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Note

Preparation of acetylated derivatives of modified chito-oligosaccharides by the depolymerisation of partially *N*-acetylated chitosan with nitrous acid

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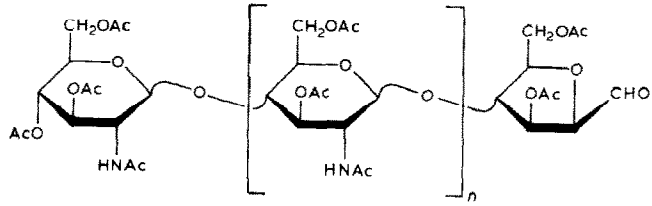
Oligosaccharides have been prepared from chitin [a (1→4)-linked 2-acetamido-2-deoxy-β-D-glucan] and chitosan (its *N*-deacetylated product) by partial hydrolysis with concentrated hydrochloric acid[1], phosphoric acid[2], trichloroacetic acid[3], and formic acid[4], and by acetolysis[5], but the yields were generally low (<16%). Nitrous acid-deaminative cleavage[6–11] is a well known reaction for the structural analysis of mucopolysaccharides. We now report its use for the preparation of oligosaccharides, in relatively good yields, from partially *N*-acetylated chitosan. The products are chito-oligosaccharide derivatives, since they have 2,5-anhydro-D-mannose as the reducing end-groups.



2 $n = 0$
3 $n = 1$
4 $n = 2$
5 $n = 3$
6 $n > 4$

Partially *N*-acetylated chitosan (d.s. for NAc, 0.48) was prepared in quantitative yield[12]. The nitrous acid-deaminative cleavage was performed in aqueous 20% acetic acid and the products were acetylated. Column chromatography on silica gel of the resulting amorphous mixture (83%) gave six fractions (Table I). Compounds **1–5** each gave a single spot in t.l.c., and **6** gave a tailing spot. The total yield of **2–6** was 62%. Compound **1** was identified (g.l.c.) as acetylated 2,5-anhydro-D-mannose

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TABLE I

CHROMATOGRAPHY OF ACETYLATED, MODIFIED CHITO-OLIGOSACCHARIDES

Fraction number	*Yield (%)*[a] *(g)*	R_{GlcNAc}[b]	*Compound*
37–80	0.98 (14)	1.8–1.4	**1**
81–89	0.07 (1)	1.0	**1, 2**
90–109	1.03 (15)	0.96	**2**
110–139	0.22 (3)	0.91, 0.62	**2, 3**
140–180	0.66 (10)	0.53	**3**
181–209	0.36 (5)	0.53, 0.30	**3, 4**
210–264	0.73 (11)	0.30	**4**
265–289	0.66 (10)	0.64, 0.34	**4, 5**
290–340	0.45 (7)	0.34	**5**
340–400	1.04 (15)	~0.00	**6**

[a]The acetylated mixture (6.9 g) was eluted from a column (3 × 63 cm) of silica gel (see Experimental). [b]T.l.c.; acetone–benzene (1:1) for fractions 37–264, and acetone–benzene (3:1) for fractions 265–400.

by comparison with an authentic sample[9]. Compounds **2–5** had i.r. absorptions for *O*- and *N*-acetyl, and consisted of 2-amino-2-deoxy-D-glucose and 2,5-anhydro-D-mannose as revealed by component analysis. 2,5-Anhydro-D-mannitol (g.l.c. of the acetylated product), but no 2-amino-2-deoxy-D-glucitol, was detected in acid hydrolysates of borohydride-reduced **2–6**, indicating that the reducing end-group in each parent compound was 2,5-anhydro-D-mannose. An aldehyde proton signal at δ 9.50 was detected in the ^{1}H-n.m.r. spectra ($CDCl_3$) of the acetylated products. As shown in Table II, the structures were supported by the elemental analyses.

The d.p. of each of the compounds **2–5** was determined from the molar ratio of 2-amino-2-deoxy-D-glucose and 2,5-anhydro-D-mannose (1.0, 2.1, 3.0, and 4.2, respectively) and by gel filtration, after *O*-deacetylation, on Sephadex G-25 (Fig. 1). The data indicated that **2–5** were di-, tri-, tetra-, and penta-saccharides, and that **6** was a mixture of saccharides (d.p. >6).

TABLE II

DATA FOR THE ACETYLATED, MODIFIED CHITO-OLIGOSACCHARIDES

Compound	$[\alpha]_D$ *(c, temp.)*[a] *(degrees)*	*Formula*	*Calc. (%)*			*Found (%)*		
			C	*H*	*N*	*C*	*H*	*N*
1	+36 (0.9, 17°)					n.d.[b]		
2	+16 (1.3, 16°)	$C_{24}H_{33}NO_{15} \cdot 0.82\,H_2O$	48.83	5.92	2.37	48.73	5.89	2.38
3	+1 (1, 24°)	$C_{36}H_{50}N_2O_{22} \cdot 0.96\,H_2O$	49.13	5.95	3.18	48.98	5.95	3.09
4	−8 (1, 24°)	$C_{48}H_{67}N_3O_{29} \cdot 1.26\,H_2O$	49.16	5.98	3.58	49.21	6.03	3.49
5	−13 (1, 24°)	$C_{60}H_{84}N_4O_{36} \cdot 1.03\,H_2O$	49.50	5.96	3.65	49.50	5.92	3.80
6	−12 (1, 27°)					n.d.		

[a]In chloroform. [b]Not determined.

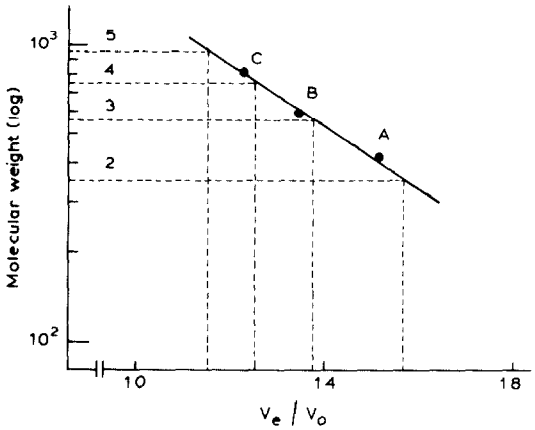

Fig. 1. Estimation of the molecular weights of **2–5** by gel filtration on a column (1.4 × 142 cm) of Sephadex G-25 by elution with distilled water. The calibration curve was obtained by plotting V_e/V_o against the log mol. wt. of standard *N*-acetylated chito-oligosaccharides (A, 424; B, 627; C, 830) Compounds **2–5** were estimated to be di-, tri-, tetra-, and penta-saccharides, respectively.

EXPERIMENTAL

General methods. — I.r. spectra (KBr or Nujol) were recorded with a Hitachi 215 grating spectrometer, n.m.r. spectra with a 60-MHz Hitachi R-24 spectrometer, and specific rotations with a JASCO Dip-181 digital polarimeter. G.l.c. was performed at 175–180° (column) and 280° (injection port) with a Shimadzu GC-5A gas chromatograph equipped with a hydrogen flame-ionisation detector and a glass column (3 mm × 2.5 m) packed with 3% of ECNSS-M on Chromosorb-W (AW-DMCS); nitrogen was the carrier gas at 60 mL/min, and the analytical data were computed with a Shimadzu C-EB Chromatopac. T.l.c. was performed on Silica Gel 60G (Merck). Column chromatography was performed on a column (3 × 63 cm) of Kieselgel 60 (Merck) which was previously activated at 125° for 2 h. Acid hydrolysis was performed with 3M HCl at 100° for 18 h. Neutral sugars were analysed by the phenol–sulfuric acid method[13] with D-mannose as the standard. Hexosamine was analysed by the Elson–Morgan method[14] with 2-amino-2-deoxy-D-glucose hydrochloride as the standard. An authentic sample of 2,5-anhydro-D-mannose was prepared from 2-amino-2-deoxy-D-glucose hydrochloride[9].

Chitosan. — Flonac-N [a commercial product of chitosan (crab shell), Kyōwa Yushi Co., Ltd.) was treated with aqueous 45% NaOH containing $NaBH_4$ (0.1 g/100 mL) at 110° for 5 h. The product, $[\alpha]_D^{19}$ −10° (*c* 0.6, aqueous 10% acetic acid), had a negligible signal for NAc (~2 p.p.m.) in the ^{1}H-n.m.r. spectrum (D_2O–DCO_2D, 9:1), and its elemental analyses agreed, within 0.3%, with the theoretical values for completely *N*-deacetylated chitin. The observed C/N ratio from the elemental analyses was 6.09 (calc. 6.00).

Partially N-*acetylated chitosan.* — A solution of chitosan in aqueous 2% acetic acid was diluted with methanol (2 vol.). Acetic anhydride (0.52 mol/GlcN)

was added with vigorous stirring at room temperature[12]. The product (d.s. for NAc, 0.48), isolated in a quantitative yield, had $[\alpha]_D^{29}$ −16° (*c* 0.8, aqueous 10% acetic acid); ν_{max}^{KBr} 1660 and 1560 cm^{-1} (C=O and NH of NAc).

Anal. Calc. for $[C_6H_{10}NO_4(C_2H_3O)_{0.48}(H)_{0.52} \cdot 0.42\ H_2O]_n$: C, 44.25; H, 6.83; N, 7.42. Found: C, 44.24; H, 6.82; N, 7.40.

Nitrous acid-deaminative depolymerisation. — To a solution of the partially *N*-acetylated chitosan (8.8 g) in aqueous 20% acetic acid (300 mL) was added $NaNO_2$ (9.5 g), and the mixture was stirred at room temperature for 30 min, then stored at room temperature for 18 h, filtered, neutralised with 6M NaOH, and concentrated to dryness at <45° *in vacuo*. The residue was stirred with acetic anhydride–pyridine (1:1, 80 mL) at room temperature for 3 days. The mixture was poured into ice–water (~300 mL) and extracted with chloroform (3 × 100 mL), and the combined extracts were concentrated *in vacuo* to afford an amorphous mixture (6.9 g) of acetylated products.

A solution of this mixture in chloroform (~ 30 mL) was applied to a column (3 × 63 cm) of silica gel and eluted with benzene–ethyl acetate (3:1) at 17 mL/h (10-mL fractions). Each fraction was subjected to t.l.c. (Table I). Six compounds were isolated, and re-chromatographed on the column of silica gel (Table II).

A portion (36–102 mg) of **2–5** was *O*-deacetylated with 0.1M NaOH at room temperature for 18 h to afford a syrupy, hygroscopic, water-soluble compound, which had i.r. absorptions at 1650 and 1540 cm^{-1} (NAc) but not at 1750 and 1240 cm^{-1} (OAc). Each *O*-deacetylated product gave a single spot in t.l.c. The molecular weights of **2–5** were estimated (Fig. 1) by gel chromatography on a column (1.4 × 142 cm) of Sephadex G-25, and by plotting V_e/V_0 against the log mol. wt. of standard *N*-acetylated chito-oligosaccharides (mol. wts. 424, 627, and 830, Seikagaku Kogyo Co., Ltd.). The elutates were monitored by u.v. absorption at 194 nm.

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