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A comparison of the results of sequential hydrazinolysis–nitrosation and alkali-mediated cleavage–nitrosation of the *O*-linked oligosaccharides of gastric mucus glycoproteins*†

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ABSTRACT

Analysis of the oligosaccharides released from pig gastric mucus glycopolypeptides by hydrazinolysis showed that degradation had occurred. Nitrosation of the products followed by reduction gave a mixture that had a low content of 2,5-anhydro-D-talitol, which implied destruction of much of the terminal reducing 2-amino-2-deoxy-D-galactose. Under the conditions of hydrazinolysis, cellobiose was largely unchanged but laminaribiose gave a complex mixture that probably contained glucose hydrazone (^{13}C-n.m.r. data). In order to avoid degradation, the hydrazinolysis–nitrosation sequence should be applied to the reduced oligosaccharides released on cleavage with alkali.

INTRODUCTION

The mixtures of oligosaccharides that are obtained from glycoproteins by chemical or enzymic methods present a formidable challenge in the elucidation of structure. The identification of common sequences in such mixtures by regiospecific degradation has some merit, especially if fragment oligosaccharides that are of key importance can be identified readily, for example, serological determinants that originate from non-reducing termini of the oligosaccharide chains. Nitrosation of oligosaccharides that contain amino sugar residues can achieve such regiospecific degradations in reasonably good yield[1].

Alkaline sodium borohydride has been used since 1971 for the cleavage, by β-elimination, of the serine- and threonine-linked ("*O*-linked") oligosaccharides of glycoproteins, to give alkali-stable alditol derivatives[2]. Hydrazinolysis has been used widely to release the asparagine-linked ("*N*-linked") oligosaccharides of glycoproteins

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and yields hydrazone derivatives. The extent of degradation of carbohydrate during hydrazinolysis can be reduced by the use of hydrazinium sulphate as a catalyst[3].

Montreuil, in 1975, reported[4] that *O*-linked oligosaccharides of glycoproteins were not released by hydrazinolysis, and Amerongen *et al.*[5] used hydrazinolysis to isolate the *N*-linked oligosaccharides from mouse submandibular mucin which also contained *O*-linked oligosaccharides. However, the lability of *O*-galactosylserine linkages in extensin towards hydrazinolysis was reported in 1973 by Lamport *et al.*[6].

A detailed study of hydrazinolysis and alkali-mediated cleavage for the release of oligosaccharides from pig gastric mucus glycoprotein, coupled with regiospecific degradation *via* nitrosation, is now reported.

RESULTS AND DISCUSSION

The starting material was the mixture of glycopolypeptides prepared[7] by exhaustive proteolytic digestion of mucus glycoprotein, isolated from the stomach of freshly slaughtered pigs. After dialysis and gel-permeation chromatography, the glycopolypeptides had the monosaccharide proportions shown in Table I. The principal amino acids were serine, thereonine, and proline. Treatment of this mixture with 0.05M sodium hydroxide–M sodium borohydride (15 h, 45°) gave a product with the monosaccharide proportions shown in Table I, and chromatography on Bio-Gel P-4 gave the results shown in Fig. 1 (assay by the phenol–sulphuric acid method[8]). Fractions 12–18 (22% recovery) contained the products of higher molecular weight together with small proportions of reduced oligosaccharides, as shown by the presence of 2-acetamido-2-deoxy-D-galactitol (GalNAcol) and also polypeptide and glycopolypeptide. When the sodium borohydride was omitted, the polypeptide product was present in fractions 12–18.

H.p.l.c. of fractions 19–36 (59% recovery) revealed a complex mixture. A less

TABLE I

Monosaccharide composition of pig gastric mucus glycopolypeptides and the alkali-mediated cleavage product

Monosaccharide	*Moles/mole of* D-*galactose*			
		Alkali mediated cleavage product		
	Glycopoly-peptides	*Unfractionated*	*Fractions 12–18*	*Fractions 19–36*
L-Fucose	0.49	0.51	0.53	0.43
D-Galactose	1.00	1.00	1.00	1.00
2-Acetamido-2-deoxy-D-glucose	0.94	0.96	1.0	0.93
2-Acetamido-2-deoxy-D-galactose	0.71	0.28	0.38	0.19
2-Acetamido-2-deoxy-D-galactitol	–	0.24	0.05	0.38
N-Acetylneuraminic acid	0.01	0.07	0.03	0.02

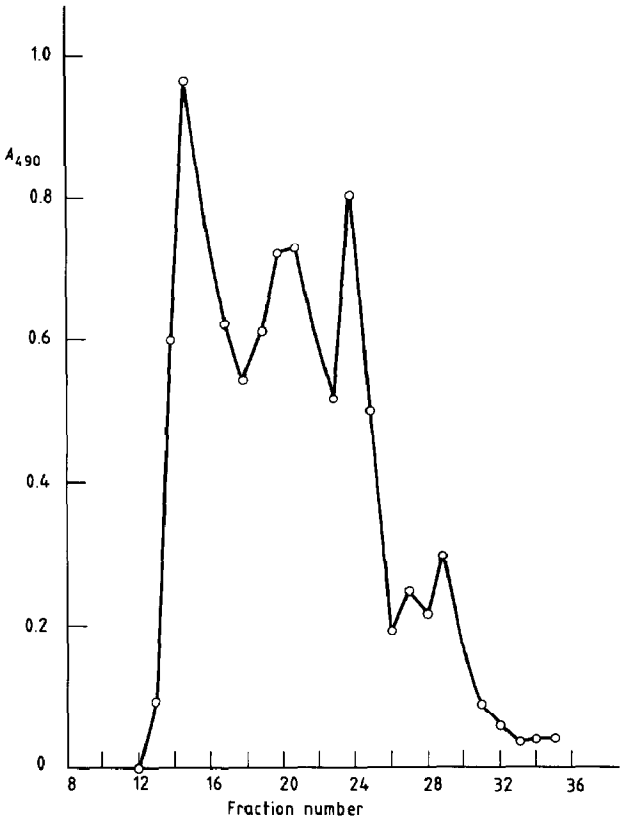


Fig. 1. Gel-permeation chromatography of reduced oligosaccharides from glycopolypeptides on a column (85 × 1.5 cm) of Bio-Gel P-4 by elution with 0.1M acetic acid (9.6 mL/h).

complex mixture was obtained by *N*-deacetylation by hydrazinolysis, followed by degradation with nitrous acid.

However, the total recovery was only 74% after alkaline-borohydride treatment and there was further loss of carbohydrate, as non-degraded glycopolypeptide, during the chromatography on Bio-Gel P-4.

Hydrazinolysis has two potential advantages, namely (*a*) polypeptides are degraded to amino acid hydrazides, which can be separated from the released oligosaccharides by chromatography on Bio-Gel P-4; and (*b*) concomitant *N*-deacetylation of *N*-acetylhexosamine and *N*-acetylneuraminic acid residues will give a product amenable to degradation by nitrosation. The product of hydrazinolysis of pig gastric mucus glycopolypeptide should be a mixture of oligosaccharide hydrazones, if the sugar–amino acid linkage is cleaved, and the terminal residue in each product should be 2-amino-2-deoxy-D-galactose hydrazone. Whereas 2-amino-2-deoxy-D-glucose hydrazone (and hence, by analogy, 2-amino-2-deoxy-D-galactose hydrazone) was not converted readily into the aldose[9], the *N*-acetylated derivative was hydrolysed readily to the *N*-acetylated aldose by a cation-exchange (H^+) resin[10]. Hydrazinolysis of 2-amino-2-

deoxy-D-galactose gave 1-deoxy-D-tagatose hydrazone as by-product in a reaction which paralleled that of 2-amino-2-deoxy-D-glucose, which gave 1-deoxy-D-fructose hydrazone[11].

Hydrazinolysis of the mixture of glycopolypeptides in the presence of hydrazinium sulphate (10 h, 105°) gave, after chromatography on Bio-Gel P-4, a mixture of

TABLE II

Monosaccharide composition of the product of hydrazinolysis–nitrosation–reduction

Monosaccharide	*Moles/mole of* D-*galactose*
L-Fucose	0.54
D-Galactose	1.0
2-Acetamido-2-deoxy-D-glucose	0.13
2-Acetamido-2-deoxy-D-galactose	–
2,5-Anhydro-D-mannitol	0.65
2,5-Anhydro-D-talitol	0.09

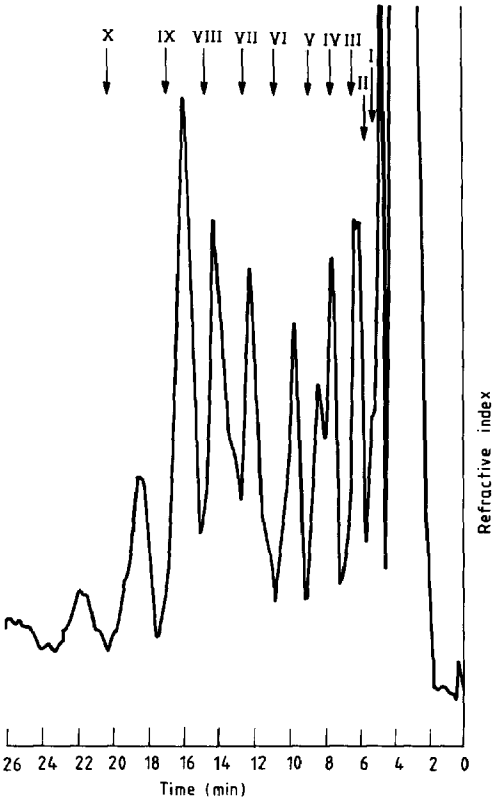


Fig. 2. H.p.l.c. of the reduced oligosaccharides, after hydrazinolysis and *N*-reacetylation, on a column[12] (25 cm × 4.6 mm) of Partisil PAC 10μ. Arrows indicate the positions of elution of malto-oligosaccharide standards (the Roman numerals correspond to the d.p.).

oligosaccharides. Nitrosation of this mixture, followed by reduction[12] with sodium cyanoborohydride, gave a product with the monosaccharide proportions shown in Table II. The very low proportion of 2,5-anhydro-D-talitol, which originated from the reducing 2-amino-2-deoxy-D-galactose end-group, implied that degradation of this end group had occurred during hydrazinolysis. The major mono-, di-, and tri-saccharides, isolated by chromatography, were shown to be 2,5-anhydro-D-mannitol, galactosyl–2,5-anhydro-D-mannitol, and fucosylgalactosyl–2,5-anhydro-D-mannitol, respectively.

The nitrosation product, after removal of cations and boric acid, gave an acidic solution (pH 2) due to hydrazinium sulphate, which was incompletely separated from the monosaccharides on Bio-Gel-P4, and to nitrate which resulted from some nitrite decompostion. An anion-exchange (AcO^-) resin was used to remove the acids. Carboxylic acids were also retained quantitatively and could be eluted by 2M acetic acid. Removal of sodium ions using a cation-exchange (H^+) resin was performed at 5–7° in order to avoid cleavage of sensitive glycosides.

That hydrazinolysis caused substantial degradation was shown by chromatography (Fig. 2) of the oligosaccharides released by alkali-mediated cleavage, after subsequent exposure to the hydrazinolysis conditions followed by *N*-acetylation. The

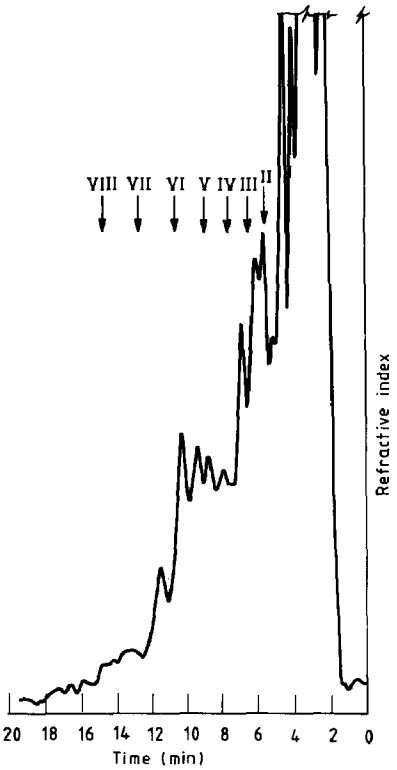
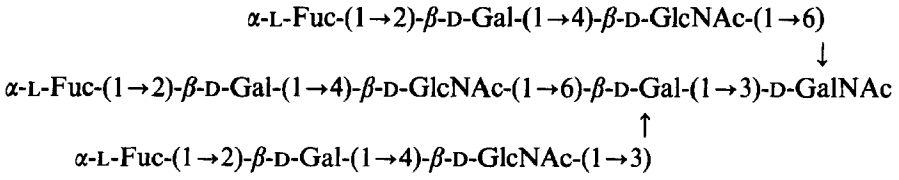


Fig. 3. H.p.l.c. of oligosaccharides released by hydrazinolysis followed by *N*-reacetylation; conditions as in Fig. 2.

result shown in Fig. 2 was similar to that obtained before hydrazinolysis. Fig. 3, on the other hand, shows that a complex mixture of smaller oligosaccharides was formed by hydrazinolysis of the glycopolypeptides.

These results imply that the O-linkages in mucus glycopolypeptides are cleaved during hydrazinolysis, presumably by a β-elimination mechanism, and that the resulting oligosaccharides are degraded from the reducing hydrazone end-group. A typical pig gastric mucus oligosaccharide, such as **1**[13], can be degraded by two successive peeling (β-elimination) reactions, which cleave the (1→3) linkages, to form three smaller oligosaccharides.



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Further evidence for the type of degradation was provided by subjecting cellobiose and laminaribiose to the conditions of hydrazinolysis. The products were analysed by ^{13}C-n.m.r. spectroscopy of the hydrazine solutions because aldose hydrazones exist mainly in acyclic forms whereas, in aqueous solution, tautomerisation occurs which complicates the spectra. The ^{1}H-n.m.r. spectra were useful for the detection of certain by-products. Hydrazinolysis of cellobiose gave mainly the hydrazone together with small proportions of other compounds, some of which were identified provisionally as 1-deoxy-4-*O*-β-D-glucopyranosyl-D-fructose hydrazone (δ 154.9 for $C{=}NNH_2$, 13.9 for $CH_3C{=}NNH_2$, 1.78 for $CH_3C{=}NNH_2$) and the corresponding 1-deoxy- and 1,2-dideoxy-alditols. Such products are formed by the hydrazinolysis of aldoses[14]. In contrast, the product of hydrazinolysis of the (1→3)-linked disaccharide laminaribiose gave a complex ^{13}C.n.m.r. spectrum; the region 147–155 p.p.m. ($C{=}NNH_2$) contained five major and two minor signals together with signals corresponding to D-glucose hydrazone and laminaribiose hydrazone. The signal at δ 13.9 (for $CH_3C{=}NNH_2$) was relatively more intense than in the spectrum of the products from cellobiose. The ^{1}H-n.m.r. spectrum contained several intense signals in the region $\delta \sim 1.8$ and weak signals corresponding to 1-deoxyalditol and 1,2-dideoxyalditol derivatives.

In an attempt to improve the recovery of carbohydrate in the alkali-mediated cleavage, the glycopolypeptides (fractions 12–18, Fig. 1) were treated with alkaline borohydride a second time. Reduced oligosaccharides were released and the material of higher molecular mass (20%) contained small proportions of arabinose and glucuronic acid. These monosaccharides, which were present at too low a level in the initial mixture of glycopolypeptides to be detected, may have originated from a polysaccharide analogous to that found[15] in human gastric mucus.

Extension of the treatment with alkaline borohydride from 15 to 24 h improved

the recovery from 70% to 82%. When an *N*-reacetylation step was incorporated in order to avoid any loss of *N*-deacetylated products on the cation-exchange resin, the recovery after reaction for 30 h was 95.6%. Chromatography on Bio-Gel P-4 then gave a 76% recovery of the reduced oligosaccharides and a profile similar to that in Fig. 2. *N*-Deacetylation of these oligosaccharides by hydrazinolysis, followed by degradation by nitrosation, gave neutral and acidic products in the ratio 4:1, and their monosaccharide proportions are shown in Table III. The acidic products originated from oligosaccharides that contained neuraminic acid[16]. Chromatography of the neutral product gave mainly 2,5-anhydro-D-mannitol and 2,5-anhydro-D-talitol (which co-

TABLE III

Monosaccharide composition of the product of alkali-mediated cleavage–nitrosation–reduction

Monosaccharide	*Moles/mole of* D-*galactose*	
	Neutral	*Acidic*[a]
L-Fucose	0.42	0.45
D-Galactose	1.0	1.0
2-Acetamido-2-deoxy-D-glucose	0.06	0.09
2-Acetamido-2-deoxy-D-galactose	–	–
2,5-Anhydro-D-mannitol	0.58	0.38
2,5-Anhydro-D-talitol	0.10	0.08
2-Deoxy-D-*lyxo*-hexitol	0.07	0.05

[a] A small peak in the NANA region was thought to be due to the nitrosation product from NANA[16].

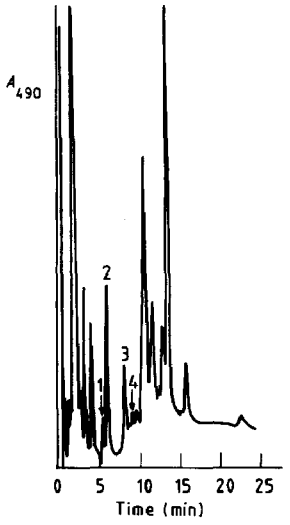

Fig. 4. H.p.l.c. of the benzoylated products of nitrosation of the reduced oligosaccharides on a column[20] (12.5 × 0.4 cm) of Spherisorb C8 5μ by elution with aqueous 70% acetonitrile for 5 min, then a concave gradient to 95% acetonitrile during 15 min: peaks: 1, 2,5-anhydro-D-talitol; 2, 2,5-anhydro-D-mannitol; 3, 2-deoxy-D-*lyxo*-hexitol; 4, galactitol.

eluted), disaccharide, and trisaccharide plus smaller proportions of other products. G.l.c. of the trimethylsilylated products on 2% Dexil and h.p.l.c. of the benzoylated products (Fig. 4) were also carried out. The latter method identified 2-deoxy-D-*lyxo*-hexitol, which resulted from a hydride shift in the 2-amino-2-deoxy-D-galactitol[17], and the 2,5-anhydrides of D-mannitol and D-talitol. 2,5-Anhydro-D-talitol would arise from non-reducing 2-acetamido-2-deoxy-D-galactose end-groups and implied the presence of some blood-group A activity in the pig gastric mucus oligosaccharides.

Earlier model experiments[17] suggested that, in the nitrosation step, 2-amino-2-deoxy-D-galactitol would be cleaved from a monosaccharide (D-galactose in pig gastric mucus glycoproteins) attached at O-3, by rearrangement and fragmentation pathways. The exposed D-galactose end-groups would then be converted into the alditol in the subsequent reduction step. Thus, such oligosaccharides as **1** should yield free galactitol. Fig. 4 implied a low ratio of galactitol to 2-deoxy-D-*lyxo*-hexitol. The behaviour of a substituted 2-amino-2-deoxy-D-galactitol derivative upon nitrosation may not parallel exactly that of the alditol because such reactions are sensitive to the conformation of the amine. The fact that the nitrosation reactions do not give exclusively one product means that minor products are formed which contribute to the chromatography profiles.

The reduced oligosaccharides, released from human gastric mucus glycopolypeptides with H-secretor and non-secretor blood-group activity, could be differentiated qualitatively[18] by chromatography and by analysis of the products formed after *N*-deacetylation (by hydrazinolysis) and nitrosation. Since minor by-products can arise from the 2-amino-2-deoxy-D-galactitol end-groups upon nitrosation, further work is required in order to characterise fully the mixtures obtained.

The apparently successful use of hydrazinolysis to release oligosaccharides that are *N*-glycosylically linked to asparagine[4] is fortuitously dependent on the fact that the *N*-linked monosaccharide moiety is not 3-substituted. An *N*-linked oligosaccharide in which the reducing terminal residue is substituted at O-3 would undergo degradation during hydrazinolysis. Such degradations destroy the core oligosaccharide, but sequential hydrazinolysis–nitrosation can provide information on the structure of the non-reducing terminus (or termini) of glycoprotein oligosaccharides. Hydrazine-induced degradations can be avoided by using the reduced oligosaccharides released by alkali-mediated cleavage; these are stable towards hydrazinolysis (apart from the desired *N*-deacetylation).

EXPERIMENTAL

General. — Monosaccharide composition was determined by acid-catalysed methanolysis followed by g.l.c. of the trimethylsilylated products[19]. Sequential hydrazinolysis, nitrosation, reduction, and chromatography was carried out as reported[12], except that Bio-Gel P-4 was used instead of Sephadex G-15 for gel-permeation chromatography. H.p.l.c. of benzoylated derivatives was carried out on a C_8 reverse-phase column[20] and amino acids were analysed as the dansyl derivatives[21]. N.m.r. spectra were recorded with a Bruker WM250 spectrometer with sodium 2,2,3,3-tetradeuterio-3-

trimethylsilylpropionate (TSP) as the reference for solutions in D_2O. ^{13}C-N.m.r. measurements were made with proton-noise decoupling and the number of attached protons was deduced from DEPT or proton off-resonance decoupled spectra.

Isolation of reduced oligosaccharides: modified procedures. — A solution of dry pig gastric mucus glycopolypeptides (105.1 mg) and sodium borohydride (1.62 g) in 0.05M sodium hydroxide (40 mL) was kept for 30 h at 45°. Glacial acetic acid was added dropwise to the stirred cooled solution to pH 6–7, followed by methanol (20 mL) and then acetic anhydride (5 × 666 μL) during 1 h at room temperature. After 75 min, the solution was extracted with ether (3 × 15 mL), and the combined extracts were washed with water (15 mL). The combined aqueous layers were applied to a column of Amberlite IR-120 (H^+) resin (100 mL, cooled to 5–7°), which was eluted with water (800 mL). Boric acid was removed from the lyophilised eluate as methyl borate to give a white residue (100.5 mg), which was eluted from a column (85 × 1.5 cm) of Bio-Gel P-4 with 0.1M acetic acid at 9.1 mL/h. Fractions (4.55 mL) were analysed for carbohydrate and combined as follows: 10–12 (13% recovery), 13 (6%), and 14–32 (76%). Monosaccharide analysis, after lyophilisation, showed fractions 14–32 to contain the reduced oligosaccharides. The other fractions contained no GalNAcol and hence contained glycopolypeptides and polypeptides.

Hydrazinolysis. — *(a) Of 2-amino-2-deoxy-*D*-galactose.* A degassed solution of dry 2-amino-2-deoxy-D-galactose hydrochloride in anhydrous hydrazine was heated for 30 h at 100°. Hydrazine was removed from the cooled solution *in vacuo* over conc. H_2SO_4 and the residue was analysed by ^{13}C- and ^1H-n.m.r. spectroscopy; ^{13}C, δ 157.8 ($C{=}NNH_2$, 1-deoxy-D-tagatose hydrazone), 150.4 ($CH{=}NNH_2$, GalN hydrazone), 145.8, 143.8, 140.1 (C=N, unidentified), 94.3 (C-1, 2-amino-2-deoxy-β-D-galactopyranosylhydrazine), 12.5 ($CH_3C{=}NNH_2$); ^1H, 7.72, 7.55 (2 s, unidentified), 7.35 (d, J 4 Hz, $CH{=}NNH_2$), 1.85 (s, $CH_3C{=}NNH_2$).

(b) Of cellobiose. A degassed solution of dry cellobiose (49 mg) and hydrazinium sulphate (Analar, 19 mg) in anhydrous hydrazine (0.4 mL) was heated for 10 h at 105°. The product was shown by ^{13}C-n.m.r. spectroscopy to be mainly cellobiose hydrazone: δ 148.4 (C-1, *syn* isomer), 147.7 (C-1, *anti* isomer). Weak absorptions were also detected at δ 154.9 (quat. C), 31.5 (CH_2), and 13.9 (CH_3). The ^1H-n.m.r. spectrum, recorded after removal of hydrazine *in vacuo* at room temperature over conc. H_2SO_4 and dissolution of the residue in D_2O, contained very weak signals at δ 1.23 (d, J 6.5 Hz, CH_3CHOH) and 0.95 (t, J 7.4 Hz, CH_3CH_2).

(c) Of laminaribiose. Dry laminaribiose (45 mg), obtained[22] from laminarin, was hydrazinolysed as in (*b*) and the resulting solution was shown by ^{13}C-n.m.r. spectroscopy (90 MHz), to contain a complex mixture of compounds. The spectrum included signals for D-glucose hydrazone [δ 148.2, 75.6, 74.5 (×2), 73.3, 66.5], laminaribiose hydrazone [δ 146.9, 107.0, 83.3, 80.3, 79.5, 77.1, 74.8, 74.2, 73.0, 72.6, 66.1, and 63.5], and other signals at δ 155.0, 152.7, 151.9, 151.5, 150.0 (all $C{=}NNH_2$), and 13.9 ($CH_3C{=}NNH_2$). The ^1H-n.m.r. spectrum (D_2O) contained weak signals at δ 1.25 (d, J 6.5 Hz) and 0.96 (t, J 7.4 Hz).

Modified nitrosation–reduction procedure. — To a cooled (ice) solution of *N*-deacetylated oligosaccharides (x mg), prepared by hydrazinolysis, and sodium nitrite ($3.6x$ mg) in distilled water ($0.07x$ mL) was added glacial acetic acid ($0.014x$ mL) in three equal portions during 30 min, the flask being kept stoppered between additions. The solution was allowed to warm to room temperature (1 h), then purged with N_2 for 15 min, and sodium cyanoborohydride ($1.85x$ mg) was added to the ice-cooled solution, which was then kept for 1 h at room temperature. To the ice-cooled solution was added Amberlite IR-120 (H^+) resin ($0.2x$ mL, wet volume), and, when the effervescence had subsided (CAUTION; HCN evolved), the mixture was added to more resin ($0.3x$ mL) in a jacketed column cooled to 5–7°. The column was eluted with cold water (10 bed volumes), the eluate was passed directly through a column of Amberlite IRA-400 (AcO^-) resin (2 mL) and lyophilised, and boric acid was removed from the residue as methyl borate. Acidic oligosaccharides were eluted from the anion-exchange resin with 2M acetic acid (50 bed volumes) and the eluate was lyophilised.

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