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Note

Carbohydrate structures of quail ovomucoid. Characterization of degraded oligosaccharides produced during alkaline hydrolysis of the asparaginyl–*N*-acetylglucosamine linkage*

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Alkaline treatment in the presence of sodium borohydride is a widely used procedure to isolate the carbohydrate components of glycoproteins. There are marked differences in the stability of the various types of carbohydrate–peptide linkages to alkaline conditions. Thus, incubation for 18 h in a mixture of 0.1M potassium hydroxide and M potassium borohydride at 45° cleaved the *O*-glycosyl linkage present in mucin-type glycoproteins quantitatively[1]. The *N*-glycosyl linkage between the 2-acetamido-2-deoxy-β-D-glucosyl and the asparaginyl residue is known to be very stable and the cleavage of this linkage occurs only with high concentrations of sodium hydroxide–sodium borohydride, as described by Lee and Scocca[2] who used one molar concentrations for 4–6 h at 100°. Under these conditions, the authors reported the complete cleavage of the linkage, with concomitant production of a terminal 2-amino-2-deoxyglucitol residue. However the conditions of Lee and Scocca[2] were shown by Austen and Marshall[3] to lead to the formation of 2-acetamido-2-deoxymannitol, thus indicating the modification of the 2-acetamido-2-deoxyglucose residue in reducing terminal position. We report herein the structure analysis of degraded oligosaccharides produced by alkaline hydrolysis, as determined by m.s. and 400-MHz ^{1}H-n.m.r.

The neutral oligosaccharide alditols, liberated from quail ovomucoid glycopeptides after alkaline hydrolysis, were separated into 14 fractions by alkylamine-

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silica column chromatography, and the structures of oligosaccharide-alditol Fractions 1, 3, 4, 6, 7, 11, and 13 were determined[4].

The low yield of both 2-amino-2-deoxyglucitol (identified by g.l.c. analysis) and 2-amino-2-deoxy-1,3,5,6-tetra-*O*-methylglucitol residues (identified by methylation analysis), and the observation in the f.a.b.m.s. spectra of pseudomolecular ions corresponding to oligosaccharides with a 129 amu lower than expected suggested the presence, in some fractions, of degradation products formed during alkaline hydrolysis.

The major oligosaccharide alditol obtained from quail ovomucoid is the mannotriosyl-di-*N*-acetylchitobiitol[4,5], which is one of the oligosaccharides modified in the reducing terminal position by the alkaline borohydride treatment. Thus, two peaks were obtained by alkylamine h.p.l.c.[4]; both showed the same carbohydrate composition except for 2-acetamido-2-deoxyglucose content (3:1:1 and 3:0.82:0.38 mannose:2-acetamido-2-deoxyglucose:2-acetamido-2-deoxyglucitol for oligosaccharide alditol Fractions 3 and 2, respectively)[4].

Analysis of permethylated oligosaccharide alditol Fractions 2 and 3 (see Table I and Fig. 1) clearly showed the loss of 2-acetamido-2-deoxy-1,3,5,6-tetra-*O*-methylglucitol relative to 2,4-di-*O*-methylmannose in Fraction 2 (Fig. 1, Peak 3), as well as the occurrence of a new peak (arrow in Fig. 1A), which was identified as tri-*O*-methyltetritol, in agreement with fragments at *m/z* 45, 89, and 161 in the e.i.m.s. spectrum (Fig. 2A; Scheme 1). These results were confirmed by further analysis of the c.i.m.s. (Fig. 2B), which showed the presence of pseudomolecular ions at *m/z* 207 $(MH)^+$ and 224 $(M + NH_4)^+$.

In the g.l.c. pattern of the oligosaccharide alditol of Fraction 3, we observed the presence of a small peak of 2-amino-2-deoxy-1,3,5,6-tetra-*O*-methylmannitol (Fig. 1), reported earlier by Austen and Marshall[3], after alkaline hydrolysis under the conditions of Lee and Scocca[2].

The heterogeneity of oligosaccharide alditols produced during alkaline hydrolysis was clearly demonstrated by the f.a.b.m.s. analysis of Fraction 2, which

TABLE I

MOLAR RATIOS OF MONOSACCHARIDE METHYL ETHERS PRESENT IN THE METHANOLYZATES OF PERMETHYLATED FRACTIONS 2 AND 3

Methyl ethers of monosaccharides	*Fraction (molar ratios)*[a]	
	2	*3*
1,3,4-Tri-*O*-Me-tetritol	0.30[b]	
2,3,4,6-Tetra-*O*-Me-Man	1.73	1.94
2,4-Di-*O*-Me-Man	1.0	1.0
3,6-Di-*O*-Me-GlcNAcNMe	0.85	1.00
1,3,5,6-Tetra-*O*-Me-GlcNAcNMe-ol	0.42	1.10

[a]Values are given relative to 2,4-di-*O*-methylmannose (1.0). [b]Because of the relatively high volatility of this methyl ether, the value is lower than expected.

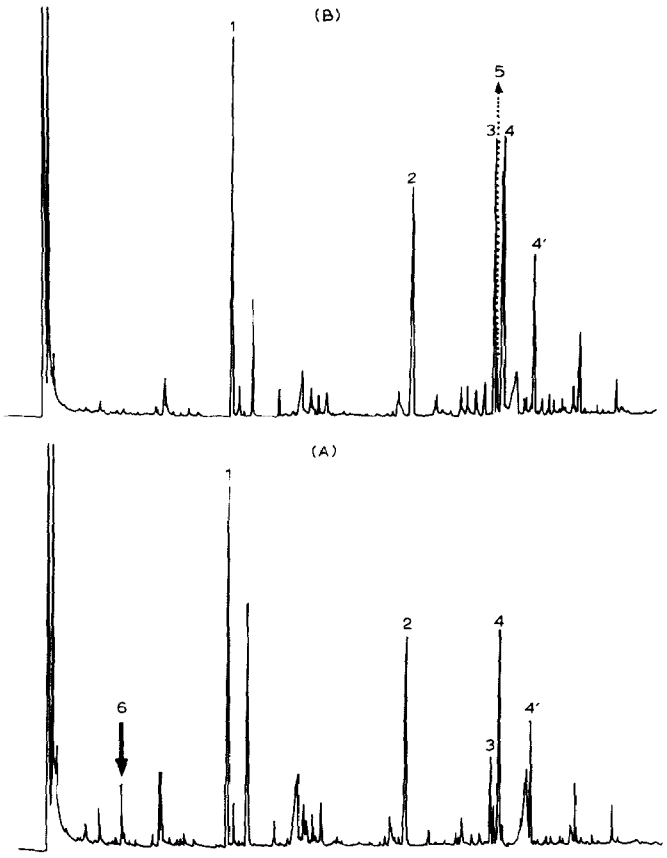

Fig. 1. Gas–liquid chromatogram of partially methylated and acetylated methyl glycosides obtained after methanolysis of permethylated oligosaccharide alditol Fractions 2 (A) and 3 (B); *O*-acetyl derivatives of (1) 2,3,4,6-tetra-*O*-methylmannose, (2) 2,4-di-*O*-methylmannose, (3) 2-deoxy-1,3,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)glucitol, (4) 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucose, (4′) 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucose, (5) 2-deoxy-1,3,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)mannose, and (6) 1,3,4-tri-*O*-methyltetritol.

showed two peaks at *m/z* 942 $(M + Na)^+$ and 1058 $(M + Na)^+$ corresponding to $Man_3GlcNAcol$ and $Man_3GlcNAc$-tetritol, respectively (Fig. 3A). The major components were accompanied by ions at *m/z* 994 and 878, which are produced by fission of two methanol molecules from the pseudomolecular ions at *m/z* 1058 and 942, respectively. For Fraction 3, the pseudomolecular ion at *m/z* 1188 $(M + Na)^+$ was accompanied by an ion 316 amu lower than that produced by fission of the chitobiose linkage (Fig. 3B), as shown by Egge *et al.*[6].

The structure of these oligosaccharide alditols was deduced from the e.i.m.s. spectra of permethylated oligosaccharide alditol Fractions 2 and 3 (Fig. 4). Fraction 2 (Fig. 4A) showed characteristic fragments at *m/z* 147 (tetritol), 207 ($Me–O–CH=O^+$ – tetritol), and 392 (GlcNAc-tetritol) for the oligosaccharide having a tetritol residue in reducing terminal position. The presence of the fragment at

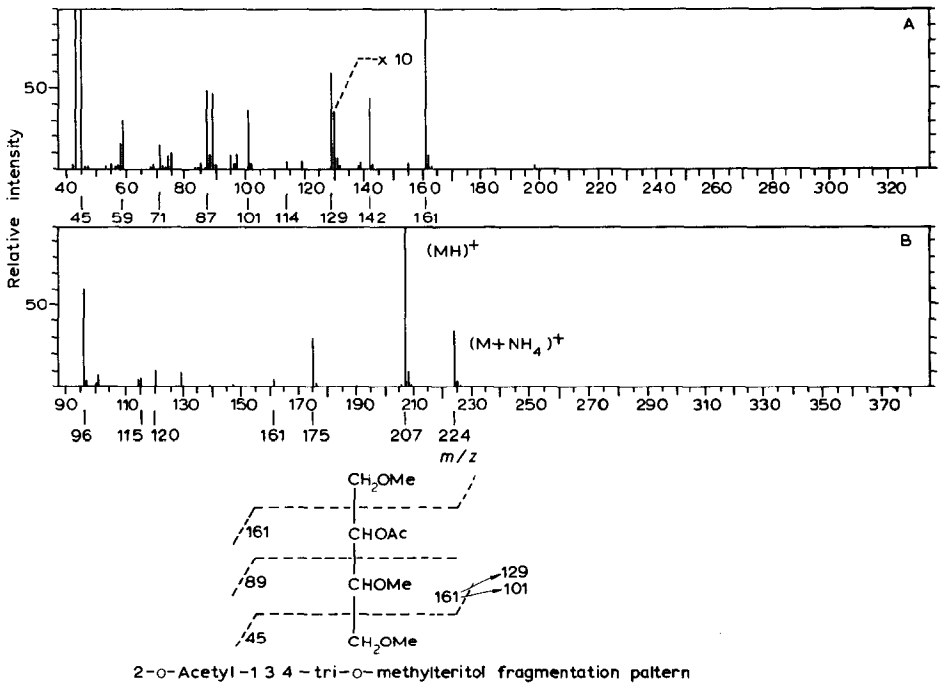

Fig. 2. Mass spectra of 2-*O*-acetyl-1,3,4-tri-*O*-methyl-tetritol obtained by (A) e.i.m.s. and (B) c.i.m.s.

m/z 276 (aminodeoxyhexitol) and the absence of a peak at *m/z* 521 (chitobiitol residue) indicated that, in this oligosaccharide, one of the two 2-acetamido-2-deoxyglucose residues of the chitobiose unit had been lost. In both cases, ions at *m/z* 219 and 187 demonstrated the presence of hexoses at the nonreducing terminal positions. These results were confirmed by f.a.b.m.s. analysis. The e.i.m.s. data of Fraction 3 were in agreement with the structure of a mannotriosyl-di-*N*-acetyl-chitobiitol unit: *m/z* at 276 (aminodeoxyhexitol), 521 (chitobiitol), and 219 and 187 (hexoses at the nonreducing terminal position). The absence of a peak at *m/z* 423 (dihexosyl fragments) revealed the branched structure of the mannotriose unit (Fig. 4B).

The heterogeneity of the products formed by alkaline hydrolysis of the GlcNAc-Asn linkage was also demonstrated by 400-MHz, ^{1}H-n.m.r. spectroscopy of Fractions 2 and 3 (ref. 4) (Table II). ^{1}H-N.m.r. analysis of Fraction 3 revealed the heterogeneity of the reducing 2-acetamido-2-deoxyhexose-(*1*) residue by the presence of two signals, one for *N*-acetyl protons of 2-acetamido-2-deoxyglucitol at δ 2.056 and the other at δ 2.036 for the *N*-acetyl proton of 2-acetamido-2-deoxymannitol, as shown by methylation analysis. ^{1}H-N.m.r. analysis of Fraction 2 showed clearly the loss of a 2-acetamido-2-deoxyglucitol-(*1*) residue from the chitobiose unit by the absence of *N*-acetyl proton signals at δ 2.056 and 2.036, by the absence of an H-2 signal at δ 4.242, and by the presence of an *N*-acetyl proton signal at δ 2.059

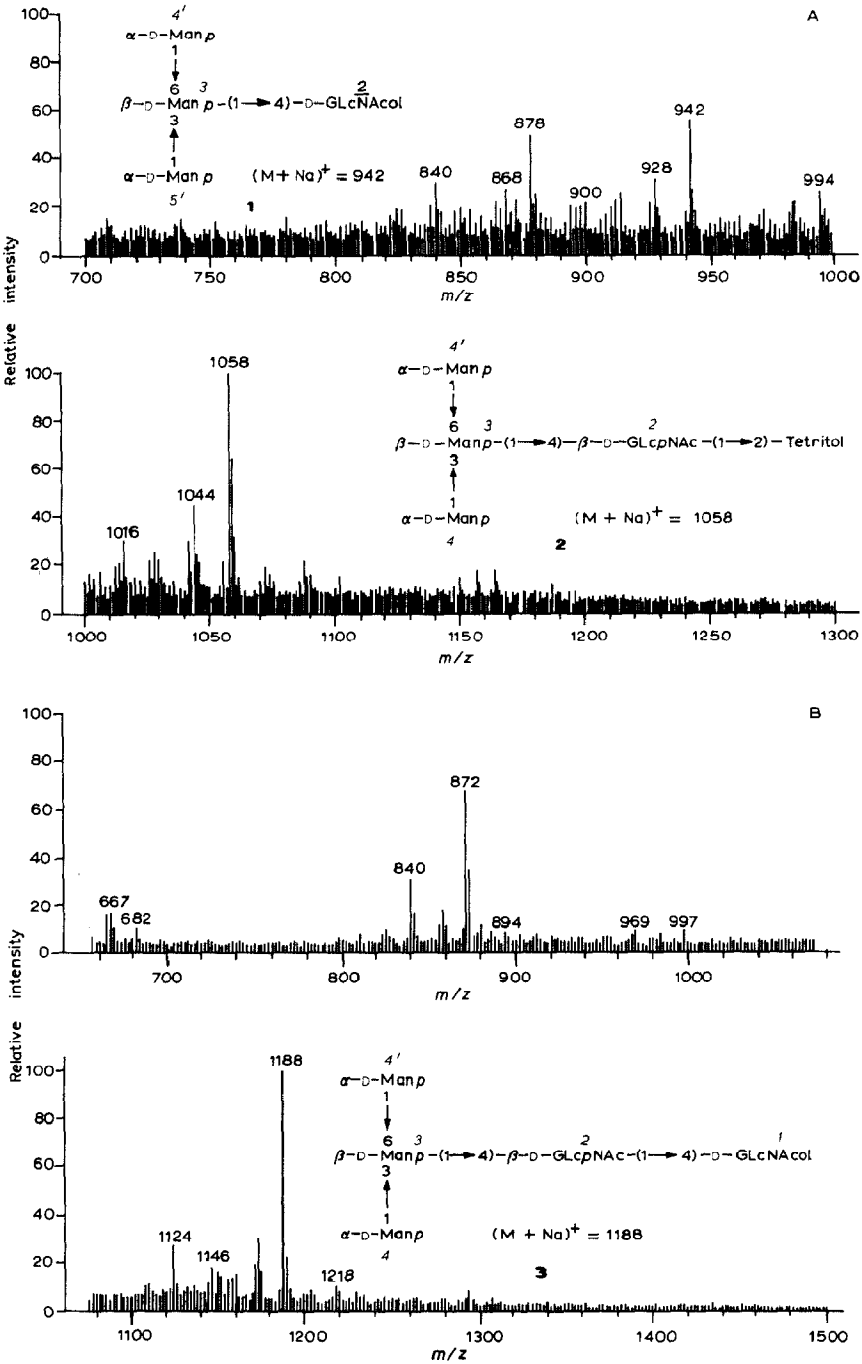

Fig. 3. F.a.b.m. spectra of permethylated oligosaccharide alditol Fractions 2 (A) (structures **1** and **2**) and 3 (B) (structure **3**).

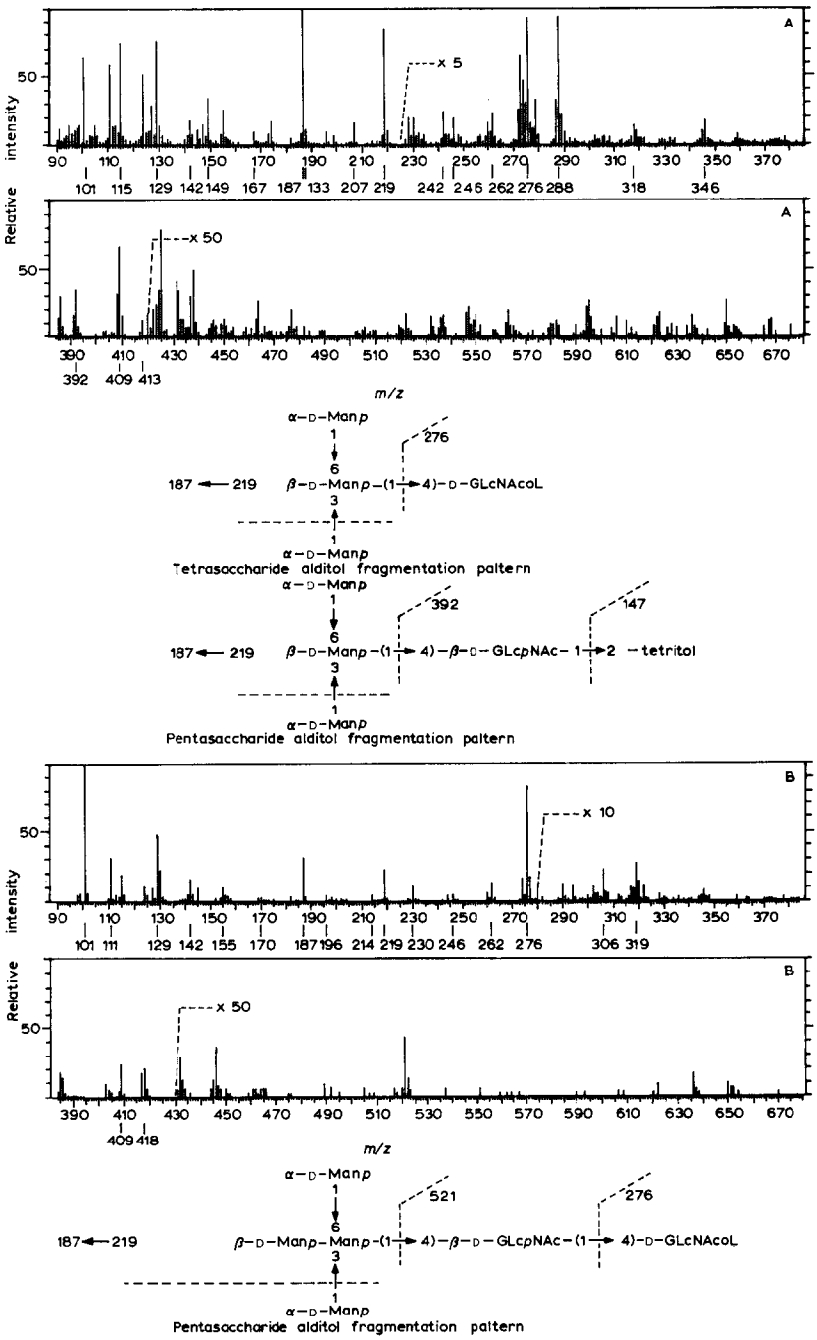

Fig. 4. E.i.m.s. Spectra of permethylated oligosaccharide alditol Fractions 2 (A) and 3 (B).

TABLE II

^{1}H-CHEMICAL SHIFTS OF STRUCTURAL-REPORTER GROUPS PROTONS OF CONSTITUENT MONOSACCHARIDES FOR THE NEUTRAL OLIGOSACCHARIDE ALDITOL FRACTIONS 2 AND 3 ISOLATED FROM QUAIL OVOMUCOID

Residue	*Reporter group*	*Fraction*	
		2[a]	*3*[b]
GlcNAc(*1*)ol	H-2	[c]	4.242
GlcNAc(*1*)ol–ManNAc(*1*)ol	NAc	[c]	2.056–2.036
GlcNAc(*2*)	H-1	4.620	4.639
	NAc	2.075	2.076
GlcNAc(*2*)ol[2]	NAc	2.059	
Man-(*3*)	H-1	4.781	4.788
	H-2	4.253	4.258
Man-(*4*)	H-1	5.102	5.105
	H-2	4.067	4.068
Man-(*4'*)	H-1	4.914	4.915
	H-2	3.974	3.974

[a]See structures **1** and **2**. [b]See structure **3**. [c]Not present.

from the 2-acetamido-2-deoxyglucitol-*2* residue. The presence of a tetritol instead of a 2-acetamido-2-deoxyglucitol-*1* residue affected considerably the chemical shift of H-1 of 2-acetamido (*2*) deoxyglucitol (*2*) residue ($\Delta\delta$ +0.02 p.p.m.).

These results clearly demonstrate that cleavage of the glucosamine–asparagine linkage with sodium hydroxide M sodium borohydride, as well as hydrazinolysis[7,8], gives reduced oligosaccharides and byproducts. This heterogeneous mixture affects the chromatographic separations of oligosaccharide alditols and perturbs m.s. and n.m.r. analyses.

EXPERIMENTAL

The preparation of quail ovomucoid and h.p.l.c. of the oligosaccharide alditols released by alkaline hydrolysis[2] were described previously[4].

The carbohydrate composition of the oligosaccharide alditol fractions was determined by g.l.c. after methanolysis and pertrifluoroacetylation[9]. Permethylation of the oligosaccharides was performed according to Ciucanu and Kerek[10], and the partially methylated monosaccharides were identified by g.l.c.–m.s.[11].

The permethylated oligosaccharide alditol fractions were submitted to fast-atom-bombardment mass-spectrometry[12,13] (f.a.b.m.s.) in a Kratos MS 50 RF high-resolution spectrometer, equipped with a DS 90 (DGDG/30) data system. The cooper tip was first loaded with a 0.1% solution of sodium acetate in methanol (1 μL) and, after drying, thioglycerol (3–4 μL) and the sample solution (0.5–1.0 μL; 5 μg/μL of methanol) were applied. The cooper tip was bombarded by Xe atoms with a kinetic energy equivalent to 7.3 kV; the mass spectrometer was operated at 8 KeV accelerating potential. Spectra were recorded in the positive-ion mode. The

mass range 650 to 1500 was scanned at 10s/decade with mass resolution of 3500. The mass numbers indicated for Fraction 2 are lower by one mass unit from the theoretical. The mass values were calculated on the basis of C = 12.011, H = 1.008, O = 15.999, and N = 14.007.

Electron-impact mass spectra (e.i.m.s.) were obtained with a Riber R-1010 spectrometer under the following operating conditions: ionization energy, 70 eV; ionization current, 300 μA; ion-source temperature 150°. Ammonia was used for chemical-ionization mass spectrometry (c.i.m.s.).

For ^{1}H-n.m.r. spectroscopic analysis, the oligosaccharide alditol fractions were repeatedly treated with D_2O at room temperature with intermediate lyophilization. Spectra were recorded with a Bruker AM-400 WB spectrometer operating in the F.t. mode at a probe temperature of 27° and equipped with a Bruker Aspect 3000 Computer. Chemical shifts (δ) are expressed relative to the signal of sodium 4,4-dimethyl-4-silapentane-1-sulfonate (indirectly to the signal of acetone in D_2O, δ 2.225)[14].

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REFERENCES

1 D. AMINOFF, W. D. GATHMANN, C. M. MACLEAN, AND T. YADOMA, *Anal. Biochem.*, 101 (1980) 44–53.
2 Y. C. LEE AND J. R. SCOCCA, *J. Biol. Chem.*, 247 (1972) 5753–5758.
3 B. M. AUSTEN AND R. D. MARSHALL, in *Méthologie de la Structure et du Métabolisme des Glycoconjugués*, CNRS, Paris, 1973, 219–230.
4 J. M. ALONSO, P. BOULENGUER, J. M. WIERUSZESKI, Y. LEROY, J. MONTREUIL, AND B. FOURNET, *Eur. J. Biochem.*, 177 (1988) 187–197.
5 S. HASE, K. OKAWA, AND T. IKENAKA, *J. Biochem. (Tokyo)*, 91 (1982) 735–737.
6 H. EGGE, J. PETER-KATALINIC, J. PAZ PARENTE, G. STRECKER, J. MONTREUIL, AND B. FOURNET, *FEBS Lett.*, 156 (1983) 357–362.
7 P. W. TANG AND J. M. WILLIAMS, *Carbohydr. Res.*, 113 (1983) c13–c15.
8 J. C. MICHALSKI, J. PETER-KATALINIC, H. EGGE, J. PAZ PARENTE, J. MONTREUIL, AND G. STRECKER, *Carbohydr. Res.*, 134 (1984) 177–189.
9 J. P. ZANETTA, W. C. BREKENRIDGE, AND G. VINCENDON, *J. Chromatogr.*, 69 (1972) 291–304.
10 I. CIUCANU AND F. KEREK, *Carbohydr. Res.*, 131 (1984) 209–217.
11 B. FOURNET, G. STRECKER, Y. LEROY, AND J. MONTREUIL, *Anal. Biochem.*, 116 (1981) 489–502.
12 A. DELL, H. R. MORRIS, H. EGGE, G. STRECKER, AND H. NICOLAI, *Carbohydr. Res.*, 115 (1983) 41–52.
13 A. DELL, J. E. OATES, H. R. MORRIS, AND H. EGGE, *Int. J. Mass Spec. Ion. Phys.*, 46 (1983) 415–418.
14 J. F. G. VLIEGENTHART, L. DORLAND, AND H. VAN HALBEEK, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 209–374.