Carbohydrate Research, 239 (1993) 107–115
Elsevier Science Publishers B.V., Amsterdam

Specificity of mild periodate oxidation of oligosaccharide-alditols: relevance to the analysis of the core-branching pattern of O-linked glycoprotein oligosaccharides

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(Received March 18th, 1992; accepted June 20th, 1992)

ABSTRACT

The specificity of mild periodate oxidation of 3- and 6-substituted 2-acetamido-2-deoxy-D-galactitols and 4- and 6-substituted D-glucitols has been investigated. The products were reacted with 1,2-di-palmitoyl-*sn*-glycero-3-phosphoethanolamine and the derivatives were analysed by application of liquid secondary-ion mass spectrometry directly to the TLC plates. There was > 95% specificity of cleavage of the C-4–C-5 bond (*threo*-diol) for the GalNAcol derivatives. The major sites of oxidation for the Glcol derivatives also involved *threo*-diols. For α-Neu5Ac-(2 → 6)-GalNAcol, ~ 30% of the products of oxidation involved the sialic acid side chain, and ~ 60% were cleaved at the C-4–C-5 bond of the GalNAcol moiety. The mild periodate oxidation reaction forms part of a strategy for determining the patterns of branching of the cores of O-linked glycoprotein oligosaccharides and other oligosaccharide-alditols.

INTRODUCTION

Cleavage of vicinal diols by periodate oxidation is a well-established reaction in carbohydrate chemistry[1]. Under controlled conditions of concentration, temperature, time, and pH, periodate selectively oxidises vicinal diols in the glucitol[2] (Glcol), 2-acetamido-2-deoxyglucitol[3], and 2-acetamido-2-deoxygalactitol[4] (GalNAcol) moieties of oligosaccharide-alditols and the glycerol side chain of sialic acid[5]. The selective oxidation of the C-4–C-5 bond of 3,6- and 3-substituted GalNAcol is part of a strategy for determining the patterns of branching and the sequences of O-linked oligosaccharides released by treatment of glycoproteins[6] with alkaline borohydride and for the preparation of neoglycolipid probes[4]. Although acyclic vicinal *threo*-diols are oxidised more rapidly than *erythro*-diols[7], the selectivity for monosubstituted alditols needs to be examined quantitatively. In

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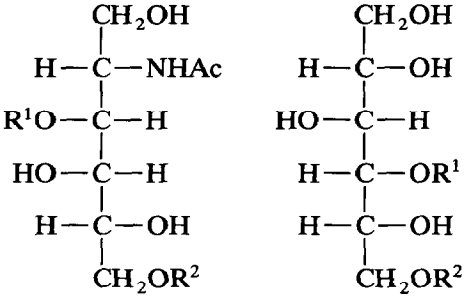
addition, the successful application of the strategy to the 6-substituted GalNAcol core of O-linked glycoprotein oligosaccharides and to substituted hexitols produced from glycoprotein oligosaccharides and other sources requires optimised oxidation conditions which, as far as possible, limit cleavage to a single diol bond.

Using a variety of oligosaccharide-alditols, the mild periodate oxidation reaction has been assessed by characterising the aldehyde-containing products as their 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) derivatives[6,8] by the application of liquid secondary-ion mass spectrometry (LSIMS) directly to the surface of high performance (HP) TLC plates[9].

RESULTS AND DISCUSSION

The products of mild periodate treatment of the 2-acetamido-2-deoxy-D-galactitol (**1**) and D-glucitol (**2**) derivatives were converted directly without purification into DPPE derivatives and subjected to HPTLC. Since the derivatisation proceeded in high yield (> 95%), HPTLC should reflect the quantitative distribution of the products. The component(s) in each TLC band (bands a–t, Fig. 1) was identified from its LSI-mass spectrum, the site(s) of cleavage was deduced, and the distribution of products was determined (Table I).



1 R^1 or $R^2 = H$ **2** R^1 or $R^2 = H$

Monosubstituted GalNAcol derivatives (**1**).—Of the two possible sites for mild periodate oxidation in β-Gal-(1 → 6)-GalNAcol, only the *threo*-4,5-diol was cleaved to any extent (> 95%) to give a product (band b, Fig. 1, lane 2) with $[M - H]^-$ at *m/z* 896 and a major fragment ion with *m/z* 734 arising from cleavage of the glycosidic bond, together with satellite ions at *m/z* 716 and 762 (ref. 8) (Fig. 2a). The non-substituted oxidation fragment consisting of C-1/4 (band c, Fig. 1) was identified by $[M - H]^-$ at *m/z* 835. Cleavage of the 3,4-diol would have given products with $[M - H]^-$ at *m/z* 929 and 805, and their absence is consistent with the reduced rate of reaction of acyclic vicinal *erythro*-diols[7].

The product of oxidation of β-Gal-(1 → 3)-GalNAcol has been reported[6]. In the present study, the product of cleavage of the C-4–C-5 bond was determined quantitatively (band a, Fig 1, lane 1, $[M - H]^-$ at *m/z* 997) as > 95% of the total

TABLE I

LSI-mass spectral data of the DPPE derivatives of fragments from the periodate oxidation of alditols, the deduced cleavage sites, and the distribution of the products

Alditol	TLC band (Fig. 1)	$[M-H]^-$ (m/z)	Major fragment ions (m/z)	Composition [a]	Deduced bond cleavage	Distribution of cleavage sites (%)
β-Gal-(1 → 3)-GalNAcol	a	997	835	Gal.(C-1/4).P	C-4–C-5	> 95
β-Gal-(1 → 6)-GalNAcol	b	896	734	Gal.C-6,5.P	C-4–C-5	> 95
	c	835		(C-1/4).P	C-4–C-5	–[b]
α-Glc-(1 → 4)-α-Glc-(1 → 4)-Glcol	d	1148	986, 824	Glc.Glc.(C-1/5).P	C-5–C-6	8
	e	1118	956, 794	Glc.Glc.(C-6/3).P	C-2–C-3	92
α-Glc-(1 → 6)-α-Glc-(1 → 6)-Glcol	f	1148		Glc.Glc.(C-6/2).P	C-1–C-2	1
	g	1118	956, 794	Glc.Glc.(C-6/3).P	C-2–C-3	29
	h	1088	926, 764	Glc.Glc.(C-6/4).P	C-3–C-4	68
	i	1058	896, 734	Glc.Glc.C-6,5.P	C-4–C-5	2
	j	764		(C-1/3).P	C-3–C-4	–
α-Neu5Ac-(2 → 6)-GalNAcol (3)	k	1156		P.(C-8′/1′).GalNAcol	C-8′–C-9′	22
		1126		P.(C-7′/1′).GalNAcol	C-7′–C-8′	8
	l	1025	734	Neu5Ac.C-6,5.P	C-4–C-5	60
	m	835		(C-1/4).P	C-4–C-5	–
α-GalNAc-(1 → 3)-GalNAcol (4)	o	1038	835	GalNAc.(C-1/4).P	C-4–C-5	> 95
α-GalNAc-(1 → 6)-GalNAcol (5)	p	937	734	GlcNAc.C-6,5.P	C-4–C-5	
	q	937	734	GalNAc.C-6,5.P	C-4–C-5	> 95
	r	835		(C-1/4).P	C-4–C-5	–

[a] Fragment ions are indicated in brackets as carbon numbers, e.g., (C-1/4) for alditols or as (C-7′, 8′) for sialic acid. P represents the 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine moiety. [b] Dashes denote accompanying unsubstituted cleavage products.

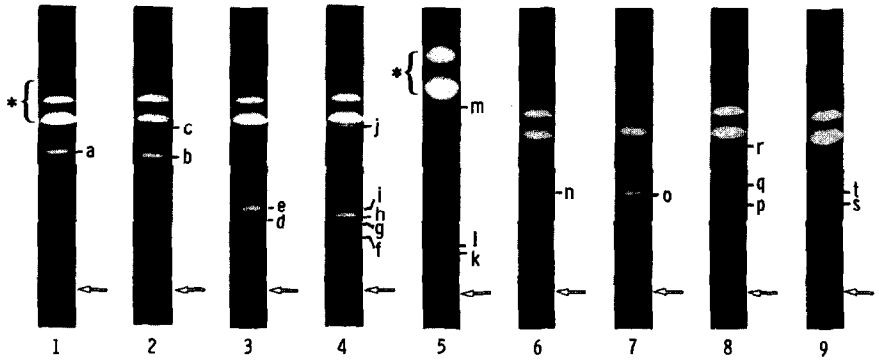


Fig. 1. TLC of the DPPE derivatives of the products of oxidation of 1, β-Gal-(1 → 3)-GalNAcol; 2, β-Gal-(1 → 6)-GalNAcol; 3, maltotri-itol; 4, isomaltotri-itol; 5, α-Neu5Ac-(2 → 6)-GalNAcol (**3**); 6, β-GlcNAc-(1 → 3)-GalNAcol (**7**); 7, α-GalNAc-(1 → 3)-GalNAcol (**4**); 8, α-GalNAc-(1 → 6)-GalNAcol (**5**); and 9, β-Gal-(1 → 3)[β-GlcNAc-(1 → 6)]-GalNAcol (**6**): the arrow indicates the origin, a–t indicate the positions of the bands identified, and * denotes the DPPE reagent and side products. Lane 5 was developed under more polar conditions than the other lanes.

products. The DPPE derivative of the terminal C_2 fragment (i.e., 2-hydroxyacetaldehyde) was not detected and may have been obscured by the reagent region. There was negligible cleavage of the C-5–C-6 bond under the conditions

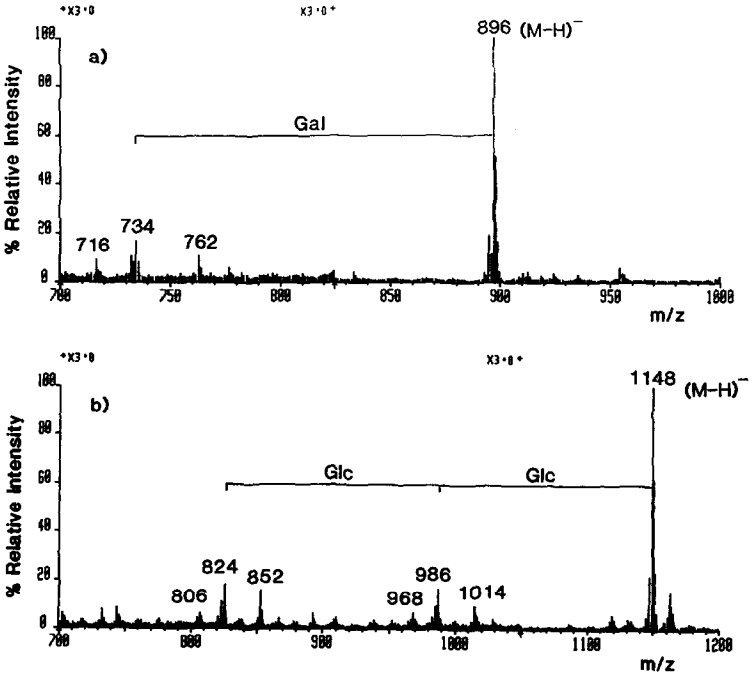


Fig. 2. Negative-ion LSI-mass spectra of the DPPE derivatives of the products of mild periodate oxidation of (a) β-Gal-(1 → 6)-GalNAcol (Fig. 1, band b), and (b) maltotri-itol (Fig. 1, band e).

used, in agreement with the reduced susceptibility of terminal vicinal diols in hexitols[10].

Monosubstituted Glcol derivative (**2**).—Of the three potential sites for mild periodate oxidation in the 4-substituted Glcol, maltotri-itol, only the *threo*-2,3-diol was cleaved efficiently (~ 92% of the products) to give a DPPE derivative (band e, Fig. 1, lane 3) with $[M - H]^-$ at *m/z* 1118. Band d (Fig. 1, lane 3) with $[M - H]^-$ at *m/z* 1148 and major fragment ions *m/z* 986 and 824 (Fig. 2b) indicated ~ 8% cleavage of the terminal vicinal diols. In order to differentiate the extent to which C-1–C-2 and C-5–C-6 bonds were affected, maltotriose was reduced with $NaBD_4$ and the product was treated with periodate. LSIMS of the DPPE derivative showed the single band to have $[M - H]^-$ shifted from *m/z* 1148 completely to *m/z* 1149, together with shifts of the ions with *m/z* 986 and 824 to 987 and 825, respectively, indicating that cleavage was limited to the C-5–C-6 bond.

The 6-substituted Glcol, isomaltotri-itol, has four potential bonds for oxidation under mild periodate conditions. Cleavage of the C-3–C-4 and C-2–C-3 bonds led to DPPE derivatives (bands h and g, respectively, Fig. 1, lane 4) with $[M - H]^-$ at *m/z* 1088 and 1118, respectively. These cleavages, involving vicinal *threo*-diols, accounted for 97% of the products and most of the remainder arose from cleavage of the C-4–C-5 bond (band i, Fig. 1, lane 4) with cleavage of the C-1–C-2 bond being almost undetectable (band f, Fig. 1, lane 4). The presence of band j ($[M - H]^-$ at *m/z* 764), due to the non-substituted fragment from fission of the C-3–C-4 bond, supported the selective cleavage of this bond. The ring structures were preserved as indicated by the sequence-determining fragment ions in the LSI-mass spectra of derivatives (e.g., Figs. 2a and b). This finding substantiated the earlier reports based on indirect evidence that ring vicinal diols were not oxidised[2,3].

These results are in accord with the reported rates[11] of cleavage of the bonds in D-glucitol, namely, 3,4 (*threo*) > 2,3 (*threo*) > 4,5 (*erythro*) > 5,6 > 1,2, with limited amounts of periodate (molar ratio of 1 : 4 for periodate–carbohydrate).

α-Neu5Ac-(2 → 6)-GalNAcol (**3**).—In **3**, the C-3–C-4 and C-4–C-5 bonds of the GalNAcol moiety and the C-7′–C-8′ and C-8′–C-9′ of the α-Neu5Ac moiety are candidate sites for oxidation[5]. Under the conditions used, the products formed resulted from cleavage of the C-4–C-5 bond (band l, Fig. 1, lane 5; $[M - H]^-$ at *m/z* 1025 and fragment ion at *m/z* 734) and the C-7′–C-8′ and C-8′–C-9′ bonds (band k, Fig. 1, lane 5; $[M - H]^-$ at *m/z* 1126 and 1156, respectively). The major product was from cleavage of the C-4–C-5 bond, which represented ~ 60% of the total, and was accompanied by the non-substituted fragment (band m, $[M - H]^-$ at *m/z* 835). The bands of the products from cleavage of the side chain of sialic acid were unresolved in TLC (band k) and amounted to ~ 30% of the total products, with the LSI-mass spectrum indicating a ratio of ~ 1 : 3 for cleavage of the C-7′–C-8′ and C-8′–C-9′ bonds, and establishing the slow rate of cleavage of the 7′,8′-*erythro*-diol than the terminal 8′,9′-diol. The remaining 10% was reflected by several faint bands which could be detected only by heavy loading of the TLC plate.

Analysis of the pattern of branching of the monosubstituted GalNAcol core.—The mild periodate procedure has been applied extensively to determine the pattern of branching of the 3,6-disubstituted GalNAcol core[6]. In the present study, the application to the monosubstituted GalNAcol core was demonstrated further for two recently established but less common monosubstituted core-type sugars, namely, α-GalNAc-(1 → 3)-GalNAcol[12] (**4**) and α-GalNAc-(1 → 6)-GalNAcol[13] (**5**).

As expected, the product from **4** showed a single band in TLC (band o, Fig. 1, lane 7) derived from cleavage of the C-4–C-5 bond with $[M - H]^-$ at m/z 1038 and a fragment ion at m/z 835 indicating HexNAc 3-linked to GalNAcol. A major TLC band (band q, Fig. 1, lane 8), with a greater mobility than that of band o from **4**, was produced from α-GalNAc-(1 → 6)-GalNAcol (**5**). The $[M - H]^-$ ion at m/z 937 and the fragment ion at m/z 734 clearly indicated HexNAc 6-linked to GalNAcol. Again, a single product with cleavage of the C-4–C-5 bond was obtained for **5**. The presence of a weak band (band p, Fig. 1, lane 8) reflected the known contaminant β-GlcNAc-(1 → 6)-GalNAcol in the original preparation[13]. The presence of the non-reducing terminal GalNAc for both **4** and **5** was confirmed by methylation analysis of the material in bands o and q (data not shown).

As indicated above, the contaminant [β-GlcNAc-(1 → 6)-GalNAcol] of **5** gave a derivatised oxidation product (band p, Fig. 1, lane 8; $[M - H]^-$ at m/z 937 and fragment ion at m/z 734) with an LSI-mass spectrum identical to that of the derivative in band q from **5**, but with a major difference in mobility in TLC. In order to confirm the identity of the derivative in band p, the well-characterised trisaccharide[13] β-Gal-(1 → 3)-[β-GlcNAc-(1 → 6)-]-GalNAcol (**6**) was submitted to the oxidation and derivatisation procedure. The resulting two bands corresponded to the β-Gal-(1 → 3) and β-GlcNAc-(1 → 6) substituent bands (bands s and t, respectively, Fig. 1, lane 9) from cleavage of the C-4–C-5 bond. Band s showed a TLC mobility and mass spectrum identical to those of band p from **5**.

Similarly, β-GlcNAc-(1 → 3)-GalNAcol (**7**) gave a single-product, β-GlcNAc-(1 → 3)-derived band (band n, Fig. 1, lane 6) with an LSI-mass spectrum identical to that from the α-GalNAc-(1 → 3) substituent of band o and it had a slightly higher mobility in TLC. Hence, the four isomeric HexNAc-substituted GalNAcol cores can be differentiated readily on the basis of their mass spectra and/or mobility in TLC, the order of the latter being α-GalNAc-(1 → 6) > β-GlcNAc-(1 → 3)- > α-GalNAc-(1 → 3)- > β-GlcNAc-(1 → 6).

Thus, by using a molar excess of periodate, only acyclic vicinal diols are oxidised and with selectivity. This situation contrasts with the multiple cleavage of vicinal diols[14] under normal conditions of periodate oxidation. The selectivity found for the vicinal diols confirmed the rates *threo* > *erythro* > terminal described for hexitols[7,10,11].

The two products of cleavage of the C-4–C-5 bond (*threo*) of β-Gal-(1 → 6)-GalNAcol by mild periodate oxidation were both detected, whereas the C-3–C-4 bond (*erythro*) remained intact (absence of bands with $[M - H]^-$ at m/z 929 and

805). This finding demonstrated the almost quantitative specificity of the reaction for the C-4–C-5 bond in 6-substituted GalNAcol, not documented hitherto. The detection of small unsubstituted fragments may be obscured by the bands for the reagent and, in practice, only unsubstituted C_3 or larger fragments were evident (e.g., bands c, j, m, and r, Fig. 1).

The mild periodate treatment of alditols has several potential applications. As demonstrated[6], it can be used to oxidise O-linked glycoprotein oligosaccharides released by treatment with alkaline borohydride to give reducing structures suitable for conjugation to lipids and subsequent use as probes, and for identification of oligosaccharide sequences and GalNAcol core-branching patterns by LSIMS. The present investigation has shown that a single bond in 6-substituted GalNAcol is cleaved, as observed[6] for 3- and 3,6-substituted GalNAcol, thus completing the range of core structures released from O-linked oligosaccharide chains. In addition, sialic acid-containing oligosaccharides may be only partially side-chain degraded under the conditions used and sufficient selective oxidation of the GalNAcol occurs to facilitate the use of the reaction in synthesis of neoglycolipid probes for carbohydrate-recognition studies[15]. Although oligosaccharide monosubstituted glucitols have multiple candidate oxidation sites, under mild conditions the degree of specificity exhibited will be valuable for studies of oligosaccharide-alditols derived from biological sources.

EXPERIMENTAL

β-Gal-(1 → 3)-GalNAc and β-Gal-(1 → 6)-GalNAc were purchased from Bio-Carb Chemicals, and maltotriose and isomaltotriose from Sigma. α-GalNAc-(1 → 6)-GalNAcol and α-Neu5Ac-(2 → 6)-GalNAcol were prepared[13] from bovine submaxillary mucin by treatment with alkaline borohydride. α-GalNAc-(1 → 3)-GalNAcol and β-Gal-(1 → 3)-[β-GlcNAc-(1 → 6)-]GalNAcol were kindly provided by Drs. T. Feizi and E.F. Hounsell (M.R.C. Clinical Research Centre) and were prepared from human meconium glycopeptides[12].

TLC was performed on Silica Gel 60 aluminium-backed HPTLC plates (5 μm, Merck). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) was purchased from Sigma. All other chemicals and solvents were of analytical grade.

Reduction of sugars.—A solution of each sugar (1 mg) in 0.05 M NaOH–0.2 M $NaBH_4$ (1 mL) was kept at room temperature overnight, then acidified to pH 5 with 1:1 HOAc–H_2O, and washed through a column (1 mL bed volume) of AG50W-X8 (H^+) resin with H_2O; 5 mL of the eluate was collected and freeze-dried. Boric acid was removed from the residue by repeated evaporation of MeOH therefrom and the reduced product was identified by TLC and LSIMS.

Mild periodate oxidation and conjugation to DPPE.—The procedure was essentially as described[6]. The molar ratio of alditol to periodate was kept at 1:2. Thus, for example, maltotri-itol (50 μg, ~ 100 nmol) was oxidised with freshly made $NaIO_4$ solution (35 μL, 1.25 mg/mL in 40 mM imidazole buffer, pH 6.5) at 0°C in

the dark for 5 min. Aqueous butane-2,3-diol (3 μL, 14.5 mg/mL) was added followed, after 40 min, by DPPE solution (700 μL, 1 mg/mL in 1:1 $CHCl_3$–EtOH), and the mixture was kept at 50°C for 2 h. Ethanolic sodium cyanoborohydride (32 μL, 10 mg/mL) was added and the reaction was continued for 16 h. The overall yield of periodate oxidation and conjugation was 65%. The mixture was desalted when necessary, as described[6].

TLC and TLC–LSIMS.—Each of the above reaction mixtures (typically 1–2 nmol of the carbohydrate-DPPE derivatives) was applied as a 5-nm band to an aluminium-backed HPTLC plate and developed in 130:50:9 $CHCl_3$–MeOH–H_2O. The bands were located under UV light (366 nm) after spraying with 0.001% of primulin in 4:1 acetone–H_2O, and each was excised together with the aluminium backing to give a strip typically 1.5 × 5.5 mm.

Each strip was attached to the LSIMS probe tip by an electro-conducting adhesive[8,9]. Extraction solvent (25:25:8 $CHCl_3$–MeOH–H_2O) and matrix (2:2:1 diethanolamine–tetramethylurea–*m*-nitrobenzyl alcohol) were added to the surface of the silica gel prior to LSIMS.

LSIMS was carried out on a VG ZAB-2E instrument fitted with a caesium ion gun operated at 25 keV and an emission current of 0.5 μA. Negative-ion mass spectra were acquired at 30 s/decade, using the VG Analytical 11-250J data system in the continuum acquisition mode.

Quantification.—The sample and standard solutions of compounds containing hexose at various concentrations were subjected to TLC, then detected with 0.2% orcinol in 83.3:5.6:11.1 EtOH–H_2O–H_2SO_4 at 105°C for 5 min, and scanned with a Shimadzu CS-9000 flying-spot scanner at 550 nm.

DPPE derivatives were detected after TLC by spraying with 0.001% primulin in 4:1 acetone–H_2O, drying, soaking in phosphate-buffered saline for 30 min, drying, and scanning at an excitation wavelength of 420 nm, with the emission being monitored through a filter with a cut off below 450 nm.

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