Characterization of mannoooligosaccharide caps in mycobacterial lipoarabinomannan by capillary electrophoresis/electrospray mass spectrometry

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A new analytical approach based on capillary electrophoresis-electrospray mass spectrometry (CE/ESI-MS) has provided new insight into the characterization of mannoooligosaccharide caps from lipoarabinomannans (LAMs), which are key molecules in the immunopathogenesis of tuberculosis. This analytical approach requires oligosaccharide labeling with the fluorophore 1-aminopyrene-3,6,8-trisulfonate (APTS) by reductive amination at the reducing termini. Optimization of the separation and ionization conditions, such as the choice of capillary electrophoresis (CE) electrolyte buffers, is presented and discussed. Anionic separation of the mono and oligosaccharide APTS derivatives was finally achieved with aqueous triethylammonium formate buffer. It was found that in contrast to the triethylammonium phosphate buffer, the triethylammonium formate buffer was appropriate for CE/ESI-MS coupling analysis of APTS-carbohydrate derivatives. In this case, negative ESI-mass spectra of APTS-carbohydrate adducts showed mainly (M-2H)2– pseudomolecular ions and some sequence fragment ions allowing their non-ambiguous structural characterization at the picomolar level. This analytical approach was successfully applied to more complex mixtures of carbohydrates released by mild acid hydrolysis of the lipoarabinomannans from Mycobacterium bovis BCG. The APTS-mannoooligosaccharide cap adducts were separated by CE and their structural characterization achieved by CE/ESI-MS analyses. Mannoooligosaccharide caps were routinely determined from CE/ESI-MS analyses. Mannoooligosaccharide caps and fatty acid residues are required in binding of the ManLAM to the mannose receptor (Schlesinger et al., 1995; Venisse et al., 1995), which mediates the adhesion of virulent mycobacteria to the macrophages, and also in the recognition process of the ManLAMs by the TcR CD4+CD8+ cell (Sieling et al., 1995).

PI-LAMs and ManLAMs appear to be pivotal mycobacterial antigens. It is now clear that their immunological activities are modulated by both the caps and the anchor. For example, mannoooligosaccharide caps and fatty acid residues are required in binding of the ManLAM to the mannose receptor (Schlesinger et al., 1995; Venisse et al., 1995), which mediates the adhesion of virulent mycobacteria to the macrophages, and also in the recognition process of the ManLAMs by the TcR CD4+CD8+ cell (Sieling et al., 1995). The ManLAMs from M. bovis BCG, M. tuberculosis, and M. leprae are capped by identical mannoooligosaccharides composed of one, two, and three Manp unit among which the disaccharide form is the major motif. However, they differ in the mannoooligosaccharide capping frequency (Khoo et al., 1995). More recently, in the case of M. bovis BCG, the presence of so-called parietal and cellular ManLAMs has been reported (Nigou et al., 1997). They differ in their mannoooligosaccharide capping frequency as well as in the structure of the anchor lipid part (Nigou et al., 1997).

The presence of the mannoooligosaccharide caps was directly determined from the intact ManLAMs using nuclear magnetic resonance (NMR) spectrometry 2D 1H-13C heteronuclear experiments (Venisse et al., 1993). However, this approach is limited by the need for purified LAMs in millimolar amounts and the absence of data concerning the mannoooligosaccharide structures. Their location, sequence, and number of monosaccharide units were determined by degradative methods involving their release by hydrolysis under mild acidic conditions, fractionation by gel filtration, reducing end tagging by aminobenzoate ethyl ester

Introduction

Lipoarabinomannans (LAMs) are complex glycoconjugates found in the mycobacterial cell walls (Puzo, 1993; Brennan and Nkaido, 1995). Like the lipopolysaccharides (LPSs) of Gram-negative bacteria, LAMs are amphipatic molecules. Chemically, LAMs consist of a bipartite structure (Puzo, 1993; Brennan and Nkaido 1995) assigned to a polysaccharide core and a hydrophobic anchor referred to as phosphatidyl- myo-inositol. The polysaccharide core can be subdivided into two homopoly-saccharides, according to their monosaccharide composition namely, mannan and arabinan. The former consists of a segment of α-Manp-(α→6) with side chains formed by a single α-d-Manp attached to the C2. Arabinan is composed of a linear chain of α-Araf-(α→3) with oligoarabinoferanosyl side chains branched at the C3. The phosphatidyl- myo-inositol anchor is located at the reducing end of the mannan domain. All the LAMs investigated to date share this bipartite structure, but differ at their reducing ends in the structure and composition of the fatty acids from the phosphatidyl- myo-inositol anchor (Nigou et al., 1997) and in the motifs capping the arabinofuranosyl side chains. These latter motifs have been identified as mannoooligosaccharides (Chatterjee et al., 1992; Venisse et al., 1993), and as phosho-my-o-inositol leading to the classification of LAMs into liparabinomannan with mannosyl capping (ManLAMs) and phosphoinositol-lipoarabinomannan (PI-LAMs), respectively. In the case of ManLAMs from Mycobacterium bovis BCG, succinyl residues were located on the C2 of 3,5-di-O-linked-α-Araf residues (Delmas et al., 1997). ManLAMs have been isolated from different strains of Mycobacterium tuberculosis (Chatterjee et al., 1992), M. bovis BCG (Venisse et al., 1993), and Mycobacterium leprae (Khoo et al., 1995) whereas PI-LAMs were identified in the case of a non-virulent mycobacterial species: Mycobacterium smegmatis (Gilleron et al., 1997).

PI-LAMs and ManLAMs appear to be pivotal mycobacterial antigens. It is now clear that their immunological activities are modulated by both the caps and the anchor. For example, mannoooligosaccharide caps and fatty acid residues are required in binding of the ManLAM to the mannose receptor (Schlesinger et al., 1995; Venisse et al., 1995), which mediates the adhesion of virulent mycobacteria to the macrophages, and also in the recognition process of the ManLAMs by the TcR CD4+CD8+ cell (Sieling et al., 1995).

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The presence of the mannoooligosaccharide caps was directly determined from the intact ManLAMs using nuclear magnetic resonance (NMR) spectrometry 2D 1H-13C heteronuclear experiments (Venisse et al., 1993). However, this approach is limited by the need for purified LAMs in millimolar amounts and the absence of data concerning the mannoooligosaccharide structures. Their location, sequence, and number of monosaccharide units were determined by degradative methods involving their release by hydrolysis under mild acidic conditions, fractionation by gel filtration, reducing end tagging by aminobenzoate ethyl ester.
Results and discussion

Electrophoretic separation of APTS-labeled mono- and oligosaccharides

The polysaccharidic core of the LAMs is exclusively composed of neutral monosaccharides assigned to α-D-Man (aldopentose) and α-D-Araf (aldohexose) which do not significantly absorb in the UV light. A derivatization procedure was therefore applied, and the fluorescent dye, 1-aminopyrene-3,6,8-trisulfonate (APTS) was selected (Evangelista et al., 1995). The optimal excitation of 490 nm for APTS-labeled oligosaccharides matched with the 488 nm of the Ar-ion laser which equipped the CE instrument (Guttman, 1996). Moreover, this excitation value was close to the fluorescence value of 520 nm allowing a lower limit of detection. Other related fluorescent dyes, such as 8-amino-naphtalene-1,3,6-trisulfonate (ANTS) and disulfonate (ANDS) have also been widely used successfully for oligosaccharide labeling (Chiesa and Horváth, 1993). However, their fluorescence characteristics are less satisfactory than those of the APTS and require a He-Cd laser (Paulus and Klockow, 1996). All these fluorescent dyes are routinely attached to the reducing termini of each carbohydrate by reductive amination. The fluorophore labeling efficiency was greater than 90%, and no labeling selectivity was observed during derivatization process of the carbohydrate under investigation as had previously been reported by Guttman et al. (1996).

The electropherogram in Figure 1A illustrates the separation, at low pH (pH 2.4), of the APTS-derivatized arabinose, mannose, maltose, maltotriose, and maltotetraose conducted in uncoated fused-silica capillaries. In this case, 500 fmol of each derivative was injected by hydrodynamic pressure, but up to 200 amol of APTS-monosaccharide could be routinely detected by this method. The low pH value was selected to suppress ionization of the surface silanol groups leading to a value for electroendosmotic flow which was almost zero (Ofner and Chiesa, 1994). Thus, the three negative charges arising from the sulfonic groups, which were still present even in acid media, provided the driving force for electrophoretic mobility of the oligosaccharide. However, under the acidic conditions used, the secondary amino group carried a positive charge leading to an overall charge of two negative charges. Under these conditions, the samples were introduced at the cathode and flowed toward the anode. The running electrolyte was prepared by dissolving different amounts of phosphoric acid in deionized water and adjusting the pH by the addition of triethylamine (TEA). When used as a buffer additive, TEA increases separation reproducibility by masking the interaction between the labeled-carbohydrate and the inner surface of the silica capillary (Chiesa and Horváth, 1993; Chiesa and O’Neill, 1994). In Figure 1A, triethylammonium phosphate buffer was the running electrolyte (pH 2.4) with a final concentration of phosphoric acid of 50 mM and the TEA concentration approximately 30 mM. As expected from the literature data, the electropherogram depicted in Figure 1A shows good resolution of the APTS-derivatives investigated. Arabinose-APTS (peak 1, 5.78 min) migrated faster than mannose-APTS (peak 2, 6.08 min).
Both types of APTS-monosaccharide derivatives moved faster than the APTS-maltoligosaccharide derivative mixture composed of maltose (peak 3), maltotriose (peak 4), and maltotetraose (peak 5) APTS-derivatives (6.73, 7.60, and 8.58 min respectively). The use of such acidic conditions also offered the advantage that the APTS excess appeared first at 4.90 min and was well isolated from the APTS-analytes. Since the net charge was the same for all the carbohydrate APTS derivatives, it can be seen, in agreement with the literature data that the migration velocity decreased with increasing molecular size (591 Da, 621 Da, 756 Da, and 945 Da respectively).

The amount of TEA needed to titrate phosphoric acid to pH 2.4 (∼30 mM) was sufficient to impart a positive charge to the inner wall of the capillary, so that an electroendosmotic flow toward the anodic end was generated when the voltage was applied thus enhancing the speed of analysis. To verify this point, the amount of TEA was decreased from 30 mM to 10 mM, leading to a final pH of 1.9. Under these conditions, the electropherogram depicted in Figure 1B showed a small decrease of ~2% in the electrophoretic mobilities of the APTS-carbohydrate derivatives due to reduction of the electroendosmotic flow.

As depicted in Figure 1A,B, all these APTS-carbohydrate adducts were very well resolved under such conditions, in agreement with the fact that CE had been shown to be particularly useful for the separation of closely related oligosaccharides and the subject of such applications had been addressed in recent reviews (Novotny and Sudor, 1993; Oefner and Chiesa, 1994; Paulus and Klockow, 1996).

**Capillary zone electrophoresis-electrospray ionization mass spectrometry of APTS-carbohydrate derivatives**

The mixture of arabinose, mannose, maltose, maltotriose, and maltotetraose APTS-derivatives was subjected to analysis by CE/ESI-MS in negative mode. The optimized CE separation conditions described above and the triethylammonium phosphate buffer were selected. Under these conditions, the ionization process by electrospray led to an unstable total ionic current (TIC). Moreover, the mass spectra recorded were characterized by a high ionic background and the absence of the expected pseudomolecular ions from the analytes. We postulated, that this phenomenon arose from the incompatibility of the phosphate buffer with the electrospray ionization process. To overcome this problem, other buffers composed of more volatile acids known to be more suitable for ESI/MS were investigated (Johnson and Tomlinson, 1996; Wahl and Smith, 1994). Thus, acetic and formic acids were selected as buffers for the CE separation of APTS-carbohydrate derivatives and the pH was always adjusted using TEA. As depicted in Figure 2A, good separation within less than 9 min was obtained using triethylammonium acetate buffer at pH 3.5. Decreasing the pH to 2.7 did not significantly improve either the resolution or the analysis time. Figure 2B shows the electropherogram of the carbohydrate APTS-derivatives using triethylammonium formate buffer at pH 2.7. Compared to the acetate buffer, it can be observed that the resolution remained similar whereas the analysis time was faster. So, both triethylammonium acetate and formate buffers, which allowed good APTS-carbohydrate adducts separation, were selected for CE coupling with ESI/MS.

Figure 3B shows the electropherogram of the reconstructed total ion current (TIC) trace from CE/ESI-MS analysis obtained from 15 pmol of arabinose-APTS derivative using the triethylammonium acetate buffer adjusted to pH 3.5 as running electrolyte, while Figure 3A depicts the UV trace. A sheath liquid, delivered coaxially to the CE, was used for the ionization process allowing completion of the CE capillary electric field effect, carriage of the analytes from the capillary end and finally the improved formation of highly charged droplets. Different solvent mixtures were checked and a mixture of isopropanol/water (75/25, v/v) at a flow rate of 1–5 µl/min was used. However, as previously noted, (Johnson and Tomlinson, 1996) the position of the CE capillary appeared to be the major parameter improving the ionization process. The optimum position was empirically determined from the ESI current stability to be located between 0.1 and 0.5 mm beyond the ESI needle.

The shorter migration time of the arabinose-APTS (2.6 min) observed in the CE-UV detection (Figure 3A) compared to that of the TIC electropherogram (11.4 min; Figure 3B) as well as the increased resolution arose mainly from the difference in capillary...
Fig. 3. Analysis of the arabinose-APTS. (A) CE/UV profile (254 nm). Conditions CE: capillary L = 80 cm, UV detection (254 nm) at 20 cm, triethylammonium acetate buffer (1% v/v acetic acid, 30 mM TEA). Peak assignment: 1 (APTS, 2.40 min); 2 (Ara-APTS, 2.53 min). (B) TIC profile from CE/ESI-MS. Conditions CE are the same as in (A), Conditions MS: ESI voltage 4 kV, sheath liquid (isopropanol/water 75/25 v/v, 3 µl/min), sheath gas (N₂, 30 psi). (C) Negative ESI mass spectrum of arabinose-APTS (peak 2).

The extracted mass spectrum taken at the crest of peak 2 is presented in Figure 3C and recorded in negative mode. The mass spectrum was dominated by ions at m/z 590.0 and 294.5 corresponding to singly and doubly charged deprotonated molecular ions (M-H)⁻ and (M-2H)²⁻, respectively. These latter ions were more abundant than the singly charged one. The presence of peaks of lower intensity at m/z 612.4 and 305.1 assigned to (M+Na⁺-2H)²⁻ and (M+Na⁺-3H)³⁻ respectively can also be observed. Finally, it can be noted the absence of triply charged ions. Similar CE/ESI-MS experiments were carried out on the arabinose-APTS-derivative but using triethylammonium formate buffer as the CE electrolyte. In this case, the abundance of the single and doubly charged ions was increased by a factor of 2 and significant mass spectra were obtained with up to 200 femol of arabinose-APTS derivative.

The triethylammonium formate CE buffer (pH 2.7) was also investigated for analysis of the APTS-derivatives of arabinose, mannose, maltose, maltotriose, and maltotetraose. The TIC electropherogram (Figure 4A), compared to the LIF electropherogram (Figure 2B), indicated a drastic decrease in the CE chromatographic resolution. This point was reflected by the unresolved arabinose and mannose APTS-derivatives (peak 1 and 2) appearing as one peak at 9 min. However, the TIC profile showed distinct peaks for the maltose (peak 3), maltotriose (peak 4), and maltotetraose (peak 5) APTS-derivatives. All the mass spectra were dominated by ions corresponding to singly and doubly charged pseudomolecular ions (M-H)⁻ and (M-2H)²⁻ with no significant presence of fragment ions (Table I). It should be noted that the two extracted mass spectra of the unresolved peak at 9 min exhibited pseudomolecular ions indicating, as shown by single ion monitoring, that the Ara- and Man-APTS derivatives could be separated (Figure 4B,C). Indeed, the mass spectra recorded at 8.90 min was dominated by the ions at m/z 294.6 typifying Ara-APTS derivative (Figure 4B) whereas the mass spectra recorded at 9.20 min exhibited ions at 309.6 which characterize the Man-APTS derivative (Figure 4C).

The extracted mass spectrum of the peak at 10.05 min assigned to the maltotetraose derivative (Figure 4D, Table I) was quite different to that of the other compounds. It was dominated by the doubly and triply charged pseudomolecular ions at m/z 552.9 and m/z 368.4 respectively and the presence in very low amounts of single charge pseudomolecular ions at m/z 1107.0. Apart from these pseudomolecular ions, fragment ions were also observed at m/z 471.6 arising from the pseudomolecular ions by the loss of an anhydro-monosaccharide.

The mannooligosaccharide caps were released by mild acid hydrolysis (0.1N HCl, 30 min, 110°C) from 2 µg of cellular ManLAMs of M.bovis BCG (Nigou et al., 1997). Under these conditions, preferential ManLAMs cleavages occurred in the arabinan domain leading mainly to the formation of Ara, mannooligosaccharide caps and the mannan core. The reaction subproducts were then labeled by APTS, as described in the Material and methods section, and then analyzed by CE using triethylammonium formate buffer. The separation was controlled by LIF and UV detection. Figure 5A depicts the UV electropherogram profile showing the presence of six major compounds characterized by migration times of 4.17 min (peak 1), 4.25 min (peak 2), 4.30 min (peak 3), 4.60 min (peak 4), 4.97 min (peak 5), and 5.37 min (peak 6), respectively. The most abundant compound migrated at 4.17 min (peak 1) and was unambiguously assigned to Ara-APTS.
Characterization of mannooligosaccharide caps

in agreement with the fact that ManLAM mild acid hydrolysis released mainly Ara (Venisse et al., 1993). The compound at 4.25 min (peak 2) which represented less than 10% of Ara (peak 1), was assigned, by coinjection, to the Man-APTS derivative. Other carbohydrate-APTS derivatives were observed with highest migration times of 4.30 min (peak 3), 4.60 min (peak 4), 4.97 min (peak 5) and 5.37 min (peak 6) similar to those of maltose, maltotriose, and maltotetraose APTS-derivatives, respectively. Thus, the structures of these carbohydrate-APTS adducts were tentatively assigned to Ara-Man, Ara-Ara, Ara-Man-Man or Ara-Ara-Man, and Ara-Man-Man-Man or/and Ara-Ara-Man-Man.

Table I. CE/ESI-MS analysis of APTS-labeled mono- and oligosaccharide standards

<table>
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<tr>
<th>Peak</th>
<th>Migration time (min)</th>
<th>Measured mass (M-H)</th>
<th>Calculated mass (Da)</th>
</tr>
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<td>1</td>
<td>8.90</td>
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<td>591.01</td>
</tr>
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<td>2</td>
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</tr>
<tr>
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<td>9.40</td>
<td>782.3</td>
<td>783.08</td>
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<tr>
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<td>945.13</td>
</tr>
<tr>
<td>5</td>
<td>10.05</td>
<td>1106</td>
<td>1107.18</td>
</tr>
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</table>

Peak assignment and migration time. Characteristic negative ions were extracted from the TIC electropherogram (Figure 4A). Calculated mass corresponded to the exact molecular mass.
In order to support these assignments, this mixture was then analyzed by CE/ESI-MS. The presence on the reconstructed total ion current profile (Figure 5B, Table II) of five intense peaks of interest with migration times at 13.30, 13.60, 14.05, 14.60, and 15.01 min was observed. Again, a drastic decrease in separation efficiency compared to that of the CE-UV electropherogram was apparent (Figure 5A). However, the ESI extracted mass spectra recorded in negative mode were characteristic of well separated compounds (Table II). This point, was reflected by the two mass spectra extracted at 13.25 and 13.40 min from the unresolved peak at 13.3 min. Indeed, the mass spectrum (peak 1, 13.25 min) was dominated by the doubly charged pseudomolecular ions at \(m/z\) 294.6 characteristic of the Ara-APTS derivative (Table II), while the mass spectrum (13.40 min) revealed a major signal at \(m/z\) 309.6 typifying Man-APTS. Likewise two different mass spectra were extracted from the next peak 3 in the TIC profile (Figure 5B) at 13.6 min. One, extracted at 13.47 min with major ions at \(m/z\) 360.3 assigned to (M-2H)\(^{-2}\) typified Ara-Ara-APTS (Table II), whereas the other showed ions for which the masses were not in agreement with the expected oligosaccharide-APTS adducts. The next peaks of the TIC profile at 14.05 min (peak 4) and 14.60 min (peak 5) corresponded from their extracted mass spectra to the single oligosaccharide APTS-derivative. Indeed, the mass spectra of peak 4 and peak 5 characterized Man-Ara-APTS [ions (M-2H)\(^{-2}\) at \(m/z\) 375.6], and Man-Man-Ara-APTS ions [M-2H]\(^{-2}\) at \(m/z\) 456.6 (Figure 5C), respectively. The localization of the mannose unit at the nonreducing end was supported by the presence of a fragment ion at \(m/z\) 375.6 (Figure 5C) arising from the loss of an anhydro-Man. Finally, the extracted mass spectrum of the last peak at 15.01 min was complex, exhibiting three types of ions at \(m/z\) 522.9, 344.0, and 291.0. The ions at \(m/z\) 522.9 were assigned to the (M-2H)\(^{-2}\)-pseudomolecular ions typifying the Man-Man-Ara-APTS derivative (Table II). However, we were unable to identify the remaining ions and it is likely from their high abundance that they were not produced by cleavage. Also, the expected Man-Man-Ara structure previously described for the BCG parietal ManLAM was in this case not observed (Venisse et al., 1993).

### Materials and methods

#### Capillary zone electrophoresis with LIF detector

Capillary zone electrophoresis separations were performed on a PACE capillary zone electrophoresis system (Beckman Instruments, Inc.) with the cathode on the injection side and the anode on the detection side (reverse polarity). The samples were injected by applying 0.5 psi pressure for 5 s. The separations were monitored on a 470 cm × 50 µm (I.D) uncoated fused-silica capillary column (Sigma, Division Supelco, St. Quentin, France) with a Beckman laser-induced fluorescence (LIF) detection system using a 4 mW argon-ion laser with an excitation wavelength of 488 nm and an emission filter of 520 nm. The temperature of the capillary in the PACE instrument was maintained at 25°C. The LIF detection was determined at a capillary length of 40 cm. The electropherograms were acquired and stored on an Dell/Pentium computer using the system Gold software package (Beckman Instruments, Inc.). The capillary was flushed with HCl 0.1N. Separations were carried out using different buffers, prepared from aqueous solutions of phosphoric acid, acetic acid and formic acid at low pH < 4 adjusted with an aqueous solution of TEA.

#### Capillary zone electrophoresis-electrospray ionization mass spectrometry analysis

All these experiments were carried out using the Beckman CE described above via a floated FinniganMat ESI source to a FinniganMat triple quadrupole instrument (TSQ-700). The CE spectrum of peak 1 (13.25 min) revealed a major signal at \(m/z\) 309.6 typifying Man-APTS. Likewise two different mass spectra were extracted from the next peak 3 in the TIC profile (Figure 5B) at 13.6 min. One, extracted at 13.47 min with major ions at \(m/z\) 360.3 assigned to (M-2H)\(^{-2}\) typified Ara-Ara-APTS (Table II), whereas the other showed ions for which the masses were not in agreement with the expected oligosaccharide-APTS adducts. The next peaks of the TIC profile at 14.05 min (peak 4) and 14.60 min (peak 5) corresponded from their extracted mass spectra to the single oligosaccharide APTS-derivative. Indeed, the mass spectra of peak 4 and peak 5 characterized Man-Ara-APTS [ions (M-2H)\(^{-2}\) at \(m/z\) 375.6], and Man-Man-Ara-APTS ions [M-2H]\(^{-2}\) at \(m/z\) 456.6 (Figure 5C), respectively. The localization of the mannose unit at the nonreducing end was supported by the presence of a fragment ion at \(m/z\) 375.6 (Figure 5C) arising from the loss of an anhydro-Man. Finally, the extracted mass spectrum of the last peak at 15.01 min was complex, exhibiting three types of ions at \(m/z\) 522.9, 344.0, and 291.0. The ions at \(m/z\) 522.9 were assigned to the (M-2H)\(^{-2}\)-pseudomolecular ions typifying the Man-Man-Ara-APTS derivative (Table II). However, we were unable to identify the remaining ions and it is likely from their high abundance that they were not produced by cleavage. Also, the expected Man-Man-Ara structure previously described for the BCG parietal ManLAM was in this case not observed (Venisse et al., 1993).

### Conclusion

This study has demonstrated that APTS-derivatized oligosaccharides may be analyzed by direct coupling of CE with ESI-MS. APTS-oligosaccharides have already been used for sensitive quantitative and qualitative analysis by CE with LIF detection. According to these results, on-line CE/ESI-MS provides a powerful analytical approach for the separation and characterization of unusual oligosaccharides. This, was shown by the structural characterization of the ManLAMs manno-oligosaccharide caps, which became more reliable after analysis of the ESI spectra. This strategy can be routinely applied to 5 nM of LAM so that it may be used in cap analysis of ManLAM obtained from other mycobacterial species, and also in order to screen banks of BCG \textit{bovis} mutants with truncated ManLAMs. Preliminary studies by on-line CE/ESI on large APTS-oligosaccharide derivatives indicated a loss of resolution and decrease of the ionization process. This problem might be overcome by using a nanoelectrospray system, instead of an electrospray, and an Iontrap mass spectrometer. An alternative approach based on off-line coupling between CE and matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF/MS) can also be proposed as it has recently been established that APTS tagging increases oligosaccharide ionization by MALDI-TOF (Suzuki et al., 1997).

<table>
<thead>
<tr>
<th>Peak</th>
<th>Migration time (min)</th>
<th>Measured mass (M-H)(^{-})</th>
<th>Measured mass (M-2H)(^{-2})</th>
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<td>522.9</td>
<td>APTS-Ara-Ara-Man-Man</td>
<td>1047.15</td>
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</tbody>
</table>

Peak assignment and migration time. Characteristic negative ions were extracted from the TIC electropherogram (Figure 5B). Calculated mass corresponded to the exact molecular mass.
ESI spray current using a micrometer head adapter kit for CE/MS (FinniganMat). The outlet end of the capillary was maintained at 4 kV which was the voltage used during the ESI. The voltage allowing electrophoretic separation of the analytes was the difference between the CE (20 kV) and the ESI (4 kV). The sheath liquid (isopropanol/water, 75/25 v/v) was supplied at a flow rate of 3 µl/min and a sheath gas (nitrogen, 30 psi) were delivered coaxially to the CE capillary. In all cases, the sheath liquid should be degassed daily by sonication. During the CE/ESI-MS experiments, the CE current and ESI current spray should be monitored and used diagnostically. Electropherograms of the APTS-carbohydrate derivatives were obtained using the mixture of acetic or formic acid adjusted to low pH with triethylamine. The UV absorption (254nm) was determined at a capillary length of 20 cm. Mass spectra were acquired in the negative mode using dwell times of 2 ms per 1 Da step in full mass scan mode.

**Samples preparation**

Monosaccharides and glucose oligomers (maltose, maltotriose, maltotetraose) were purchased from Sigma, trisodium APTS from Interchim (Paris). The APTS-derivatized oligosaccharide samples were used directly after derivatization or stored at -20 °C. All buffer solutions were filtered through a 0.2 µm pore size filter.

The carbohydrate solutions were dried through the gyrovap. The dried sugars (around 5 nmol) were then labeled as a result of reductive amination by the adding of 0.5 µl of 0.2 M APTS in 15% acetic acid and the same volume of NaBH₃CN 1 M in THF. The reaction was incubated for 90 min at 35°C and then diluted with deionized water in order to stop the reaction.

Cellular ManLAMs were extracted and purified as described previously (Nigou et al., 1997). Five nmoles of ManLAMs was hydrolyzed (0.1 M HCl, 110°C, for 30 min). The reaction products were mixed with 0.5 µl of 0.2 M APTS in 15% acetic acid and the same volume of NaBH₃CN 1 M in THF.

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**Abbreviations**

ABEE, aminobenzoate ethyl ester; ANDS, 3-amino-naphtalene-2,7-disulfonic acid; ANTS, 8-amino-naphtalene-1,3,6-trisulfonic acid; APTS, 9-aminopyrene-1,4,6-trisulfonate or 1-aminopyrene-3,6,8-trisulfonate; BCG, bacille de Calmette et Guérin; CE, capillary electrophoresis; CE/ESI-MS, capillary electrophoresis/electrospray ionization-mass spectrometry; FAB, fast atom bombardment ionization mode; FAB-MS/MS, fast atom bombardment–tandem mass spectrometry; HPLC, high performance liquid chromatography; LAM, lipoarabinomannan; LIF, laser induced fluorescence detection; MALDI-TOF/MS, matrix-assisted laser desorption ionization time-of-flight/mass spectrometry; ManLAM, lipoarabinomannan with mannosyl capping; NMR, nuclear magnetic resonance; PI-LAM, phospho-inositol lipoarabinomannan; TEA, triethylamine; TIC, total ion current.
References


