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MASS-SPECTRAL STUDIES OF ISOMERIC D-RIBOFURANOSYLRIBITOL DISACCHARIDES FROM THE CAPSULAR POLYSACCHARIDES OF *Haemophilus influenzae* TYPE b AND *Escherichia coli* K 100

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ABSTRACT

Two isomeric D-ribofuranosylribitols, derived from capsular polysaccharides of *Haemophilus influenzae* type b and *Escherichia coli* K 100, were methylated or acetylated, and the products analyzed by gas–liquid chromatography–mass spectrometry. The marked difference in the mass spectra of the methyl ethers of these disaccharides allowed clear distinction between 1- and 2-*O*-D-ribofuranosylribitol. The base peak, *m/e* 101, for the methylated 1-*O*-D-ribofuranosylribitol was characteristic for this disaccharide; its isomer, the (1 → 2)-linked species, has a base peak at *m/e* 57. The difference in the base peaks is attributable to fragmentation of the methylated ribitol, as both spectra display common ions characteristic of the methylated D-ribofuranosyl group. For the acetylated disaccharides, the mass spectra displayed common ions characteristic of the acetylated D-ribofuranosyl group. However, no ions similar to those found for the methylated ribitol allowed ready differentiation between the two acetates. Instead, their spectra displayed similar ions, differing somewhat in relative abundance; the M—1 ion, *m/e* 577, was obtained for both. Comparison of the relative abundance of *m/e* 139, 259, and 303 in the spectra of the two acetates did allow distinction between them.

INTRODUCTION

Haemophilus influenzae type b is a serious pathogen, responsible for a variety of invasive diseases of childhood, most commonly meningitis[1]. In addition to being a known virulence factor[2], its capsular polysaccharide is serologically and immunogenically cross-reactive[3] with the capsular polysaccharide of *Escherichia coli* K 100, a non-invasive organism that is not associated with disease in humans[4]. Structurally, both polysaccharides are phosphoric diester-linked D-ribofuranosylribitol polymers that differ solely in the position of the glycosidic linkage. The dimeric unit of *Haemophilus*

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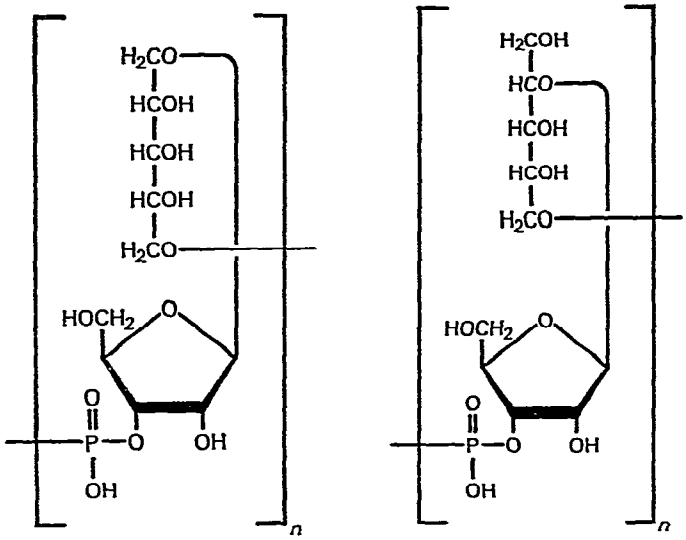

influenza type b polymer (**1**) is 1-*O*-*β*-D-ribofuranosylribitol[5,6] and that of *Escherichia coli* K 100 polymer (**2**) is 2-*O*-*β*-D-ribofuranosyl-D-ribitol[7].

During the course of structural studies on these capsular polysaccharides, the mass spectra of the fully methylated and of the fully acetylated disaccharides were examined, and characteristic fragment-ions were discerned that allowed distinction between the two isomers. With regard to the acetates, a structure could not be assigned after analyzing only one isomer. Analysis of the mass spectra of both acetates, and comparison with the mass spectra of the methyl ethers permitted structural assignment.

EXPERIMENTAL

Derivatization. — The D-ribofuranosylribitol disaccharides were prepared from the capsular polysaccharides of *Haemophilus influenzae* type b and *Escherichia coli* K 100 as described[7]. Samples were acetylated by heating in an excess of 1:1 (v/v) acetic anhydride–pyridine for 15 min at 150°. The excess of the reagents was then removed by evaporation at room temperature under a stream of nitrogen. Samples were methylated by using the procedure of Kärkkäinen[8]. For analysis, the derivatized disaccharides were dissolved in a suitable volume of acetone.

Mass spectrometry. — Mass spectra were recorded with an LKB 2091 Gas Chromatograph–Mass Spectrometer equipped with an LKB 2130 Data System. Electron-impact, mass spectra were scanned from samples introduced *via* a 25-m, glass-capillary column, wall-coated with SE-30 as the stationary phase (LKB 2101-402). A 2-*μ*L sample was injected through a glass splitter, at 200°, set at a 1:50 split ratio, onto the column maintained at 150°. After an initial, 4-min, isothermal period, the column temperature was elevated at $20°.min^{-1}$ to a final temperature of 300°. The flow of

helium through the column at 150° was 6.2 mL.min^{-1}. The jet separator was at 200°, and the ion-source temperature was 180°; the ionizing potential was 70 eV, and the ionizing current was 50 μA.

RESULTS AND DISCUSSION

Gas–liquid chromatographic analysis. — The total-ion tracing for the g.l.c. of permethylated 2-*O*-β-D-ribofuranosyl-D-ribitol is presented in Fig. 1. The elution

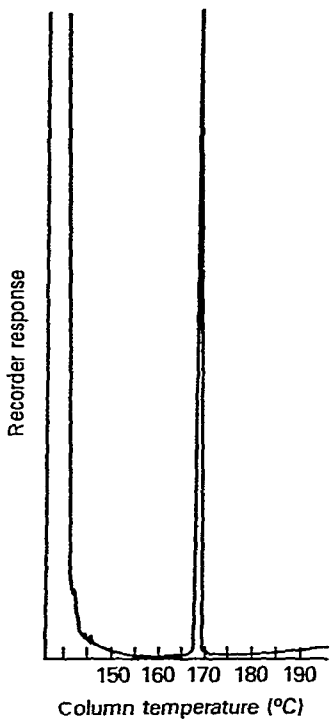

Fig. 1. Total-ion tracing for separation of hepta-*O*-methyl-2-*O*-β-D-ribofuranosyl-D-ribitol on a capillary column of SE-30.

temperature of the methylated disaccharides was 168° at the peak maximum, with a 4-s peak-width at half peak-height. Under the same column conditions, the peracetylated disaccharides were eluted at 280°, and displayed a slightly broader (11-s at half peak-height), but still symmetrical, peak.

Mass-spectral analysis of the methylated disaccharides. — Fragmentation patterns of the permethylated 1- and 2-*O*-β-D-ribofuranosylribitols were similar to those either reported[8] or predicted[9,10]. The intensities of the fragment ions of the permethylated 1-*O*-β-D-ribofuranosylribitol, presented in Fig. 2A, are similar to those reported[8]. Fig. 2B presents the mass spectrum of permethylated 2-*O*-β-D-ribofuranosyl-D-ribitol. Common to both spectra are the fragment ions at *m/e* 175, 143 (175–32), and 111 (143–32), the D-ribofuranosyl ions formed by cleavage of the glycosidic

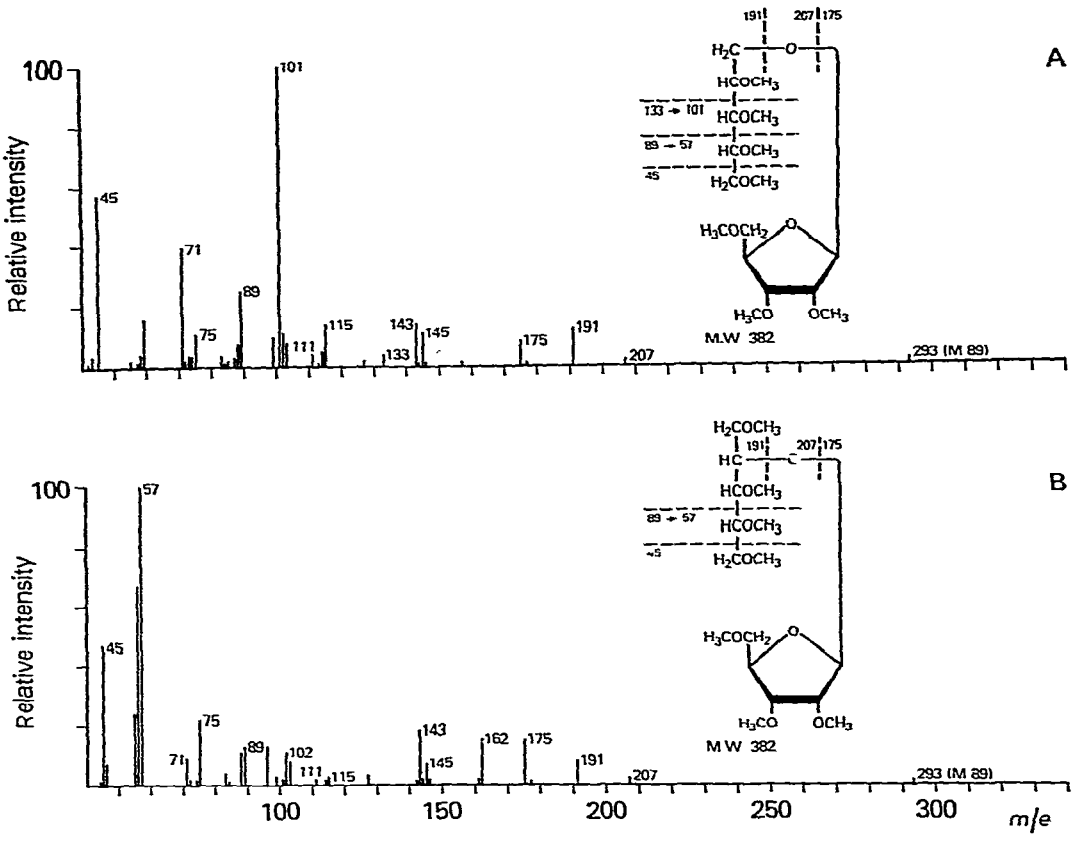

Fig. 2. Mass spectra of the permethylated D-ribofuranosylribitol isomers. [A, Hepta-*O*-methyl-1-*O*-*β*-D-ribofuranosyl-D-ribitol; B, hepta-*O*-methyl-2-*O*-*β*-D-ribofuranosyl-D-ribitol. The mass-spectral conditions are described in the Experimental section. Schematic representations of the fragmentation patterns are indicated on the structural formulas.]

bond followed by elimination of one molecule, and two molecules, of methanol. The molecular ion and the M–45 ion were not present in these spectra, but the ion at *m/e* 293 (0.8%) coincides with the M–89 ion.

The most striking difference between the spectra of the two methylated isomers was that of the base peaks. The base peak for 1-*O*-*β*-D-ribofuranosylribitol was *m/e* 101. Presence of *m/e* 133 and the intensity of *m/e* 101 indicated primary cleavage of the C-2–C-3 bond in the methylated ribitol residue; the ion resulting from this cleavage, *m/e* 133, loses methanol, to yield the base peak, *m/e* 101. Degradation of the ribofuranosyl oxonium ion does not significantly contribute to the relative intensity of *m/e* 101 (compare Figs. 2A and 2B).

In the mass spectrum of methylated 2-*O*-*β*-D-ribofuranosyl-D-ribitol, the base peak is *m/e* 57. Fragmentation of the C-2–C-3 bond and formation of the *m/e* 133 ion would be unlikely in the methyl ethers of 2- or 3-substituted ribitols[10]. The lack of significant intensity at *m/e* 101 and 45 indicates that little bond cleavage at C-2–C-3 and C-4–C-5 occurred relative to cleavage at C-3–C-4; the latter cleavage in the

2-substituted D-ribitol would yield *m/e* 89, which rapidly loses methanol to yield *m/e* 57.

Additionally, comparison of the intensities for *m/e* 175 and 191 for each spectrum suggests contribution to the abundance of *m/e* 175 by the permethylated, 2-*O*-substituted D-ribitol. The *m/e* 191 ion results from cleavage of the glycosidic bond without retention of the glycosidic oxygen atom by the methylated ribitol residue. This fragmentation is common to both methylated isomers. The *m/e* 207 ion is formed by cleavage of the D-ribofuranosyl group from the ribitol residue with retention of the glycosidic oxygen atom by the ribitol residue. In the 2-*O*-substituted D-ribitol, this ion could form a furanoid ring through bond formation between that glycosidic oxygen atom (at C-2) and C-5 of the ribitol residue. Elimination of methanol from this ion would result in an oxonium ion, *m/e* 175. Thus, contributions to the abundance of *m/e* 175 could result from fragmentations of both the D-ribofuranosyl group and the ribitol residue. This cyclization of the ribitol would be difficult to envisage for the permethylated 1-*O*-substituted ribitol. The five-membered-ring oxonium ion would be thermodynamically favored over the analogous ion having a six-membered ring[10].

Other, low-intensity, but characteristic, fragmentations for these methylated disaccharides include the ribitol ion, *m/e* 191, losing methanol in two steps to yield, first, *m/e* 159 (191—32) and, second, *m/e* 127 (159—32). Cleavage of the C-1–C-2 bond of the furanoid ring, with subsequent loss of acetic acid, results in *m/e* 115 (175–60).

Mass-spectral analysis of the acetylated disaccharides. — The fragmentation patterns for the peracetylated 1-*O*-β-D-ribofuranosyl-D-ribitol and 2-*O*-β-D-ribofuranosyl-D-ribitol are explicable by the principles set forth by Biemann *et al.*[11] and Kochetkov and Chizhov[10]. The spectra of the peracetylated 1-*O*-β-D-ribofuranosyl-D-ribitol (Fig. 3A) and 2-*O*-β-D-ribofuranosyl-D-ribitol (Fig. 3B) both display an M–1 ion at *m/e* 577. The more-intense *m/e* 505 (M–73) results from loss of acetoxylated C-5 from either the D-ribofuranosyl group or the ribitol residue. More so than with the methylated derivatives, various fragmentation series common to both spectra made difficult the interpretation of the spectra. Only comparison of the relative intensities for certain ions provided the clue to understanding the structure of each acetylated isomer.

Both spectra display ions indicative of an acetylated D-ribofuranosyl group. Cleavage of the glycosidic bond, and loss of the acetylated ribitol residue leads to the A series of fragments[11]. Ion A_1 (*m/e* 259) is the D-ribofuranosyl oxonium ion, which loses two molecular proportions of acetic acid, from C-3 and then from C-2, to yield A_2 (*m/e* 139). The A_2 ion subsequently eliminates ketene, to yield A_3 (*m/e* 97), an oxonium ion.

Elimination of C-1 and the ether oxygen atom in the D-ribofuranosyl oxonium ion, with simultaneous elimination of acetic acid, produces the first ion of the B series, B_1 (*m/e* 170). Ketene elimination from B_1 results in B_2 (*m/e* 128), and, finally, the loss of acetic acid from B_2 results in B_3 (*m/e* 68). The C series of fragmentations results from C-2, C-3, and C-4 of the D-ribofuranosyl group, and differs in mass, due to sequential, ketene eliminations (C_1, *m/e* 157; C_2, *m/e* 115; C_3, *m/e* 73). The D series of fragmentations arises due to elimination of ketene, followed by loss of acetic acid from the A_1

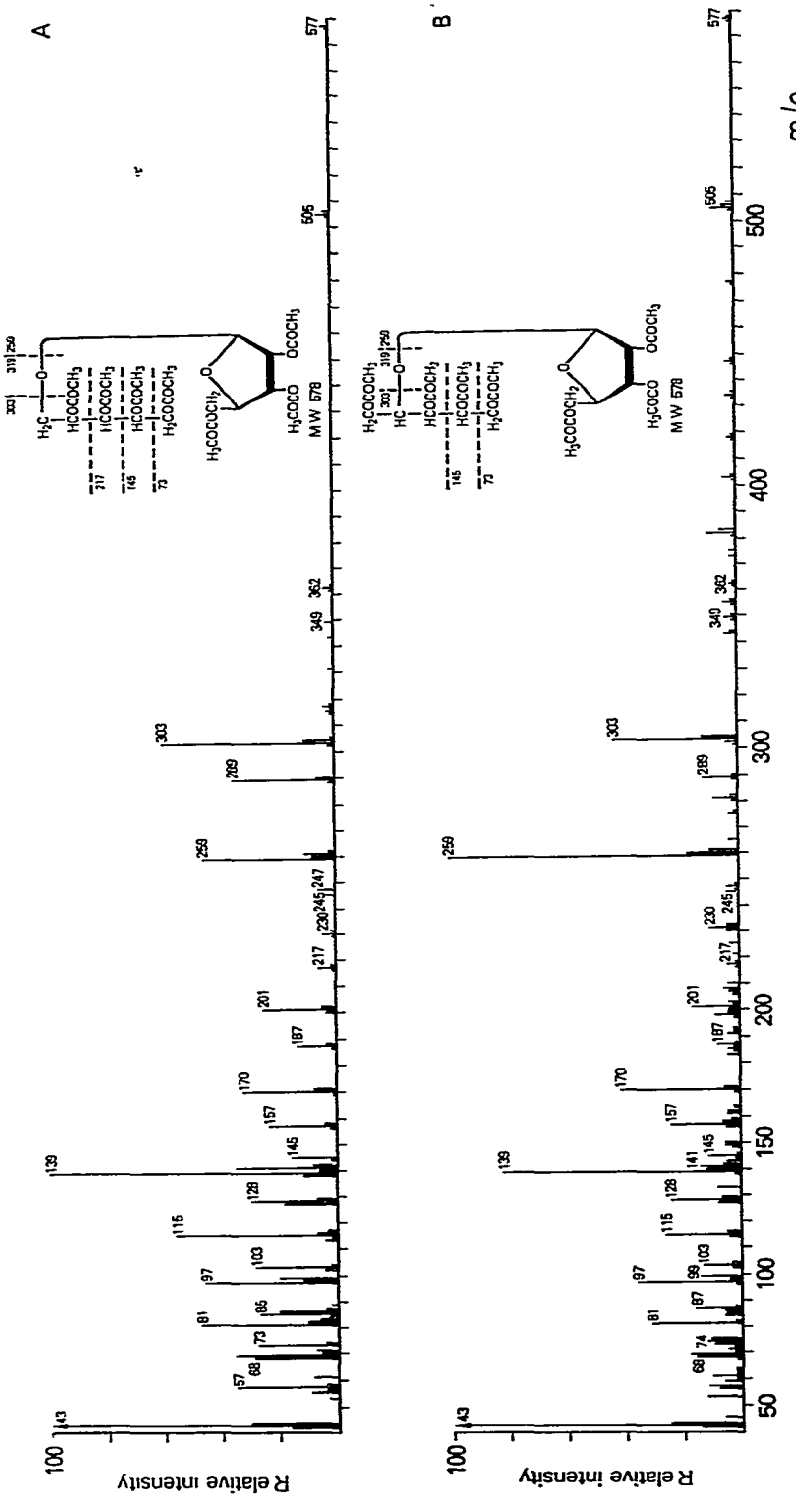

Fig. 3. Mass spectra of the peracetylated D-ribofuranosylribitol isomers. [A, Hepta-*O*-acetyl-1-*O*-*β*-D-ribofuranosyl-D-ribitol; B, hepta-*O*-acetyl-2-*O*-*β*-D-ribofuranosyl-D-ribitol. The base peak was chosen as the next most abundant ion[11] to *m/e* 43. Mass-spectral conditions are described in the Experimental section. Schematic representations of the fragmentation patterns are indicated on the structural formulas.]

ion (D_1, *m/e* 259; D_2, *m/e* 217; D_3, *m/e* 157). The E series, characteristic of polyacetates, results from the formation of tri- and di-acetyloxonium ions (E_1, *m/e* 145; E_2, *m/e* 103; E_3, *m/e* 85). Ions of similar intensity and mass establish the D-ribofuranosyl group in both of the peracetylated disaccharides.

The fragmentation of the acetylated, substituted ribitols commences with the *m/e* 319 ion, the result of cleavage of the glycosidic bond and retention of the glycosidic oxygen atom on the ribitol acetate. For the acetylated, 1-substituted ribitol, rapid loss of C-1 as formaldehyde leads to an ion having *m/e* 289. Through elimination of ketene, and loss of acetic acid, this ion can form a five-membered ring, *m/e* 187; further loss of ketene and acetic acid then yields *m/e* 85. In addition to this fragmentation pathway, *m/e* 245 can result from loss of C-5 from the ribitol. Contributions from the second pathway do not appear to be significant.

For the acetylated, 2-substituted D-ribitol, the *m/e* 319 ion can more readily cyclize, and lose acetic acid, to form *m/e* 259 (A_1 ion). Through loss of two molecular proportions of acetic acid, *m/e* 259 gives rise to *m/e* 139 (A_2 ion). As the *m/e* 319 ion can more readily cyclize in the 2-substituted D-ribitol derivative, the abundance of the *m/e* 259 and 139 ions (relative to that of *m/e* 289) appear to be greater for the peracetylated 2-*O*- than for the 1-*O*-substituted ribitol (see Fig. 3). The abundance of *m/e* 289 could also arise through a fragmentation pathway similar to that observed for its permethylated counterpart. Cleavage of the C-3–C-4 linkage of the ribitol of the 2-*O*-β-D-ribofuranosyl-D-ribitol could lead to formation of *m/e* 433 and 145 (E_1 ion). The *m/e* 433 could sequentially lose acetic acid and ketene, to yield *m/e* 331 (433–102), with the latter ion losing another molecular proportion of ketene, to yield *m/e* 289 (331–42).

REFERENCES

1 J. B. Robbins, R. Schneerson, M. Argaman, and Z. T. Handzel, *Ann. Intern. Med*, 78 (1973) 259–269.
2 B. D. Davis, R. Dulbecco, H. N. Eisen, H. S. Ginsberg, and W. B. Wood, *Microbiology* (2nd edn.), Harper and Row, New York, 1973, pp. 633–635.
3 R. L. Myerowitz, Z. T. Handzel, R. Schneerson, and J. B Robbins, in S. H. W. Sell and D. T. Karzon (Eds.), *Hemophilus influenzae*, Vanderbilt University Press, Nashville, 1973, pp. 71–78.
4 J. B. Robbins, R. Schneerson, J. C. Parke, T.-Y. Liu, Z. T. Handzel, I. Orskov, and F. Orskov, in R. F. Beers and E. Bassett (Eds), *The Role of Immunological Factors in Infectious, Allergic, and Autoimmune Processes*, Raven Press, New York, 1976, pp. 103–120.
5 R. M. Crisel, R. S. Baker, and D. E. Dorman, *J. Biol. Chem*, 250 (1975) 4926–4930.
6 P. J. Garegg, B. Lindberg, and B. Samuelsson, *Carbohydr. Res.*, 58 (1977) 219–221.
7 W. Egan, F.-P. Tsui, R. Schneerson, and J. B. Robbins, *J. Biol. Chem.*, in press.
8 J. Kärkkäinen, *Carbohydr. Res.*, 14 (1970) 27–33.
9 P. Branefors-Helander, C Erbing, L. Kenne, and B Lindberg, *Acta. Chem. Scand., Ser. B* 30 (1976) 276–277.
10 N. K. Kochetkov and O. S. Chizhov, *Adv. Carbohydr. Chem*, 21 (1966) 39–93.
11 K. Biemann, D. C. DeJongh, and H. K. Schnoes, *J. Am. Chem. Soc.*, 85 (1963) 1763–1771.