Structural Features of the Arabinan Component of the Lipoarabinomannan of Mycobacterium tuberculosis*

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The recent availability of pure lipoarabinomannan (LAM) from Mycobacterium spp. has resulted in its implication in host-parasite interaction, which events may be mediated by the presence of a phosphatidylinositol unit at the reducing end of LAM. Herein we address the structure of the antigenic, nonreducing end of the molecule. Through the process of 13C NMR analysis of the whole molecule and gas chromatography/mass spectrometry of alditol acetates derived from the differential per-O-alkylated lipopolysaccharide, the majority of the arabinosyl residues were recognized as furanosides. Second, through analysis of per-O-alkylated oligoarabinosyl arabinobiframents of partially hydrolyzed LAM, it was established that the internal segments of the arabinoxylan component consists of branched 3,5-linked α-D-arabinofuranosyl (Araf) units with stretches of linear 5-linked α-D-Araf residues attached at both branch positions, whereas the nonreducing terminal segments of LAM consist of either of the two arrangements, β-D-Araf-(1→2)-α-D-Araf-(1→5)-α-D-Araf or β-D-Araf-(1→2)-α-D-Araf-(1→5)-α-D-Araf. Since this latter arrangement also characterizes the terminal segments of the peptidoglycan-bound arabinogalactan of Mycobacterium spp., we propose that mycobacteria elaborate unique terminal arabinan motifs in two distinct settings. In the case of the bound arabinogalactan, these motifs provide the nucleus for the esterified mycolic acids, entities which dominate the physicochemical features of mycobacteria and their peculiar pathogenesis. In the case of LAM, these motifs, non-mycolylated, are the dominant B-cell antigens responsible for the majority of the copious antibody response evident in most mycobacterial infections.

Within Mycobacterium spp. there exists a dominant soluble polysaccharide, termed lipoarabinomannan (LAM), highly antigenic, excreted in copious quantities, and with many of the biological attributes long associated with the likes of the O-antigenic lipopolysaccharides (1), such as suppression of T-lymphocyte activation (2, 3), inhibition of γ-interferon activation of macrophages (4), induction of the release of tumor necrosis factor (5, 6), and a generalized inhibition of antigen presentation by antigen presenting cells (7). So pervasive and profound are some of these biological effects that it has been argued, on the one hand, that LAM is the key factor in the intracellular survival of mycobacteria and in much of the pathogenesis of mycobacterioses (1) and, on the other, that it contains endotoxin or endotoxic activity (8), despite the absence of chemical evidence of ketodeoxyoctonate. In fact, Hunter et al. (9) and Hunter and Brennan (10), following earlier evidence that LAM is phosphorylated (11) and acylated (9), attributed to it many of the physicochemical properties of the O-antigenic lipopolysaccharides, such as a “membrane anchor” in the form of phosphatidylinositol (9, 10), alkali-labile phosphodiester groups identified as 1-phospho-myoinositol (9), a mannan “core,” and oligoarabinosyl side chains. The older literature had clearly demonstrated that the arabinan segments of LAM were the basis of its profound B-cell antigenicity in that serological activity was ablated by the action of endoarabinases and mild acid hydrolysis (12, 13) and the fact that lipomannan, essentially an arabinose-free version of LAM, was not antigenic (14). In this present report, we address the issue of the structural basis of the antigenicity of LAM, revealing novel oligoarabinofuranosyl arrangements, some of which had been recognized previously as part of the peptidoglycan-bound D-arabinobio-d-galactan of mycobacteria (15). We speculate that these motifs provide the structural basis of the dominant immunogenicity of LAM, whereas the majority, when appearing as the terminal nonreducing segments of arabinogalactan, provide the templates for esterified mycolic acid, the one entity that dominates the physical properties of mycobacteria, their peculiar pathogenesis and persistence.

EXPERIMENTAL PROCEDURES

Purification of LAM—The isolation of LAM-containing fractions from Mycobacterium tuberculosis H37Ra and primary resolution on columns of DEAE-Sephacel in detergent-containing buffer have been described (9, 10). Furthermore to these steps, preparations of LAM, recovered from columns of DEAE-Sephacel and highly pure according to polyacrylamide gel electrophoresis (9), were dialyzed, concentrated on an Amicon flow cell (Amicon 8200; Danvers, MA; 10 kDa molecular mass cut-off membrane), precipitated with 85% ethanol, redissolved in 0.01 M Tris HCl (pH 7.4) containing 0.1% Triton X-100 and applied to a HYDROPORE AX HPLC column (121.4 mm x 25 cm, Rainin, Woburn, MA) equilibrated in the same buffer. The column was eluted with the same buffer followed by a sharp gradient of 0-0.1 M NaCl fractions (10 ml) were collected, analyzed for carbohydrate (10), and positive fractions were re-examined by polyacrylamide gel electrophoresis. Pure lipomannan was eluted with 0.01 M NaCl, followed by the mannosyl phosphatidylinositol which emerged with...
Analysis of Fragments—The strategy and exact protocols for the degradation of pure LAM was passed through a column (2 ml) of Extracti Gel-D (Pierce Chemical Co.) and eluted with H₂O. Pure LAM thus obtained was the subject of the analyses described below.

Procedure for Determination of Ring Form of Glycosyl Residues in LAM—Pure LAM (10-15 mg) was methylated with CH₃I as described (15,16). Partial acid hydrolysis with 2 M CF₃COOH, reduction with NaB[¹H]₄, ethylation with C₂[¹H]₄I, complete hydrolysis, further reduction with NaB[¹HI₄, and acetylation were accomplished as described previously (16). The resulting partially O-acetylated, partially O-pentadecanoylthylated, and partially O-methylated alditols were analyzed by GC/MS as described (15,16).

Partial Degradation of Per-O-acetylated LAM and Resolution and Analysis of Fragments—The strategy and exact protocols for the random partial depolymerization of per-O-Me-LAM followed by reduction with NaB[¹H]₄, and per-O-deuterioethylation have been described (15). Exact procedures for the separation of the per-O-acetylated oligoarabinosyl arabinobiose and subsequent GC/MS analysis have been described (15). Selected HPLC fractions were also separately hydrolyzed, reduced, acetylated, and analyzed by GC/MS (15) in order to confirm substitution arrangements on individual partially O-acetylated alditol acetates. The per-O-acetylated oligoarabinobiosyl arabinobiose was the object of ¹H NMR analyses and was dissolved in hexadeuterioacetone. ¹³C NMR analysis on intact LAM was conducted in [¹H]₂O (15). NMR was performed on a Bruker AC-300 or -500 NMR at the Colorado State University Department of Chemistry Central Instrument Facility or at the Regional NMR Center.

RESULTS

Ring Form of Ara Residues in LAM—In the only other contemporary analysis of LAM (11), it was concluded that substantial amounts of the Ara residues were in the pyranose ring form. In the light of the fact that all Ara residues within arabinogalactan are Ara (15-17), we wished to re-address the matter. Pure LAM (15 mg) was per-O-methylated as described (16) and an aliquot completely hydrolyzed with 2 M CF₃COOH at 120 °C for 1 h, reduced, acetylated, and analyzed by GC/MS and GC (Table I). Such conventional methylation analysis can establish the ring form of a glycosyl residue only if an O-methyl substituent appears on C-4 in the case of sugars in the pyranose form or on C-5 for glycofuranoses. All of the mannosyl residues of LAM after methylation contain a methoxyl group at C-4 (Table I) and thus are pyranosyl, an observation consistent with earlier reports (11,13). In addition, identification of 2,3,5-tri-O-Me-Ara and 3,5-di-O-Me-Ara (Table I) pointed to the presence in LAM of t-Araf and 2-linked Ara, respectively. However, methylation analysis could not help determine the nature of the ring form of the majority of arabinosyl residues, those devoid of 5-O-Me groups. In order to address this matter, the native, pure molecule was first subjected to DEPT ¹³C NMR analysis (Fig. 1). The cluster of signals at 109 ppm can only result from C-1 of α-Araf residues; identical signals in the case of arabinogalactan arise from 5-linked-α-Araf residues (15). The C-1 resonances of 2-linked-α-Araf residues are known to be slightly upfield from the C-1 resonances of 5-linked-α-Araf, and occur at ~107 ppm (15). The mannosyl residues are known to be in the α configuration (11), and, indeed, the three signals at 104, 101, and 97 ppm are attributable to 6-linked-α-Manp, t-α-Manp, and 2,6-linked-α-Manp; the 2,6-linked-α-Manp signal is upfield, at ~97 ppm, due to substitution at C-2; the assignment of the 6-linked-α-Manp and t-α-Manp may be reversed. The C-1 of t-β-Araf residues is known to absorb at ~102 ppm (15) and, in view of the fact that this signal is noticeably more intense than those at ~104 and ~97 ppm, the presence of t-β-Araf in LAM was at once suspected. The C-5 resonance of t-β-Araf shown previously to occur at 64.1 ppm (15) was also evident in LAM. Thus, ¹³C NMR analysis confirmed the furanosyl ring form of the 2-linked Ara and the t-Ara as first suggested by methylation analysis.

In order to further corroborate the evidence for the dominance of Ara in LAM, the experimental approach successfully applied previously to the related question in the context of arabinogalactan (16,18) was applied. Per-O-methylated LAM was partially hydrolyzed, reduced with NaB[¹H]₄, ethylated, fully hydrolyzed, again reduced with NaB[¹H]₄, and O-acetylated. The effect of this series of reactions on a hypothetical 4-linked Ara and 5-linked Ara is illustrated in Fig. 2. Thus a hypothetical 4-linked Ara residue cleaved by the partial

### Table I

| Glycosyl linkage composition of LAM from M. tuberculosis |
|--------------------------------------------------------|
| Product identified                                      |
| Deduced linkage and ring form                           |
| Mole %                                                 |
| Ara 1, 4                                               | 1,4  | 2.35 | t-Araf | 7 |
| Ara 1                                                | 1.2 | 2.5 | 2-Ara | 9 |
| Ara 1, 4, 5                                         | 2.5 | 2-Ara/4-Arap⁴ | 43 |
| Ara 1                                                | 1.2 | 3  | 2,5-Ara/2,4-Arap⁵ | 1 |
| Ara 1                                                | 1.3 | 2  | 3,5-Ara/3,4-Arap⁶ | 12 |
| Man 1                                                | 1.5 | 2.3, 4, 6-Manp | 11 |
| Man 1                                                | 1.56 | 2.34 | 6-Manp | 6 |
| Man 1                                                | 1.25, 6 | 3.4 | 2,6-Manp | 9 |
| Man 1                                                | 1.25 | 3.46 | 2-Manp | 0.6 |

⁴ Shown to be predominantly 5-linked Ara with a very small amount of 4-linked Ara (see text).
⁵ Proof that the product is 2,5-linked Ara rather than 2,4-linked Ara is given in Footnote 2.
⁶ Proof that the product is 3,5-linked Ara rather than 3,4-linked Ara is given in the text.

![Fig. 1. The ¹³C NMR (DEPT) spectrum of LAM from M. tuberculosis H37Ra.](image-url)

The assignment of resonances is discussed in the text.
higher temperature allowed hydrolysis of about one-third of the Manp residues and thus presumably one-third of any Arap residues that may be present. Each sample was reduced with NaB[14]H₄, pentadeterioethyleted, completely hydrolyzed, reduced with NaB[14]H₄, acetylated, and analyzed by GC/MS (Figs. 3 and 4). Information leading to identification of the products is provided in Table II.

At the lower temperature (75 °C), the derivatives expected from 5-linked Araf (A and E, Figs. 2 and 3) were produced in large amounts. Thus, 5-linked Araf as opposed to 4-linked Araf predominates in LAM. Analysis of other products, e.g. F, G, and J (Table II), demonstrated the presence of 3,5-linked Araf rather than 3,4-linked Arap in LAM. Under the 95 °C partial hydrolysis conditions, few partially alkylated partially acetylated arabinitols were detected; obviously, the majority of the arabinofuranosides were cleaved to monosaccharides at this temperature. However, quantitatively minor amounts of two products, Y (4-O-Ac-1,5-di-O-C₆[14]H₄)-2,3-di-O-CH₃-arabinitol) and Z (1,5-di-O-Ac-2,3-di-O-C₆[14]H₄)-4-O-Me-arabinitol) (Fig. 2; Table II; Fig. 4) were observed, indicative of the presence of 4-linked Arap. Nevertheless, the amounts were extremely low, suggestive of the existence of only a few residues of 4-linked Arap, at most. No derivatives indicative of 3,4-linked Arap were detected by this analysis (Table II), corroborating results obtained from the application of the milder temperature. Therefore, we conclude that the 2-O-Me-Ara formed during methylation analysis (Table I) resulted from a 3,5-linked Araf only and not from 3,4-linked Arap, and that the 2,3-di-O-Me-Ara resulted mainly from 5-
linked Ara and, perhaps, from one or two residues of 4-linked Ara.

Arrangement of Ara Residues in LAM as Derived from Structural Elucidation of Oligoarabinofuranosyl Fragments—The per-O-Me-LAM was hydrolyzed with 2 M CF₃COOH, this time at 75 °C for 45 min, reduced with NaBH₄, and penta-0-deuterioethylated; these partial acid hydrolysis conditions were such that the more acid-stable Manp linkages were not cleaved. The resulting per-0-Me-LAM was hydrolyzed with CF₃COOH, this time at 75 °C for 45 min, reduced with NaBH₄, and applied to reverse phase HPLC (Waters, Milford, MA) and analyzed by GC/MS. The application of this series of degradation, derivatizations, separations, and mass spectrometric analyses to a portion of the arabinan segment of LAM is illustrated in Fig. 5.

Conclusions as to the position of the O-C₂[H]₅ units are key to the structural interpretations, in that they indicate the original point of attachment of other glycosyl residues. Thus, the O-C₂[H]₅ groups at C-2 of glycosyl residue "a" (Fig. 5D) unequivocally established that this residue was originally substituted at C-2 by another glycosyl unit. The presence of 2,5-linked Ara rather than 2,4-linked Ara in LAM.

Identification of the partially 0-alkylated alditol acetates arising from per-O-Me-LAM of M. tuberculosis and structural conclusions thereof

| Product identified | GC peak (Fig. 3 or 4) | Glycosyl residue | Position(s) of linkage and ring form | Position cleaved by partial acid hydrolysis |
|--------------------|----------------------|-----------------|------------------------------------|------------------------------------------|
| A                   | Ara                  | O-Ac            | 5-Ara²                             | 1 and 5                                  |
| B                   | Ara                  | O-Me            | 3,5-Ara⁵                           |                                          |
| C                   | Ara                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| D                   | Ara                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| E                   | Ara                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| F                   | Ara                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| G                   | Ara                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| H                   | Ara                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| I                   | Ara                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| J                   | Ara                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| K                   | Ara                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| L                   | Ara                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| M                   | Ara                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| N                   | Man                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| P                   | Man                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| Q                   | Man                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| R                   | Man                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| S                   | Man                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| T                   | Man                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| U                   | Man                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| V                   | Man                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| W                   | Man                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |
| X                   | Man                  | O-C₂[H]₅        | 5-Ara⁴                             | 1                                         |

² Derivatives of 3-O-Me-Ara (Table I), which could establish its ring form, was observed under either set of acid hydrolysis conditions, since this residue is present in extremely small amounts. Consequently, its ring form has not been rigorously determined. However, partial acid hydrolysis showed that this residue was cleaved at the same rate as the unambiguously assigned 3,5-di-O-Araf, suggesting the presence of 2,5-linked Ara rather than 2,4-linked Ara in LAM.

³ The observation that an internal 2-linked Ara results in both ald and acyl derivatives of 2,5-linked Ara in LAM, and, perhaps, from one or two residues of 4-linked Ara.
alditols (15, 19) was applied to a total of 25 such fragments of LAM and the results compared with those derived from a similar analysis of the peptidoglycan-bound arabinogalactan (15), the structures shown in Table III emerged.

The Major Arabinan Structural Motifs of LAM—Examination of the structures of these 25 individual oligosaccharide fragments allowed the recognition of four families of oligosaccharides and thereby four major structural motifs (Fig. 7), three of which, motifs A, B, and D, had been recognized previously as part of the peptidoglycan-bound arabinogalactan (15). Thus, proof of the existence of compounds 5, 6, 10, 11, 18, 19, and 21 support the case for motif A; compounds 7, 8, 14, 15, 16, 17, and 22 point to motif B; and compounds 4, 13 and 25 prove the existence of motif D. The presence of structural motif C, which was not encountered previously within arabinogalactan, was deduced in part from the existence of arabinosyl alditol 3 which would have demonstrated that at least some of the 2-linked Araf residues are glycosidically linked to a linear (nonbranched) 5-linked Araf. A key product, compound 12, confirmed this linkage pattern, i.e. the attachment of a 2-linked Araf at C-5 of a 5-linked Araf and, most importantly, indicated that the unit was part of a terminal motif in that a t-Araf was glycosidically linked to C-2 of the 2-linked Araf. The formulation of motif C (Fig. 7) was further aided by the recognition of compound 24 and further confirmed by the structures of compounds 1, 20, and 23.

Assignment of Anomeric Configurations—Both of the 2-linked Araf and 5-linked Araf residues within motif C (Fig. 7) are α, based on 1H NMR analysis of compound 20 (Table IV). The t-Ara in motif C is β as demonstrated by 1H NMR analysis of compound 12 (Fig. 6 and Table IV). The 2-Araf residues on both C-3 and C-5 of the branched Araf of structural motif A and the 5-Araf on both C-3 and C-5 of the branched Araf of motif B are in the α configuration as demonstrated by 1H NMR analysis of compounds 5-8 (Table IV). The t-Araf residues that are part of motifs A and C are in the β configuration as demonstrated by 1H NMR analysis of compound 1 (Table IV). Finally, the branched 3,5-linked-Araf residues in motifs A and B (Fig. 7) are both in the α configuration according to 1H NMR analysis of compound 9 (Table IV). Thus, assignment of all of the anomeric configurations shown in Fig. 7 was accomplished.

The Application of 13C NMR to the Arabinan Segments of LAM—Previously, we had shown how the application of 13C NMR analysis to solubilized peptidoglycan-bound arabinogalactan allowed assignment of C-1 of the t-Araf units of structural motif A, to δ 101.9 and δ 101.8; C-1 of the 2-linked Araf residue of the same motif was assigned to δ 106.8 and δ 106.6; and C-2 was assigned to δ 88.2 and δ 87.9 (15). The 13C NMR spectrum of LAM was compared in Fig. 8 to that of solubilized arabinogalactan (15) in the range δ 85 to δ 115. Thus, the resonances corresponding to C-1 (δ 106.8 and δ 106.6) and C-2 (δ 88.2 and δ 87.9) of the 2-linked Araf unit of motif A are readily apparent. In addition, resonances from C-1 (δ 106.9) and C-2 (broadening signal at δ 87.9) of the 2-linked Araf unit of the closely related structural motif C are also evident. Also, the resonances of the t-Araf units of both motifs A and C were demonstrable between δ 101.7 and δ 101.9; however, they are somewhat obscured by resonances arising from the mannopyranosyl residues. Nevertheless, the presence of motifs A and C in LAM is confirmed by the use of 13C NMR.

DISCUSSION

Some years ago, in reviewing the large body of early elegant work on the structure of the cell wall of mycobacteria, we
### Table III

**Structures of some 25 oligarabinol alditol fragment derived from per-O-Me-LAM**

| Compound No.* | Structure* | Structural proof* | Compound No.* | Structure* | Structural proof* |
|---------------|------------|-------------------|---------------|------------|-------------------|
| 1             | t-Araf-(1→2)-Araf/ | Identical to compound 1 (15) | 15             | -5-Araf-(1→5)-Araf/ (1→3)-Araf | Identical to compound 14 (15) |
| 2             | t-Araf-(1→5)-Araf/ | Footnote d, this table | 16             | -5-Araf-(1→3)-Araf/ (1→5)-Araf | Identical to compound 15 (15) |
| 3             | t-Araf-(1→5)-Araf/ | Identical to compound 3 (15) | 17             | -5-Araf-(1→5)-Araf/ (1→5)-Araf | Identical to compound 16 (15) |
| 4             | t-Araf-(1→5)-Araf/ | Identical to compound 4 (15) | 18             | -2-Araf-(1→5)-Araf/ (1→5)-Araf | Identical to compound 17 (15) |
| 5             | t-Araf-(1→5)-Araf/ | Identical to compound 5 (15) | 19             | -2-Araf-(1→5)-Araf/ (1→5)-Araf | Identical to compound 18 (15) |
| 6             | t-Araf-(1→5)-Araf/ | Identical to compound 6 (15) | 20             | -2-Araf-(1→5)-Araf/ (1→5)-Araf | Footnote f, this table |
| 7             | t-Araf-(1→5)-Araf/ | Identical to compound 7 (15) | 21             | -2-Araf-(1→5)-Araf/ (1→5)-Araf | Identical to compound 19 (15) |
| 8             | t-Araf-(1→5)-Araf/ | Identical to compound 8 (15) | 22             | -5-Araf-(1→3)-Araf/ (1→5)-Araf | Identical to compound 20 (15) |
| 9             | t-Araf-(1→5)-Araf/ | Identical to compound 9 (15) | 23             | t-Araf-(1→5)-Araf/ (1→5)-Araf/ | Footnote g, this table |
| 10            | t-Araf-(1→5)-Araf/ | Identical to compound 10 (15) | 24             | -5-Araf-(1→5)-Araf/ (1→5)-Araf | Footnote h, this table |
| 11            | t-Araf-(1→5)-Araf/ | Identical to compound 11 (15) | 25             | -5-Araf-(1→5)-Araf/ (1→5)-Araf | Identical to compound 24 (15) |
| 12            | t-Araf-(1→5)-Araf/ | Identical to compound 12 (15) |               |            |                   |
| 13            | t-Araf-(1→5)-Araf/ | Identical to compound 13 (15) |               |            |                   |

*These are listed in chronological order as they emerge from the HPLC column. The compounds were then further resolved and analyzed by GC/MS (15).

The nomenclature used is illustrated in the following examples: compound 1-5-Araf-(1→5)-Araf is 2,3-di-O-methyl-5-pentadenterythritol arabinofuranose-(1→5)-2,3-di-O-methyl-1,4-di-O-pentadenterythritol arabinoolit.

Thus, the arrows outside the parentheses point to the position of prior glycosidic linkage (now occupied by a C₂H₅ substituent).

Structural data leading to the proposed structure are provided in Ref. 15. Number in parentheses represents reference.

*Compound 2: GC Rp (retention time), 9.7 min; HPLC Rp, 21.5 min. El/MS ions at m/z 101, 143 (aA₃), 175 (aA₄), 230 (aA₅), 290 (aA₆), and 368 and 312 (alditol cleavages).

*Compound 12: GC Rp, 16.8 min; HPLC Rp, 25.8 min. El/MS ions at m/z 101, 143 (aA₃), 175 (aA₄), 335 (aA₅), 335 (aA₆), 230 (aA₇), 276 (aA₈), 290 (aA₉), 428 (alditol cleavage), and 472 (alditol cleavage). Glycosyl linkage composition: 1,4-di-O-Ac, 2,3,5-tri-O-Me-arabinol, 1,4,5-tri-O-Ac, 5-di-O-Me-arabinol, and 5-O-Ac, 2,3-di-O-Me, 1,4-di-O-C₅H₁₀H₂-arabinol in the ratio of 1:1:1.

*Compound 20: GC Rp, 17.3 min; HPLC Rp, 35.0 min. El/MS ions at m/z 120, 162 (aA₁), 194 (aA₂), 230 (aA₃), 290 (aA₄), 354 (aA₅), and 491 (alditol cleavage). Glycosyl linkage composition: 1,4-di-O-Ac, 3,5-di-O-Me, 2-O-C₅H₁₀H₂-arabinol, 1,4,5-tri-O-Ac, 2,3-di-O-Me-arabinol, and 5-O-Ac, 2,3-di-O-Me, 1,4-di-O-C₅H₁₀H₂-arabinol. The ratio could not be obtained.

*Compound 24: GC Rp, 23.1 min; HPLC Rp, 40.5 min. El/MS ions at m/z 101, 143 (aA₃), 175 (aA₄), 300 (aA₅), 300 (aA₆), 230 (aA₇), 290 (aA₈), 436 (cald J₈), 450 (cald J₉), and 610 (cald J₁₀).
concluded that the "common antigen of *Mycobacterium*, *Cor-
rynebacterium*, and *Nocardia* species" is arabinogalactan
"constructed on a main chain of galactopyranose and arabi-
nofuranose (20)." This summation has proved to be incorrect
on two scores. First, Vilkas et al. (21), we (16), and re- 
cently Gruber and Gray (17) have established that the arabinogalac-
tan heteropolysaccharide does not contain galactopyranose
residues but instead is composed entirely of D-galactofuran-
syl and D-arabinofuranosyl units. A significant point of the
present work is that the majority of the arabinosyl residues
of LAM are also in the furanose form. The evidence, which
again is contrary to what prevails in the literature, is based
on the fact that ¹³C NMR of holistic LAM showed abundant
signals assignable to Araf and no evidence of Araf. In addi-
tion, methylation, followed by partial hydrolysis and sub-
sequent steps, showed the dominance of 5-linked Araf and only
minor amounts of 4-linked Araf. Above all, the ring form of
the Ara units in the linear di- and trisaccharides were all
clearly furanose. The presence of 3,5-linked Araf in *M. tuber-
culosus* LAM is unequivocal in light of the data presented in
Table II and in view of the structures of compounds 6, 8, 9,
11, 16, 17, and 19 (Table III) and also considering the rate of
acidic cleavage of the 2-O-Me-Araf substituent as compared
with other residues. Furthermore, even though we do detect
small amounts of 4-linked Araf, the ¹³C NMR analysis (see
peak 109, Fig. 8) as well as the data in Tables II and III
require that 5-linked Araf vastly predominates over 4-linked
Araf. In a previous thorough study of the composition of
LAM from *Mycobacterium smegmatis* (11), the presence of
substantial amounts of 4-linked Araf was suggested, and data
were presented to the effect that the 2-O-Me-Ara arising from
the permethylated product is indicative of 3,4-linked Araf rather
than 3,5-linked Araf. Indeed, we² have confirmed that
mild acid hydrolysis of LAM prior to any methylation leads
to copious amounts of 1,5-di-O-acetyl-2,3,4-tri-O-methyl ara-
binitol, apparently from t-Araf, and to some 1,3,5-tri-O-
acetyl-2,4-di-O-methyl arabinitol, expected from 3,4-linked
Araf residues upon cleavage of a substituent at C-4. Thus,

\[
\text{Motif A}
\]

\[
\begin{align*}
\text{t} & \text{-Ara}-(1 \rightarrow 2) \text{-Ara}-(1 \rightarrow 3) \text{-Ara}-(1 \rightarrow 5) \text{-Ara}-(1 \rightarrow 6) \text{-Ara}-(1 \rightarrow 7)
\end{align*}
\]

\[
\text{Motif B}
\]

\[
\begin{align*}
\text{t} & \text{-Ara}-(1 \rightarrow 2) \text{-Ara}-(1 \rightarrow 3) \text{-Ara}-(1 \rightarrow 4) \text{-Ara}-(1 \rightarrow 5) \text{-Ara}-(1 \rightarrow 6)
\end{align*}
\]

\[
\text{Motif C}
\]

\[
\begin{align*}
\text{t} & \text{-Ara}-(1 \rightarrow 2) \text{-Ara}-(1 \rightarrow 3) \text{-Ara}-(1 \rightarrow 4) \text{-Ara}-(1 \rightarrow 5) \text{-Ara}-(1 \rightarrow 6)
\end{align*}
\]

\[
\text{Motif D}
\]

\[
\begin{align*}
\text{t} & \text{-Ara}-(1 \rightarrow 2) \text{-Ara}-(1 \rightarrow 3) \text{-Ara}-(1 \rightarrow 4) \text{-Ara}-(1 \rightarrow 5) \text{-Ara}-(1 \rightarrow 6)
\end{align*}
\]

**Fig. 7.** Structures of the four major structural motifs that
represent the majority of the structure of the arabinan seg-
ment of LAM. All ring forms are furanoid and all arabinosyl residues
are D.

### Table IV

| Compound No. | Per-O-alkylated oligoarabinosyl arabinitol | Chemical shift of H-1 (δ) | J₁₂ (Hz) | Assignment |
|--------------|-------------------------------------------|--------------------------|-----------|------------|
| 1            | t-Araf-(1→2)-Araf                         | 5.29                     | 4.2       | t-β-Araf   |
| 12           | t-Araf-(1→2)-Araf-(1→5)-Araf             | 5.23                     | 4.3       | t-β-Araf²  |
| 6'           | →2-Araf-(1→5)-Araf                       | 4.95                     | brs²      | →2-α-Araf  |
| 8'           | →5-Araf-(1→5)-Araf                       | 5.00                     | brs²      | →5-α-Araf  |
| 5²           | →2-Araf-(1→5)-Araf                       | 4.99                     | brs²      | →2-α-Araf  |
| 7²           | →5-Araf-(1→5)-Araf                       | 5.00                     | brs²      | →5-α-Araf  |
| 20           | →2-Araf-(1→5)-Araf                       | 5.00                     | brs²      | →2-α-Araf  |
| 13²          | →5-Araf-(1→5)-Araf                       | 5.04                     | brs²      | →5-α-Araf  |
| 9            | →5-Araf-(1→5)-Araf                       | 5.10                     | brs²      | →5-α-Araf  |

(a) Assignment of β to the t-Araf and α to the 2-linked Araf was based on analysis of compounds 1 and 20.

(b) brs, broad singlet.

(c) Compounds 6 and 8 co-eluted from the HPLC, and thus it is not known which anomeric signal results from 6 and which results from 8. The assignment of α in both cases is unambiguous.

(d) Compounds 5 and 7 co-eluted from the HPLC, and thus it is not known which anomeric signal results from 5 and which results from 7. The assignment of α in both cases is unambiguous.

(e) Compounds 20 and 13 co-eluted from the HPLC and NMR analysis resulted in a broad singlet at 6 5.00 and δ 5.04 in a ratio of about 3:1. Individual assignments cannot be made, but all of the glycosyl residues must be α-Araf.
FIG. 8. A comparison of the $^{13}$C NMR spectra of (A) LAM and (B) base solubilized cell wall arabinogalactan between 111 and 87.

FIG. 9. A model representing the major known structural features of LAM. The arabinan structural motifs A-D established in this report may be linked together in arrangements other than those shown here. The other structural features of LAM shown here were established previously (10). Details of the attachment of the arabinan segments to the mannan core are unknown as is the nature of the attachment of the mannan to the phosphatidylinositol anchor. Other groups such as succinyl, lactyl (11), and inositol phosphate (9, 10) are also part of LAM.

The long-held contention (20) that arabinogalactan is the dominant B-cell antigen of mycobacteria may well be re-evaluated in the light of present work. In a crucial set of studies, Misaki et al. (13) digested the cell walls of several mycobacterial species with alkali, resulting in cleavage of the esterified mycolates and the generation of a solubilized highly purified arabinogalactan. When tested against antiserum raised against whole bacteria, or partially purified cell walls, the arabinogalactan was serologically active. Removal of the majority of arabinofuranosyl through the action of a specific arabinofuranosidase ablated most of the antigen-antibody binding activity (12, 13, 22), clearly indicating that the multiple branched oligoarabinofuranosyl units represent the responsible determinants or epitopes. Similar evidence from others, combined with the demonstration that soluble, extracted, apparently demycyolylated arabinogalactans are antigenic (23), has led to the widely held belief that arabinogalactan is one of the most powerful immunogens of Mycobacterium spp. However, in the light of the evidence that the same terminal branched oligoarabinosyl motifs, the obvious antigen determinants (15), are shared by peptidoglycan-bound arabinogalactan and LAM, the role of arabinogalactan in the immunogenicity of mycobacteria must be questioned. It is more likely that LAM, which presents the naked terminal branched pentaarabinosyl unit and its linear arabinosyl variation to the immune system, represents the source of the majority of anti-arabinosyl antibodies in natural and experimental infections. Recently, we have established that the terminal arabinofuranosyl arrangements on the peptidoglycan-bound arabinogalactan are the foci for the attachment of the majority of mycolyl groups (15), an observation in accord with earlier work (24, 25) and are, accordingly, expected to be non-immunogenic. On the other hand, LAM, since its generation as a pure product in its native lipopolysaccharide state, has been shown to be highly immunogenic (9) and a powerful antigen in binding to the copious antibodies in cases of human lepromatous leprosy, tuberculosis (10) and bovine tuberculosis (14). Thus, it would appear that mycobacteria, in their biosynthetic economy and exceptional penchant for survival, can produce the same oligoarabinosyl motif for dual purposes, as
B-cell immunogens, in the context of LAM, antibodies to which are copious but ultimately nonneutralizing and non-deleterious to these intracellular parasites, and, in the context of peptidoglycan-bound arabinogalactan, as the fulcrum for mycolate attachment, the entities apparently most responsible for the intracellular survival of mycobacteria.

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