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DETERMINATION OF POLYSACCHARIDE LINKAGE AND BRANCHING BY REDUCTIVE DEPOLYMERIZATION. GAS–LIQUID CHROMATOGRAPHY AND GAS–LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY REFERENCE DATA*

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ABSTRACT

A series of partially methylated anhydro-D-alditol acetates has been prepared and analyzed by g.l.c. and g.l.c.–m.s. The reference compounds were obtained by reductive cleavage of permethylated and partially methylated standard methyl glycosides, and standard di-, tri-, and tetra-saccharides of known composition and linkage. Structural characterization included retention times on g.l.c. using DB-225 capillary columns and mass spectrometry in both the chemical- and electron-ionization modes. This compiled library allows further evaluation of the applicability of the reductive cleavage procedure for determining linkage or branching (or both) of new and more complex oligosaccharides. The technique has been applied to microgram amounts of a bacterial-membrane oligosaccharide and to a saccharide obtained from the urine of a mannosidosis patient. The procedures are simple, reliable, and applicable to small amounts of isolated biological materials.

INTRODUCTION

A complete structural analysis of a polysaccharide requires the determination of linkage, anomeric configuration, and branching, as well as the usual characterization of each monomer and the linear sequence. Problems of linkage determination have classically been approached by permethylation of the sample and determination of the products of hydrolysis or methanolysis[1–3]. The essential features of the well established alditol acetate procedure[1] entail permethylation, hydrolysis, reduction, and acetylation. Component sugars and location of acetyl groups (indicating linkage and branching) are determined by chromatographic comparison with standards, by mass spectrometry, or by a combination of both. These chemical procedures, however, lack the sensitivity, and in some cases the specificity, to ap-

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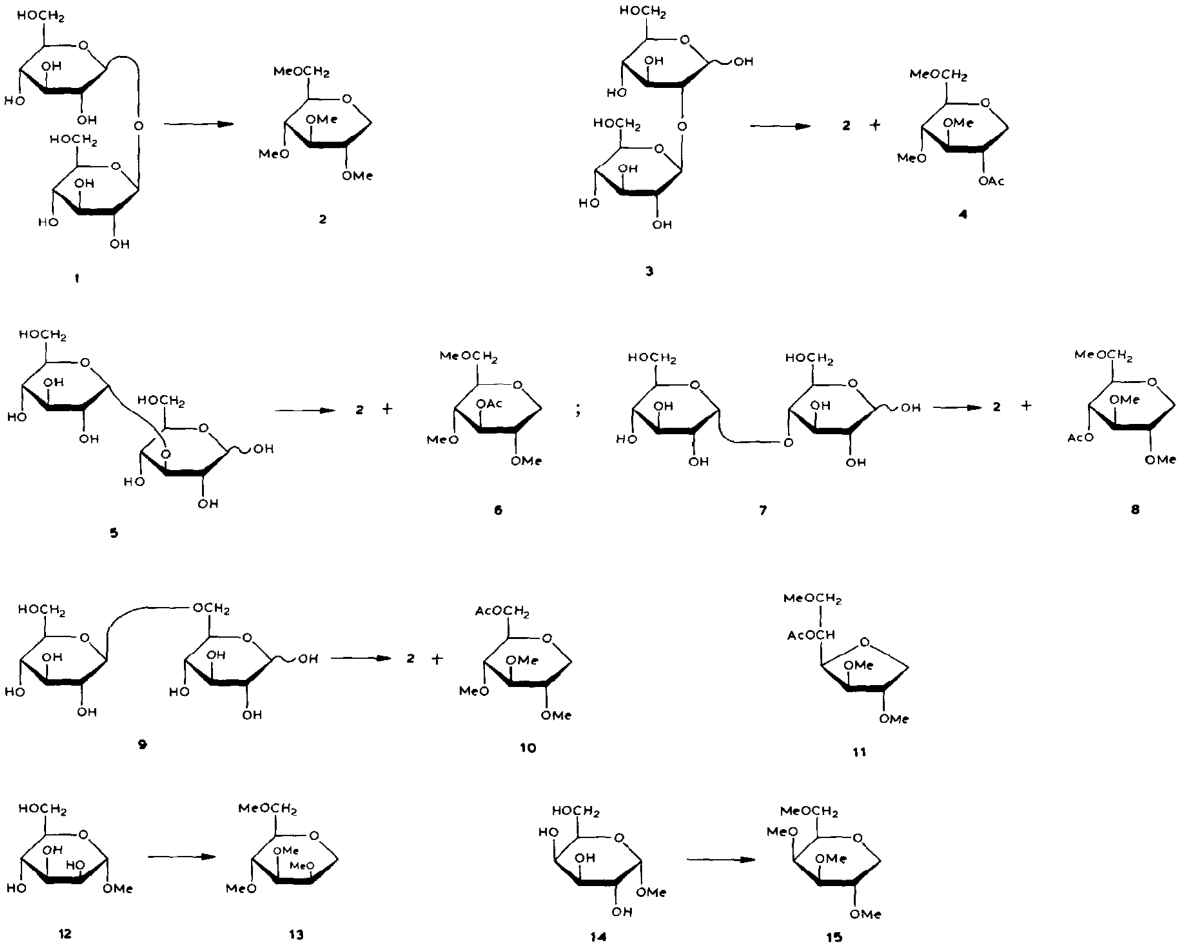
proach many current polysaccharide problems. In particular, the acid-catalyzed degradation of the polymer and the extensive processing of the sample necessary to generate alditol acetates do not allow manipulation of small amounts of sample.

An alternative and more direct approach has recently been introduced by Rolf and Gray[4] by reduction of the glycosidic linkage to yield anhydroalditols. Organosilane reduction of ether linkages (by use of triethylsilane with trifluoroacetic acid as a catalyst under anhydrous conditions) was first introduced by Loim *et al.*[5]. Doyle *et al.*[6] studied the reduction of aldehydes and ketones with boron trifluoride as a catalyst, and discussed two new transformations, reductive elimination and acylation–reduction. Research into the kinetics and mechanism of reductive cleavage has been reported[7,8] and initial evidence suggests that, under anhydrous conditions, the native pyran or furan ring form is preserved[7–9]; the method was successfully applied to the determination of linkages in *Saccharomyces cerevisiae* mannans[9]. The introduction of these procedures for the study of polysaccharides offers a most interesting new approach that may circumvent the complications of earlier procedures.

To improve the sensitivity for the characterization of the oligosaccharides, we devised a three-step process, the first of which entails partial depolymerization leading to the generation of a family of smaller, overlapping oligosaccharides[10]. The second step is the purification of these complex mixtures, the detectability during l.c. separation being increased by fluorescent derivatization[10,11]. The third and final step encompasses composition and linkage analysis of these purified materials, which requires a series of reference standards. Anhydroalditols being not commercially available, we carried out the methylation, reductive cleavage, and acetylation steps on known mono-, di-, tri-, and tetra-saccharides, and characterized the products by g.l.c. and g.l.c.–m.s. In all cases, the compounds obtained proved to be consistent in amount and distribution when analyzed by g.l.c., and their mass spectra, in both the electron- and chemical-ionization modes, provided the expected molecular weights and concordant fragmentation patterns. The reduction, derivatization, and structural characterization were carried out at the microgram level.

EXPERIMENTAL

Materials. — D-Mannose, D-galactose, methyl α-D-galactopyranoside (**14**), methyl α-D-mannopyranoside (**12**), 2-deoxy-2-acetamido-D-glucose, 6-*O*-β-D-glucopyranosyl-D-glucopyranose (gentiobiose) (**9**), 4-*O*-β-D-glucopyranosyl-D-glucopyranose (cellobiose), 4-*O*-α-D-glucopyranosyl-D-glucopyranose (maltose) (**7**), α-D-glucopyranosyl α-D-glucopyranoside (trehalose) (**1**), *O*-α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl β-D-fructofuranoside (raffinose), *O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl β-D-fructofuranoside (stachyose) (**32**) were purchased from Pfanstiehl (Waukegan, IL 60085); 2,4-di-*O*-methyl-D-glucose, 3,4-di-*O*-methyl-D-glucose, and 2,3-di-*O*-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methyl-D-glucose from Supelco (Bellefonte, PA 16823); 3-*O*-α-D-glucopyranosyl-D-glucopyranose (nigerose) (**5**) was purchased from Sigma Chemical Co. (St. Louis, MO 63178), 2-*O*-α-D-glucopyranosyl-D-glucopyranose (kojibiose) (**3**) from Koch-Light Laboratories Ltd. (Coinbrook, Bucks., United Kingdom), and *O*-α-D-glucopyranosyl-(1→3)-β-D-fructofuranosyl α-D-glucopyranoside (melezitose) from Calbiochem–Behring Co. (La Jolla, CA 92037).

The synthetically prepared, partially methylated methyl mannopyranosides (**16**–**19**) and the urinary mannosidosis trisaccharide sample (**38**) were obtained from Dr. C. Warren, and the bacterial oligosaccharide samples from Dr. E. Kennedy.

Permethylation and partial methylation. — The methylation of the samples was performed by the general procedure of Hakomori[12], as modified by Sanford and Conrad[13]. Complete permethylation was obtained by use of the previously described conditions[10] with an excess of reagents. Partially methylated sugars were obtained by use of less than the equivalent amount of the dimethyl sulfoxide anion.

Reductive cleavage and acetylation. — Boron trifluoride etherate (90 μL), triethylsilane (100 μL), and trifluoroacetic acid (20 μL) were combined at 0° and brought into a homogeneous solution in dichloromethane (dried over CaH_2). This reducing agent (100 μL) was added to a dried residue of permethylated oligosaccharide (10–100 μg of material). The solution was heated for 1 h at 45° in a temperature block. To terminate the reductive cleavage reaction and to derivatize all liberated hydroxyl groups, acetic anhydride (50 μL) was added and the solution warmed to 40° for 10 min. Owing to water solubility resulting in a loss of low-molecular-weight reaction products, acetylation must precede the extraction. The product was purified by extraction with dichloromethane from an aqueous solution by the addition of water (250 μL) and dichloromethane (250 μL). The reducing agent was made up fresh before each analysis and an excess of reagent always used.

Gas–liquid chromatography. — G.l.c. was carried out on 30-m, fused silica, capillary columns coated with three different liquid phases, DB-1, DB-1701, and DB-225 (J & W Scientific, Rancho Cardova, CA 95670). The retention times for the DB-225 column are reported in Table I. The gas chromatograph was a Hewlett–Packard (Avondale, PA 19311) model No. 5792, equipped for flame-ionization detection. The gas chromatographic conditions were as follows. For the DB-1 and DB-1701 columns, initial temperature 75°, with a 2-min hold, and then programmed to 300°, at a rate of 10°/min. For the DB-225 column, initial temperature 75°, with a 2-min hold, and then rapidly brought to 200° at a rate of 29°/min. After a 5-min hold, the peaks of interest were eluted with a temperature program of 3°/min to the final temperature of 240°. The retention times were determined relative to the alkane $C_{16}H_{34}$ and are listed in Table I.

Gas–liquid chromatography–mass spectrometry. — The mass spectrometer utilized in this study was a Finnigan-MAT 312 (San Jose, CA 95134), double-focusing instrument fitted with a combined electron- and chemical-ionization source. For g.l.c.–m.s. analysis, the columns were directly coupled to the mass spectrometer, and the peaks eluted were ionized by either chemical or electron

TABLE I

RETENTION TIMES OF THE ANHYDROALDITOL STANDARDS[a]

Compound	R_t
1,5-Anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol (**2**)	1.16
1,5-Anhydro-2,3,4,6-tetra-*O*-methyl-D-mannitol (**13**)	1.39
1,5-Anhydro-2,3,4,6-tetra-*O*-methyl-D-galactitol (**15**)	1.26
2,5-Anhydro-1,3,4,6-tetra-*O*-methyl-D-hexitol from D-fructose	
Peak 1 (**33**)	1.21
Peak 2 (**33**)	1.23
2-*O*-Acetyl-1,5-anhydro-3,4,6-tri-*O*-methyl-D-glucitol (**4**)	1.57
3-*O*-Acetyl-1,5-anhydro-2,4,6-tri-*O*-methyl-D-glucitol (**6**)	1.78
4-*O*-Acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-glucitol (**8**)	1.59
6-*O*-Acetyl-1,5-anhydro-2,3,4-tri-*O*-methyl-D-glucitol (**10**)	1.54
5-*O*-Acetyl-1,4-anhydro-2,3,6-tri-*O*-methyl-D-glucitol (**11**)	1.73
2-*O*-Acetyl-1,5-anhydro-3,4,6-tri-*O*-methyl-D-mannitol (**20**)	1.52
3-*O*-Acetyl-1,5-anhydro-2,4,6-tri-*O*-methyl-D-mannitol (**21**)	1.71
4-*O*-Acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-mannitol (**22**)	2.12
6-*O*-Acetyl-1,5-anhydro-2,3,4-tri-*O*-methyl-D-mannitol (**23**)	1.96
2-*O*-Acetyl-1,5-anhydro-3,4,6-tri-*O*-methyl-D-galactitol (**28**)	1.87
3-*O*-Acetyl-1,5-anhydro-2,4,6-tri-*O*-methyl-D-galactitol (**29**)	1.68
4-*O*-Acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-galactitol (**30**)	1.50
6-*O*-Acetyl-1,5-anhydro-2,3,4-tri-*O*-methyl-D-galactitol (**31**)	1.77
3-*O*-Acetyl-2,5-anhydro-1,4,6-tri-*O*-methyl-D-hexitol from D-fructose	
Peak 1 (**34**)	1.47
Peak 2 (**34**)	1.49
2,6-Di-*O*-acetyl-1,5-anhydro-3,4-di-*O*-methyl-D-glucitol (**35**)	2.30
3,6-Di-*O*-acetyl-1,5-anhydro-2,4-di-*O*-methyl-D-glucitol (**36**)	2.58
4,6-Di-*O*-acetyl-1,5-anhydro-2,3-di-*O*-methyl-D-glucitol (**37**)	2.19

[a] Relative to hexadecane.

ionization. The reagent gas used while in the chemical ionization mode was ammonia with an ion-source temperature of 125°. Electron-ionization voltage was 70 eV and the ion-source temperature 200°.

RESULTS AND DISCUSSION

*Characterization of 1,5-anhydro-*D*-glucitol compounds.* — To obtain a complete set of reference compounds for D-glucose, the disaccharides trehalose (**1**), kojibiose (**3**), nigerose (**5**), maltose (**7**), and gentiobiose (**9**) were permethylated, reductively cleaved, and acetylated. These products provided the reference standards 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol (**2**), and the complete series of 1,5-anhydromono-*O*-acetylper-*O*-methyl-D-glucitol derivatives **4**, **6**, **8**, and **10**.

For each individual disaccharide, the reductive cleavage products were analyzed by g.l.c. in a 30-m fused silica column. Complete g.l.c. separation of **4**, **8**, and **10** in a DB-1 or DB-1701 capillary column was not possible because of the coelution of **8** and **10** from the former, and of **4** and **8** from the latter column. Separation of all components, however, was achieved on a 30-m DB-225 capillary column and this column was adopted for routine use.

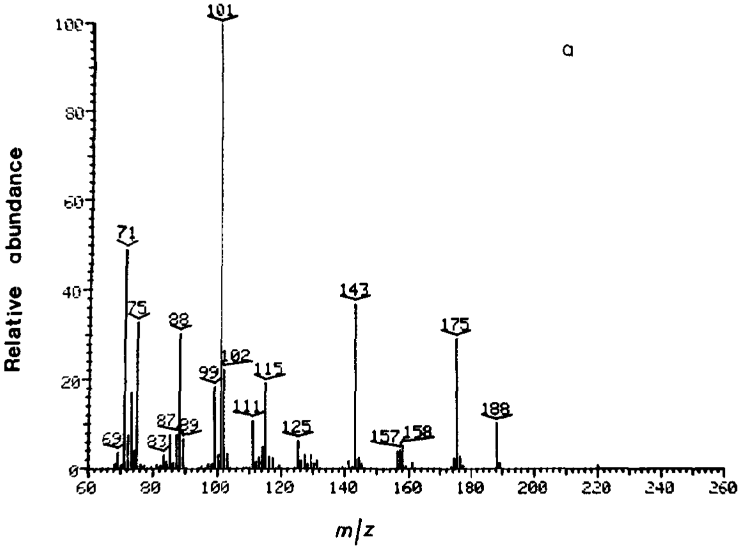
Under the conditions established, a clear g.l.c. profile was obtained showing two peaks, one for the product resulting from the terminal group of the original disaccharide and one for the monoacetylated product derived from the reducing residue. For **1**, the g.l.c. profile showed only one peak with a retention time indicative of **2**, the expected single compound from a (1→1)-linked disaccharide. These results indicated that all linkages were cleaved quantitatively. Cleavage of the (1→4)-linked disaccharides cellobiose and maltose proved to be very sensitive to trace amounts of water which, when present, would generate[8] as by-product 5-*O*-acetyl-1,4-anhydro-2,3,6-tri-*O*-methyl-D-glucitol (**11**).

Characterization of compounds eluted by g.l.c. was performed by mass spectrometry (g.l.c.–m.s.) in both the chemical-ionization (c.i.) mode to confirm molecular weight (degree of acetylation) and, subsequently, the electron-ionization (e.i.) mode to determine the structure. Ammonia was used as the reagent gas in the c.i. mode and the spectra provided one major ion, that of the ammonium adduct, $(M + NH_4)^+$. Since these spectra are of little comparative value, they are not reproduced herein.

The e.i. mass spectra of **2**, **4**, **6**, **8**, **10**, and **11** are shown in Fig. 1. Although a detailed study of the fragmentation was not attempted, the interpretation is straightforward in terms of consecutive losses of the ring substituent(s). Preliminary observations indicate that the same general fragmentation pathways as those previously observed for permethylated methyl glycosides[14,15] and for partially methylated, acetylated methyl glycosides[16] were obtained (with the exclusion of those fragments involving the C-1 group). The degree of acetylation and position of acetyl group may, thus, be deduced from previous data. All of the e.i. spectra described herein are sufficiently different for an unambiguous localization of the *O*-acetyl groups.

*Characterization of 1,5-anhydro-*D*-mannitol and 1,5-anhydro-*D*-galactitol compounds.* — As it was not possible to obtain a series of variously linked disaccharide standards composed of D-galactose and D-mannose, the starting compounds were derived from three different sources. For characterization of the 1,5-anhydrotetra-*O*-methylhexitols (the reductive cleavage products of terminal residues), the known methyl glycosides **12** and **14** were permethylated and reductively cleaved to give 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-mannitol (**13**) and -D-galactitol (**15**).

The 1,5-anhydromono-*O*-acetyltri-*O*-methyl-D-mannitol derivatives were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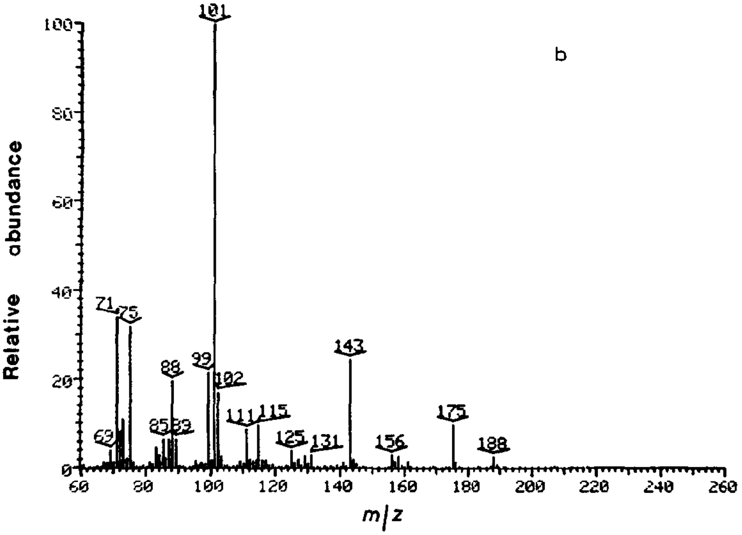
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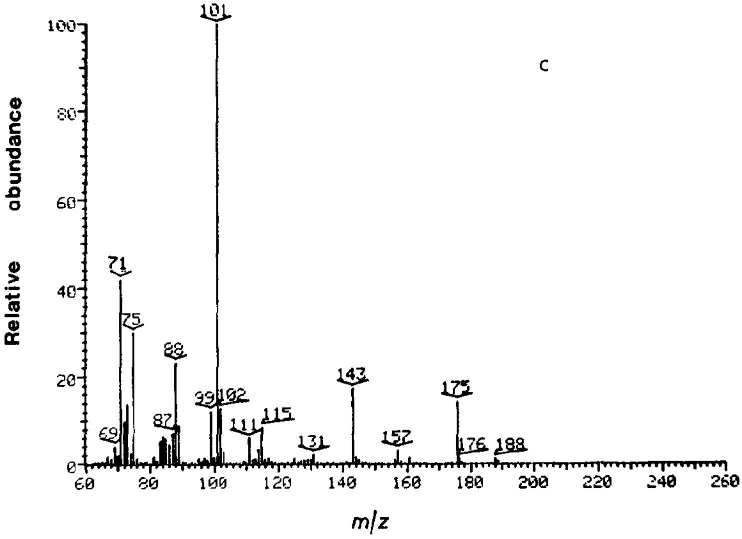
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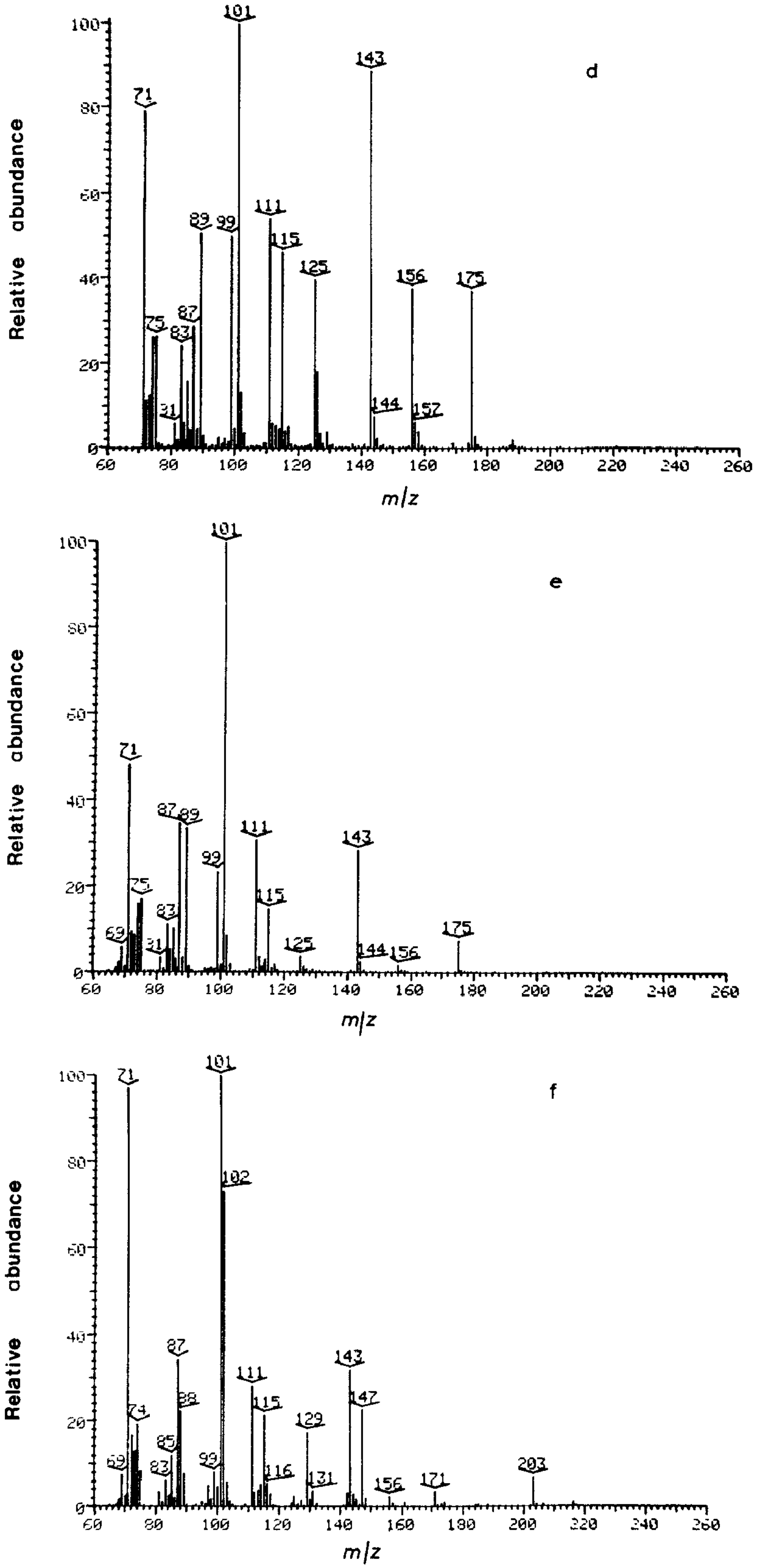
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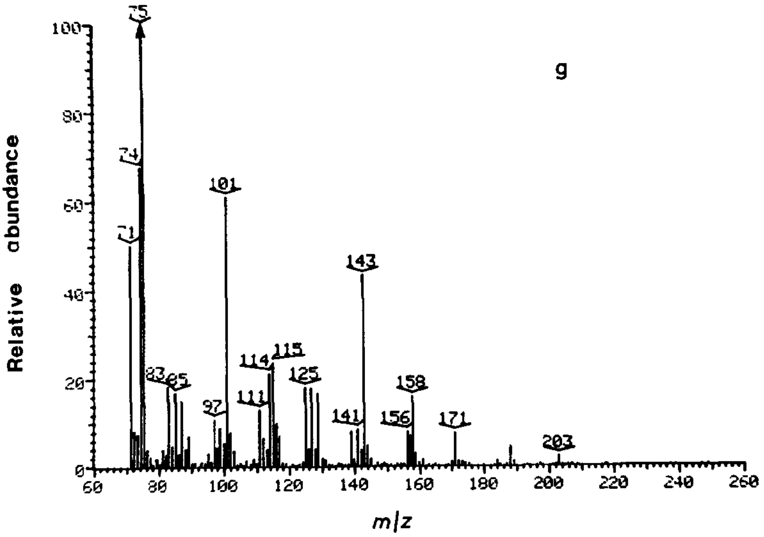
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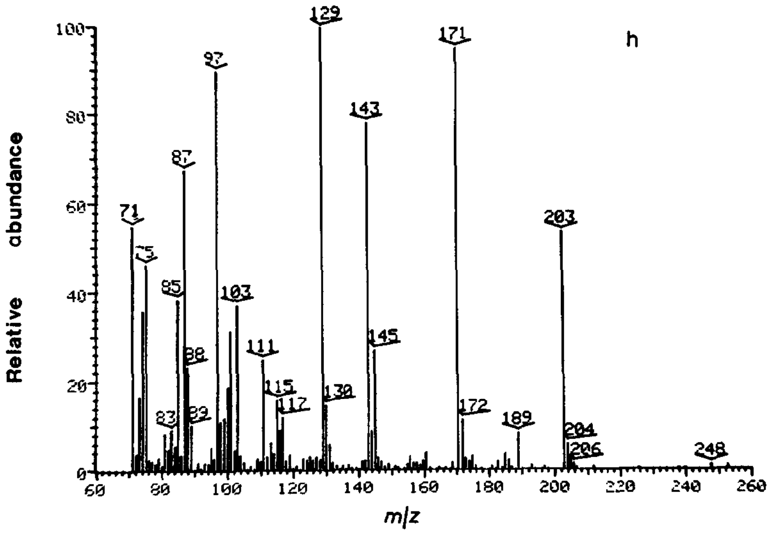
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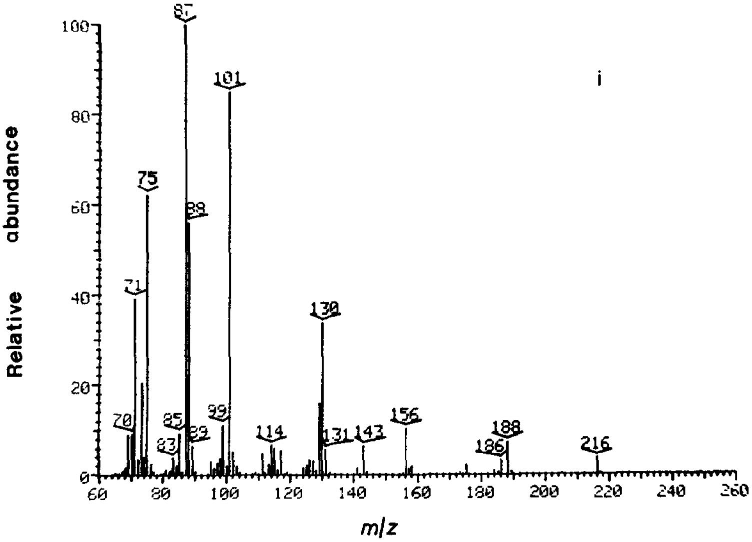
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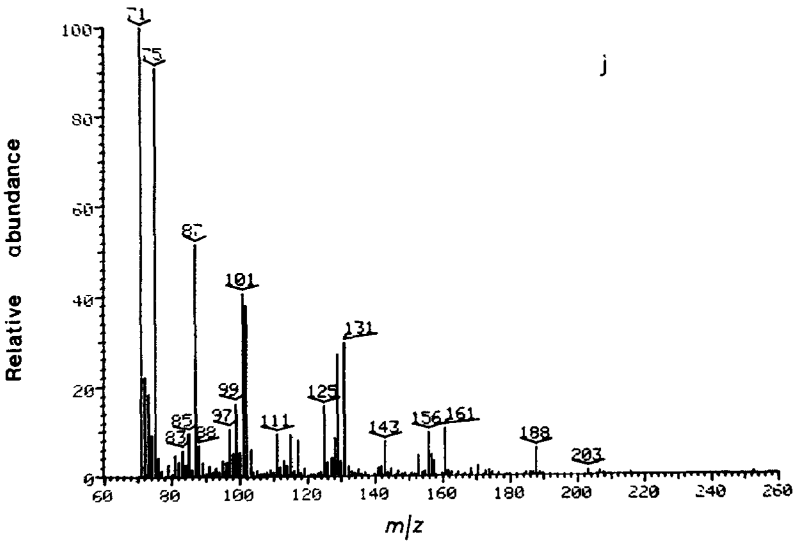
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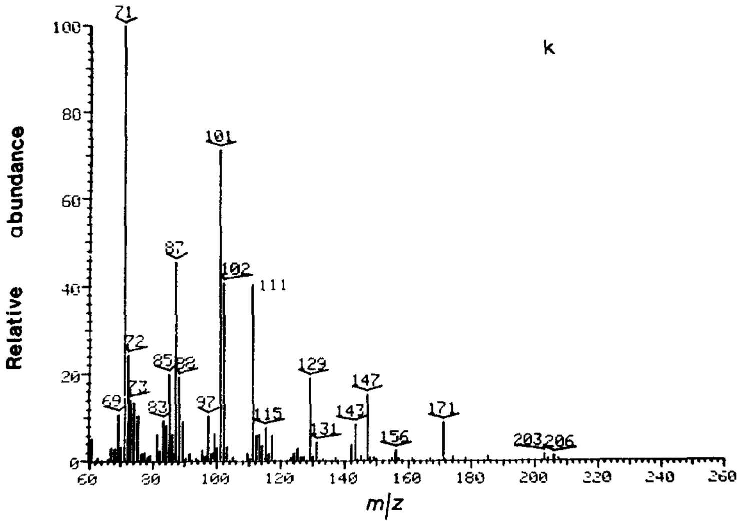
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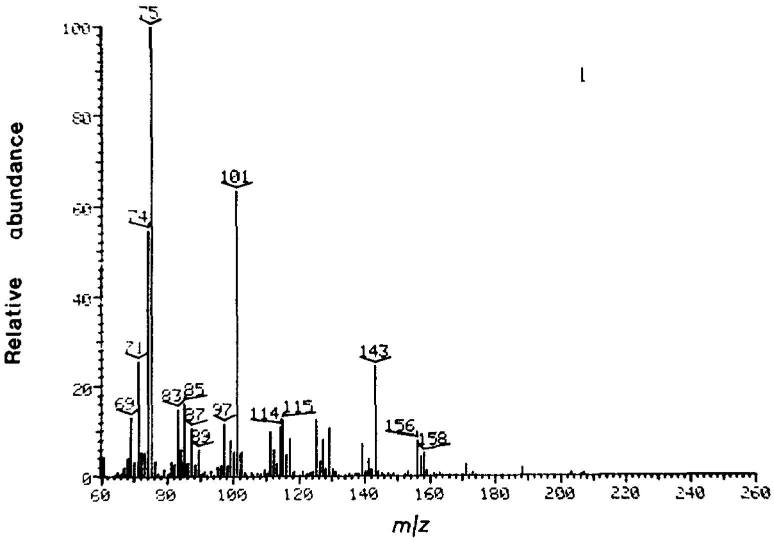
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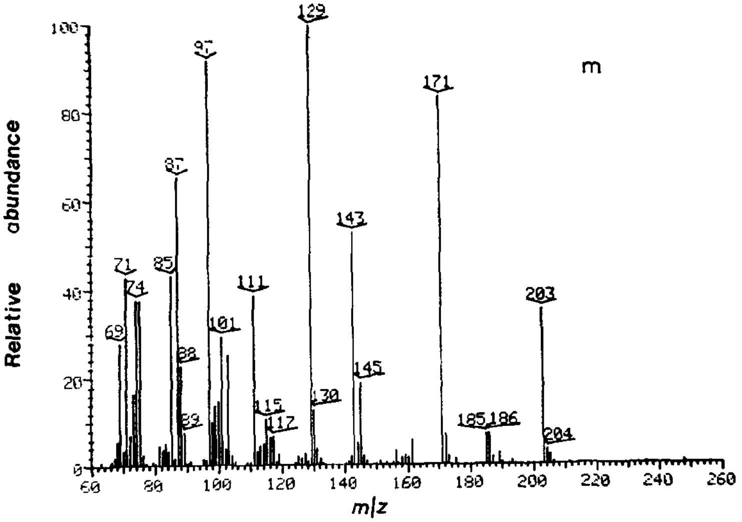
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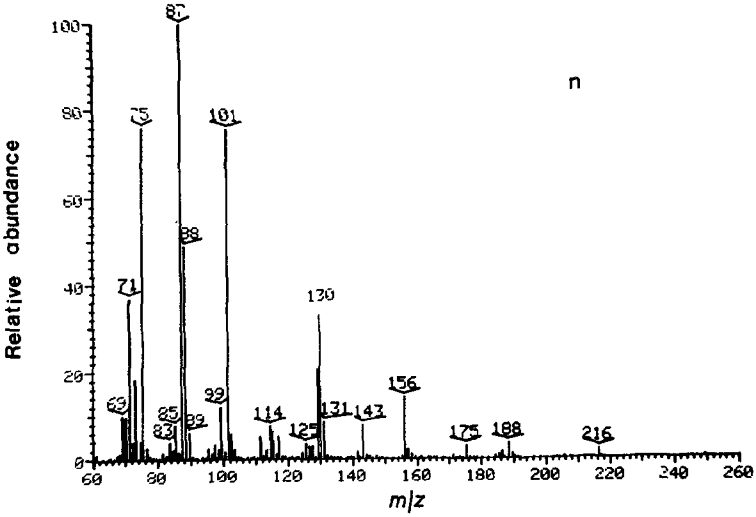
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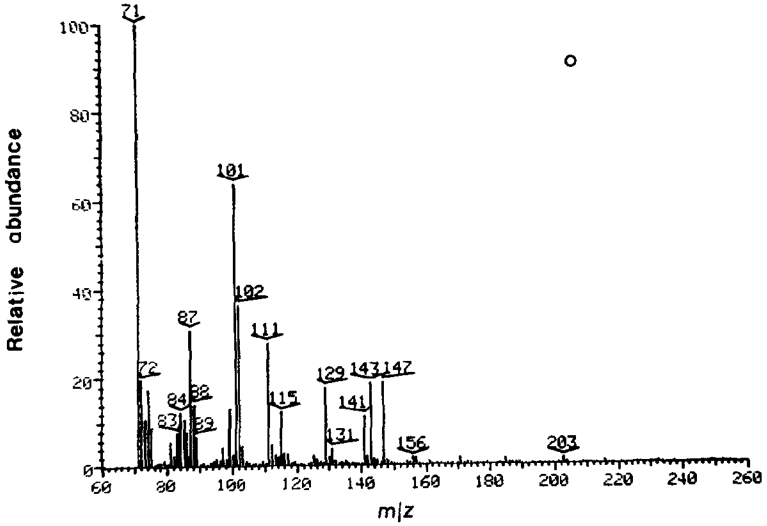
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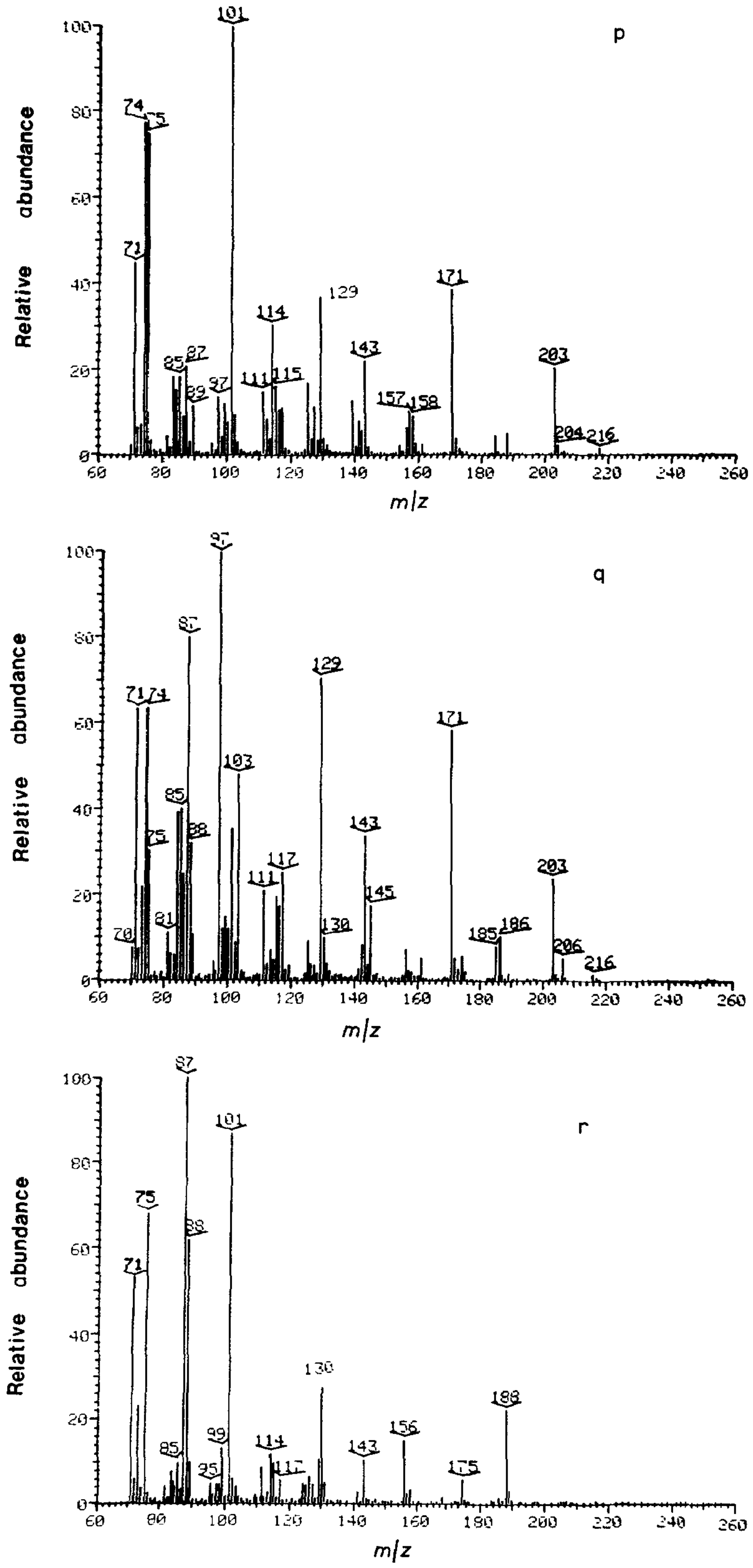
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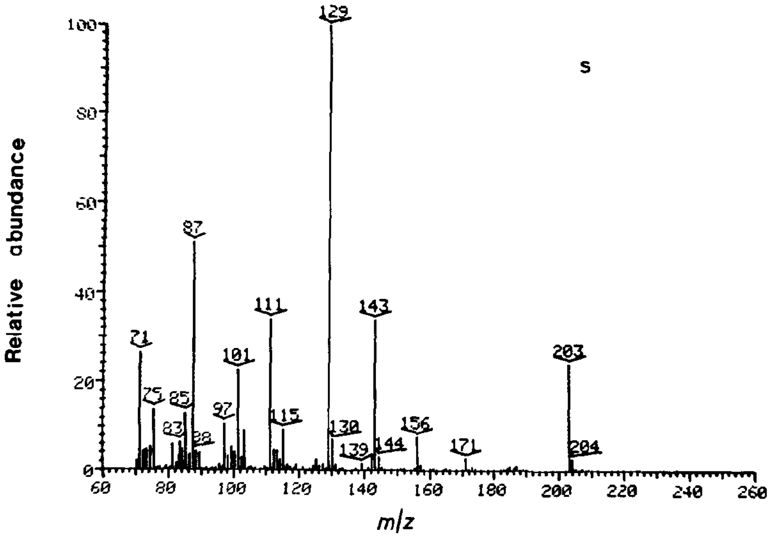
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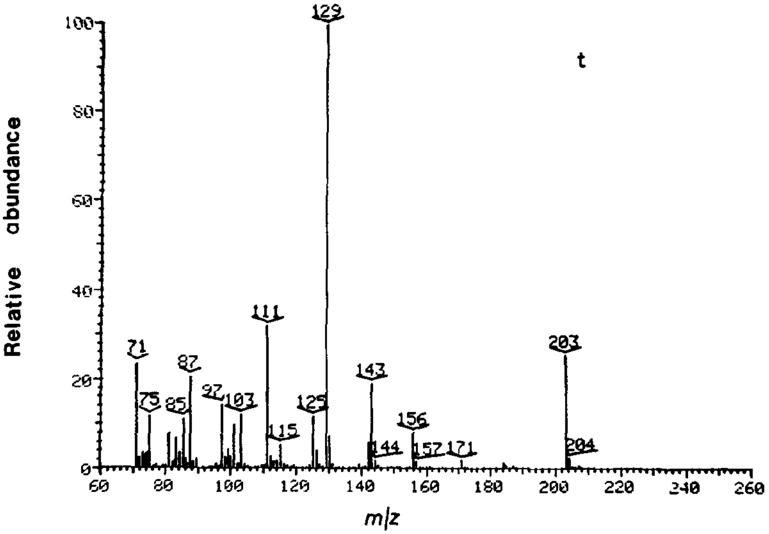
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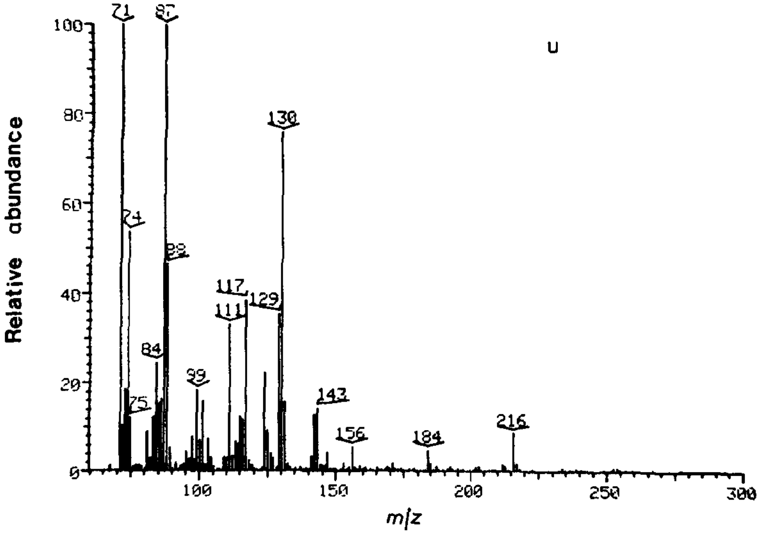
u
Relative abundance
m/z
0
20
40
60
80
100
100
150
200
250
300
71
87
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74
88
117
129
111
84
99
75
143
156
184
216

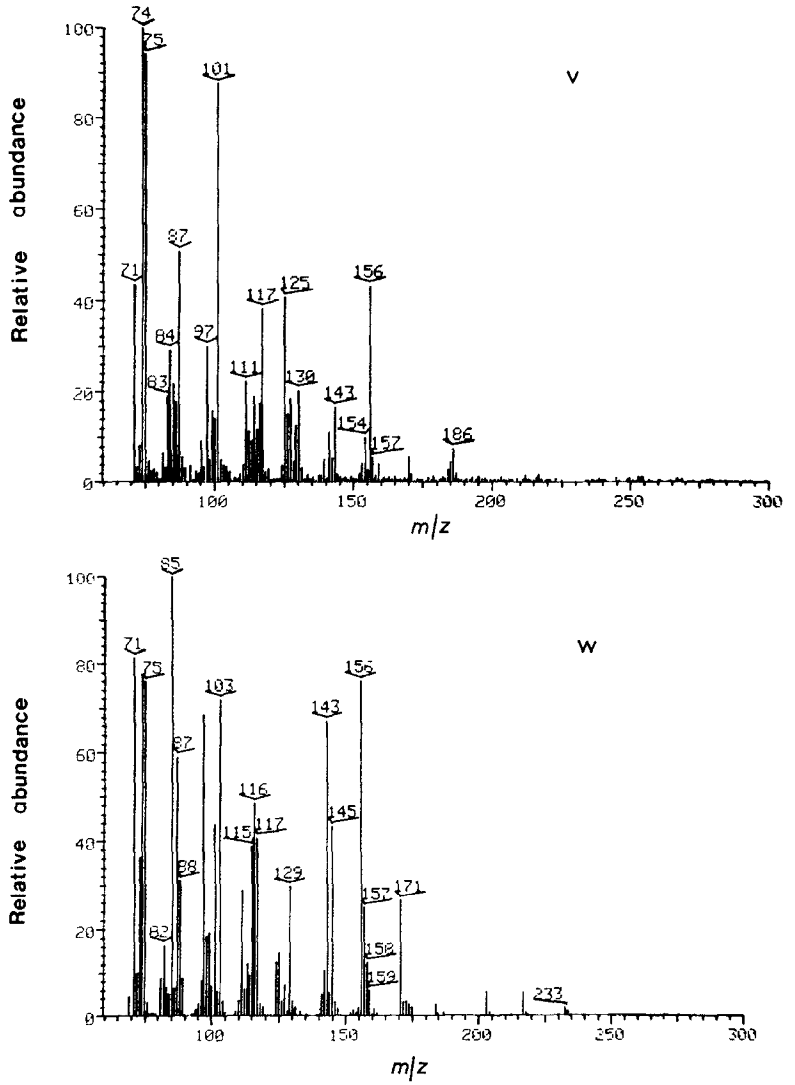
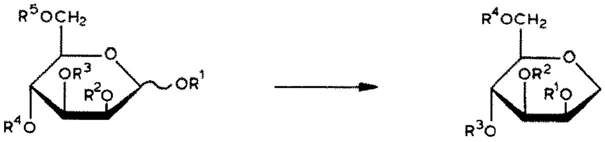
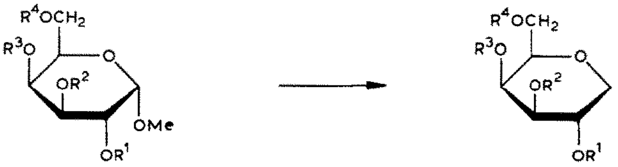


Fig. 1. E.i. mass spectra of partially methylated and acetylated anhydro-D-hexitols: (a) 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol (**2**); (b) 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-mannitol (**13**); (c) 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-galactitol (**15**); (d) 2,5-anhydro-1,3,4,6-tetra-*O*-methyl-D-hexitol, Peak 1 (**33**) and (e) Peak 2 (**33**); (f) 2-*O*-acetyl-1,5-anhydro-3,4,6-tri-*O*-methyl-D-glucitol (**4**); (g) 3-*O*-acetyl-1,5-anhydro-2,4,6-tri-*O*-methyl-D-glucitol (**6**); (h) 4-*O*-acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-glucitol (**8**); (i) 6-*O*-acetyl-1,5-anhydro-2,3,4-tri-*O*-methyl-D-glucitol (**10**); (j) 5-*O*-acetyl-1,4-anhydro-2,3,6-tri-*O*-methyl-D-glucitol (**11**); (k) 2-*O*-acetyl-1,5-anhydro-3,4,6-tri-*O*-methyl-D-mannitol (**20**); (l) 3-*O*-acetyl-1,5-anhydro-2,4,6-tri-*O*-methyl-D-mannitol (**21**); (m) 4-*O*-acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-mannitol (**22**); (n) 6-*O*-acetyl-1,5-anhydro-2,3,4-tri-*O*-methyl-D-mannitol (**23**); (o) 2-*O*-acetyl-1,5-anhydro-3,4,6-tri-*O*-methyl-D-galactitol (**28**); (p) 3-*O*-acetyl-1,5-anhydro-2,4,6-tri-*O*-methyl-D-galactitol (**29**); (q) 4-*O*-acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-galactitol (**30**); (r) 6-*O*-acetyl-1,5-anhydro-2,3,4-tri-*O*-methyl-D-galactitol (**31**); (s) 3-*O*-acetyl-2,5-anhydro-1,4,6-tri-*O*-methyl-D-hexitol (Peak 1) (**34**), and (t) Peak 2 (**34**); (u) 2,6-di-*O*-acetyl-1,5-anhydro-3,4-di-*O*-methyl-D-glucitol (**35**); (v) 3,6-di-*O*-acetyl-1,5-anhydro-2,4-di-*O*-methyl-D-glucitol (**36**); and (w) 4,6-di-*O*-acetyl-1,5-anhydro-2,3-di-*O*-methyl-D-glucitol (**37**). Ionization voltage, 70 eV; ion-source temperature, 200°; and accelerating voltage, 3 kV.



16 $R^1 = R^3 = R^4 = R^5 = Me, R^2 = H$
17 $R^1 = R^2 = R^4 = R^5 = Me, R^3 = H$
18 $R^1 = R^2 = R^3 = R^5 = Me, R^4 = H$
19 $R^1 = R^2 = R^3 = R^4 = Me, R^5 = H$

20 $R^1 = Ac, R^2 = R^3 = R^4 = Me$
21 $R^1 = R^3 = R^4 = Me, R^2 = Ac$
22 $R^1 = R^2 = R^4 = Me, R^3 = Ac$
23 $R^1 = R^2 = R^3 = Me, R^4 = Ac$



24 $R^1 = H, R^2 = R^3 = R^4 = Me$
25 $R^1 = R^3 = R^4 = Me, R^2 = H$
26 $R^1 = R^2 = R^4 = Me, R^3 = H$
27 $R^1 = R^2 = R^3 = Me, R^4 = H$

28 $R^1 = Ac, R^2 = R^3 = R^4 = Me$
29 $R^1 = R^3 = R^4 = Me, R^2 = Ac$
30 $R^1 = R^2 = R^4 = Me, R^3 = Ac$
31 $R^1 = R^2 = R^3 = Me, R^4 = Ac$

obtained from a series of methyl tri-*O*-methyl-D-mannopyranosides (**16–19**), prepared in conjunction with ongoing studies on urinary mannosidosis oligosaccharides. The positions of the methyl groups were previously established by the alditol acetate procedure[1]. The e.i.–m.s. of **20–23*** provided unique spectra for each positional isomer. These spectra, however, were identical to the corresponding 1,5-anhydro-D-glucitol analogs **4**, **6**, **8**, and **10**.

The analogous 1,5-anhydro-D-galactitol compounds **28–31** were generated by partial methylation of methyl α-D-galactopyranoside (**14**) under conditions that resulted in the formation of a series of methyl tri-*O*-methyl-α-D-galactosides (**24–27**). The reaction mixture was acetylated and the positions of methylation determined by g.l.c. and g.l.c.–m.s. The retention times and e.i. mass spectra were, therefore, compared with published data for acetylated methyl *O*-methyl-D-galactosides[2], giving the degree of methylation and position of the methyl groups. The mixture was then submitted to reductive cleavage, and the products were purified and identified by g.l.c.–m.s. The individual components of the mixture were characterized by the similarity of their e.i. mass spectrum with that of the 1,5-anhydro-D-mannitol and 1,5-anhydro-D-glucitol analog. The 1,5-anhydro-D-galactitol compounds **28–31** had the same distribution of mono-*O*-acetyltri-*O*-methyl

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*After submission of this manuscript, the mass spectra of **13**, **17**, **19**, and **23** have been reported[17] in tabular form. The e.i.–m.s. fragments and relative intensities are comparable, although instrumental operating parameters were not reported.

TABLE II

PEAK-AREA DISTRIBUTION OF METHYL MONO-*O*-ACETYLTRI-*O*-METHYL-D-GALACTOPYRANOSIDES FORMED BY PARTIAL METHYLATION AND SUBSEQUENT ACETYLATION OF METHYL α-D-GALACTOPYRANOSIDE[a]

Derivative	*Total distribution (%)*	
	Methyl α-D-galactopyranoside derivative	*1,5-Anhydro-D-galactitol derivative*
2-*O*-Ac, $(OMe)_3$	24 (**24**)	22 (**28**)
3-*O*-Ac, $(OMe)_3$	41 (**25**)	37 (**29**)
4-*O*-Ac, $(OMe)_3$	17 (**26**)	18 (**30**)
6-*O*-Ac, $(OMe)_3$	18 (**27**)	23 (**31**)

[a] As compared with the relative amounts of reductively cleaved products, *i.e.*, mono-*O*-acetyl-1,5-anhydro tri-*O*-methyl-D-galactitol derivatives. The peak areas were determined by g.l.c. using flame-ionization detection. The components were characterized by g.l.c.–m.s.

groups as the previously characterized starting methyl galactosides (Table II). The 6-*O*-acetyl derivative **31** was also shown to be identical by g.l.c.–m.s. with the corresponding compound obtained by reductive cleavage of stachyose (**32**).

Reductive cleavage of standard tri- and tetra-saccharides. — Reductive depolymerization of permethylated raffinose, stachyose (**32**), and melezitose, followed by acetylation produced results consistent with their known structures. The results obtained for permethylated stachyose are presented in Fig. 2. The presence of D-fructose in **32** was clearly indicated by a characteristic g.l.c. doublet. The retention times of the doublet proved to be identical with those of the doublet obtained on reductive cleavage of permethylated D-fructose and raffinose. Mass spectral analysis of each peak, and their similarity, supported the structures of 2,5-anhydro-1,3,4,6-tetra-*O*-methyl-D-hexitol isomers (**33**)*. The other three peaks of the stachyose g.l.c. profile were easily identified as the D-galactitol derivatives **15** and **31**, and the D-glucitol derivative **10**. These data were consistent with structure **32**, but gave no information on the linkages Gal→Gal or Gal→Glc.

Reductive cleavage of permethylated melezitose, followed by acetylation and g.l.c.–m.s. provided three g.l.c. peaks. One peak, the D-glucitol derivative **2**, originated from the terminal group. The other two peaks were eluted as a doublet and had identical mass spectra, indicating a single point of acetylation. This is consistent with the doublet obtained from D-fructose and corresponds to 3-*O*-acetyl-2,5-anhydro-1,4,6-tri-*O*-methyl-D-hexitol isomers (**34**). Thus, the structure Glc-(1→3)-Fru-(2→1)-Glc may be deduced directly from the g.l.c.–m.s. data. The generation of two g.l.c. peaks for D-fructose and neuraminic acid[19] is consistent with the reported mechanism of reductive cleavage which involves a cyclic oxonium ion and the formation of products independent of the configuration of the starting

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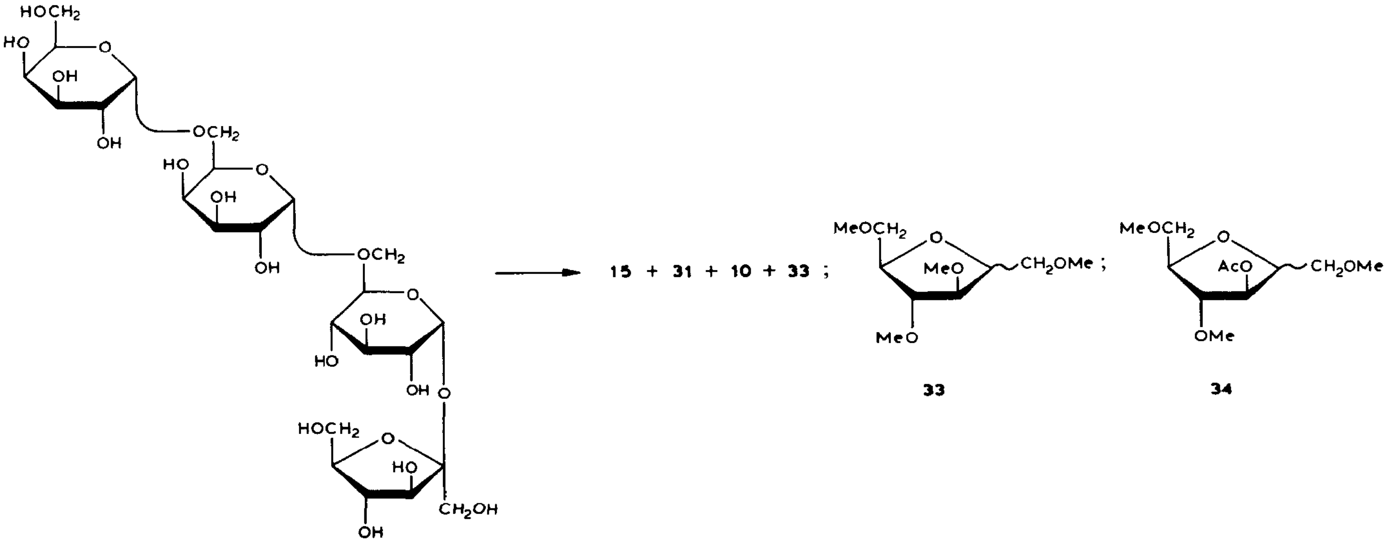
*After submission of this manuscript, a report giving similar results for D-fructofuranosyl residues and assigning a structure to each of the chiral products was published[18]. The e.i.–m.s. fragments and relative intensities are comparable to those described herein, although instrumental operating parameters were not reported.


HOCH2
HO
OH
OH
OCH2
HO
OH
OH
OCH2
OH
HO
OH
O
HOCH2
HO
CH2OH
OH
15 + 31 + 10 + 33 ;
MeOCH2
MeO
CH2OMe ;
MeO
MeOCH2
AcO
CH2OMe
OMe

32

33

34

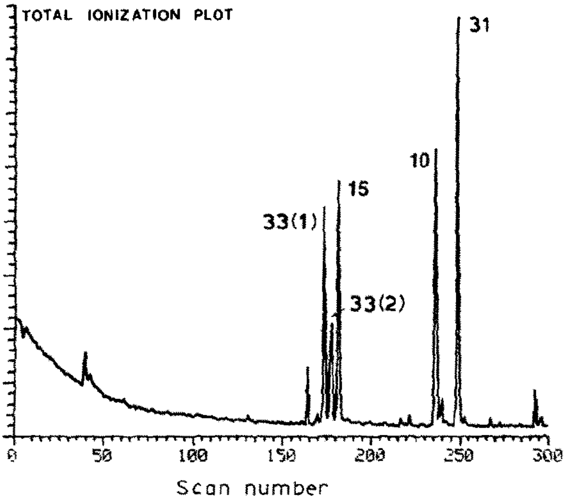
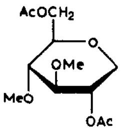


Fig. 2. G.l.c.-m.s. analysis of the reductively cleaved, acetylated products of permethylated stachyose. Gas chromatography conditions as described in the Experimental section; DB-225 capillary column.

glycoside[7]. Thus, the two isomeric peaks must represent the *Re* and *Se* hydride addition products. The successful chromatographic resolution of these dimers is probably facilitated by the bulky CH_2OMe- and CO_2Me-, from the D-fructosyl and neuraminyl derivatives, respectively.

Characterization of di-O-acetyl-1,5-anhydrodi-O-methylhexitol branch residues. — The characterization of branch residue(s) in oligosaccharides requires the preparation of a reference series of di-*O*-acetyl-1,5-anhydrodi-*O*-methylhexitols. Six isomeric structures are required for each branch residue. To date, we have obtained reference data for three D-glucose branch residues. The commercially available starting compounds were prepared as methyl glycosides, and then acetylated and reductively cleaved to provide the reference compounds **35–37**. The retention times and mass spectra are presented in Table I and Fig. 1, respectively.

Study of the structure of a membrane oligosaccharide. — A complex series of conjugated, D-glucose-containing oligosaccharides is located in the periplasmic space of gram-negative bacteria[20]. Deconjugation and purification by liquid chromatography have shown the oligosaccharides to consist of three highly branched chains of seven, eight, and nine residues. To study the linkage and branching pattern of these compounds, fractions were reduced, permethylated, reductively cleaved, acetylated, and analyzed by g.l.c.–m.s. The g.l. chromatograms showed four identical peaks for each of the oligomers with small variations in concentration. Three of the g.l.c. peaks were easily identified as **2**, **4**, and **35**. The fourth g.l.c. peak was a minor constituent and identified as 2-*O*-acetyl-1,3,4,5,6-penta-*O*-methylglucitol deriving from the reducing-end residue. This 2-*O*-acetylglucitol compound had been previously characterized when these oligomers



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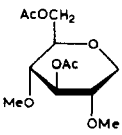

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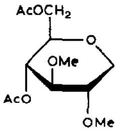

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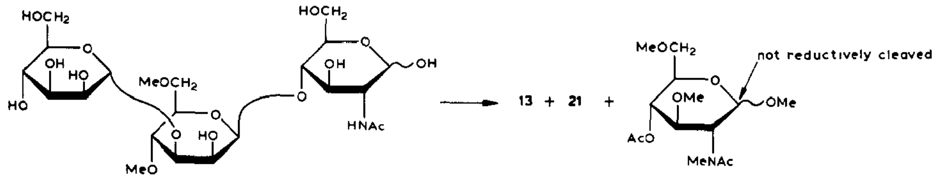

38 **39**

were analyzed by the alditol acetate procedure. Confirmation of this assignment was supported by reductive cleavage and alditol acetate analysis of kojibiitol[19]. Thus, each oligomer was found to consist of a terminal D-glucosyl group and D-glucosyl residues linked at O-2 and branching at O-2 and -6. Furthermore, the characterization of the reducing-end fragment indicates the adjacent D-glucosyl residue to be linked at O-2.

Study of the structure of a mannosidosis trisaccharide. — Mannosidosis is the consequence of a lysosomal enzyme defficiency that results in the excretion of high concentrations of D-mannose-containing oligosaccharides. The structure of the oligosaccharide observed in highest concentration has been shown[21] to be Man-(1→3)-Man-(1→4)-GlcNAc (**38**). Isolation of a fraction of this material by l.c., followed by permethylation and reductive cleavage resulted in the formation of three compounds that were identified by g.l.c.–m.s. as **13**, **21**, and methyl 4-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucopyranoside (**39**). The spectrum of the last-named compound proved to be identical with that described earlier[2]. It is noteworthy that the methyl glycoside linkage of the amino sugar was not reductively cleaved under the conditions established for the reduction of other glycoside linkages; this observation was supported by control studies with permethylated 2-acetamido-2-deoxy-D-glucose. It remains to be determined whether this is a consequence of diminished C-1 electrophilicity or stereochemical inhibition. This lack of reactivity may be helpful for the analysis of amino sugar-containing oligosaccharides.

In conclusion, two sets of data have been obtained for further evaluation of the applicability of reductive cleavage for the analysis of linkages in oligosaccharides. The aldohexitol residues yielded single g.l.c. peaks as the corresponding 1,5-anhydroalditol derivative and the ketose residues yielded a pair of 2,5-anhydrohexitol derivatives for all components reported herein. All compounds exhibited excellent g.l.c. characteristics in 30-m capillary columns containing the liquid phase DB-225. These excellent g.l.c. properties are necessary for more complex oligosaccharides. The mass spectra of each positional isomer in the methylated mono- and di-acetate series provided unique patterns for an unambiguous assignment of the location of acetylation point and, hence, information on the linkage position. These same positional isomers provided identical mass spectra within the hexose series. The combined methods of partial reductive depolymerization, fluorescence derivatization combined with liquid-chromatographic separation, and oligomer characterization by complete reductive cleavage and g.l.c.–m.s. show considerable promise for a more specific and sensitive analytical approach to polysaccharide-structure determination[10].

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