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N-FORMYLKANSOSAMINYL-(1→3)-2-*O*-METHYL-D-RHAMNOPYRANOSE: THE TYPE-SPECIFIC DETERMINANT OF SEROVARIANT 14 OF THE *Mycobacterium avium* COMPLEX

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ABSTRACT

Significant occurrences of pulmonary and disseminated infections due to "atypical" mycobacteria have again focused attention on the *Mycobacterium avium* serocomplex. An examination of the major surface glycopeptidolipid antigen of one of the more prominent and one of the first described serovariants, the Boone strain (serovariant 14), has shown that it is characterized by a unique terminal disaccharide unit, *N*-formylkansosaminyl-(1→3)-2-*O*-methyl-D-rhamnopyranose. The structure of the entire oligoglycosyl hapten is *N*-formylkansosaminyl-(1→3)-2-*O*-methyl-α-D-rhamnopyranosyl-(1→3)-2-*O*-methyl-α-L-fucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-6-deoxy-L-talose. We had previously described a 4,6-dideoxy-2-*O*-methyl-3-*C*-methyl-4-(2-methoxy propanamido)-L-mannopyranose (an *N*-acylkansosamine) as the characteristic sugar of the trehalose-containing lipo-oligosaccharide antigens of strains of *Mycobacterium kansasii*. Thus, a derivative of this sugar is now found to occur in other mycobacteria as part of other antigens. The other distinguishing feature of the structure is the presence of D-rhamnopyranose, which occurs only sparingly in Nature.

INTRODUCTION

The resurgence of interest in "atypical" mycobacteria stems from their occurrence as opportunistic pathogens in many patients with acquired immunodeficiency syndrome (AIDS)[1–4], although they have long been associated with pulmonary and other organ infections[5]. Foremost among these mycobacteria are serovariants of the *Mycobacterium avium–Mycobacterium intracellulare* complex (the *M. avium* complex)[1]. The surface antigens that differentiate these serovariants are glycopeptidolipids, related to "mycoside C", and, accordingly, composed of a glycosylated lipopeptide "core", fatty acyl-D-Phe-D-alloThr-D-Ala-L-alaninyl-*O*-(3,4-di-*O*-methyl-α-L-rhamnopyranoside), to which a haptenic oligosaccharide is linked at the threonine substituent; this oligoglycosyl unit is the source of type specificity[4,6–8]. We have been examining these haptens from several aspects, notably structurally, because,

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although mere tri-, tetra-, or penta-saccharides possessing an invariant α-L-rhamnopyranosyl-(1→2)-6-deoxy-L-talose reducing-end unit, they show extraordinary structural diversity and novelty at the nonreducing termini.

Early studies on the distribution of serovariants of the *M. avium* complex in the U.S., Great Britain, and Australia showed that serovariant 14 is among the most common of those isolated from sputa and cervical lymph-nodes[9]. In a later study[10], it was shown that, of 690 *M. avium* serovariants isolated from patients, 28 were of the Boone serovariant, ranking it tenth in frequency of isolation. However, serovariant 14 has rarely been isolated from cases of disseminated mycobacterioses associated with AIDS; as pointed out previously, the majority are of the serovariant 1, 4, or 8 designation[1,4]. In this communication, we describe the specific hapten of serovariant 14, which may best exemplify the diversity of structures created by these mycobacteria as they contend with the immune system and other environments.

RESULTS AND DISCUSSION

Isolation of the hapten as an oligoglycosyl alditol.— The characteristic glycopeptidolipid (GPL) of serovariant 14 had previously been shown on t.l.c plates against other GPLs from other members of the *M. avium* complex[11]. The GPL was purified, subjected to reductive β-elimination, and the released oligoglycosylalditol applied to Sephadex G-15 in order to remove salt from the sugar[12]. Surprisingly, the oligoglycosylalditol emerged as two peaks (see Fig. 1). The explanation of this phenomenon, unique among *M. avium* serovariants, arose from subsequent work (see later); an *N*-formyl- and an *N*-deformyl-oligoglycosylalditol had been generated

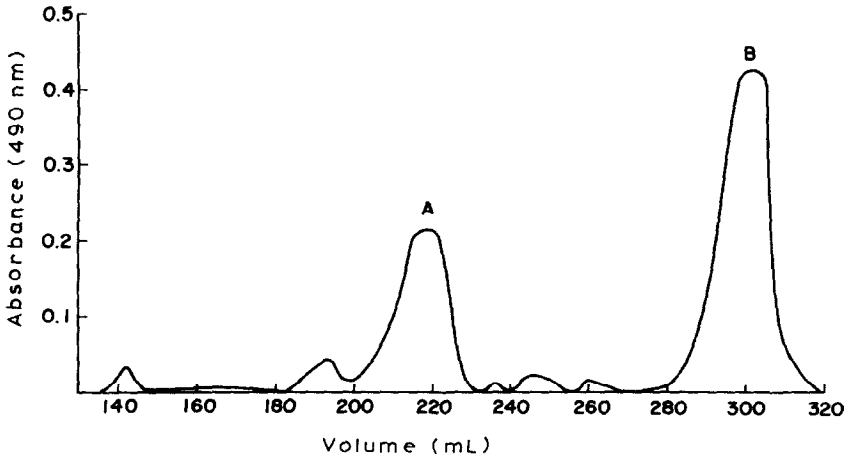

Fig. 1. Gel filtration of the oligoglycosylalditols released from the characteristic GPL of serovariant 14. Water-soluble material (~ 100 mg) after alkali-catalyzed β-elimination of the pure GPL was applied to a column (2.5 × 150cm) of Bio-Gel P-2, and eluted with water. Fractions (2 mL) were assayed for carbohydrate with phenol-H_2SO_4 (ref. 8).

and were being separated.

Glycosyl composition of the oligoglycosyl alditol. — The sugar composition of the oligoglycosylalditol was established by conventional g.l.c.-m.s. of the alditol acetates on an SP-2340 capillary column. Rhamnose, 2-*O*-methyl rhamnose, 2-*O*-methyl fucose, and 6-deoxytalose were readily identified; the same sugars had previously been encountered[13] in the oligosaccharide hapten from serovariant 20 in which they were present in the ratios of 1:0.8:1.1:1.2, respectively. The 6-deoxytalose residue was recovered as 1,3,4-tri-*O*-acetyl-2,5-anhydro-6-deoxytalitol, 2,3,5-tri-*O*-acetyl-1,4-anhydro-6-deoxytalitol, and the expected 1,2,3,4,5-penta-*O*-acetyl-6-deoxytalitol; the appearance of these products on g.l.c., the basis of their identification, and the reason for their production have previously[4] been presented. Precedent[6] would indicate that the 6-deoxytalitol is present as such, *i.e.*, as the alditol residue in the oligoglycosylalditol. This point was confirmed as g.l.c.-m.s. of the 6-deoxytalitol acetate showed fragments (*m/z* 103, 128, 129, 170, 187, 217 and 231) attributable to the presence to two ^{1}H atoms on C-1, whereas all of the other sugar derivatives produced corresponding fragments having on C-1 a single ^{2}H atom, arising from the $NaB(^{2}H)_4$ used in reduction of the glycosyl residues to the corresponding alditol acetates.

Structure of the unique amino sugar. — F.a.b.-m.s. analysis of the oligoglyco-

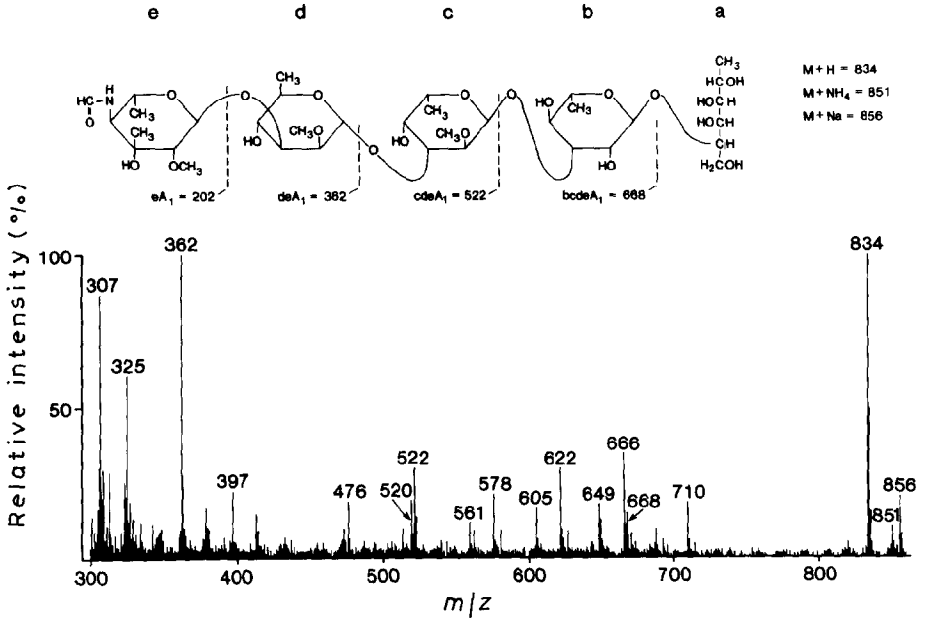

Fig. 2. F.a.b.-m.s. of the oligoglycosylalditol in peak A of (Fig. 1). The formation of the structurally significant ions is depicted in the top half of the Figure; the ion present at *m/z* 202 is not shown.

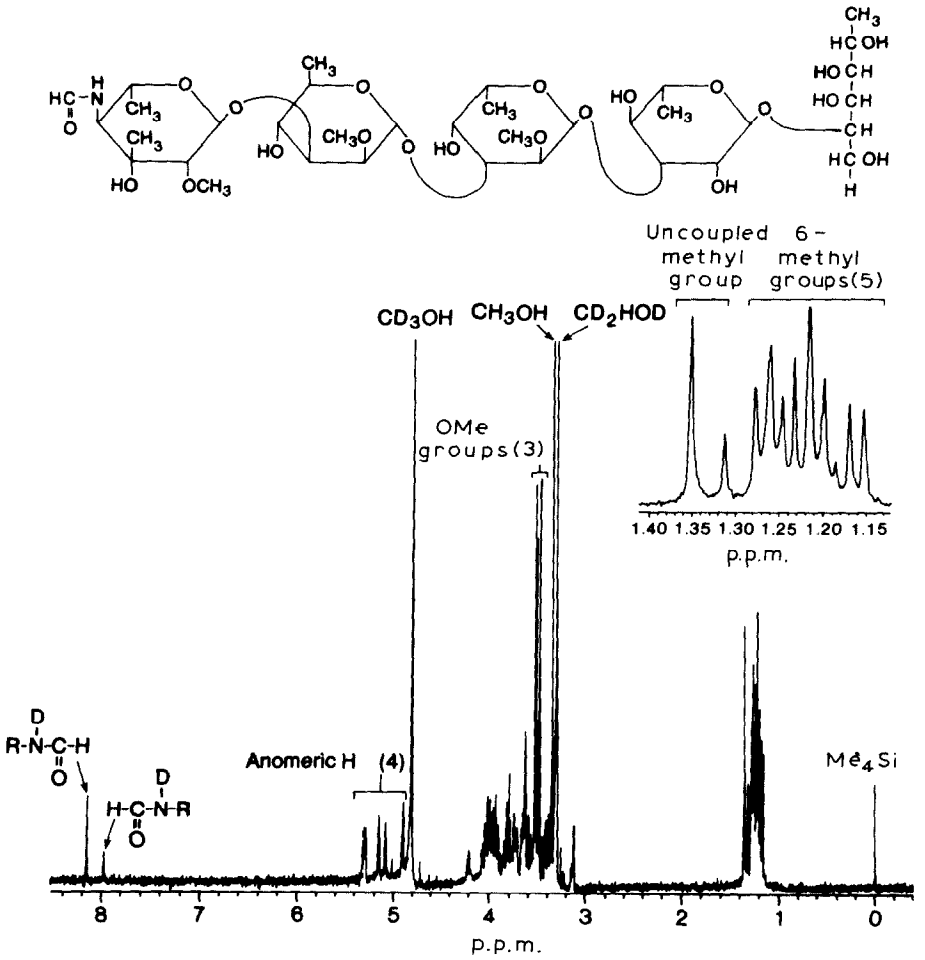

Fig. 3. The 360-MHz, ^{1}H-n.m.r. spectrum of the oligoglycosylalditol in peak A (of Fig. 1).

sylalditol itself (see Fig. 2) suggested a mol. wt. of 833. Clearly, the oligoglycosylalditol was larger than the triglycosylalditol implied by the glycosyl group analysis. In fact, the mol. wt. of 833 suggested a tetraglycosylalditol in which the additional, unknown residue was a nitrogenous sugar of 210 mass units. Indeed, the ^{1}H-n.m.r. spectrum of the purified oligoglycosylalditol (see Fig. 3) further suggested the presence of an *N*-formyl group; the proton of the *N*-formyl group is present in two environments, *E* and *Z*, thus absorbing at two chemical shifts (δ 8.0 and 8.2) due to hindered rotation of the formyl C–N bond[14]. An uncoupled CH_3 group attached to C-3 of a non reducing aminoglycosyl residue is also present in two different chemical environments, absorbing at δ1.31 and 1.35, undoubtedly a reflection of the *E* and *Z* forms of an *N*-formyl group on C-4. The presence of five CH_3 groups attached to C-5

of the corresponding number of 6-deoxyhexosyl or hexitol residues, each split by ~6 Hz, is apparent from the absorbances lying between δ1.15 and 1.29. The resonances of four anomeric protons were obvious between δ4.95 and 5.40. Accordingly, the preliminary evidence from f.a.b.-m.s. and ^{1}H-n.m.r. spectroscopy suggested, at the outset, the presence of a novel dideoxy-*C*-(formamido)-*C*-methyl-*O*-methyl residue (see Fig. 2) as part of a pentasaccharide hapten.

That the reductive β-elimination conditions used to release the oligosaccharide hapten from the original glycolipid also resulted in removal of an *N*-formyl group from about half of the population of oligoglycosylalditols was indicated by the following observations. The *N*-deformylated oligoglycosylalditol (Peak B, Fig. 1) was ninhydrin-positive, was devoid of those signals, at δ8.0 and 8.2, in the ^{1}H-n.m.r. spectrum attributable to an *N*-formyl group, and had as determined by f.a.b.-m.s. a mol. wt. of 805, 28 mass units less than the *N*-formyloligoglycosylalditol (Peak A, Fig. 1); the fact that the *N*-deformyl product was eluted later was probably due to ion retardation. A portion of the *N*-deformyloligoglycosylalditol was *N*-acetylated, hydrolyzed, reduced with $NaBH_4$, and the products *O*-acetylated. The resulting alditol acetates were analyzed by g.l.c.-m.s. on DB-1 capillary column. Among the readily recognizable alditol acetates was one whose mass spectrum (*m/z* 117, 158, and 132) was consistent with that of a 4-acetamido-1,5-di-*O*-acetyl-4,6-dideoxy-3-*C*-methyl-2-*O*-methylhexitol[15,16], and was identical to that of the 4-acetamido-1,5-di-*O*-acetyl-dideoxy-3-*C*-methyl-2-*O*-methyl mannitol (*N*-acetylkansosamine*) previously described[15]. A comparison of the chromatographic properties of the unknown 4-acetamido-1,5-di-*O*-acetyl-4,6-dideoxy-3-*C*-methyl-2-*O*-methylhexitol and the corresponding derivative of the origianal *N*-acetylkansosamine showed identical retention-times on both a DB-1 and an SP-2340 capillary column.

Thus, the evidence implies that the type-specific sugar of serovariant 14 is a 4,6-dideoxy-4-formamido-3-*C*-methyl-2-*O*-methylmannose (*N*-formylkansosamine). This is the third example of a naturally occurring derivative of 4-amino-4,6-dideoxy-3-*C*-methylmannose in Nature. The other two are *N*-(*R*)-2-methoxypropanokansosamine {4,6-dideoxy-4[(*R*)-2-methoxypropanamido]-3-*C*-methyl-2-*O*-methyl-L-mannopyranose} from *Mycobacterium kansasii*[15,16] and L-sibirosaminide (4,6-dideoxy-3-*C*-methyl-4-(methylamino)-L-mannose) from *Streptosporangium sibiricum*, an actinomycete phylogenetically related to *Mycobacterium*[18,19]. Serovariants 17 and 25[(ref. 7)], of the *M. avium* complex contain other 6-deoxyamino sugars; however, these are not 3-*C*-methylated and are not in the *manno* configuration, as determined by H-2–H-1 coupling experiments and, accordingly, are not derivatives of 4-amino-4,6-dideoxy-3-*C*-methyl mannose.

* The trivial name originally given to this sugar was *N*-acylkansosamine[15]. This was subsequently inadvertently changed to *N*-acylkanosamine[16]. Because the name kanosamine was already associated with the 3-amino-3-deoxy-D-glucose from the antibiotic kanamycin (*cf.*, ref. 17), we propose reverting to the original name, kansosamine. We thank Dr. Hans H. Baer for pointing out the discrepancy.

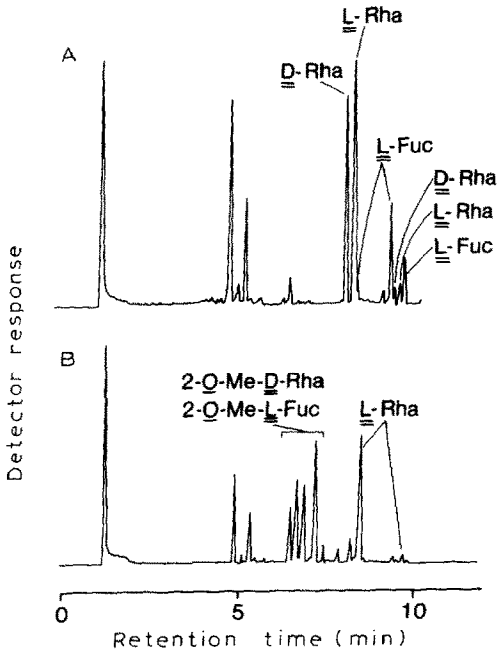

Fig. 4. G.l.c. of the per-*O*-(trimethylsilyl)ated 2-*R*-(-)-butyl glycosides prepared from (A) the glycosyl residues of the *O*-de-methylated and (B) the non-*O*-de-methylated tetraglycosylalditol. G.l.c. was conducted on a DB-1 capillary column, temperature-programmed for 2 min at an initial temperature of 150° followed by a 2°/min rise to 190°. The D-rhamnose and L-fucose derivatives produced after *O*-de-methylation were eluted later than the 2-*O*-methyl-3,4-di-*O*-Me_3Si 2-*R*-(−)-butyl glycosides produced from the non-*O*-de-methylated tetraglycosylalditol.

Absolute configuration of glycosyl units. — When applied to the products from other serovariants, the procedure[20] for elucidation of absolute sugar configuration, involving g.l.c. of the per-*O*-(trimethylsilyl)ated butyl glycosides after demethylation of the intact oligoglycosylalditol with lithium in ethylenediamine[4,21], had consistently shown that all 6-deoxyhexoses were in the L enantiomeric configuration[4,7,22]. However, routine application of this procedure to the demethylated oligoglycosylalditol from serovariant 14 produced unexpected results (see Fig. 4A). A D-rhamnose residue was obviously present, in addition to the L-rhamnose unit invariably present in like products from other serovariants; the fucose residue, as before[4,7,22], was the L enantiomer. On account of the unexpectedness of the finding of a D-rhamnose residue, the analysis was repeated, but on the per-*O*-acetyl-2-(+)octyl derivatives[23]. Again, the two enantiomers of rhamnose were shown to coexist. Because the analyses were applied to the *O*-demethylated pentaglycosylalditol, it was not evident which rhamnose unit was in the D configuration, although, should the principle of an invariant, nonreducing, "linkage"disaccharide, α-L-rhamnosyl-(1→2)-6-deoxytalose, in all GPL antigens, be adhered to, the 2-*O*-methyl-rhamnose unit seemed the more likely candidate. Nevertheless, the per-*O*-(trimethylsilyl)ated

butyl glycosides were derived from the pure pentaglycosylalditol with its full complement of *O*-CH_3 groups, and were examined by g.l.c. (see Fig. 4B). It was obvious that the rhamnose residue was L, and, accordingly, the 2-*O*-methylrhamnose residue must be D. Because the original 6-deoxytalose residue was present as a 6-deoxytalitol unit in the tetraglycosylalditol, its absolute configuration could not be so determined*. Neither was the procedure applicable to the (dideoxy)-4-formamido-3-*C*-methyl-2-*O*-methylmannosyl unit.

D-Rhamnose occurs only sparingly in Nature[24]. For instance, the O-antigen of *Pseudomonas capacia* is exceptional, in that it is a homopolysaccharide composed of D-rhamnopyranosyl residues[25]. The older literature[26] contains convincing evidence of a D-rhamnose- and a 6-deoxy-D-talose-containing, capsular polysaccharide in a Gram-negative, motile rod, designated GS. Also, the lipopolysaccharide of the purple, sulfur bacterium *Chromatium vinosum* contains D-rhamnose[27,28], and that from the photosynthetic prokaryote *Rhodospirillum tenue* Eu1 contains 2-*O*-methyl-D-rhamnose[29]. Incidentally, the glycosyl substituent of mycoside B, the species-specific, phenol-phthiocerol-containing glycolipid of *Mycobacterium bovis*, had been reported as 2-*O*-methyl-β-D-rhamnopyranose[30]; however, Chatterjee *et al.*[31] presented evidence that it is, in fact, a 2-*O*-methyl-α-L-rhamnopyranose.

Glycosyl linkage and sequence. — The results of methylation analysis of the pentaglycosylalditol are summarized in Table I. As with the haptens from all other serotypes studied, the 6-deoxytalitol residue was obviously 2-linked. In addition, the 2-*O*-methyl-D-rhamnose, the L-rhamnose, and the 2-*O*-methyl-L-fucose were all 3-linked pyranosyl residues. The 6-deoxy-4-formamido-3-*C*-methyl-2-*O*-methylhexosyl residue was not amenable to conventional methylation analysis. However, it apparently occupies the nonreducing terminus because methylation analysis indicated that none of the other sugars occupy this position. F.a.b.-m.s. (see Fig. 2) and other evidence, discussed later, support this contention.

F.a.b.-m.s. (see Fig. 2) also provided information on the sequence of sugars. Among the low-mass 2-thioglycerol peaks was an ion at *m/z* 202 (not shown in Fig. 2) attributable to the terminal amino sugar. Prominent fragment-ions at *m/z* 362, 522, and 668, combined with the results of methylation analysis (see Table I) suggested the sequence, *N*-formylkansosaminyl-(1→3)-6-deoxy-2-*O*-methyl-hexosyl$_1$-(1→3)-6-deoxy-2-*O*-methylhexosyl$_2$-(1→3)-rhamnosyl-(1→2)-6-deoxytalitol.

As a prelude to solving the riddle of the full sequence of the glycosyl units, it was reasoned that the rhamnose, the 6-deoxytalitol and the amino sugar should all have distinctive masses upon trideuteriomethylation, and, accordingly, their locations could be unambiguously assigned, given an informative f.a.b.-mass spectrum. However, the 2-*O*-methyl-D-rhamnose and 2-*O*-methyl-L-fucose units, having the same molecular weight, could not be so differentiated, just as they could not be distinguished by methylation analysis. In an effort to resolve this dilemma,

*By examining the 6-deoxytalose isolated from the native glycolipids from several serovariants, D. Chatterjee has established that it is the L enantiomer.

TABLE I

GLYCOSYL LINKAGE ANALYSIS ON THE OLIGOGLYCOSYLALDITOL FROM THE SPECIFIC GPL OF SEROVARIANT 14

Nature of O-*acetyl*-O-*methyl*-O-*trideuteriomethyl derivative*	*Parent Oligoglycosyl Alditol*	*Compound* **6** *(Fig. 5 and Table I)*	*Compound* **3** *and* **4** *(Fig. 5 and Table I)*
	Mole %		
2-*O*-Ac-6-deoxy-1,3,4,5-penta-*O*-CD_3-talitol	10[a]	12[a]	8[a]
1,3,5-Tri-*O*-Ac-2-*O*-CH_3-4-*O*-CD_3-rhamnitol	27	0	0
1,3,5-Tri-*O*-Ac-2,4-di-*O*-CD_3-rhamnitol	27	27	38
1,3,5-Tri-*O*-Ac-2-*O*-CH_3-4-*O*-CD_3-fucitol	29	38	7
1,5-Di-*O*-Ac-4-*O*-CD_3-2,3-di-*O*-CH_3-fucitol	0	0	41
1,5-Di-*O*-Ac-4-*O*-CD_3-2,3-di-*O*-CH_3-rhamnitol	0	22	9
3-*O*-Ac-2,4-di-*O*-CD_3-1,5-di-*O*-CH_3-rhamnitol	0	0	?[b]

[a]Present in less than expected amounts, presumably due to volatility of the 6-deoxy-tetra-*O*-methylhexitol derivatives. [b]Should be present in small amounts in compound **3**.

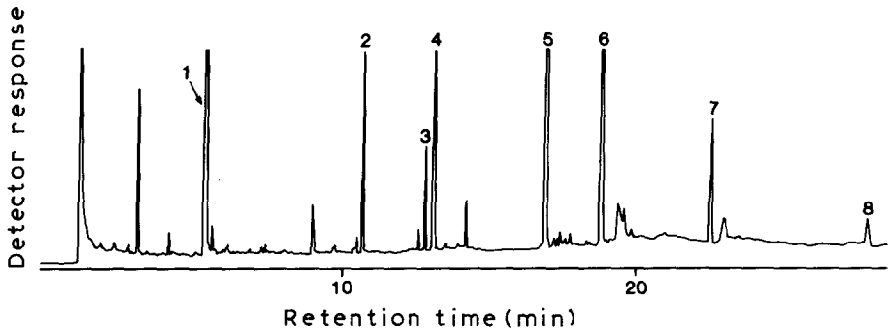

Fig. 5. G.l.c. of the partially *O*-methylated, partially *O*-(trideuteriomethyl)ated glycosylalditols derived form the original tetraglycosylalditol by *O*-(trideuteriomethyl)ation, partial acid hydrolysis, sodium borodeuteride reduction, and *O*-methylation. G.l.c. was performed on a DB-1 capillary column, temperature-programmed for 4 min at an initial temperature of 200°, followed by an 8°/min rise to 340°, and then held for 10 min at 340°. The identity of the compounds eluted in each numbered peak is given in Table II, and the stucturally relevant fragment-ions obtained during g.l.c–m.s. analyses are also listed therein. The mass spectrum of the compound eluted in Peak 5 is presented in Fig. 6.

the parent tetraglycosylalditol was (trideuteriomethyl)ated [*i.e.*, $C(^2H)_3$ groups inserted], subjected to partial hydrolysis, reduced with $NaB(^2H)_4$ and *O*-methylated [*i.e.*, $C(^1H)_3$ groups inserted]. The resulting complex mixture of partial *O*-$C(^1H)_3$, partial *O*-$C(^2H)_3$-glycosylalditols was subjected to g.l.c. (see Fig. 5) and g.l.c.–m.s. The mass fragmentation[32,33] of each of the seven glycosylalditols allowed a sequence to be proposed for each. Such an analysis, as applied to compound **5** is shown in Fig. 6. The evidence suggests that compound **5** is an alditol containing the nonreducing, terminal aldobiose, *i.e.*, per(trideuteriomethyl)ated *N*-formylkansosaminyl-(1→3)-6-deoxy-2-*O*-methylhexosyl$_1$-(1→3)-6-deoxy-1,2,5-tri-*O*-methyl-hexitol.

Data from the mass spectra of the other glycosylalditols separated by g.l.c. are summarized in Table II, and the structural information deduced is included. Obviously, at this stage of the analyses, the order of the 2-*O*-methylrhamnose and 2-*O*-methylfucose units could not be resolved. However, it is obvious from the data in Table II that analysis of the component residues and of any one of compounds **2**, **3**, **4**, **5**, or **6** as the partial *O*-acetyl-*O*-methyl-*O*-(trideuteriomethyl)alditols, would establish the order of the 2-*O*-methyl-D-rhamnose and 2-*O*-methyl-L-fucose residues.

Accordingly, the same mixture of per-*O*-alkylated oligosaccharide alditols that had been subjected to g.l.c.–m.s. as per Fig. 5 was now subjected to h.p.l.c. Individual fractions were collected, and analyzed by g.l.c. as described in Fig. 5. Compound **6** was obtained in pure form, and another fraction was collected which contained a mixture of compounds **3** and **4** in the ratio of 1:4. Analysis of the partially *O*-acetylated *O*-methylated *O*-(trideuteriomethyl)ated alditols (see Table I) clearly showed that the terminal glycosyl residue is 2-*O*-methylrhamnose and the penultimate glycosyl unit is 2-*O*-methylfucose. This conclusion was confirmed by

TABLE II

IDENTIFICATION AND INTERPRETATION OF THE MAJOR EI-MS IONS USED IN ANALYSIS OF PEAKS 1–8 FROM FIGURE 5[a]

Peak	*m/z Values for the appropriate ion*										*Proposed structure*
	aJ_2	aJ_1	bA_1	bA_2							
1	217	277	195	163							3-CH_3-2,4-CD_3-rhamnosyl-(1→2)-1,3,4,5-CD_3-6-deoxytalitol
2	209	272	236	201							*N*-formylkan[b]-(1→3)-1,2,5-CH_3-4-CD_3-6-deoxyhexitol$_1$
	aJ_2	aJ_0	abJ_2	abJ_1	cA_1	cA_2	cbA_1				
3	(212)[c]	258	(389)	(449)	192	160	369				2,3-CH_3-4-CD_3-6-deoxyhexosyl$_1$-(1→3)-2-CH_3-4-CD_3-6-deoxyhexosyl$_2$-(1→3)-2,4-CD_3-1,5-CH_3-rhamnitol
4	217	263	397	457	192	160	372				2,3-CH_3-4-CD_3-6-deoxyhexosyl$_2$-(1→3)-2,4-CD_3-rhamnosyl-(1→2)-1,3,4,5-CD_3-6-deoxytalitol
5	209	255	(386)	(449)	236	201	413				*N*-formylkan-(1→3)-2-CH_3-4-CD_3-6-deoxyhexosyl$_1$-(1→3)-1,2,5-CH_3-4-CD_3-6-deoxyhexitol$_2$
	aJ_2	aJ_0	abJ_2	abJ_0	$abcJ_2$	$abcJ_1$	dA_1	dA_2	cdA_1	$dcbA_1$	
6	217	263	(397)	443	(574)	634	192	160	369	549	2,3-CH_3-4-CD_3-6-deoxyhexosyl$_1$-(1→3)-2-CH_3-4-CD_3-6-deoxyhexosyl$_2$-(1→3)-2,4-CD_3-rhamnosyl-(1→2)-1,3,4,5-CD_3-6-deoxytalitol
7	212	258	(389)	435	(566)	(629)	236	201	413	590	*N*-formylkan-(1→3)-2-CH_3-4-CD_3-6-deoxyhexosyl$_1$-(1→3)-2-CH_3-4-CD_3-6-deoxyhexosyl$_2$-(1→3)-1,5-CH_3-2,4-CD_3-rhamnitol
	aJ_2	aJ_0	abJ_2	abJ_0	eA_1	eA_2	edA_1	$edcA_1$	$edcbA_1$		
8	217	263	(397)	443	236	201	413	590	770		*N*-formylkan-(1→3)-2-CH_3-4-CD_3-6-deoxyhexosyl$_1$-(1→3)-2-CH_3-4-CD_3-6-deoxyhexitol$_2$-(1→3)-2,4-CD_3-rhamnosyl-(1→2)-1,3,4,5-CD_3-6-deoxytalitol

[a]The nomenclature is that of Kochetkov and Chizhov[32]. [b]*N* Formylkan = 4,6-dideoxy-4-*N*-formyl-*N*-(trideuteriomethyl)-3-*O*-(trideuteriomethyl)-3-*C*-methyl-2-*O*-methylmannosyl. [c]Ions in parentheses were not detected.

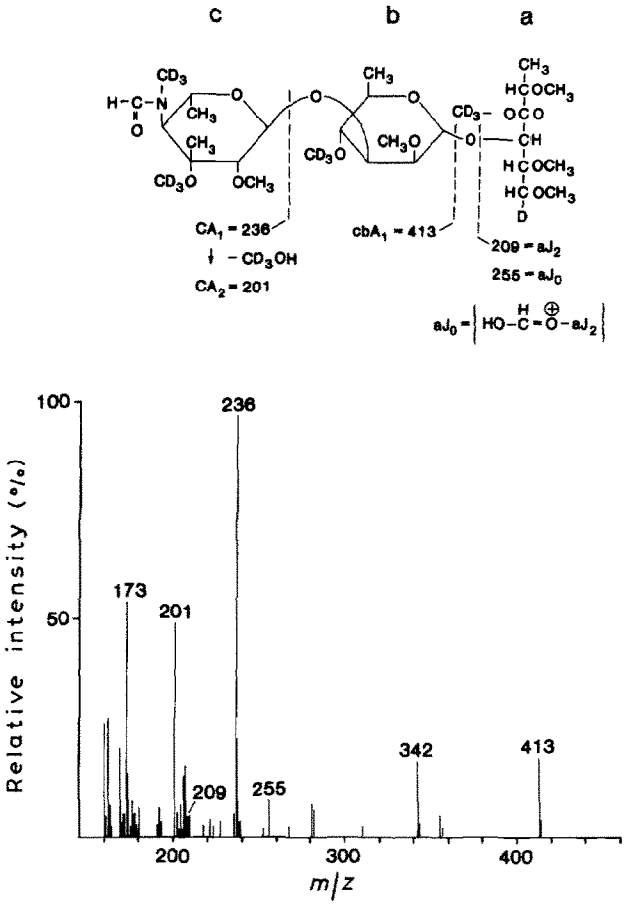

Fig. 6. The mass spectrum of the partially *O*-methylated, partially *O*-(trideuteriomethyl)ated diglycosylalditol from Peak 5 in Fig. 5. The formation of the structurally significant ions is depicted in the top half of the figure. Ions containing the amino sugar preponderate, as may be seen from the low intensities of the non-amino sugar-containing ions at *m/z* 209 and 255. The aJ_0 ion[33] is the depicted aJ_2 ion plus C-1, H-1, and O-1 from residue b and O-1 and H-2 from residue C-2.

the results of the analysis of the mixture of compounds **3** and **4** (see Table I). Thus, the sequence of sugars in the original tetraglycosylalditol is *N*-formylkansosaminyl-(1→3)-2-*O*-methyl-D-rhamnopyranosyl-(1→3)-2-*O*-deoxy-L-fucopyranosyl-(1→3)-L-rhamnopyranosyl-(1→2)-6-deoxy-talitol.

The anomeric configurations of the glycosyl residues were determined by ^{1}H-n.m.r. spectroscopy (see Fig. 3). Because all of the anomeric protons appear at $\delta >$ 4.9, all of the sugar units must be in the α- configuration[34,35]. Therefore, with the exception of the D and L configuration of the terminal amido sugar and the 6-deoxy-talitol residue, the complete structure of the oligosaccharide alditol was established as 4,6-dideoxy-4-formamido-3-*C*-methyl-2-*O*-methyl-α-mannosyl-(1→3)-2-*O*-

methyl-α-D-rhamnopyranosyl-(1→3)-2-*O*-methyl-α-L-fucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-6-deoxy-L-talitol.

EXPERIMENTAL

Serovariant 14. — The strain of *M. avium* complex serovariant 14 used throughout this work, once called the Boone serotype[9], was the original Edgar Boone strain [36]. More recently, its authenticity was reaffirmed by seroagglutination against pedigree rabbit antiserum[11] and by t.l.c.[37] and ELISA[38] of the characteristic antigen.

Extraction of GPL antigen and preparation of the oligoglycosylalditol. — Bacilli (100 g, dry weight) were obtained from growths on 7H11 broth[8]. Procedures for extraction and purification of the major polar GPL antigen, and preparation of the hapten as the oligoglycosylalditol have been described[4,7]. Those reductive-elimination conditions designed to cleave the oligosaccaride hapten from the GPL itself[4,12] also served to remove the *N*-formyl group from about half of the population of ensuing oligoglycosylalditols. The precise conditions were 0.45M NaOH and 1.85M $NaBH_4$ in 5:6 (v/v) ethanol–water for 23 h at 60°.

Analytical procedures. — Methods for hydrolysis of the oligoglycosylalditol, preparation of alditol acetates, and permethylation or per(deuteriomethyl)ation, have been described[4,7]. Analytical procedures designed for this particular study were as follows. *N*-Acetylation of the *N*-formyl-free amino group in the oligoglycosylalditol was accomplished by treating the material (2 mg) for 15 min at room temperature in 250 μL of CH_3OH, 50 μL of pyridine, and 50 μL of acetic anhydride. The reaction mixture was blown dry with N_2, resuspended in toluene, and re-evaporated to dryness. The acetamido sugar was then cleaved from the oligoglycosylalditol with M HCl for 18 h at 65°. The resulting sugars were reduced with $NaB(^2H)_4$ in the usual fashion. Subsequent *O*-acetylation of the mixture of alditols, including the acetamidoalditol, was accomplished with 100 μL of acetic anhydride and 100 μL of pyridine for 18 h at room temparature; the product was extracted into $CHCL_3$.

In order to cleave the intact per-*O*-$C(^2H)_3$-oligoglycosylalditol, 3 mg in 2M CF_3CO_2H (250 μL) was heated for 2 h at 60°. The sample was blown dry with N_2 at 50°, and the product reduced with $NaB(^2H)_4$ (2.5 mg) in 1:1 C_2H_5OH–NH_4OH (250 μL) for 1 h at room temperature. The borate was removed by repeated evaporation in 1:9 AcOH–MeOH, and by desalting on a 1-mL column of Dowex 50 (H^+) resin. The resulting mixture of partially alkylated glycosylalditols was then *O*-methylated as described previously[4]. This mixture of per-*O*-alkylated glycosylalditols was separated on an International Business Machines (IBM) (Wallingford, CT) 3 μm octadecyl (C-18) reversed phase, h.p.l.c. column (4.5 × 100 mm) pre-equilibrated with 3:7 MeOH–H_2O. One min after injection, a gradient of 30–65% Me_3COH in H_2O was applied during 30 min. The flow rate was 1 mL /min. The eluate was collected in 0.5-mL fractions, and examined by g.l.c. Routine g.l.c. and g.l.c.–

m.s. were conducted on a fused-silica Durabond-1 capillary column for 2 min at 160°, followed by 4°/min, to a final temperature of 240°, as described[4], or, by use of the temperature programme described in the text. A fused-silica SP-2340 column[4] was also used. Procedures for f.a.b.-m.s. and ^{1}H-n.m.r. spectroscopy have also been described in detail[4,7]. The sensitive, chemical procedures developed by one of us[39] for sequencing the glycosyl residues in complex polysaccharides were applied as described, except that partial acid hydrolysis of per-*O*-alkylated oligosaccharidealditols was monitored by t.l.c., not by g.l.c.-m.s.

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