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THE BEHAVIOR OF CHITIN TOWARDS ANHYDROUS HYDROGEN FLUORIDE. PREPARATION OF β-(1→4)-LINKED 2-ACETAMIDO-2-DEOXY-D-GLUCOPYRANOSYL OLIGOSACCHARIDES*

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ABSTRACT

Fluorohydrolysis of chitin in anhydrous hydrogen fluoride led to β-(1→4)-linked 2-acetamido-2-deoxy-D-glucopyranosyl oligosaccharides in almost quantitative yield. The average d.p. depended on both reaction time and temperature, and was conveniently monitored by ^{13}C-n.m.r. spectroscopy and gel-exclusion chromatography. Preparative fractionation of oligosaccharides of chitin (d.p. 2–10) was conveniently achieved by gel-exclusion chromatography in Bio-Gel P-4.

INTRODUCTION

It has been shown previously that cellulose, xylan, and polysaccharides in wood are dissolved readily by anhydrous hydrogen fluoride[2,3]. It has also been found that this dissolution is accompanied by a rapid transformation of cellulose and xylan into their respective glycosyl fluorides which, subsequently, on removal of hydrogen fluoride, were converted into complex mixtures of water-soluble reversion products. The same reversion oligosaccharides have been obtained by treatment of the monomeric sugars, D-glucose and D-xylose, with hydrogen fluoride. This behavior has been ascribed to a fast equilibrium in acidic medium between the glycosyl fluoride and the corresponding oxocarbenium ions whose stability should be enhanced in hydrogen fluoride, in agreement with the known propensity of this reagent to stabilize carbonium and oxonium ions[4]. This reactivity has further been confirmed with keto-hexoses and keto-hexosans[1,5] where formation of such reversion products, as ketose dianhydrides, was considerably

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enhanced in hydrogen fluoride as compared to other mineral acids. Since the stability of oxocarbenium ions depends on electronic factors at adjacent positions, one could expect that fluorolysis of glycosaminoglycans in hydrogen fluoride could proceed at a rate slower than that for cellulose and would, therefore, allow a convenient access to β-(1→4)-linked 2-acetamido-2-deoxy-D-glucopyranosyl oligosaccharides from chitin. Such oligosaccharides may be of synthetic value, or of use as model compounds for studies of the conformational behavior and metal-chelating properties of the corresponding natural polymers.

RESULTS AND DISCUSSION

Chitin, a β-(1→4)-linked polysaccharide consisting mainly of 2-acetamido-2-deoxy-D-glucopyranose units was found to be readily soluble in hydrogen fluoride and, when mixed with 5 to 10 parts of this reagent at 20°, homogeneous solutions were obtained in the course of ~10 min.

The reaction products could be isolated from the hydrogen fluoride solution by precipitation with ether. The composition of the products obtained from a series of experiments was studied by ^{13}C-n.m.r. spectroscopy. After a 1-h reaction with hydrogen fluoride, the product gave a spectrum (Fig. 1A) that showed a signal at δ 102 corresponding to β-C-1 of chitin and double signals for both C-6 (δ 61.6) and C-2 (δ 56.1) ascribable to terminal and non-terminal 2-acetamido-2-deoxy-D-glucopyranosyl units of chitin oligosaccharides. This indicated that a partial cleavage of chitin had taken place. After a 2-h (Fig. 1B), and a 7-h reaction (Fig. 1C), increasing signals were seen at δ 90 and 95, corresponding to α-C-1 and β-C-1, respectively, of reducing end-groups. A doublet centered at δ 106.8 p.p.m. indicated that some 2-acetamido-2-deoxy-α-D-glucopyranosyl fluoride residues had been formed ($J_{C\text{-}1,F}$ 221 Hz). The presence of a signal at δ 102.3, after a 7-h treatment with hydrogen fluoride (Fig. 1C), indicated that the degradation of chitin is slow, as compared to that of cellulose[2,3], and further experiments showed that 24 h at 20° were required for complete cleavage of chitin. Of interest is the apparent stability of the *N*-acetyl substituent, whose methyl group signal at δ 23.1 appeared to show an unchanged intensity after the reaction in hydrogen fluoride.

In order to observe more closely the reaction of chitin with hydrogen fluoride, ^{13}C-n.m.r. spectra were measured directly on the hydrogen fluoride solutions at various time intervals (Fig. 2). A spectrum recorded after a 0.5-h contact with hydrogen fluoride (Fig. 2A) showed an unchanged β-D-(1→4)-linked polysaccharide structure (β-C-1 at δ 98.1) together with a small proportion of other products. After 24 h at 20°, chitin was completely degraded and the spectrum (Fig. 2D) showed mainly one product, assumed to be the glucofuranosyl oxazolinium ion **2** (Scheme 1). The characteristic signals of this ion are at δ 114.0 for C-1, 13.6 for H_3C–C^+, and 80.6 for C-4, the last named indicating a furanose ring. An identical spectrum was obtained for a solution of 2-acetamido-2-deoxy-D-glucose (**4**) in hydrogen fluoride. A spectrum recorded after a 16-h reaction with

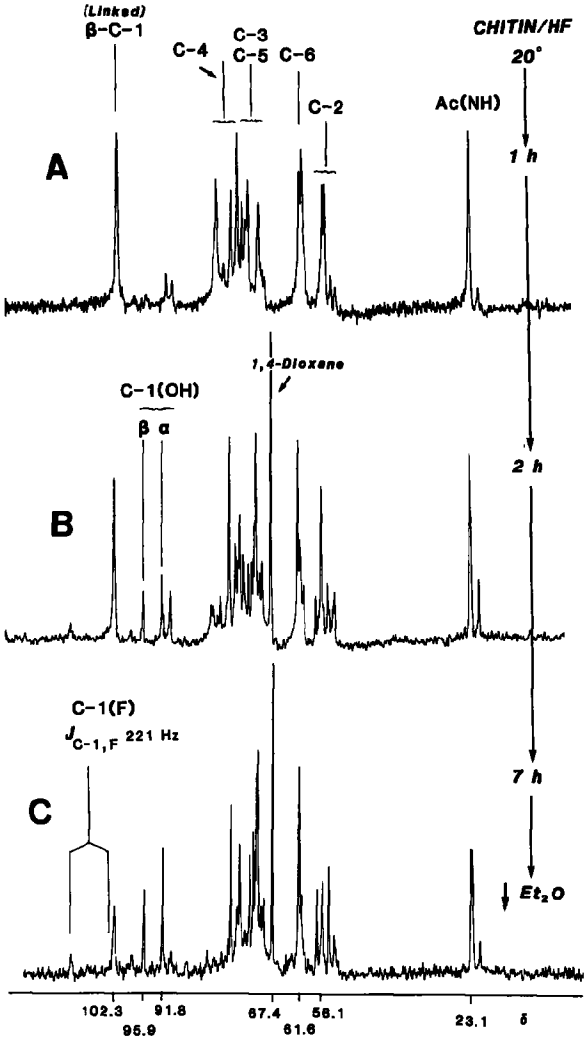


Fig. 1. ^{13}C-N.m.r. spectra, at 22.63 MHz, of solutions in deuterium oxide of reaction products obtained by treatment of chitin in HF after various reaction times at 20°: A - 1 h-reaction time; B - 2 h-reaction time; C - 7 h-reaction time.

hydrogen fluoride (Fig. 2C) showed, in addition to the ion **2**, a second oxazolinium ion, as seen from the signal at δ 109.5 assumed to arise from C-1 of a monomeric ion having a pyranose structure **1**. The latter ion would be the expected initial obtained in hydrogen fluoride from the cleavage of (1→4)-linked oligomers. This ion may then undergo a subsequent ring-contraction to give **2**, in analogy with the previously observed behavior of pyranose derivatives in hydrogen fluoride[6]. The spectrum obtained after a 4-h reaction with hydrogen fluoride (Fig. 2B) showed two rather large signals at δ 106.4 and 100.3. The product giving these signals is probably the ion **1**, preponderantly the dimer (n = 1). This is in agreement with the

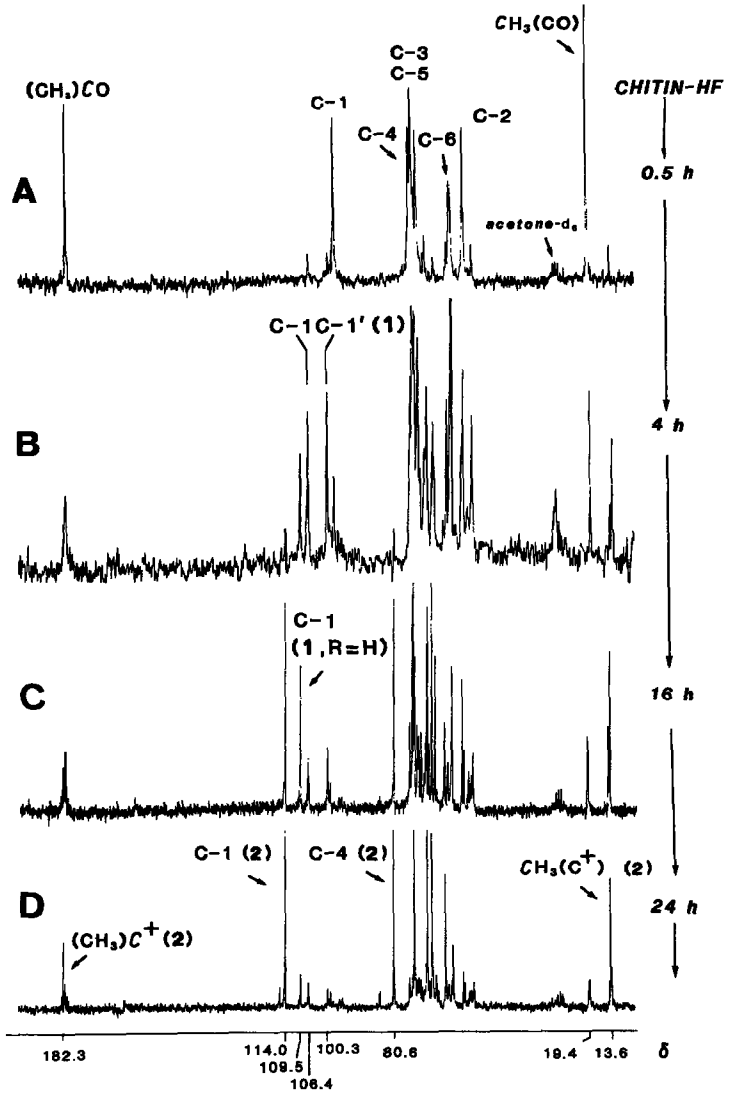
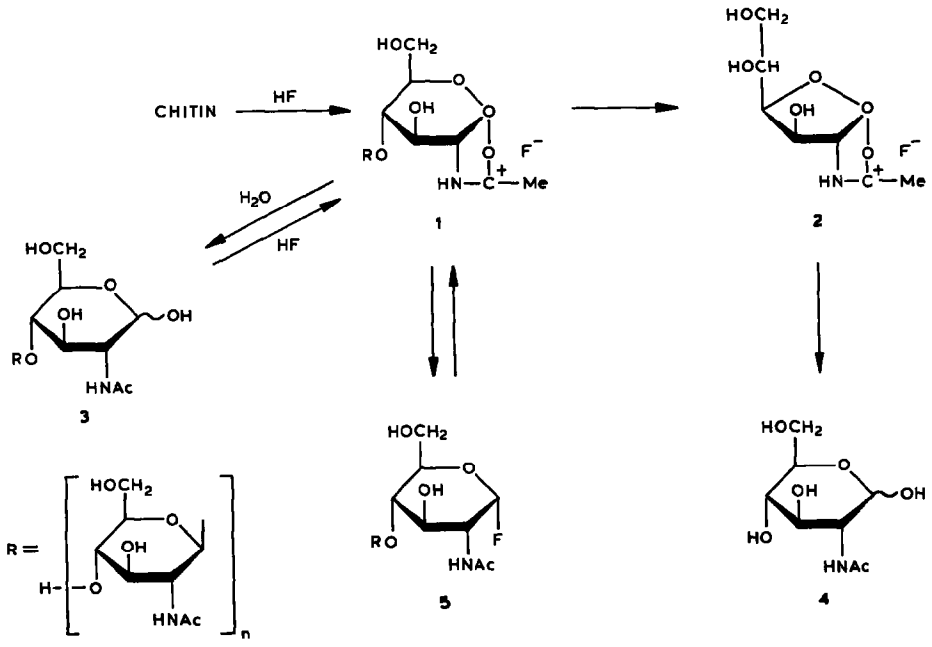


Fig. 2. ^{13}C-N.m.r. spectra at 25.182 MHz of solutions of chitin in anhydrous hydrogen fluoride at 20°. The spectra were measured after: (A) 0.5 h, (B) 4 H, (C) 16 h, and (D) 24 h. Numbers in bracket refer to Scheme 1.

analytical data (Fig. 3) which showed that the dimer is the preponderant product obtained after a 4-h reaction with hydrogen fluoride. Hydrolysis of the products present in the hydrogen fluoride solutions during work-up led to the products isolated as shown in Fig. 1.

Precipitation with ether of a 2-h reaction mixture of chitin in hydrogen fluoride, followed by dissolution in water of the resulting dried precipitate in order to hydrolyze any glycosyl fluoride residue (**5**), led to a mixture of chitin oligo-



Scheme 1. Proposed mechanism for the fluorohydrolysis of chitin.

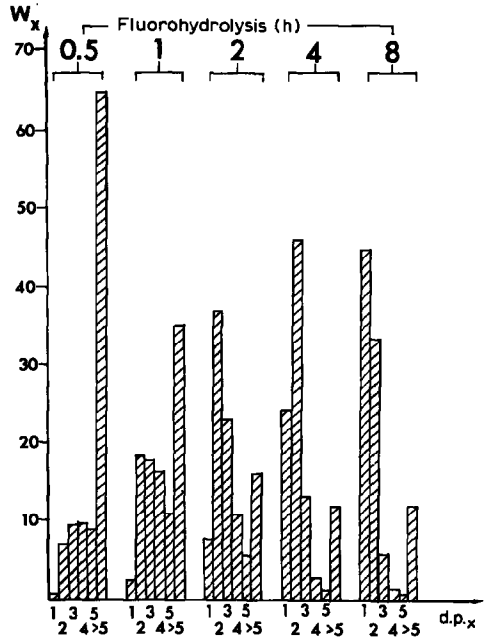


Fig. 3. 2-Acetamido-2-deoxy-D-glucopyranosyl oligosaccharides distribution as a function of fluorohydrolysis time. The product distribution was monitored by use of a Bio-Gel P-4 analytical column, with buffered 50mM ammonium acetate (pH 4.5) as eluent.

saccharides which showed, in the ^{13}C-n.m.r. spectrum, signals at δ 91.8 and 95.9 indicative of the presence of a significant proportion of α and β anomers of reducing 2-acetamido-2-deoxy-D-glucopyranose units. From a comparison of the absorbance intensities, in i.r. spectroscopy[7], of the amide and hydroxyl groups at 3450 and 1655 cm^{-1}, respectively, the *N*-acetyl content of the sample did not appear to be reduced as compared to that of the original chitin, and this agreed with previous observations by ^{13}C-n.m.r. spectroscopy.

This mixture was subjected to preparative gel-permeation chromatography on Bio-Gel P-4 (Pharmacia) using an aqueous buffered ammonium acetate solution as eluent[8]. The results showed that, by use of this gel, the separation of the oligomers is independent of the pH, the concentration of ions, and the temperature of the eluent. Comparison of the separations obtained on Bio-Gel P-2, P-4, and on the previously suggested[9] P-6 showed that P-4 gives a superior separation, which can be attributed to the high hydrodynamic volumes of the 2-acetamido-2-deoxy-D-glucose oligomers, arising in turn from their solvation in aqueous solution.

These oligomers may be considered to be electrically neutral, and pure water would be proposed as eluent. However, when this eluent was used, the chromatograms showed an abnormal elution pattern beyond d.p. 5 (Fig. 4A). This behavior might be caused by an electrostatic-exclusion process due to the presence of charged molecules in the medium. Such compounds may be calcium salts (from the chitin), partially deacetylated oligomers, or their ionic complexes. According to their charge, such products can be excluded, more or less delayed, or completely adsorbed. For this reason, the separation of the oligomers was carried out in two steps. Firstly, the mixtures were fractionated in 50mM aqueous ammonium acetate at pH 4.5 to give chromatograms as shown in Fig. 4B. In the second step, each oligomer was rechromatographed with water as eluent.

Yields, and physico-chemical and analytical data are reported in Table I for

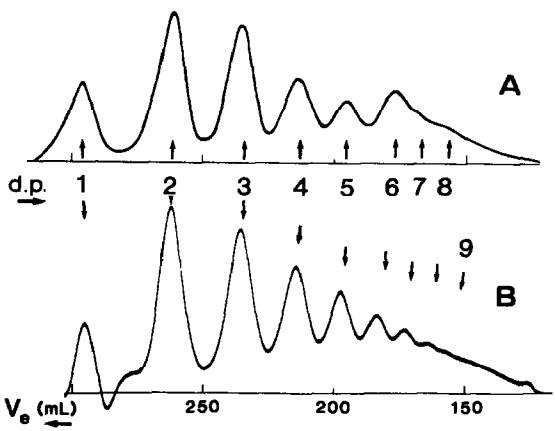


Fig. 4. Comparative elution profiles of a 2-h chitin–HF fluorohydrolysis sample in Bio-Gel P-4: (A) With water as eluent and (B) with buffered 50mM ammonium acetate (pH 4.5) as eluent.

TABLE I

YIELDS, MELTING POINTS, OPTICAL ROTATIONS, MASS SPECTROMETRY (f.a.b.), AND ANALYTICAL DATA FOR β-(1→4)-LINKED 2-ACETAMIDO-2-DEOXY-D-GLUCOPYRANOSYL OLIGOSACCHARIDES (d.p. 2–10) RESULTING FROM THE ACTION OF HYDROGEN FLUORIDE ON CHITIN FOR 2 H

Compound (d.p.)	*Yields*[a] *(%)*	*M.p. (degrees)*[b]	$[\alpha]_D^{18}$ [c] *(degrees)*	m/z *(M + H)*[d]	*Mol. Formula*	*Analytical data*						*Lit.*		*Ref.*
						Calc.			*Found*			*M.p. (degrees)*	$[\alpha]_D$[c] *(degrees)*	
						C	*H*	*N*	*C*	*H*	*N*			
2	37	239–241	+13.9 (0.82)	425	$C_{16}H_{28}N_2O_{11}$	45.28	6.64	6.60	45.85	6.99	6.11	245–247	+18 (1)	10
												260–262	+17.2 (0.5)	11
												251.5–252.5	+17 (1)	12
													+16 (2)	13
3	23	308–310	+0.6 (0.98)	628	$C_{24}H_{41}N_3O_{16}$	45.93	6.57	6.69	46.38	7.02	6.28	304–306	+2.2 (0.9)	11
												309–311	+2.5 (1.2)	11
													+0.5 (1.0)	12
													+1.8 (2.0)	13
4	10	295–305	−6.4 (0.92)	831	$C_{32}H_{54}N_4O_{21}$	46.26	6.54	6.74	46.66	7.05	6.43	290–300	−4.1 (1.0)	11
													−5 (1.0)	12
													−2.9 (2.0)	13
5	5	295–298	−12 (1.01)	1034	$C_{40}H_{67}N_5O_{26}$	46.46	6.52	6.77	46.61	6.97	6.54	285–295	−9.1 (1.0)	11
													−7.5 (2.0)	13
6	4.6	300–305	−12.6 (0.76)	1237	$C_{48}H_{80}N_6O_{31}$	46.60	6.51	6.79	46.57	6.95	6.45		−11.4 (0.8)	11
7	3		−28.3 (0.52)	1440	$C_{56}H_{93}N_7O_{36}$	46.70	6.50	6.80	46.32	6.97	6.41		−12.6 (0.3)	11
8	2.2		32.4 (0.52)	1643	$C_{64}H_{106}N_8O_{41}$	46.83	6.50	6.82	45.02	6.79	6.66			
9	1.6		−17.6 (0.68)	1846	$C_{72}H_{119}N_9O_{46}$	46.83	6.48	6.82	46.17	6.89	6.51			
10	1		−24.3 (0.39)	2049	$C_{80}H_{132}N_{10}O_{51}$	46.87	6.48	6.83	45.73	6.87	6.62			

[a]From chitin (25 g) heated with HF (100 mL) for 2 h, followed by a single chromatographic fractionation using buffered ammonium acetate as eluent. Formation of 10% of 2-acetamido-2-deoxy-D-glucose in this reaction. [b]Freeze-dried in water. [c]For a solution in water, at equilibrium, *c* in parentheses. [d]In f.a.b.$^+$ ionisation mode; internal fragmentation not given.

TABLE II

^{13}C-N.M.R. SPECTRAL DATA FOR A SOLUTION OF β-(1→4)-LINKED 2-ACETAMIDO-2-DEOXY-D-GLUCOPYRANOSYL OLIGOSACCHARIDES (d.p. 2–5) IN DEUTERIUM OXIDE

$[\beta$-D-(1→4)-GlcNAc$]_n$	*Chemical shifts (δ)*[a]					
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
n = 2	91.26(α)	54.43(α)	70.13(α)	80.76(α)	70.79(α)	60.90(α)
	95.65(β)	56.94(β)	73.34(β)	80.35(β)	75.38(β)	61.02(β)
	102.30(*2*)[b]	56.44(*2*)[b]	74.32(*2*)[b]	70.60(*2*)[b]	76.72(*2*)[b]	61.41(*2*)[b]
n = 3	91.29(α)	54.46(α)	70.10(α)	80.56(α)	70.85(α)	60.85(α)
	95.66(β)	57.00(β)	73.30(β)	80.08(β)	75.43(β)	61.00(β)
	102.10(*2*)[b]	55.86(*2*)[b]	73.02(*2*)[b]	80.12(*2*)[b]	75.33(*2*)[b]	60.85(*2*)[b]
	102.29(*3*)[b]	56.43(*3*)[b]	74.30(*3*)[b]	70.60(*3*)[b]	76.75(*3*)[b]	61.42(*3*)[b]
n = 4	91.29(α)	54.46(α)	70.10(α)	80.58(α)	70.85(α)	60.85(α)
	95.66(β)	57.00(β)	73.30(β)	80.11(β)	75.42(β)	61.00(β)
	102.10(*2,3*)[b]	55.84,	72.95(*2,3*)[b]	79.90(*2,3*)[b]	75.35(*2,3*)[b]	60.81(*2,3*)[b]
		55.90(*2,3*)[b]	72.99			
				80.16		60.88
	102.29(*4*)[b]	56.43(*4*)[b]	74.31(*4*)[b]	70.60(*4*)[b]	76.76(*4*)[b]	61.43(*4*)[b]
n = 5	91.28(α)	54.46(α)	70.10(α)	80.56(α)	70.84(α)	60.82(α)
	95.65(β)	56.99(β)	73.30(β)	80.10(β)	75.35(β)	61.00(β)
	102.08(*2,3,4*)[b]	55.89(*2,3,4*)[b]	72.98(*2,3,4*)[b]	79.88(2C)		60.82(*2,3,4*)[b]
				80.10(1C)		
	102.29(*5*)[b]	56.43(*5*)[b]	74.30(*5*)[b]	70.60(*5*)[b]	76.75(*5*)[b]	*61.42(5)*[b]

[a]Relative to 1,4-dioxane (δ 67.4) as internal standard. [b]Number(s) in brackets indicates the 2-acetamido-2-deoxy-D-glucopyranosyl unit, numbered from the reducing end, to which belongs the assigned carbon atom(s).

the entire series of oligosaccharides. ^{13}C-N.m.r. spectroscopy assignments of oligosaccharides of d.p. 2–5 are summarized in Table II. Data for both anomers of *N,N'*-diacetylchitobiose are in good agreement with those found in the literature[9,14,15]. Assignments for the C-chemical shifts of *N,N',N''*-triacetylchitotriose were obtained by substracting the signals of the *N,N'*-diacetylchitobiose spectrum, as previously suggested for the interpretation of ^{13}C-n.m.r. spectra of cello-oligosaccharides[16]. As expected, higher homologs displayed homomorphous patterns, with the signals corresponding to the two terminal units decreasing in relative intensity, and the signals corresponding to the internal repeating units gradually increasing. Slight variations were detected for C-4 atoms involved in the interglycosidic linkages. The spectra of the oligomers of d.p. 6–10 are not reported in Table II. They showed largely the same pattern as described above, but they were more complicated. This may be due to a change in conformations with increasing d.p.

The fast-atom-bombardment mass-spectrometry-ionisation (f.a.b. m.s.) technique afforded a further useful characterization of this series of β-(1→4)-linked 2-acetamido-2-deoxy-D-glucopyranosyl oligosaccharides. The pseudo-molecular, protonated ion, $[MH]^+$, or the corresponding cationized species, was readily detected for the whole series of oligomers by use of an acidified glycerol matrix (Table I). In addition, each sample, excepting those of d.p. 9 and 10, exhibited a $[MH-H_2O]^+$ ion, together with signals at *m/z* $[MH - (221)]^+$, followed by fragments at

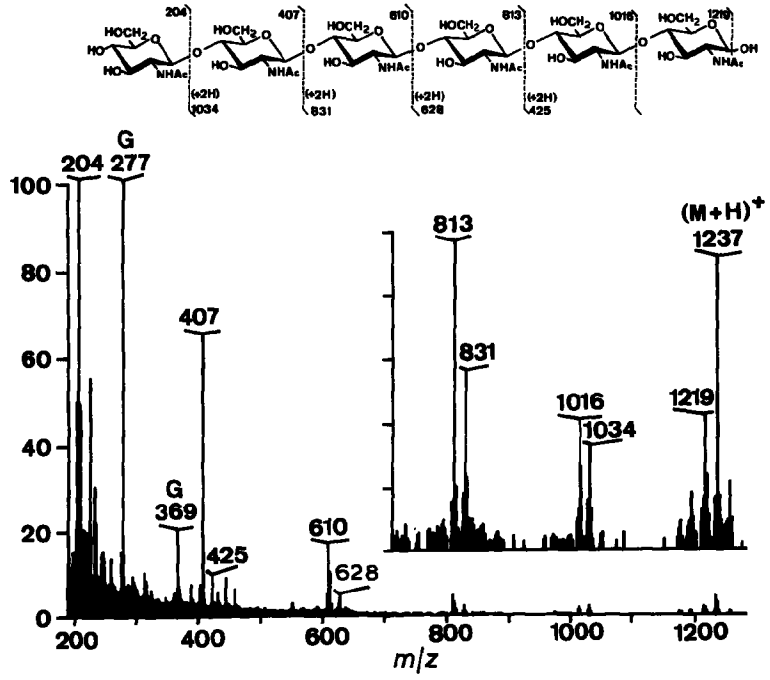


Fig. 5. F.a.b. mass spectrum of *N*-acetylchitohexaose.

intervals of *m/z* 203 corresponding to the sequential fragmentation of the glycoside bonds, with the positive charge remaining associated with the larger part of the chain, as shown in Fig. 5 for the hexaose oligosaccharide. This behavior is in contrast with that of cello- and malto-oligosaccharides, where the pseudo-molecular ions were the only detectable signals in the f.a.b. mass spectra[17], and where the sequential fragmentation just described could only be induced by collisional-activation techniques[18]. Such propensity to ionization may possibly be explained through formation of cyclic ions similar to ion **1**, which was observed in hydrogen fluoride solution as discussed earlier.

In conclusion, hydrogen fluoride fluorohydrolysis of chitin, associated with gel-exclusion separation techniques, afforded a more convenient way of preparing β-(1→4)-linked 2-acetamido-2-deoxy-D-glucopyranosyl oligosaccharides than the previously described methods using conventional mineral acids or acetolysis reagents[9,11–13,19,20]. A main advantage of this process lies in (a) that it does not result in any apparent *N*-deacylation nor *N,O*-acyl migration, as might be expected from previous observations in the peptide series[21]; and (b) that degradation products[22] are not found. A further point of interest is related to the possibility of selecting fluorolysis conditions compatible with optimized yields in given oligosaccharides. An illustration of the oligosaccharide distribution as a function of time of reaction with hydrogen fluoride is given in Fig. 2.

EXPERIMENTAL

General methods. — Melting points were determined with a Zeiss microscope hot-stage, and are corrected. Optical rotation was measured with a Perkin–Elmer 241 instrument. I.r. spectra of chitin were recorded with a Perkin–Elmer 598 instrument from films made from a solution (10 mg/0.5 mL) of the polymer in *N*-dimethylacetamide containing 5% LiCl, then air-dried on a glass plate, washed once with methanol, and dried *in vacuo*. For β-(1→4)-linked 2-acetamido-2-deoxy-D-glucose oligosaccharides, pressed discs of intimately ground solid samples (1 mg) with KBr (150 mg) were used. The ^{13}C-n.m.r. spectra were recorded with Bruker WH-90, WP-100, and AM-500 instruments. Spectra for HF solutions (Fig. 2) were obtained by use of Teflon tubes that fitted tightly inside a 10-mm glass sample tube [($^2H_6$)acetone was used as lock substance and internal reference taken at δ 29.2]. Spectra of isolated oligosaccharides (Tables II and Figs. 2 and 4) were recorded for solutions in D_2O (internal 1,4-dioxane, δ 67.40). Mass spectra in the f.a.b.(+)–m.b.s.a. ionisation mode were recorded for oligosaccharides of d.p. 2–7 on a double-focusing Kratos–AEI MS-50 apparatus (Manchester), fitted with a 1.2-T magnet, and operating at the full accelerating potential (6 kV); a f.a.b. 11 NF, Ion Tech atom-gun; and a MAT SS 200 Finnigan (DEC–PDP 11-34) computer. F.a.b. mass spectra of oligosaccharides of d.p. 8–10 were recorded with a ZAB–HF instrument (VG Analytical, Manchester), operating at 8 kV and fitted with a DS 11-250 (PDP 11-24) computer. With both equipments, the gas used was xenon. The

samples (5 μg) were dissolved in glycerol (thioglycerol for d.p. 8–10, 0.5 μL), on a copper target, and 0.1M HCl (1 μL) was added.

The anhydrous HF was a commercial product obtained in steel cylinders. Prior to use, it was kept in polyethylene bottles at 0°. All reactions with HF were conducted in polyethylene bottles. Chitin was purchased from Sigma (St Louis, MO). It originated from crab shells and was practical grade. The *N*-acetyl content was higher than 90% of the theory as determined by i.r. spectroscopy. Ultrafiltration was performed with Amicon equipment on YCO5 6.2-cm (diam.) membranes and 200-mL cells. Microanalysis were obtained on compounds dried under vacuum (60°, 70 Pa, 24 h).

Chitin fluorolysis. — Chitin (25 g) was suspended in HF (100 mL) at 0° and the suspension stirred while the temperature was allowed to rise to 20°. After 10 min, a clear solution was obtained which was kept for the appropriate time at 20°, and then cooled to 0°. Precipitation with cold ether (−70°, 600 mL) led to a colorless precipitate which was washed several times with ether, and then dried. It was suspended in water (1.5 mL) and filtered (Millipore 0.45 μm). The filtrate was concentrated (500 mL), and then freeze-dried to a solid residue (21.84 g).

Gel-permeation chromatography of chitin and oligosaccharides. — The equipment consisted of a Milton–Roy controlled-volume pump (minipump A, 350 bars; Dosapro, Pont St-Pierre, France) operating at a flow rate of 20 mL/h for analytical purposes and 100 mL/h for preparative work. This was fitted to either a 1.5 × 210 cm (analytical), or two 5 × 100 cm (preparative K 50/100) glass columns (Pharmacia, Uppsala), filled with Bio-Gel P-4 (200–400 mesh; Pharmacia). A refractive-index detector (Waters, model R 401) was used on line as concentration detector. The eluent was a 50mM aqueous ammonium acetate solution, adjusted to pH 4.5 with acetic acid for the first purification step. The samples, dissolved in the eluent, were introduced on the top of the column by means of an injection loop (Rheodyne, Cotati, CA; model 7010). For analytical purposes, 20 or 30 mg in 0.5 mL of solvent were used, and for preparative experiments the sample was 1 g, in 5 mL of solvent, for the first separation. In the second purification step, the amount of sample varied from 700 mg for the dimer to 250 mg for the decamer, to avoid solubility problems due to the d.p. value. Fractions were collected, in the preparative scale, with an LKB (Bromma, Sweden) 2112 Redirac fraction collector with a 10-min spacing time between each collection. Fractions of similar hydrodynamic volume were combined, concentrated to 150 mL, and ultrafiltrated on YCO5 membranes with a 2-L water elution. The solutions were filtered on Millipore 0.22-μm membranes (6.2 cm diam., 200 mL cell), concentrated, and freeze-dried. The recovered fractions were rechromatographed on the same Bio-Gel column with water as eluent. Fractions corresponding to base-peak width were pooled.

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