Carbohydrate Research, 69 (1979) 181–189

COMPOSITION OF A METHYLATED, ACIDIC POLYSACCHARIDE ASSOCIATED WITH COCCOLITHS OF *Emiliania huxleyi* (LOHMANN) KAMPTNER

ANNE MARIE J. FICHTINGER-SCHEPMAN, JOHANNIS P. KAMERLING, JOHANNES F. G. VLIEGENTHART,
Laboratory of Organic Chemistry, State University of Utrecht, Utrecht (The Netherlands)

ELISABETH W. DE JONG, LEENDERT BOSCH, AND PETER WESTBROEK
Department of Biochemistry, State University of Leiden, Leiden (The Netherlands)
(Received February 6th, 1978; accepted for publication, February 24th, 1978)

ABSTRACT

The water-soluble, acidic polysaccharide isolated from the coccoliths of the alga *Emiliania huxleyi* (Lohmann) Kamptner contains residues of the following sugars: L-galactose, D-glucose, D-mannose, L-mannose, L-rhamnose, L-arabinose, D-ribose, D-xylose, 6-*O*-methyl-D-mannose, 6-*O*-methyl-L-mannose, 2,3-di-*O*-methyl-L-rhamnose, 3-*O*-methyl-D-xylose, and D-galacturonic acid. L-Mannose, 6-*O*-methyl-D-mannose, 6-*O*-methyl-L-mannose, and 2,3-di-*O*-methyl-L-rhamnose are novel constituents of a polysaccharide. In addition, the presence of sulphate groups was found. Galacturonic acid and sulphate in the polysaccharide bind Ca^{2+} ions apparently in a ratio of one mol of Ca^{2+} per mol of acidic residue. This feature is relevant for the proposed matrix function of the polysaccharide in the formation of the calcified cell-wall plates (coccoliths) of the alga.

INTRODUCTION

The alga *Emiliania huxleyi* (Lohmann) Kamptner belongs to the Coccolithophoridae, a group of mostly unicellular algae. The cell wall of this organism contains calcified (calcite) plates, called coccoliths, which are formed in a vesicle near the Golgi apparatus of the cell. Recently, the isolation of a water-soluble, acidic polysaccharide from these coccoliths was reported[1]. It was postulated that the polysaccharide, which is capable of binding Ca^{2+} ions, plays a matrix role in the calcification process by acting as a nucleator of the crystallization[1]. In this respect, the elucidation of the complete structure of the polysaccharide is of interest. We now describe the various constituent neutral and acidic monosaccharides and the nature of the strongly acidic group present in the polysaccharide.

RESULTS AND DISCUSSION

The acidic polysaccharide, isolated from the EDTA-soluble fraction of the

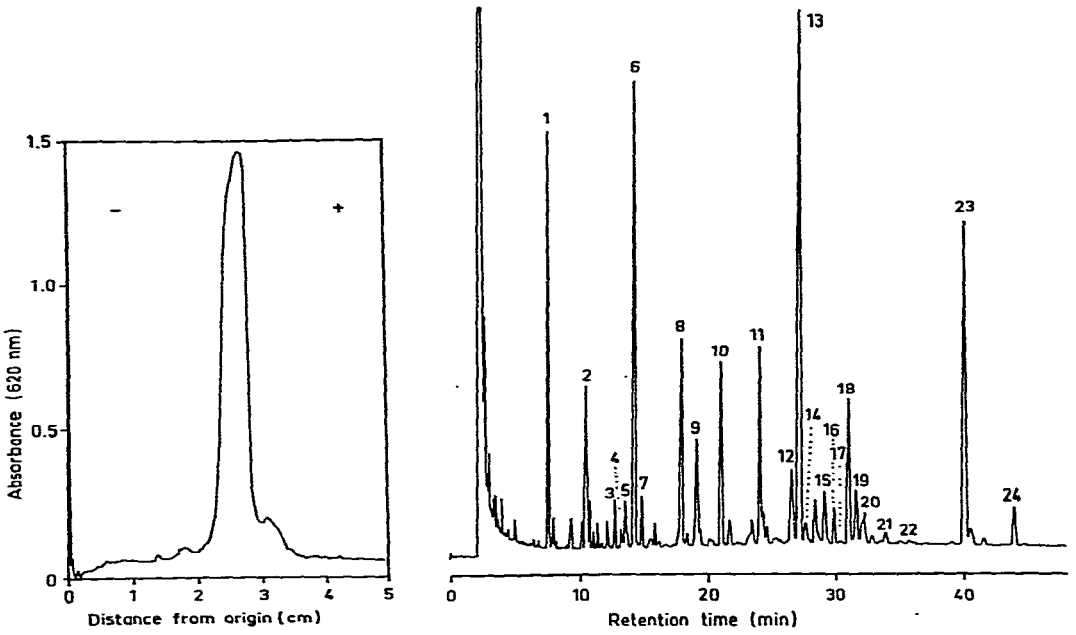

Fig. 1. Polyacrylamide gel electrophoresis[1] of the polysaccharide from coccoliths of *Emiliania huxleyi* after DEAE-Cellulose chromatography. Gels were scanned at 620 nm after staining with Alcian blue.

Fig. 2. Gas chromatogram (SE-30 capillary) of the Me_3Si derivatives of the methyl glycosides derived from the native polysaccharide of *Emiliania huxleyi* (methanolysis): 1, 2,3-di-*O*-methylrhamnose; 2, 3-*O*-methylxylose; 3 and 4, arabinose; 5 and 6, ribose; 6 and 7, rhamnose; 8 and 9, xylose; 10, 6-*O*-methylmannose; 11, 12, 18, and 19, galacturonic acid; 13 and 15, mannose; 14, 16, 17, and 20, galactose; 21 and 22, glucose; 23, mannitol (internal standard); 24, mono-*O*-acetylmannitol (by-product of the internal standard).

coccoliths of the alga *Emiliania huxleyi*, gave one peak on DEAE-cellulose chromatography, and a single spot on two-dimensional cellulose-electrophoresis[2]. As is shown in Fig. 1, polyacrylamide gel electrophoresis[1] indicates that the polysaccharide is contaminated with a few percent of another compound.

The polysaccharide had $[\alpha]_D^{20}$ +21.6° (*c* 0.32, water) and contained carbon (38.92%), hydrogen (5.22%), oxygen (48.95%), sodium (6.12%), sulphur ($\leqslant$ 1.36%), and a trace of phosphorus (0.38%). By pyrolysis–m.s.[3], a small peak at *m/e* 64 (SO_2^+ ion) could be detected, in accordance with the presence of small amounts of sulphate. Alkoxyl determinations[4] showed the presence of 5.16% of methoxyl or 7.50% of ethoxyl groups; the latter groups cannot be distinguished by the method used.

The i.r. spectrum of the sodium salt of the polysaccharide contained an intense absorption band at 1620 cm^{-1}, which shifted to 1735 cm^{-1} for the acidic form, in agreement with a carboxylate→carboxylic acid transition[5]. Both spectra contained a small absorption band at 1235 cm^{-1}, characteristic for ester sulphate (S=O stretching).

The 360-MHz ^{1}H-n.m.r. spectrum showed only one peak in the high-field region at δ 1.3, which results from the occurrence of 6-deoxy monosaccharides (CH_3 groups) in the polysaccharide (see below). No signals for *O*-acetyl groups or acetal-linked pyruvic acid were detected.

The alkaline hydroxylamine–ferric chloride method[6] (Hestrin) gave no indication of the presence of other *O*-acyl groups. To estimate the content of carboxylic groups, the polysaccharide was methanolysed, and application of the Hestrin method then indicated 0.57 μmol of methyl ester/mg of polysaccharide. This is in agreement with the presence of 0.66 μmol of galacturonic acid, determined by g.l.c. (as will be shown later). Because there are substantial proportions of neutral monosaccharides in the polysaccharide, the carbazole reaction gives ambiguous results[1,7].

The ester sulphate content determined by the turbidimetric method[8] was 0.36 μmol of sulphate/mg of polysaccharide, and the barium chloranilate test[9] gave 0.41 μmol of sulphate/mg of polysaccharide if applied to the acidic fraction from a polysaccharide hydrolysate, isolated *via* DEAE-Sephadex A-25. These values are in accordance with the elemental analysis for sulphur.

Galacturonic acid and sulphate were the only detectable acid units in a hydrolysate (HCl) of the polysaccharide after paper chromatography. As was demonstrated earlier[1], Ca^{2+}-binding assays showed the presence of 0.38 $\pm$0.04 μmol of high-affinity and 0.74 $\pm$0.11 μmol of low-affinity sites for Ca^{2+} per mg of polysaccharide. The high-affinity sites have a dissociation constant (K_d) for Ca^{2+} of (2.2 $\pm$1.0) $\times$ 10^{-5}M, and the low-affinity sites have K_d (111 $\pm$39) $\times$ 10^{-5}M.

The carbohydrate composition was determined by g.l.c. and g.l.c.–m.s. on methanolysates and hydrolysates of the polysaccharide. Methyl glycosides were

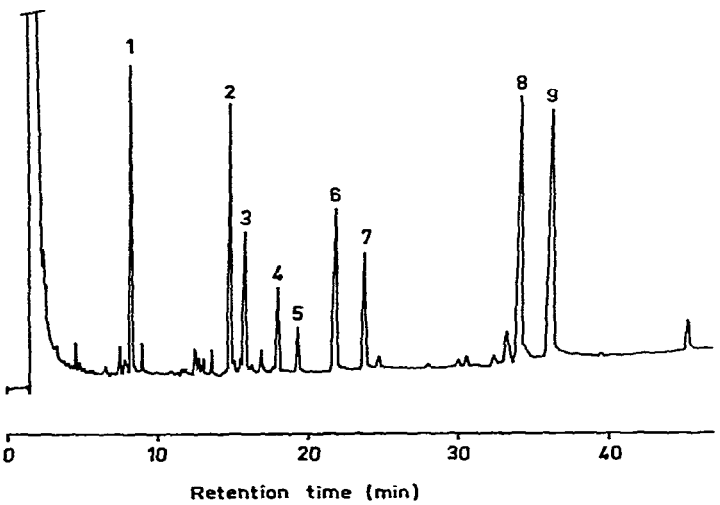

Fig. 3. Gas chromatogram (SP-1000 capillary) of the alditol acetates of the monosaccharides derived from the carboxyl-reduced polysaccharide of *Emiliania huxleyi* (hydrolysis): 1, 2,3-di-*O*-methyl-rhamnose; 2, rhamnose; 3, 3-*O*-methylxylose; 4, ribose; 5, arabinose; 6, xylose; 7, 6-*O*-methyl-mannose; 8, mannose; 9, galactose and glucose.

TABLE I

DATA FOR POLYSACCHARIDE CONSTITUENTS

Monosaccharide	T[a]	T[b]	T[c]	*μMol/mg*[d]	*Molar ratio*[e]			
					Native polysaccharide		*Carboxyl-reduced polysaccharide*	
					Methanolysis	*Hydrolysis*	*Methanolysis*	*Hydrolysis*
Galactose[f]	0.69; 0.74; 0.75; 0.80	1.05	1.09	0.10	0.4	0.5	3.3	2.8
D-Glucose	0.84; 0.89	1.05	1.18	0.03	0.1	0.5	0.8	1.0
Mannose[g]	0.68; 0.73	1.00	1.00	0.78	3.0	3.0	3.0	3.0
L-Rhamnose	0.36; 0.37	0.44	0.33	0.58[h]	2.2[h]	1.8	2.3[h]	1.7
D-Ribose	0.34; 0.36	0.53	0.39			0.6		0.5
L-Arabinose	0.32; 0.33	0.56	0.42	0.06	0.3	0.4	0.3	0.3
D-Xylose	0.45; 0.48	0.64	0.51	0.43	1.7	1.2	1.7	1.2
D-Galacturonic acid	0.60; 0.66; 0.77; 0.79	—	—	0.66	2.5	—	0	—
2,3-Di-*O*-methyl-L-rhamnose	0.19	0.24	0.18	0.22[i]	0.8[i]	1.1	0.6[i]	1.1
3-*O*-Methyl-D-xylose	0.26	0.46	0.35	0.17[i]	0.7[i]	1.2	0.6[i]	0.9
6-*O*-Methylmannose[j]	0.52	0.69	0.58	0.21[i]	0.8[i]	0.6	0.9[i]	0.8

[a]Retention times of the corresponding trimethylsilylated methyl glycosides relative to Me_3Si-mannitol on an SE-30 glass-capillary column; in some cases, only the main anomeric forms are given (temperature program, 130→200° at 1°/min). [b]Retention times of the corresponding alditol acetates relative to mannitol acetate on an SP-1000 glass-capillary column (temperature program, 165→220° at 1°/min). [c]As in *b*, but on 3% OV-225 at 215°. [d]*μ*Mol of sugar per mg of native polysaccharide, determined by quantitative sugar analysis using methanolysis (internal standard, mannitol). [e]Methanolysis values are calculated by using molar adjustment factors, whereas hydrolysis values represent uncorrected peak areas. In both cases, the molar ratio of mannose was arbitrarily taken to be 3. [f]Present as L-galactose in the native polysaccharide; after carboxyl-reduction, D-galacturonic acid gave D-galactose. [g]Mannose was determined as ~90% D-mannose and ~10% L-mannose (peak ratio). [h]Values for rhamnose and ribose could not be determined separately; both monosaccharides have the same molar adjustment factor. [i]Calculated, using the molar adjustment factor of the corresponding unmethylated sugar. [j]6-*O*-Methylmannose comprised the L form (~67%) and the D form (~33%) (peak ratio).

analysed as the corresponding Me_3Si derivatives[10], and the monosaccharides as the alditol acetates[11]. The same methods were also applied to the carboxyl-reduced polysaccharide[12]. Fig. 2 shows the gas chromatogram of the mixture of trimethylsilylated methyl glycosides derived from the native polysaccharide. The gas chromatogram of the alditol acetates obtained from the carboxyl-reduced polysaccharide is presented in Fig. 3. Table I shows the results of the various analyses. Three types of monosaccharides were detected in the solvolysis mixtures: (*a*) neutral monosaccharides, (*b*) uronic acid, and (*c*) methylated neutral monosaccharides.

(*a*) The polysaccharide contains galactose, glucose, mannose, rhamnose, ribose, arabinose, and xylose. The methanolysis values (trimethylsilylated methyl glycosides) for the different monosaccharides are corrected by the use of calculated molar-adjustment factors, whereas the hydrolysis values (alditol acetates) are not. In the latter case, only peak-area ratios are given. Small differences between the values obtained for some sugars may be due to the different solvolysis procedures. The data in Table I show that the amount of galactose increased after carboxyl-reduction of the native polysaccharide, as a result of the conversion of galacturonic acid into galactose (see below). The origin of the increase of glucose in the carboxyl-reduced polysaccharide is still unknown.

(*b*) The uronic acid was identified as galacturonic acid. Carboxyl-reduction of the native polysaccharide converted galacturonic acid into galactose, the formation of which was checked by using sodium borodeuteride. After hydrolysis, reduction, and acetylation, galactitol-6-d_2 hexa-acetate was identified by g.l.c.–m.s.

(*c*) *2,3-Di-O-methylrhamnose.* The 2,3-di-*O*-methyl-6-deoxyhexose structure of the monosaccharide was deduced from the mass spectra of the Me_3Si and Me_3Si-d_9 derivatives of the methyl glycosides[13], as well as from the mass spectra of the alditol acetates[14] obtained by reduction with sodium borohydride and borodeuteride. To establish the configuration, the monosaccharide was isolated by preparative paper chromatography. The *rhamno* configuration was determined by g.l.c. and g.l.c–m.s. analysis after demethylation[15] of the monosaccharide and its alditol. The occurrence of 2,3-di-*O*-methylrhamnose was confirmed by comparison with reference material.

3-O-Methylxylose. The 3-*O*-methylpentose structure was established in the same way as described for the 2,3-di-*O*-methyl-6-deoxyhexose. On the basis of g.l.c. retention times of the derived alditol acetate and 3-*O*-methylarabinitol acetate, the *arabino* and *lyxo* configurations were eliminated. After isolation of the 3-*O*-methylpentose by preparative paper chromatography and demethylation of the free sugar and its alditol, the *xylo* configuration was established.

6-O-Methylmannose. The structure of this monosaccharide was determined by the procedure described for the two other methylated sugars (g.l.c., g.l.c.–m.s., isolation, and demethylation), and confirmed by comparison with reference 6-*O*-methylmannose.

The absolute configurations of the monosaccharide constituents of the polysaccharide, isolated by preparative paper chromatography, were determined by

polarimetry and/or capillary g.l.c. of the trimethylsilylated (—)-2-butyl glycosides[16]. The results are included in Table I.

This study has shown that a large number of monosaccharides are present in the polysaccharide associated with the coccoliths of the alga. The occurrence of three methylated monosaccharides is remarkable. Only 3-*O*-methylxylose has been reported earlier to be a possible constituent of polysaccharides[17–19]. It is also the first time that the L configuration for mannose or its methylated derivatives has been demonstrated in Nature.

Comparison of the results from Ca^{2+}-binding assays[1] and the estimated quantities of galacturonic acid and sulphate suggest that 1 mol of acid in the polysaccharide is able to bind 1 mol of Ca^{2+}. This observation may lend support to the hypothesis that the polysaccharide serves as a nucleator of crystallization during calcification (matrix role), because of the possibility for carbonate ions to occupy the other ligand of the polysaccharide-bound Ca^{2+}.

EXPERIMENTAL

Isolation of the polysaccharide. — The alga *Emiliania huxleyi* was cultivated and the coccoliths were isolated as described previously[1]. The coccolith preparations were decalcified in 10% EDTA (pH 8.0). Insoluble residues were removed by centrifugation at 31,000 *g* and the supernatant was concentrated by filtration through an Amicon PM-10 filter. The concentrate was applied to a column of Bio-Gel P4 and eluted with 0.05M NaCl. The void volume was collected, concentrated on an Amicon PM-10 filter, dialysed against distilled water, and lyophilized (EDTA-soluble fraction). For ion-exchange chromatography, the isolated material was applied to a column of DEAE-Cellulose (Cl^- form, grade DE-52) and eluted with 0.25M NaCl in 0.01M K_2HPO_4 (pH 7.45). The combined fractions containing the polysaccharide were desalted on a column of Bio-Gel P4 and lyophilized.

Materials. — Common monosaccharides were obtained from commercial sources. 3-*O*-Methylarabinitol was a gift from the late Professor J. K. N. Jones. 2,3-Di-*O*-methylrhamnose was obtained from the cell-wall lipopolysaccharide of *Salmonella typhimurium* LT2[20] (kindly donated by Dr. J. Lönngren) after permethylation[21] and hydrolysis. 6-*O*-Methyl-D-mannose was prepared by benzylation[22] of methyl 6-*O*-trityl-α-D-mannoside, followed by detritylation[22], methylation[21], debenzylation[23], and acid hydrolysis.

General methods. — Analytical and preparative paper chromatography were performed on Whatman No. 3MM paper with ethyl acetate–acetic acid–pyridine–water (3:1:1:1). Detection of the monosaccharides was effected with a saturated solution of *o*-dianisidine in ethanol (30 min at 100°) and with naphthoresorcinol[24] (200 mg of naphthoresorcinol in 100 ml of ethanol plus 10 ml of H_3PO_4; 60 min at 50° followed by 5 min at 90°). Acidic groups were detected by spraying with the Schweppe reagent[24] (a mixture of 2 g of glucose in 20 ml of water, 2 ml of aniline in 20 ml of ethanol, and 60 ml of 1-butanol; 5 min at 100°).

Molar ester contents were determined by the alkaline hydroxylamine–ferric chloride method[6], with ethyl acetate as the standard. Turbidimetry[8] and the barium chloranilate test[9] were used for the determination of sulphate groups.

Carboxyl reduction of the native polysaccharide was performed by the method of Taylor and Conrad[12]; to obtain complete reduction, the procedure was carried out twice.

I.r. spectra were recorded for potassium bromide discs with a Perkin–Elmer Model 457 spectrophotometer. ^{1}H-N.m.r. spectra were recorded with a Bruker HX-360 spectrometer operating in the Fourier-transform mode, at a probe temperature of 25°, for solutions in D_2O with sodium 2,2-dimethyl-2-silapentane-5-sulphonate as internal standard.

Capillary g.l.c. was performed on a Varian Aerograph 2740-30-01, equipped with a flame-ionization detector. The injection port temperature and the detector temperature were 200° and 220°, respectively. The carrier-gas nitrogen flow-rate was 1 ml/min, and the make-up nitrogen flow-rate 30 ml/min. A glass-capillary column (25 m × 0.31 mm i.d.) wall-coated with SE-30 (LKB-Produkter A.B., Stockholm, Sweden) was used for the analysis of trimethylsilylated methyl glycosides; the oven temperature was programmed from 130 to 200° at 1°/min. For alditol acetates, a glass-capillary column (25 m × 0.26 mm i.d.) wall-coated with SP-1000 (LKB-Produkter A.B., Stockholm, Sweden) was used; the oven temperature was programmed from 165 to 220° at 1°/min. G.l.c. of alditol acetates was also performed at 215° on a Pye 104 instrument equipped with a flame-ionization detector and a glass column (1.60 m × 4.0 mm i.d.) packed with 3% of OV-225 on Chromosorb W HP (100–120 mesh); the nitrogen flow-rate was 40 ml/min.

G.l.c.–m.s. was performed with a Jeol JGC-1100/JMS-07 combination: ion-source temperature, 250°; accelerating voltage, 3 kV; ionizing current, 300 μA; and electron voltage, 75 eV. For trimethylsilylated methyl glycosides, a glass column (2.00 m × 2.0 mm i.d.) packed with 3.8% of SE-30 on Chromosorb W HP (100–120 mesh) was used, and for alditol acetates, a glass column (2.00 m × 2.0 mm i.d.) packed with 3% of OV-225 on Chromosorb W HP (100–120 mesh). The programming conditions for the oven temperature were dependent on the type of sample.

Solvolysis procedures. — Hydrolyses of the native and carboxyl-reduced polysaccharide (2 mg) were carried out in 5 ml of 0.25M H_2SO_4 for 17 h at 100°. After neutralization of the acid with $BaCO_3$, the liberated monosaccharides were converted into the corresponding alditol acetates by reduction with sodium borohydride or sodium borodeuteride, and subsequent treatment[25] with a mixture of acetic anhydride–pyridine (1:1) for 20 min at 100°.

Methanolyses of the native and carboxyl-reduced polysaccharide (1 mg) were performed in 1 ml of methanolic M HCl for 24 h at 85°. The methyl glycosides were analysed after trimethylsilylation[26] with hexamethyldisilazane–chlorotrimethylsilane–pyridine (1:1:5).

For the detection of acidic groups, the polysaccharide was hydrolysed with M HCl for 20 h at 100°.

Demethylation[15]. — A suspension of 1 mg of a methylated monosaccharide in 1 ml of dry dichloromethane was cooled to −80° and 0.1 ml of freshly distilled BBr_3 was added. The mixture was kept for 30 min at −80°, and then slowly brought to room temperature. After 18 h, the excess of reagent was decomposed with water, and the solution evaporated to dryness. Boric acid was removed by co-evaporation (three times) with methanol under diminished pressure; considerable formation of methyl glycoside occurred during this process. The glycosidation was completed by treatment with methanolic HCl. The resulting methyl glycosides were analysed as the corresponding Me_3Si derivatives. Methylated alditols were demethylated by the same route, and analysed as the acetates.

ACKNOWLEDGMENTS

We thank Mr. G. J. Gerwig for his skilful assistance with g.l.c., Mr. C. Versluis (Laboratory of Analytical Chemistry, University of Utrecht) for recording the mass spectra, Dr. S. K. Wadman (Wilhelmina Children's Hospital, University of Utrecht) for running two-dimensional electrophoresis, Dr. W. J. Buis (Element Analytical Section of the Institute for Organic Chemistry TNO, Utrecht) for elemental analysis and determination of methoxyl and/or ethoxyl groups, and Dr. H. L. C. Meuzelaar (FOM-Instituut voor Atoom- en Molecuulfysica, Amsterdam, The Netherlands) for pyrolysis–m.s. We thank Dr. J. Lönngren (Stockholm, Sweden) for the gift of lipopolysaccharide. This investigation was supported by the Netherlands Organization for the Advancement of Pure Research (ZWO).

REFERENCES

1 E. W. DE JONG, L. BOSCH, AND P. WESTBROEK, *Eur. J. Biochem.*, 70 (1976) 611–621.
2 N. G. G. M. ABELING, S. K. WADMAN, AND A. H. VAN GENNIP, *Clin. Chim. Acta*, 56 (1974) 297–303.
3 H. L. C. MEUZELAAR, P. G. KISTEMAKER, AND M. A. POSTHUMUS, *Biomed. Mass Spectrom.*, 1 (1974) 312–319.
4 H. ROTH, *Pregl-Roth Quantitative Organische Mikroanalyse*, Springer–Verlag, Wien, 1958, pp. 247–251.
5 C. J. POUCHERT, *The Aldrich Library of Infrared Spectra*, Aldrich Chemical Company, Inc., 1975, pp. 255 and 291.
6 J. LUDOWIEG AND A. DORFMAN, *Biochim. Biophys. Acta*, 38 (1960) 212–218.
7 M. J. SPIRO, *Anal. Biochem.*, 82 (1977) 348–352.
8 K. S. DODGSON AND R. G. PRICE, *Biochem. J.*, 84 (1962) 106–110.
9 A. WAINER AND A. L. KOCH, *Anal. Biochem.*, 3 (1962) 457–461.
10 J. R. CLAMP, T. BHATTI, AND R. E. CHAMBERS, *Methods Biochem. Anal.*, 19 (1971) 229–344.
11 J. S. SAWARDEKER, J. H. SLONEKER, AND A. JEANES, *Anal. Chem.*, 37 (1965) 1602–1604.
12 R. L. TAYLOR AND H. E. CONRAD, *Biochemistry*, 11 (1972) 1383–1388.
13 G. PETERSSON AND O. SAMUELSON, *Sven. Papperstidn.*, 71 (1968) 731–738.
14 P.-E. JANSSON, L. KENNE, H. LIEDGREN, B. LINDBERG, AND J. LÖNNGREN, *Chem. Commun. Univ. Stockholm*, No. 8, 1976.
15 J. F. W. MCOMIE, M. L. WATTS, AND D. E. WEST, *Tetrahedron*, 24 (1968) 2289–2292.
16 G. J. GERWIG, J. P. KAMERLING, AND J. F. G. VLIEGENTHART, *Carbohydr. Res.*, 62 (1978) 349–357.
17 V. S. FAREED AND E. PERCIVAL, *Carbohydr. Res.*, 53 (1977) 276–277.

18 J. WECKESSER, G. ROSENFELDER, H. MAYER, AND O. LÜDERITZ, *Eur. J. Biochem.*, 24 (1971) 112–115.
19 F. BROWN, D. J. NEAL, AND S. G. WILKINSON, *Biochem. J.*, 163 (1977) 173–175.
20 C.-G. HELLERQVIST, B. LINDBERG, S. SVENSSON, T. HOLME, AND A. A. LINDBERG, *Carbohydr. Res.*, 9 (1969) 237–241.
21 S.-I. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
22 K. MIYAI AND R. W. JEANLOZ, *Carbohydr. Res.*, 21 (1972) 45–55.
23 S. A. HOLICK AND L. ANDERSON, *Carbohydr. Res.*, 34 (1974) 208–213.
24 *Handbook of Chromatography*, Vol. II, CRC Press, 1974, pp. 127 and 132.
25 H. BJÖRNDAL, B. LINDBERG, AND S. SVENSSON, *Acta Chem. Scand.*, 21 (1967) 1801–1804.
26 J. P. KAMERLING, G. J. GERWIG, J. F. G. VLIEGENTHART, AND J. R. CLAMP, *Biochem. J.*, 151 (1975) 491–495.