetyl-chitopentaoses.

2.3. Characterization methods

2.3.1. MALDI-TOF mass spectrometry

All mass spectra were acquired with a Voyager-DE STR (AB Sciex, Framingham, MA) equipped with a nitrogen laser emitting at 337 nm with a 3 ns pulse. The instrument was operated in the linear or reflectron mode. Ions were accelerated to a final potential of 20 kV. The positive ions were detected in all cases. Mass spectra were the sum of 300 shots and an external mass calibration of mass analyzer was used (mixture of peptides from Sequazyme™ standards kit, AB Sciex). The matrix used for all experiments was 2,5-dihydroxybenzoic acid (DHB) purchased from Sigma–Aldrich and used directly without further purification. The solid matrix and chitopentaose samples were dissolved at 10 mg/mL and 1 mg/mL in water, respectively. A volume of 20 μL matrix solution was then mixed with 20 μL of chitopentaose solutions. An aliquot of 0.5 μL of each resulting solution was spotted onto the MALDI sample plate and air-dried at room temperature.

2.3.2. High resolution ESI mass spectrometry

The high resolution mass spectrum was recorded in a positive ion mode on a hybrid quadrupole time-of-flight mass spectrometer (MicroTOFQ-II, Bruker Daltonics, Bremen) with an electrospray ionization (ESI) ion source. The gas flow of spray gas is 0.6 bar and the capillary voltage is +4.5 kV. The solution was infused at 180 μL/h. The mass range of the analysis is 50–1000 *m*/*z* and the calibration was carried out with sodium formate. The solvent for HRMS is dichlomethane/MeOH/water/formic acid and for MS/MS experiment, water/acetonitrile (50/50), quadrupole selection 992.4 ± 5 *m*/*z*, and collision energy in collision cell is 45 eV.

2.3.3. Proton nuclear magnetic resonance

^{1}H NMR spectra were recorded with a Bruker ALS 300 spectrometer (300 MHz for ^{1}H) using the Bruker TopSpin 2.1 NMR software. All samples were dissolved at 10 mg/mL in D_2O and transferred to 5-mm NMR tubes. Typical conditions for the acquisition of ^{1}H NMR spectra: 300.13 MHz; 22 °C; acquisition time, 5 s; number of scans, 64 and a 30° excitation pulse-angle was used. The signal of HOD (δ 4.80 ppm at 22 °C) was used as internal calibration reference.

2.3.4. Thermo gravimetric analyses

The TGA thermograms were obtained using a TGA 2950 V5.1A Thermo Gravimetric Analyzer from Du Pont Instruments. The temperature range was from 30 to 400 °C, with an increasing rate of 2 °C/min under a flow of Helium.

2.3.5. High performance liquid chromatography

Analyses were performed on a Shimadzu HPLC Model CBM-20A. Samples were dissolved in eluent (Acetonitrile/H_2O 7/3, v/v) at 10 mg/mL and injected (20 μL) onto a Shodex Asahipak NH2P-50 column (5 μm granulometry, 250 mm × 4.6 mm) at room temperature, with a flow rate of 0.8 mL/min and a UV detection at λ = 205 nm.

2.4. Characterization data of tetra-N-acetyl-chitopentaose (D_1A_4)

^{1}H NMR (300 MHz, D_2O): δ (ppm) 5.20 (bs, 0.8H, H-1α^D), 4.65–4.50 (m, 4H, 3H-1^A, H-1^D), 4.00–3.35 (m, 29H, H-2^A, H-3^{A,D} to H-6^{A,D}), 2.80–2.70 (m,1H, H-2^D), 2.05 (m, 12H, $COCH_3$).

High resolution MS (ESI): calcd for $C_{38}H_{65}N_5O_{25}Na$: *m*/*z* 1014.3865; found 1014.3849 $[M+Na]^+$ (difference = 1.6 ppm). The MS/MS of this ion confirms the chemical structure (see Supporting Information).

The purity of D_1A_4 has been determined by HPLC analysis as >98% (see Supporting Information).

3. Results and discussions

After the characterization of the starting material, the chitopentaose hydrochloride, the influence of each parameter involved in its acetylation is below reported. For the sake of clarity, chitooligosaccharides were labelled following the nomenclature previously proposed by Haebel, Bahrke, and Peter (2007). Thus, COS were shortened as D_xA_y, where "*x*" and "*y*" correspond to the number of the repeating units of "D" (*N*-deacetylated glucosamine or GlcN) and "A" (*N*-acetylated glucosamine or GlcNAc) moieties, respectively. For example, the starting material chitopentaose

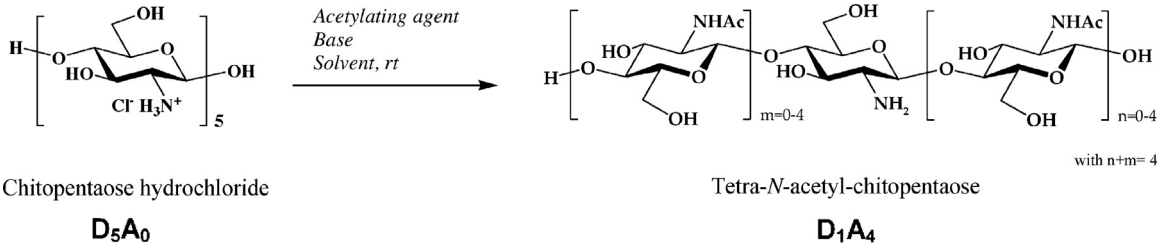

Fig. 1. Synthesis of tetra-*N*-acetyl-chitopentaose from chitopentaose hydrochloride.

hydrochloride and the targeted COS tetra-*N*-acetyl-chitopentaose will be labelled D_5A_0 and D_1A_4, respectively.

3.1. Characterization of chitopentaose starting material

In this study, the commercially available chitopentaose hydrochloride was used as starting material. In order to confirm its structure and to determine its purity, a series of analyses has been performed.

Firstly, MALDI-TOF mass spectrometry of the starting material was achieved. The resulting mass spectrum (see Supporting Information) shows the presence of a major peak at *m/z* 846.4 attributed to D_5A_0 (*m/z* monoisotopic calcd for $[C_{30}H_{57}O_{21}N_5+Na]^+ = 846.3$ mass units). However, a minor peak at *m/z* 1049.6 was also detected and identified as D_5A_1 (*m/z* monoisotopic calcd for $[C_{38}H_{70}O_{26}N_6+Na]^+ = 1049.4$ *m/z*). The proportion of each species in the sample was determined by ^{1}H NMR spectroscopy. Indeed, ^{1}H NMR spectrum of the starting material (see Supporting Information) displays the presence of a small singlet at around 2.08 ppm assigned to the *N*-acetyl protons of GlcNAc units. Thus, the molar proportion of D_5A_1 and D_5A_0 can be calculated according to Eqs. (1) and (2), respectively, taking into account signal integrals of (i) *N*-acetyl protons of GlcNAc units (A_{CH_3}), and (ii) H_2 protons of GlcN units (A_{H_2}) from 3.0 to 3.4 ppm. The result reveals that the sample is composed of 95% D_5A_0 and 5% D_5A_1 (molar %).

$$D_5A_1\,(\%) = \left(\frac{5}{3} \times \frac{A_{CH_3}}{A_{H_2}}\right) \times 100 \quad (1)$$

$$D_5A_0\,(\%) = \left(1 - \frac{5}{3} \times \frac{A_{CH_3}}{A_{H_2}}\right) \times 100 \quad (2)$$

Additionally, TGA of the starting material (see Supporting Information) shows a weight loss of around 10% up to 150 °C ascribed to the water elimination. This percentage corresponds to 6.34 molecules of water per molecule of D_5A_0. Above 150 °C, a drastic weight loss occurred due to thermal decomposition of COS. According to these results, the molar composition of the starting material was deduced as follows: $D_5A_0/D_5A_1/H_2O$ 1.0/0.05/6.34 (mol/mol/mol). This composition was subsequently used to determine the exact amount of D_5A_0 to be weighted throughout the study.

3.2. Study of the chemical N-acetylation of chitopentaose

The synthesis of the tetra-*N*-acetyl-chitopentaose (D_1A_4) was performed *via* a one-pot deprotonation/acetylation reaction, as shown in Fig. 1. This reaction required the base-mediated deprotonation of ammonium groups of the chitopentaose hydrochloride followed by a controlled *N*-acetylation of the resulting free amino groups using an acetylating agent at room temperature.

The formation of acetamido groups by *N*-acetylation of amines has been widely described in the literature, using classical acetylating agents such as acetyl chloride or acetic anhydride (Sashiwa et al., 2002). However, due to the likely similar reactivity of the 5 amino groups of the chitopoentaose, the challenge is here to enable the *N*-acetylation of only 4 amino groups. In this context, a systematic study of the main parameters involved in this reaction was carried out, namely: (i) the solvent composition which has an impact on the medium homogeneity, (ii) the base which has a direct influence on the reactivity of the chitopentaose amino groups, and finally (iii) the acetylating agent which has to be sufficiently stable in the reaction medium towards hydrolysis.

3.2.1. Influence of the solvent

An important parameter in the *N*-acetylation reaction of GlcN oligomers is the solvent. The literature shows that methanol is commonly used for the *N*-acetylation of GlcN oligomers up to tetramer (Tokuyasu, Ono, Mitsutomi, Hayashi, & Mori, 2000). But COS with higher DP require the presence of water in the solvent due to the poor solubility of these oligomers in pure methanol (Lee, Var, Shinya, Kajiuchi, & Yang, 1999). In this case, the proportion of water increases with the DP. However, regarding the *N*-acetylation reaction, the quantity of water has to remain as small as possible in order to limit the consumption of the acetylating agent by hydrolysis. This critical water/methanol ratio explains the following systematic study about the influence of the solvent composition on both the reaction medium homogeneity and the reaction products during the *N*-acetylation reaction. In this aim, a series of experiments was performed by varying only the proportion of water in methanol while keeping constant quantities of base (TEA) and acetylating agent (Ac_2O) *vs* chitopentaose (4.5 and 13.5 molar equiv., respectively; see later the explanations about the equivalent number and reactant choices).

3.2.1.1. Solubility aspects. As expected, the higher the proportion of water, the more homogeneous the medium (Table 1). Indeed, when the *N*-acetylation reaction is carried out in pure methanol (Table 1: S8, water/methanol 0/100, v/v), the reaction mixture is very heterogeneous throughout the reaction, due to the poor solubility of the starting material D_5A_0 and its partially *N*-acetylated derivatives in this solvent. Because of the presence of TEA in the reaction medium, these oligomers are actually present in their basic form, *i.e.* with free amino groups in GlcN moieties. As it was already shown, the presence of these amino groups favours the formation of intra- and inter-molecular H-bonds which in turn disfavors the solvolysis of these compounds in pure methanol (Park, Cho, Chung, Kwon, & Jeong, 2003). On the other hand, as the *N*-acetylation of the starting material progresses, the resulting acetamido groups could disturb these H-bond interactions, leading to a better homogeneity in the reaction medium, especially as the proportion of water increases. Thus, a moderate solubility of the reaction products is observed when the proportion of water is 10% v/v (Table 1: S7, water/methanol 10/90, v/v). Finally, above a proportion of 30% v/v of water (Table 1: from S1 to S6), homogeneous solutions were obtained from the beginning of the reaction and whatever the degree of *N*-acetylation of the reaction products in the mixture.

Table 1
Influence of the solvent on the *N*-acetylation reaction of chitopentaose.

Samples[a]	Water/methanol (v/v)	D_1A_4[b]	D_0A_5[b]	D_0A_5 + 1xOAc[b]	D_0A_5 + 2xOAc[b]	D_0A_5 + 3xOAc[b]	Solubility[c]
S1	100/0		■	■	■	traces	G
S2	80/20		■	■	■		G
S3	70/30	■	■	■	■		G
S4	60/40	■	■	■	traces		G
S5	40/60	■	■	■	traces		G
S6	30/70	■	■	■	traces		G
S7	10/90	■	■	traces			M
S8	0/100	■	■				L

[a] All experiments were performed at room temperature using chitopentaose hydrochloride as starting material, water/methanol mixtures as solvent, 4.5 equiv. of TEA and 13.5 equiv. of acetic anhydride *vs* chitopentaose. Black bar: detected species, and white bar: not detected species.
[b] The identification of the species was carried out by MALDI-TOF mass spectrometry. D_1A_4, tetra-*N*-acetyl-chitopentaose; D_0A_5, penta-*N*-acetyl-chitopentaose; OAc, *O*-acetyl group.
[c] Solubility of the medium at the end of the reaction: G, great; M, moderate; L, low.

3.2.1.2. Acetylation reaction products. The influence of the water/methanol ratio on the acetylation reaction products was monitored by HPLC, and the resulting chromatograms are illustrated in Fig. 2. They clearly demonstrate that the composition of the acetylation products varies according to the solvent. They show two main peaks: a peak A detected at the range of 10.0–11.0 min as retention time (Tr), and a peak B detected at Tr between 15.0 and 17.0 min. These two peaks are well separated from each other, and from the peak of the starting material D_5A_0 (Tr = 7.0–7.5 min, see Supporting Information). The area evolution of these peaks as a function of the composition of the solvent underlines that the peak A linearly increases with the amount of water in the medium, while the peak B area decreases in a linear way too (Fig. 3).

Whereas peak B was assigned to D_0A_5 after isolation by chromatography and characterization by mass spectrometry, two different compounds were identified in peak A. Indeed, after separation of peak A from the reaction medium (Table 1: S7, water/methanol 10/90, v/v), its MALDI-TOF mass spectrum showed the presence of two peaks at *m/z* 1014.4 and 1098.5. They have respectively been assigned to compounds D_1A_4 and its fully *N*-acetylated analogue bearing one additional *O*-acetyl group (labelled by $D_0A_5 + 1\times$ OAc). Unfortunately, this overlay of compounds (D_1A_4 and $D_0A_5 + 1\times$ OAc) did not allow us to determine the quantity of targeted D_1A_4 in each sample from chromatograms. In order to overcome this, all samples were analyzed by MALDI-TOF mass spectrometry and the results are reported in Table 1. The acetylation efficiency (represented by black bars in Table 1) seems to be enhanced by increasing the proportion of water in the medium. Therefore, exclusively per-*N*-acetylated compounds were detected when the water proportion was higher than 80% v/v (Table 1: S1 and S2); whereas the presence of D_1A_4 in the mixture is detected only when this proportion is below 70% v/v (Table 1: from S3 to S8).

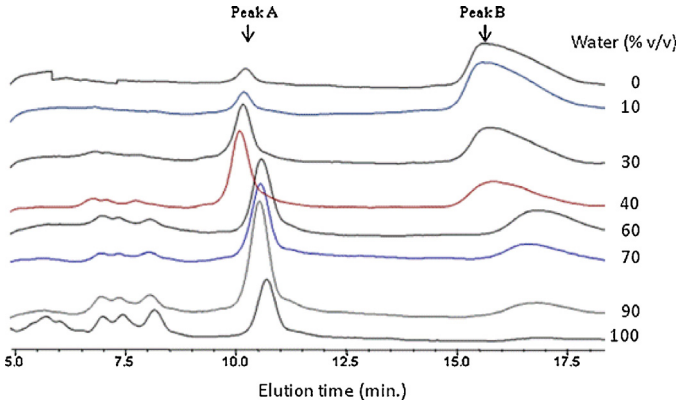

Fig. 2. HPLC chromatogram evolution of the reaction mixture *vs* the proportion of water (% v/v) in water/methanol solvent.

3.2.1.3. N- vs O-acetylation reaction selectivity. In the same way, the number of *O*-acetyl groups in *O*-acetylated D_0A_5 species (Table 1) and their proportion in reaction products appear to increase with the quantity of water (Fig. 4). Whereas at 80% (v/v) of water in

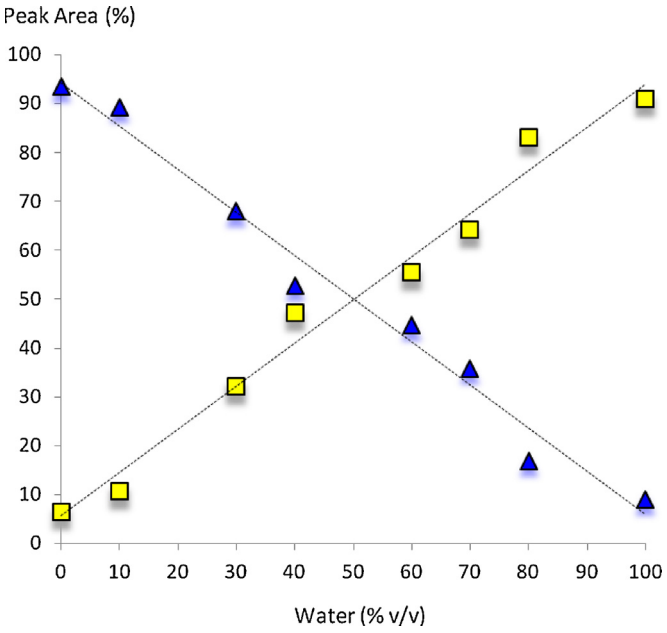

Fig. 3. % Area of (□) peak A ($D_1A_4 + D_0A_5$, $1\times$ OAc) and (▲) peak B (D_0A_5) determined from HPLC chromatograms in Fig. 2 as a function of the proportion of water (% v/v) in water/methanol solvent.

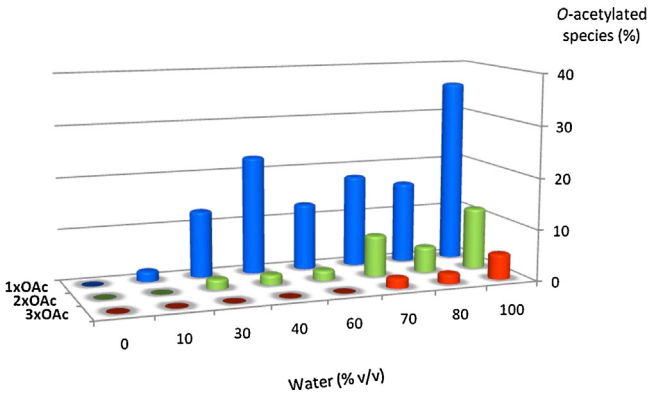

Fig. 4. Assessment of the *O*-acetylated species amount *vs* the proportion of water (% v/v) in water/methanol solvent. This proportion was determined by MALDI-TOF mass spectrometry.

water/methanol solvent (Table 1: S2), the MALDI-TOF mass spectrum revealed the presence of D_0A_5 bearing up to 3 *O*-acetyl groups, only traces of the mono *O*-acetylated derivative ($D_0A_5 + 1\times$ OAc) were detected when using only 10% of water (Table 1: S7). Indeed, MALDI-TOF mass spectra displayed peaks distant by 42 mass units (corresponding to the chemical modification of a hydroxyl group into an *O*-acetyl one) at *m/z* 1098.3 ($D_0A_5 + 1\times$ OAc), 1140.3 ($D_0A_5 + 2\times$ OAc) and 1182.3 ($D_0A_5 + 3\times$ OAc). Unfortunately, ^{1}H NMR analyses could not enable the exact quantification of these *O*-acetylated derivatives. The CH_3 hydrogen atoms of *O*-acetyl and *N*-acetyl groups have actually too similar chemical shifts between 2.1 and 2.0 ppm to be precisely separated and identified in such molecules. Despite the fact that the MALDI-TOF mass spectrometry is not a quantitative technique, this proportion was evaluated to get general trends about the *O*-acetyl group formation according to the composition of the hydro-alcoholic solvent. This proportion was obtained by dividing the intensity of the peak of each *O*-acetylated D_0A_5 species by the sum of intensities of the different *O*-acetylated D_0A_5 derivative peaks.

This enhanced *N*-/*O*-acetylation reaction with the water proportion is somewhat surprising since acetic anhydride is expected to be hydrolyzed in aqueous solution. Nevertheless, the medium homogeneity in high proportion of water (as mentioned earlier) could favour the reactivity of amino groups towards the acetylating agent, and explains thereby the unexpected high efficiency. Moreover, the hydrolysis reaction may be offset by the use of a large excess of acetic anhydride *vs* amino groups (*i.e.* 13.5 molar equiv. of acetic anhydride *vs* chitopentaose). Regarding the particular *O*-acetylation reaction, its development with water proportion, or inversely, its disappearance with methanol proportion in alkaline aqueous media could be justified by the use of the well-known TEA/methanol/water system, employed as an efficient de-*O*-acetylation method in carbohydrate chemistry (Meier, Monteiro, Baldissera, & Sa, 2010).

3.2.1.4. Solvent optimized conditions. Only two experiments S7 and S8 (Table 1) carried out with 10 and 0% of water (v/v) afforded only two main compounds in the final reaction mixture: the targeted D_1A_4 and its per-*N*-acetylated analogue D_0A_5. Their separation was performed by a cationic Dowex resin column leading to two main fractions. The first one (F1) eluted with pure water contained only the compound D_0A_5, whereas the second one (F2) eluted with a solution of $NH_4OH_{(aq)}$ (7% v/v) was exclusively composed of D_1A_4. The characterization of both fractions was carried out by MALDI-TOF mass spectrometry and ^{1}H NMR. The MALDI-TOF mass spectrum of F2 showed only one peak at *m/z* 1014.6 assigned to D_1A_4 (*m/z* monoisotopic calcd for $[C_{38}H_{65}O_{25}N_5+Na]^+ = 1014.4$ mass units). For F1, the MALDI-TOF mass spectrum showed also one peak at *m/z* 1056.6 assigned to D_0A_5 (*m/z* monoisotopic calcd for $[C_{40}H_{67}O_{26}N_5+Na]^+ = 1056.4$ mass units). The ^{1}H NMR spectrum of F2 (Fig. 5b) showed the presence of: (i) a signal at 2.75 ppm corresponding to the proton H2 of the remaining GlcN moiety; (ii) a signal at 2.04 ppm assigned to the 4 *N*-acetyl groups, and (iii) a doublet at 5.20 ppm for the anomeric proton H1α underlining the presence of a GlcNAc residue at the reducing end of the D_1A_4 oligomer, as already mentioned by Samain et al. for the Tetra-*N*,N^2,N^3,N^4-acetyl-chitopentaose (Samain et al., 1997). Concerning the ^{1}H NMR spectrum of F1 (Fig. 5c), it confirmed the absence of protons H2 of GlcN units, and the presence of a signal at 2.04 ppm corresponding to 5 *N*-acetyl groups of the D_0A_5 oligomer, in agreement with ^{1}H NMR data given in the literature (Samain et al., 1997). Consequently, the D_1A_4 oligomer (F2) was isolated in 27% mass yield from the S7 experiment (Table 1, water/methanol, 10/90, v/v), and only 7% mass yield in the case of pure methanol (Table 1: S8).

3.2.2. Influence of the base

The base in the reaction mechanism of COS *N*-acetylation plays two main roles: (i) the deprotonation of COS ammonium groups leading to more reactive amino groups, and (ii) the neutralization of acetic acid formed during the reaction. Therefore, the pK_a value of the base is expected to influence significantly the reactivity of the starting material, and consequently the composition of resulting products. Based on these considerations, experiments were performed by varying the nature and the quantity of the base, whereas the rest of the reaction parameters were kept unchanged. Experiments were carried out in water/methanol (10/90, v/v) as solvent and using 13.5 molar equiv. of acetic anhydride *vs* chitopentaose.

The results, summarized in Table 2, show the composition of the product mixture of each experiment determined by MALDI-TOF mass spectrometry. The DA was calculated by ^{1}H NMR according to Eq. (3), where "A_{CH_3}" corresponds to the integral of the *N*-acetyl proton signal and "A_{H2-H6}", to the sum of integrals of protons H2 to H6 (Fig. 5) (Asako Hirai, Odani, & Nakajima, 1991).

$$DA(\%) = \left(\frac{(1/3) \times A_{CH_3}}{(1/6) \times A_{H_2-H_6}} \right) \times 100 \quad (3)$$

Concerning the base nature, when comparing TEA (Table 2: S11, pK_a = 10.7) and pyridine (Table 2: S12, pK_a = 5.2), the former base is expected to be more efficient than the latter one for the deprotonation of ammonium groups. Unlike pyridine, the pK_a of TEA is actually higher than the COS one (p$K_a \sim$ 6–7) (Cataldo, Crea, Gianguzza, Pettignano, & Piazzese, 2009). Therefore, with TEA, the reactive basic form of chitopentaose is predominant leading to high *N*-acetylated chitopentaose derivatives. On the other hand, when pyridine is used, most of amino groups of chitopentaose are protonated providing a complex mixture of partially *N*-acetylated derivatives. In the same way, we can observe that the use of bases with higher pK_a such as DBU (Table 2: S13, p$K_a \sim$ 12) and DIPEA (Table 2: S14, p$K_a \sim$ 12) lead exclusively to the formation of D_0A_5. A similar result was obtained when TMA is employed (Table 2: S15, p$K_a \sim$ 9.8). Compared to TEA, TMA seems to be more efficient to deprotonate ammonium groups although its pK_a value is lower. This higher reactivity could be attributed to a better accessibility of the electronic doublet of the TMA nitrogen atom towards protons of COS ammonium groups.

TEA was found to be the most appropriate base for this reaction, and afforded the D_1A_4 targeted oligomer in a satisfactory extent, comparing to the other bases. Therefore, its quantity in the *N*-acetylation reaction was then optimized. The data indicate that the DA value increases with the amount of TEA (Table 2: S9, S10, S11, and S16). Acetylation of the chitopentaose hydrochloride led

Table 2
Influence of the base and acetylating agent on the *N*-acetylation reaction of chitopentaose.

Samples[a]	Reactants		Reaction products[d]						DA (%)[e]
	Base[b]	Acetylating agent[c]	D_0A_5	D_1A_4	D_2A_3	D_3A_2	D_4A_1	D_5A_0	
S9	TEA (Cat.)	Ac_2O (13.5)			■	■	■	■	29
S10	TEA (2.0)	Ac_2O (13.5)	■	■	■	■	■		56
S11[f]	TEA (4.5)	Ac_2O (13.5)	■	■					94
S12	Py (4.5)	Ac_2O (13.5)	■	■	■	■	■	■	nd
S13[g]	DBU (4.5)	Ac_2O (13.5)	■						100
S14[g]	DIPEA (4.5)	Ac_2O (13.5)	■						100
S15[g]	TMA(4.5)	Ac_2O (13.5)	■						100
S16	TEA (10)	Ac_2O (13.5)	■	■					98
S17	TEA(4.5)	Ac_2O (4.5)	■	■	■	■			nd
S18	TEA(4.5)	Ac_2O (8.0)	■	■	■	■			nd
S19	TEA(4.5)	Ac_2O (10.0)	■	■					94
S20	TEA(4.5)	AcCl (13.5)						■	2
S21	TEA(4.5)	PFPA (13.5)				■	■	■	5

[a] All experiments were performed at room temperature using chitopentaose hydrochloride as starting material, and water/methanol mixture (10/90, v/v) as solvent. Black bar: detected species, and white bar: not detected species. nd, not determined.
[b] Molar ratio of base *vs* chitopentaose hydrochloride is given in brackets.
[c] Molar ratio of acetylating agent *vs* chitopentaose hydrochloride is given in brackets.
[d] The identification of the different species was carried out by MALDI-TOF mass spectrometry.
[e] The average degree of *N*-acetylation (DA) was determined by ^{1}H NMR.
[f] Corresponding to sample S7 in Table 1.
[g] The identification of the different species was carried out by ^{1}H NMR.
Ac_2O, acetic anhydride; AcCl, acetyl chloride; Cat., catalytic; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DIPEA, *N*,*N*-diisopropylethylamine; PFPA, pentafluorophenyl acetate; Py, pyridine; TEA, triethylamine; TMA, trimethylamine.

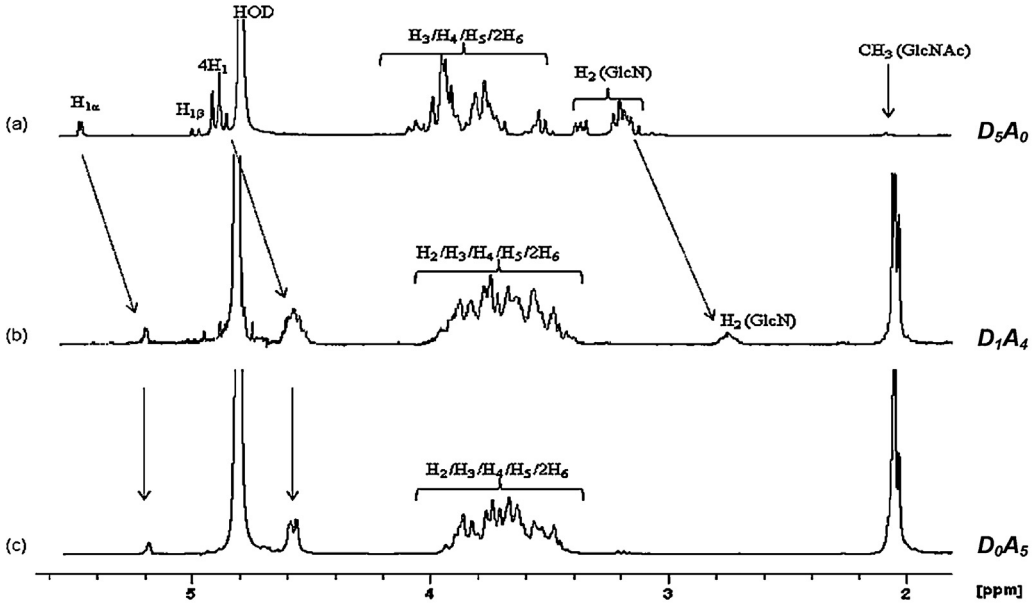

Fig. 5. ^{1}H NMR spectra (300 MHz, D_2O) of (a) the starting material chitopentaose hydrochloride D_5A_0, (b) D_1A_4 and (c) D_0A_5.

to a complex mixture of partially *N*-acetylated compounds with a DA of 29% for TEA catalytic amount and 56% for 2 molar equiv. of TEA/chitopentaose (Table 2: S9 and S10, respectively). A minimum of 4.5 molar equiv. of TEA/chitopentaose is required to increase the DA up to 94% (Table 2: S11), whereas using 10 molar equiv. (Table 2: S16), the ammonium groups seem to be completely deprotonated (DA close to 98%). Note that the two last experiments led only to D_1A_4 and D_0A_5 as products of the reaction. Their separation by cationic Dowex resin chromatography afforded D_1A_4 with 27% and 10% yields, for 4.5 and 10 molar equiv. of TEA, respectively.

3.2.3. *Influence of the acetylating agent*

The influence of the acetylating agent was studied by testing three commonly used acetylating agents, namely: acetic anhydride, acetyl chloride and pentafluorophenyl acetate (PFPA) (Kisfaludy, Mohacsi, Low, & Drexler, 1979). Among them, the best acetylation efficiency was found with acetic anhydride. Indeed, in the same experimental conditions, the reaction performed with acetic anhydride afforded COS mixture with a DA $\approx$ 95% (Table 2: S11), *vs* a DA $\leq$5% with acetyl chloride or PFPA (Table 2: S20 or S21, respectively). This result is consistent with the low stability in aqueous medium of acetyl chloride and PFPA, which are consumed by hydrolysis before reacting with amino groups of chitopentaose, leading to a very low extent of the *N*-acetylation degree. Indeed, the corresponding MALDI-TOF mass spectrum of PFPA acetylation experiment showed the presence of low partially *N*-acetylated compounds (D_3A_2, D_4A_1) and the starting material D_5A_0, whereas the one with acetyl chloride revealed the only presence of D_5A_0 after 5 h of reaction.

Based on these results, the influence of the acetic anhydride/chitopentaose molar ratio on acetylation products was achieved (Table 2). All the experiments were performed in water/methanol mixture (10/90, v/v), using 4.5 molar equiv. TEA/chitopentaose. The initial amount of acetic anhydride varied from 4.5 to 13.5 molar equiv./chitopentaose, and the determination of the composition of the product mixtures was carried out by MALDI-TOF mass spectrometry. As expected, the increase of the acetic anhydride amount (Table 2: from S17 to S19, and S11) led to the increase of the DA up to 94% (Table 2: S11). Below 10 molar equiv. of acetic anhydride (Table 2: S17 and S18), a complex mixture of *N*-acetylated derivatives identified as D_3A_2, D_2A_3, D_1A_4 and D_0A_5 was obtained. However, above 10 molar equiv./chitopentaose (Table 2: S11 and S19), only D_0A_5 and D_1A_4 species were detected, and our best D_1A_4 mass yield (27% of isolated compound after purification) was obtained with the highest amount of acetic anhydride (13.5 molar equiv./chitopentaose).

4. Conclusion

In this study, a one pot chemical synthesis of the tetra-*N*-acetyl-chitopentaose by *N*-acetylation of the chitopentaose hydrochloride under mild conditions was reported. The influence of different parameters (solvent, base and acetylating agent) involved in this reaction was investigated. Correlations between experimental parameters and final acetylated product structures have been established. These parameters were found to affect the reaction in different ways: (i) the solvent plays an important role on the selectivity between *N*- and *O*-acetylation; (ii) the base as well as the acetylating agent seem to influence drastically the degree of *N*-acetylation of chitopentaose. As a result, optimized *N*-acetylation conditions were found to be water/methanol mixture 10/90 (v/v) as solvent, acetic anhydride as acetylating agent (13.5 molar equiv./chitopentaose), and TEA as base (4.5 molar equiv./chitopentaose). In these conditions, tetra-*N*-acetyl chitopentaose was isolated by ionic chromatography in a moderate 27% mass yield. Its structural characterization underlined that the reducing end unit is fully *N*-acetylated. However, the position of the remaining GlcN unit was not determined yet. The consequent ongoing goal of this study is to define this position, which will actually be of a great interest for further controlled chemical modifications of the amino group, in particular for the access to specific bioinspired COS structures.

Supporting information available

MALDI-TOF mass spectrum, ^{1}H NMR spectrum, TGA analysis of the starting material, HPLC chromatograms of reference samples, and MS/MS mass spectrum of targeted COS tetra-*N*-acetyl-chitopentaose.

Acknowledgments

Financial support from Bayer CropScience (La Dargoire, France), the University of Lyon, and the *Agence Nationale de la Recherche* ("BIOCOS" 10-CD2I-008 research project) are greatly acknowledged. We also thank the Centre Commun de Spectrométrie de Masse (CCSM) of Université Lyon 1, for the assistance and access to the mass spectrometry facility.

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.2013.06.078.

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