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A glycomic approach reveals a new mycobacterial polysaccharide

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Abstract

Mycobacterium tuberculosis lipoarabinomannan (LAM) and biosynthetically related lipoglycans and glycans play an important role in host-pathogen interactions. Therefore, the elucidation of the complete biosynthetic pathways of these important molecules is expected to afford novel therapeutic targets. The characterization of biosynthetic enzymes and transporters involved in the formation and localization of these complex macromolecules in the bacterial cell envelope largely relies on genetic manipulation of mycobacteria and subsequent analyses of lipoglycan structural alterations. However, lipoglycans are present in relatively low amounts. Their purification to homogeneity remains tedious and time-consuming. In order to overcome these issues and to reduce the biomass and time required for lipoglycan purification, we report here the development of a methodology to efficiently purify lipoglycans by sodium deoxycholate-PAGE. This faster purification method can be applied on a small amount of mycobacterial cells biomass (10-50 mg), resulting in tens of micrograms of purified lipoglycans. This amount of purified products was found to be sufficient to undertake structural analyses of lipoglycans and glycans carbohydrate domains by a combination of highly sensitive analytical procedures, involving cryoprobe NMR analysis of intact macromolecules and chemical degradations monitored by gas chromatography and capillary electrophoresis. This glycomic approach was successfully applied to the purification and structural characterization of a newly identified polysaccharide, structurally related to LAM, in the model fast-growing species *Mycobacterium smegmatis*.

Introduction

One-third of the world's population is infected by *Mycobacterium tuberculosis* (Mtb), the aetiological agent of human tuberculosis. Mtb cell envelope constituents, such as glycolipids, lipoglycans and glycans, play an important role in bacteria-host cell interactions (Angala et al, 2014; Ray et al, 2013). Biosynthetically related lipoglycans, comprising phosphatidyl-*myo*-inositol mannosides (PIMs), lipomannan (LM) and lipoarabinomannan (LAM) play essential roles in mycobacterial physiology and are important immunomodulatory molecules of the bacilli (Briken et al, 2004; Gilleron et al, 2008; Mishra et al, 2011; Torrelles & Schlesinger, 2010; Vergne et al, 2015). LAM are amphipathic macromolecules which possess a tripartite structure, including i) a mannosyl-phosphatidyl-*myo*-inositol (MPI) anchor, ii) a polysaccharide backbone composed of a D-mannan with a linear chain of α(1→6)-linked D-Man*p* units to which are attached single α(1→2)-D-Man*p* residues and a D-arabinan, with a linear chain of α(1→5)-linked D-Ara*f* units punctuated by branching produced by 3,5-*O*-linked α-D-Ara*f* units, and finally iii) caps assigned either to small manno-oligosaccharides (ManLAM) or phosphoinositol (PILAM) (Berg et al, 2007; Mishra et al, 2011; Nigou et al, 2003). Lipid-free glycans structurally related to LAM and LM, i.e. arabinomannan (AM) and mannan, are also found in the outermost layers of the mycobacterial cell envelope (Nigou et al, 1999; Ortalo-Magne et al, 1995).

The elucidation of the complete biosynthetic pathways of these important molecules is expected to afford novel therapeutic targets (Angala et al, 2014; Berg et al, 2007; Gilleron et al, 2008). In support of this assumption, D-arabinan biosynthesis is the target of ethambutol (Deng et al, 1995), a first-line drug in the treatment of tuberculosis, as well as of benzothiazinones (Makarov et al, 2009), which are new antituberculous drug candidates in preclinical development. The characterization of biosynthetic enzymes as well as the transporters involved in the formation and localization of these complex macromolecules

largely relies on genetic manipulation of mycobacteria and subsequent analyses of lipoglycan structural alteration (Angala et al, 2014; Gilleron et al, 2008). However, lipoglycans are present in relatively low amounts (~1 mg/g of dry mycobacteria) and their purification to homogeneity remains tedious and time-consuming (Laneelle et al, 2015; Nigou et al, 2004). Conventional lipoglycan purification protocol involves several solvent extractions, followed by enzymatic digestions and two chromatographic steps: hydrophobic interaction chromatography to separate glycans and lipoglycans and then gel filtration to separate PIM, LM and LAM individually. If homogenous fractions of PIM, LM or LAM are desired and because gel filtration chromatography is not adapted to small quantities, a minimum of 1 g of dry biomass is required to start with.

In order to reduce the biomass and the time required for lipoglycan purification, we aimed at replacing the two chromatographic steps by a single step of purification using polyacrylamide gel electrophoresis (PAGE). This approach has been successful for purification of LPS and proteins (Claverol et al, 2003; Galvani et al, 2000; Hardy et al, 1997; Kurth & Stoffel, 1990; Pupo et al, 2000; Pupo et al, 2004). Typically, after PAGE migration, macromolecules are passively eluted from the gel by incubation in an elution buffer. The latter can be compatible with mass spectrometry (MS), allowing subsequent structural analysis. This approach has also proved to permit biological, including *in vivo*, studies as shown with LPS (Pupo et al, 1999).

Here, we report the development of a methodology to efficiently purify lipoglycans by sodium deoxycholate-PAGE (DOC-PAGE). This faster purification method can be applied on a small amount of mycobacterial cells biomass (10-50 mg), resulting in tens of micrograms of purified lipoglycans. This amount is sufficient, as demonstrated, to undertake structural analyses of lipoglycans and glycans carbohydrate domains by a combination of highly sensitive analytical procedures, involving cryoprobe NMR analysis of intact

macromolecules and chemical degradations monitored by gas chromatography and capillary electrophoresis. This glycomic approach was successfully applied to the structural characterization of a newly identified polysaccharide in the model fast-growing species *Mycobaterium smegmatis*.

Results and Discussion

The development of a PAGE-based methodology to efficiently purify lipoglycans required to be able: i) to quickly localize lipoglycans in the gel in order to avoid their diffusion before elution, and ii) to efficiently elute the lipoglycans from the gel. *M. smegmatis* LAM purified by the conventional protocol was used as a standard to set up and optimize the different steps.

Direct detection of fluorescently-labelled lipoglycans in gels

LAM can be routinely detected on gels using either zinc sulfate negative staining or silver nitrate staining (Venisse et al, 1993). Zinc sulfate negative staining is reversible and non-degradative (Hardy et al, 1997) and reveals LAM as a translucent area. However, the presence of zinc sulfate hinders subsequent MS analyses. Silver nitrate staining is degradative and, once stained, the chemical structure of LAM is altered. So, none of the classical staining methods of LAM is compatible with a subsequent structural analysis. We thus resorted on an alternative strategy consisting in the detection of LAM on a standard lane and its use as a migration reference to excise gel bands containing unlabelled LAM (Figure 1). However, zinc sulfate and silver nitrate staining procedures take around 2 hrs (Hardy et al, 1997; Venisse et al, 1993), a period of time during which molecules can diffuse in the gel, precluding a precise excision. To overcome this limitation, we decided to fluorescently-label LAM prior to PAGE migration. The chromophore Lucifer Yellow (LY), which bears a hydrazide function enabling its coupling to lipoglycans after periodate oxidation (Pitarque et al, 2005), was selected. The labelling procedure was optimized by focusing on three parameters: i) oxidation reaction time, ii) LAM/LY ratio and iii) coupling reaction time. After optimization, the labelling procedure was set as follows: one-hour oxidation reaction, 10 min coupling reaction using a LAM/LY ratio of 1:100 mol/mol, and 1.5 hrs dialysis against water to remove most of the probe excess. Labelling 10 µg of LAM or less was sufficient to

visualize it on the gel under UV light (Figures 1 and 2). Importantly, LY-labelling did not alter the migration of the lipoglycans. Indeed, LY-labelled LAM, when revealed by either zinc sulphate negative staining (Figure 2A) or UV (Figure 2B), showed a migration identical to that of native LAM. Altogether, this 'real-time' detection method allowed a precise excision of the gel bands containing lipoglycans immediately after migration (Figure 1), thereby reducing the risk of molecules diffusion in the gel and increasing their recovery yields.

Recovery of lipoglycans from gel by passive elution

Several methods of proteins or LPS elution from gel fragments have been described (Claverol et al, 2003; Kurth & Stoffel, 1990; Pupo et al, 1999; Pupo et al, 2000; Pupo et al, 2004). For proteins, passive elution is usually employed using an extraction solution that can contain up to 0.1 % of SDS in water (Claverol et al, 2003; Kurth & Stoffel, 1990). As for LPS, Hardy *et al.* (Hardy et al, 1997; Pupo et al, 1999) developed a grinding process which involves passing the gel pieces through 100 to 32 microns diameter pore metal filters to facilitate the elution of LPS from polyacrylamide. The recovery yields reported were in the range of 70-80 % for LPS without O-antigen chain but 10% only for O-antigen-containing LPS, which are structurally closer to mycobacterial lipoglycans (De Castro et al, 2010; Raetz & Whitfield, 2002). Based on this set of previous studies, we focused on two types of extraction solutions, one based on the use of detergents and the other on organic solvents. 20 µg of pure *M. smegmatis* LAM were loaded on the gel and submitted to DOC-PAGE migration. DOC-PAGE was selected over SDS-PAGE because lipoglycans migration was more focused (not shown). The gel band containing LAM was excised according to the migration reference provided by LY-labelled LAM (Figure 1) and cut into 0.5 mm sized pieces that were placed in the elution solution. Initially, the passive elution condition was set to 3 hours at 37 °C. The

objective was to find an extraction solution more efficient than water used as a control. Detergents are supposed to promote the disaggregation of the molecules, thereby facilitating their elution through the mesh of the gel. Two detergents were tested: SDS and DOC, both at concentrations of 0.1 % and 1 % in water. Higher concentrations make the elimination of these detergents difficult and prevent any structural analysis (Claverol et al, 2003; Kurth & Stoffel, 1990). The results presented in Table 1 show that the use of DOC did not provide any improvement as compared to water alone (yield of 4%). 1% SDS increased the recovery yield up to 9% but this remained insufficient. We thus concluded that passive elution using detergents was not a suitable method for lipoglycans and then resorted to organic solvents. Acetonitrile was first tested at concentrations of 15, 30 or 60 % in water. By dehydrating gels, acetonitrile induces a leakage of water, draining out of the gel the other molecules entrapped. However, elution yields were again comparable to those obtained with water alone (Table 1). We finally tested the volatile base triethylamine (TEA), assuming that TEA may increase mesh size, thereby facilitating the passive elution of molecules. TEA was previously used at a concentration of 5 % in water for elution of LPS (Pupo et al, 2000). Here, we tested it at 0.1, 1, 5 or 10 % in water. The recovery yield was improved by the use of 10% TEA as compared to water alone, but remained in the same range than that obtained with 1 % SDS (Table 1). Unlike SDS, however, TEA is volatile and can therefore be removed by evaporation under vacuum. After optimization of the elution time, the final conditions were set as incubation in 10 % TEA in water for 12 h at 37 °C, followed by two washes of 2 h with the same solution. The recovery yield was ~60 % as compared to ~20 % using water only (Table 1).

Structural analysis of LAM eluted from gel

We next determined whether the new purification procedure developed herein did not alter lipoglycans structure and whether it was compatible with the set of highly sensitive analytical procedures we previously developed to investigate the latter (Nigou et al, 2000). 2 mg of LAM were purified from *M. smegmatis* using the conventional protocol (LAMc). A portion of this LAM batch (~200 µg) was submitted to the new purification procedure using DOC-PAGE as described above and ~100 µg of LAM were recovered from the gel (LAMg). A comparative structural analysis of both LAM samples was then performed. MALDI-TOF mass spectrometry analysis exhibited for both deacylated LAM samples a broad unresolved peak centered at *m/z* 14,900, indicating a molecular mass around 14.9 kDa for the major molecular species (Figure 3). In addition, the intrinsic heterogeneity of LAM, as revealed by the peak width, was similar for both samples. Thus, the overall structure of LAM did not appear to be altered by the in-gel purification steps. To gain further insight into the fine structure of the polysaccharidic moiety, both LAM samples were submitted to chemical degradations, followed by capillary electrophoresis monitored by laser-induced fluorescence (CE-LIF) or gas chromatography (GC) analyses. Total acid hydrolysis showed the presence of Ara and Man, with an Ara/Man ratio of 0.8±0.1 and 0.9±0.05 for LAMc and LAMg respectively (Table 2). Acetolysis experiment monitored CE-LIF (Nigou et al, 2000) indicated that both LAM exhibited a mannan core with a similar degree of branching (Table 2). Finally, per-*O*-methylation analysis revealed the same glycosidic linkages for both LAMc and LAMg and in similar relative abundance (Table 2).

Altogether, these data demonstrated that LAM structure was not altered by the in-gel purification procedure and that it could be investigated after elution from the gel using an adapted set of sensitive procedures.

Identification and structural characterization of a new LAM-like polysaccharide in M. smegmatis mc^{2}155

Mycobacterial lipoglycans and glycans are a complex family of biosynthetically related molecules (Angala et al, 2014; Briken et al, 2004; Gilleron et al, 2008; Mishra et al, 2011; Nigou et al, 2003). They consist in LM, LAM and their MPI anchor-free counterparts mannan and AM. Interestingly, we identified in the crude fraction of lipoglycans and glycans extracted from *M. smegmatis* a previously uncharacterized compound (named X) that could be revealed by DOC-PAGE migration after alkali-treatment of the extract (Figure 4A) (Skovierova et al, 2009). Monosaccharide analysis by GC after octanolysis and trimethylsisylation showed that the crude fraction contained only D-Ara and D-Man, suggesting that compound X harboured a structure reminiscent to that of lipoglycans or glycans. Compound X was purified using the in-gel procedure described above and 100 µg could be obtained by loading 2 mg of the extract. This amount was sufficient to investigate its structure. Total acid hydrolysis followed by CE analysis indicated that compound X was composed of Ara and Man, with an Ara/Man ratio of 0.25. This ratio value was 3 to 4 fold lower than that of *M. smegmatis* LAM (Table 2). MALDI-TOF mass spectrometry analysis of permethylated compound X showed a set of peaks centred around *m/z* 6000 and separated by 204 mass units corresponding to permethylated anhydro-Man residues (Figure 4B). As previously observed for the other mycobacterial glycans and lipoglycans, compound X exhibited a Gaussian distribution of glycoforms with the major ones showing a molecular mass around 5 kDa, a value three times lower than that of LAM or AM. Compound X thus contained an average of 9 Ara and 23 Man units, indicating the presence of an arabinan domain much shorter than that of *M. smegmatis* LAM composed of a mean of ~55-70 Ara and ~20-35 Man units (Angala et al, 2014; Kaur et al, 2014; Khoo et al, 1996; Mishra et al, 2011). Per-*O*-methylation analysis revealed the presence in compound X of 6-Man*p*, 2,6-

Man*p*, t-Man*p*, 5-Ara*f*, 3,5-Ara*f*, 2-Ara*f* and t-Ara*f* residues, as observed in LAM (Table 2) (Gilleron et al, 1997). However, in agreement with other chemical analyses, the relative amount of arabinose residues was much lower in compound X than in LAM and consisted mainly in 5-Ara*f* units. In order to gain insight into the organisation of its arabinan domain, 10 µg of compound X were hydrolyzed with endoarabinanase from *Cellulomonas gelida* (Lee et al, 2006; Shi et al, 2006), followed by the analysis of the released products by CE after labelling by APTS or by MALDI-TOF MS after permethylation. As shown in Figure 5A, digestion of compound X yielded the characteristic products previously described for LAM (Shi et al, 2006), assigned to linear di-arabinofuranosides (Ara_2, peak II): Ara*f*-α(1→5)-Ara*f*-α(1→, tetra-arabinofuranosides (Ara_4, peak III): Ara*f*-β(1→2)-Ara*f*-α(1→5)-Ara*f*-α(1→5)-Ara*f*-α(1→ and biantennary hexa-arabinofuranosides (Ara_6, peak IV): [Ara*f*-β(1→2)-Ara*f*-α(1-]$_2$→3 and →5)-Ara*f*-α(1→5)-Ara*f*-α(1→ motifs. This assignment was further confirmed by MALDI-TOF MS analysis in the positive ion mode of the permethylated products (Figure 4B). The peaks at *m/z* 709.2 and *m/z* 1029.4 were assigned to $(M+Na)^+$ ions corresponding to the motifs Ara_4 and Ara_6 respectively (Figure 5B). Acetolysis experiment monitored by CE-LIF (Nigou et al, 2000) showed a ratio of Man*p*-α(1→2)-Man to Man residues of 0.75:1 (Figure 5C), indicating a degree of branching of the mannan domain of 43% (Table 2). This value is lower than that determined for *M. smegmatis* LAM (60% ± 5%) (Figure 5C; Table 2). The set of structural data obtained by chemical analyses were confirmed by NMR analysis of the entire molecule. Indeed, the anomeric region of the 2D ^{1}H-^{13}C HMQC spectrum of compound X exhibited cross peaks assigned to 6-Man*p* (VI; δ_{H-1} 4.72; δ_{C-1} 97.8), 2,6-Man*p* (VII; δ_{H-1} 4.92; δ_{C-1} 96.7), t-Man*p* (IV; δ_{H-1} 4.86; δ_{C-1} 97.8), 5-Ara*f* (II; δ_{H-1} 4.89; δ_{C-1} 106.1), 3,5-Ara*f* (I; δ_{H-1} 4.91; δ_{C-1} 106.0), and t-Ara*f* (V; δ_{H-1} 4.95; δ_{C-1} 99.3) residues (Figure 6). In agreement with chemical analyses, the signals corresponding to Ara units in compound X spectrum (Figure 6B) were of much lower relative intensity that those recorded in LAM

spectrum (Figure 6A). In addition the cross peak corresponding to 2-Ara*f* units (III; δ_{H-1} 4.98; δ_{C-1} 106.9) present in lateral arabinan chains was very faint. Of note the signal corresponding to 6-Man*p* units in compound X showed a higher relative abundance, in agreement with a weaker degree of branching of the mannan domain.

Altogether, these analyses allowed us to propose a structural model for compound X as depicted in Figure 7.

Conclusion

We herein developed a methodology to efficiently purify lipoglycans by sodium deoxycholate-PAGE that can be applied on a small amount of mycobacterial cells biomass (10-50 mg). The resulting tens of micrograms of purified lipoglycans are sufficient to undertake structural analyses of carbohydrate domains by a combination of highly sensitive analytical procedures, involving cryoprobe NMR analysis of intact macromolecules and chemical degradations monitored by gas chromatography and capillary electrophoresis. This glycomic approach was successfully applied to the purification and structural characterization of a newly identified polysaccharide in the model fast-growing species *M. smegmatis*. This polysaccharide shows a structure reminiscent to that of LAM, bearing all the structural motifs identified in this complex macromolecule, but with shorter arabinan and less branched mannan domains. However, its biosynthetic origin remains unclear. Since it was isolated after mild alkaline treatment, we investigated whether it might contain a MPI anchor in its native state. Growing bacteria with *myo*-Inositol-(2-^{3}H) resulted in a radiolabelling of LAM and LM as previously described (Hunter & Brennan, 1990), but not of compound X, suggesting that the structure of the latter does not contain *myo*-Inositol. A LM-like molecule based on a glucuronosyl diacylglycerol, instead of a MPI, anchor was previously described in corynebacteria (Tatituri et al, 2007), which along mycobacteria belongs to the suborder

Corynebacterineae. Disruption in *Corynebacterium glutamicum* of the *pimB/mgtA* gene, coding for the α-mannosyltransferase involved in the addition of the first mannosyl unit on the glucuronosyl residue, was found to result in the abrogation of the LM-like biosynthesis (Tatituri et al, 2007). Interestingly, the glycolipid 3-(*O*-α-D-glucuronopyranosyl)-1,2-diacyl-*sn*-glycerol, which corresponds to the free lipid anchor of this LM-like molecule, was also characterized in *M. smegmatis* (Wolucka et al, 1993). However, analysis of a *M. smegmatis* mutant disrupted for the orthologue of *pimB/mgtA* gene showed that compound X was still produced, indicating that the structure of the latter is not likely to be based on a glucuronosyl diacylglycerol lipid anchor. Understanding the biosynthetic origin of this newly identified polysaccharide thus requires further investigations. Its identification asks again the question of the biosynthetic link between lipoglycans on the one hand and their lipid-free glycan counterparts on the other hand. Whether the latter are generated by action of an endogenous endoglycosidase (α-mannosidase) still remains an open question (Pitarque et al, 2008).

Material and Methods

Bacterial strains and culture conditions

M. smegmatis mc^2155 was grown in Middlebrook 7H9 medium supplemented with ADC and 0.05% Tween 80.

Lipoglycans isolation and purification by the conventional protocol

LAM and LM were purified from *M. smegmatis* mc^2155 as described previously (Gilleron et al, 1997; Nigou et al, 1997). Briefly, mycobacteria were delipidated using $CHCl_3/CH_3OH$ 2:1 v/v, followed by $CHCl_3/CH_3OH$ 1:1 v/v for 2 h at 50°C. The delipidated mycobacteria were then extracted 3 times in 50% ethanol at 50°C for 2h. The ethanol/water extracts were pooled, dried and submitted to digestion by DNAse (D4263, Sigma-Aldrich), RNAse (R4875, Sigma-Aldrich), α-amylase (A6814, Sigma-Aldrich) and a cocktail of proteases [α-chymotrypsin (C4129, Sigma-Aldrich), *Streptomyces griseus* proteases (P8811, Sigma-Aldrich) and trypsin, (T3914, Sigma-Aldrich)] to remove nucleic acids, α-glucan and proteins. The digested solution was dialysed against dH_2O and dried, resulting in a fraction containing glycans and lipoglycans. This fraction was then either used for DOC-PAGE purification of lipoglycans or used for conventional purification. For conventional purification, the fraction containing glycans and lipoglycans was loaded onto an octyl-sepharose column, eluted first with 10% isopropanol and then with 80% isopropanol at a flow rate of 0.3 mL/min to separate glycans and lipoglycans. After dialysis against dH_2O of the 80% isopropanol fraction, the lipoglycans were then loaded into a gel filtration column Biogel P100 (1.5 x 50 cm) in a DOC buffer (Tris-HCl 10 mM, NaCl 200 mM, 1mM EDTA, 0,25% sodium deoxycholate, pH 8.0) at a flow rate of 1 mL/min to separate LAM from LM. The fractions were analysed by SDS-

PAGE and the fraction containing LAM or LM were pooled, dialysed against dH_2O, dried and precipitated with ethanol.

Lipoglycans labelling with Lucifer Yellow for PAGE-UV

Glycans and lipoglycans, typically 1 to 10 µg, were submitted to oxidation by sodium metaperiodate 1 M for 1 h at 4°C in the dark (Pitarque et al, 2005). The reaction was quenched by addition of sodium bisulphite 80 mM and settled for 5 min at room temperature. Oxidized glycans and lipoglycans were then labelled for 10 min at 37°C using a 5 mg/mL solution of Lucifer Yellow (861502, Aldrich) solubilized in dH_2O. Excess dye was removed by micro-dialysis against dH_2O for 2 h at room temperature. The labelled glycans and lipoglycans were then dried under speed-vacuum and submitted to DOC-PAGE migration.

DOC-PAGE passive elution of lipoglycans

Lucifer Yellow-labelled and unlabelled glycans and lipoglycans samples were loaded onto a DOC-PAGE gel 15% and submitted to electrophoresis. After migration, the gel was placed onto a glass surface and labelled lipoglycans were detected under UV (λ 254 nm). By assuming that the migration of the labelled lipoglycans was similar to that of the non-labelled one, the bands corresponding to LAM, LM or glycans were excised from the gel. The band was then cut into 0.5 mm sized pieces and placed into 1.5 mL microtubes. Lipoglycans were then passively eluted using a solution of triethylamine (TEA) 10% in dH_2O at 37°C overnight followed by 2 washes of 2 h at 37°C with fresh elution solution. The solution containing passively eluted lipoglycans were pooled, passed through a 0.22 µm non-pyrogenic filter and dried under speed-vacuum. The eluted lipoglycans were then re-suspended with dH_2O and submitted to dialysis against dH_2O for 2 h at room temperature prior to use for structural determination.

Determination of the absolute configuration of the monosaccharides

1 mg of the lipoglycans and glycans crude fraction was octanolysed by 2M trifluoroacetic in (*R*)-(−)-2-Butanol for 16 hr at 110°C (Gerwig et al, 1978). Octyl-glycosides were dried and trimethylsilylated by treating with hexamethyldisilazane/chlorotrimethylsilylane/pyridine, 4:2:1, v/v/v, for 15 min at room temperature. The resulting derivatives were solubilized in cyclohexane before analysis by GC and comparison with standards of D-Ara, L-Ara, D-Man, L-Man submitted to the same procedure.

Monosaccharide composition and mannan core branching degree determination by capillary electrophoresis

These analyses were performed as previously described (Nigou et al, 2000). Briefly, for monosaccharide composition, lipoglycans or glycans were submitted to strong acid hydrolysis with 2 M trifluoroacetic acid at 110°C for 2 h and then dried under speed-vacuum. For mannan core degree determination, lipoglycans or glycans were treated with an acetic anhydride/acetic acid/sulfuric acid (10:10:1, v/v/v) mixture for 3 h at 40°C. After extraction with water, acetolysis products were deacetylated with a methanol/20% aqueous ammonia solution (1:1, v/v) at 37°C for 18 h and dried under a stream of nitrogen. The resulting mono- or oligo-saccharides were derivatized for 90 min at 55°C using a solution of 1-aminopyrene-3,6,8-trisulfonate (APTS) at 0.2 M in 15% acetic acid and 1 M sodium cyanoborohydride solution dissolved in tetrahydrofuran. The APTS-labelled monosaccharides were suspended in dH_2O and subjected to capillary electrophoresis. Analysis was performed on a P/ACE 5000 capillary electrophoresis in reverse polarity (Beckman Coulter) equipped with a laser-induced fluorescence (LIF) detector with a 4 mW argon-ion laser (λ_{ext} 488 nm, λ_{em} 520 nm). Separations were performed using an uncoated fused-silica capillary column (50 µm internal

diameter, 40 cm effective length) and 30 mM triethylamine 1% (w/v) acetic acid running buffer at pH 3.5, at 25°C with an applied voltage of 20 kV.

Digestion of lipoglycans by the endoarabinanase from Cellulomonas gelida

10 µg of dried purified lipoglycans or glycans were treated with 4 µL of endoarabinanase from *Cellulomonas gelidas* (suspended at 5 mg/mL in Tris-HCl 10 mM pH 7.0 for 12h at 37°C) prepared as described in (Lee et al, 2006; Shi et al, 2006). The released products were purified through a 3 kDa microcon® centrifugal filter unit and analysed by CE-LIF after tagging by APTS (Nigou et al, 2000) or MALDI-TOF MS in positive ion mode after permethylation (Morelle et al, 2004).

Glycosidic linkage analysis

Glycosyl linkage composition was performed according to the modified procedure of Ciucanu and Kerek (Ciucanu & Kerek, 1984). The per-O-methylated lipoglycans or glycans were hydrolyzed using 500 µl of 2 M trifluoroacetic acid at 110°C for 2 h, reduced using 350 µl of a 10mg/ml solution of $NaBD_4$ (NH_4OH 1M / C_2H_5OH, 1:1, v/v) and per-O-acetylated using 300 µl of acetic anhydride for 1 h at 110°C. The resulting alditol acetates were solubilized in cyclohexane before analysis by GC and GC/MS.

MALDI-TOF analysis

Deacylated lipoglycans were analysed on a Voyager DE-STR MALDI-TOF instrument (PerSeptive Biosystems, Framingham, MA) using linear mode detection. Mass spectra were recorded in negative ion mode using 300 ns time delay, with a grid of 80% of full acceleration voltage (25kV) and a guide wire voltage of 0.15%. 2,5 dihydroxybenzoic acid was used as the matrix at a concentration of 10 mg/mL in 50% ethanol in dH_2O.

Methylated oligosaccharides were analysed on a 4700 Proteomics Analyser (with TOF-TOF Optics, Applied Biosystems) using the reflectron mode. Ionization was effected by irradiation with pulsed UV light (355 nm) from an Nd:YAG laser. Samples were analysed by the instrument operating at 20 kV in the positive ion modes using an extraction delay time set at 20 ns. Typically, spectra from 1,000 to 2,500 laser shots were summed to obtain the final spectrum.

NMR analysis

NMR spectra were recorded at 298K on a Bruker Avance 600 MHz spectrometer equipped with a cryogenic probe TCi (Bruker Biospin, Germany). Purified lipoglycans or glycans were exchanged in D_2O (D, 99.97% from Euriso-top, Saint-Aubin, France), with intermediate lyophilisation, and then dissolved in 0.5 ml D_2O. Samples were analysed in a 200 x 5 mm UL-5 NMR tubes (Euriso-Top, Saint-Aubin, France). Proton and carbon chemical shifts are expressed in part per million and referenced relative to internal acetone signals at δ_H/TMS 2.225 and δ_C/TMS 31.45 ppm.

All 2D NMR data sets were recorded without sample spinning. ^{1}H-^{13}C correlation spectra were acquired in the echo/anti-echo-TPPI gradient selection mode (256 data points) recorded in the proton-detected mode using the "hsqcetgpsisp2" sequence from the Topspin v2.1 software (Bruker Biospin) (Schleucher et al, 1994). The GARP sequence (Shaka et al, 1985) at the carbon frequency was used as a composite pulse decoupling during acquisition.

The HSQC spectra were recorded with a spectral width of 25,000 Hz in ^{13}C and 4807 Hz in ^{1}H dimensions in order to collect a 1024 x 256 (TPPI) point data matrix with 2 scans per t_1 value expanded to 2048 x 1024 by zero filling. The relaxation delay was 2.0 s. A sine bell window shifted by $\pi/2$ was applied in both dimensions.

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Conflict of interest statement

None declared

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Abbreviations

CE-LIF, capillary electrophoresis monitored by laser-induced fluorescence; DOC: sodium deoxycholate; HMQC, heteronuclear multiple quantum coherence; LAM, lipoarabinomannan; LM, lipomannan; LY, Lucifer Yellow; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis.

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Legends to figures

Figure 1: **DOC-PAGE-UV detection of LY-labelled lipoglycans and work-flow showing the strategy developed to purify lipoglycans from gel and to investigate their structure.**

Figure 2: DOC-PAGE migration of native and LY-labelled LAM.

5 µg of native (lane 1) and LY-labelled (lane 2) LAM were submitted to DOC-PAGE migration. The same gel was revealed first by zinc sulphate negative staining (A) and then by UV (B). std, protein ladder.

Figure 3: MALDI-TOF MS spectra of deacylated LAM from *M. smegmatis* purified by the conventional protocol (A) or by DOC-PAGE (B).

Figure 4: **DOC-PAGE (A) and MALDI-TOF MS (B) analyses of compound X.**

A) 10 µg of the *M. smegmatis* glycans and lipoglycans fraction were loaded on the gel before (lane 1) or after alkali-treatment (lane 2). The gel was revealed by zinc sulphate negative staining. B) Linear MALDI-TOF MS spectrum in the positive mode of permethylated compound X.

Figure 5. Structural analysis of compound X arabinan (A, B) and mannan (C) domains.

A, B) Digestion of arabinan domain (10 µg of compound X) by endoarabinanase from *Cellulomonas gelida* monitored by CE-LIF (A) and MALDI-TOF MS analysis (B). A) I, Internal Standard-APTS; II, Ara_2-APTS; III, Ara_4-APTS; IV, Ara_6-APTS; **a,** compound X; **b,**. LAM from *M. smegmatis*; **c**, co-injection. B) MALDI-TOF MS analysis in positive ion mode of the permethylated products of hydrolysis of LAM (upper spectrum) and Compound

X (lower spectrum) (50 µg of each). Ions at *m*/*z* 709.3 correspond to Ara_4 motif and *m*/*z* 1029.4 correspond to Ara_6 motifs.

C) Mannan core degree of branching determined by acetolysis treatment (10 µg of compound X). I, Ara-APTS; II, Man-APTS; III, Internal Standard-APTS; IV, Man-α(1→2)-Man-APTS; **a**, compound X; **b**, LAM from *M. smegmatis*; **c**, co-injection.

Figure 6: 2D ^{1}H-^{13}C HMQC NMR spectra expansion ($\delta^{1}H$: 4.60-5.15 and ^{13}C: 97.0-113.0) of *M. smegmatis* LAM (A) and compound X (B) in D_2O at 298K.

I : 3,5-α-Ara*f* ; II : 5-α-Ara*f* ; III : 2-α-Ara*f* ; IV : t-α-Man*p* ; V : t-β-Ara*f* ; VI : 6-α-Man*p* ; VII : 2,6-α-Man*p*.

Figure 7: Structural model of compound X from *M. smegmatis*.

Compound X is comprised of a linear α(1→6)-mannan domain that is substituted at almost half of the *O*-2 positions by one Man*p* unit. It contains a short arabinan chain, possibly linked at *O*-2 position of a Man*p* unit as recently described in LAM by Kaur *et al* (2014), that harbours the Ara_4 and Ara_6 motifs typically found in LAM and AM.

●, D-Man*p*; ⬠, D-Ara*f*.

Tables

Table 1: LAM recovery yield from DOC-PAGE using different elution conditions.

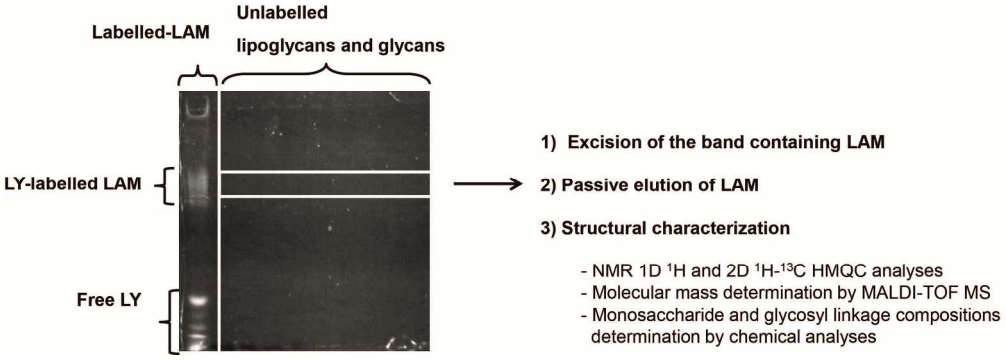
Elution solution	Time of elution (hours)	% of detergent or organic solvent	Recovery (%)[(a)]
water	3	-	4.0±0.2
	12	-	20.0±2.5
SDS in water	3	0.1 %	6.2±0.5
	3	1%	9.1±0.4
DOC in water	3	0.1%	3.5±0.3
	3	1%	3.8±0.6
ACN in water	3	15%	3.1±0.5
	3	30%	3.4±0.8
	3	60%	2.5±1.1
TEA in water	3	0.1%	4.1±0.4
	3	1%	5.3±0.2
	3	5%	5.7±0.4
	3	10%	7.8±0.3
	12	10%	60.0±4.0

[(a)] Values were determined after loading 20 µg of LAM and represent the average of 2 independent experiments

Table 2: Structural features of LAMc, LAMg and Compound X.

	LAMc	LAMg	Compound X
Ara/Man ratio[(a)]	0.8±0.1	0.9±0.05	0.25±0.08
Mannan degree of branching[(a)] **(%)**	55±8	60±5	43±5
Glycosyl linkage[(b)] **(%)**			
t-Ara*f*	4.4	4.3	0.3
2-Ara*f*	11.2	9.5	3.2
5-Ara*f*	39.3	41.5	18.8
3,5-Ara*f*	13.7	12.0	6.5
t-Man*p*	19.4	21.6	28.5
6-Man*p*	6.7	6.0	19.5
2,6-Man*p*	5.3	5.1	23.2

[(a)] Monitored by CE-LIF
[(b)] Monitored by GC



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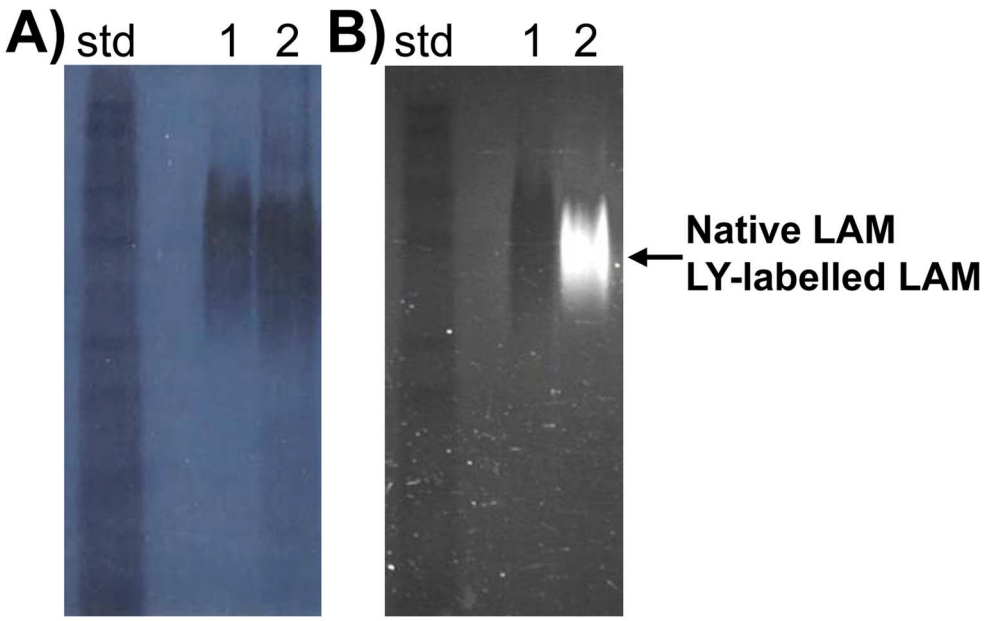

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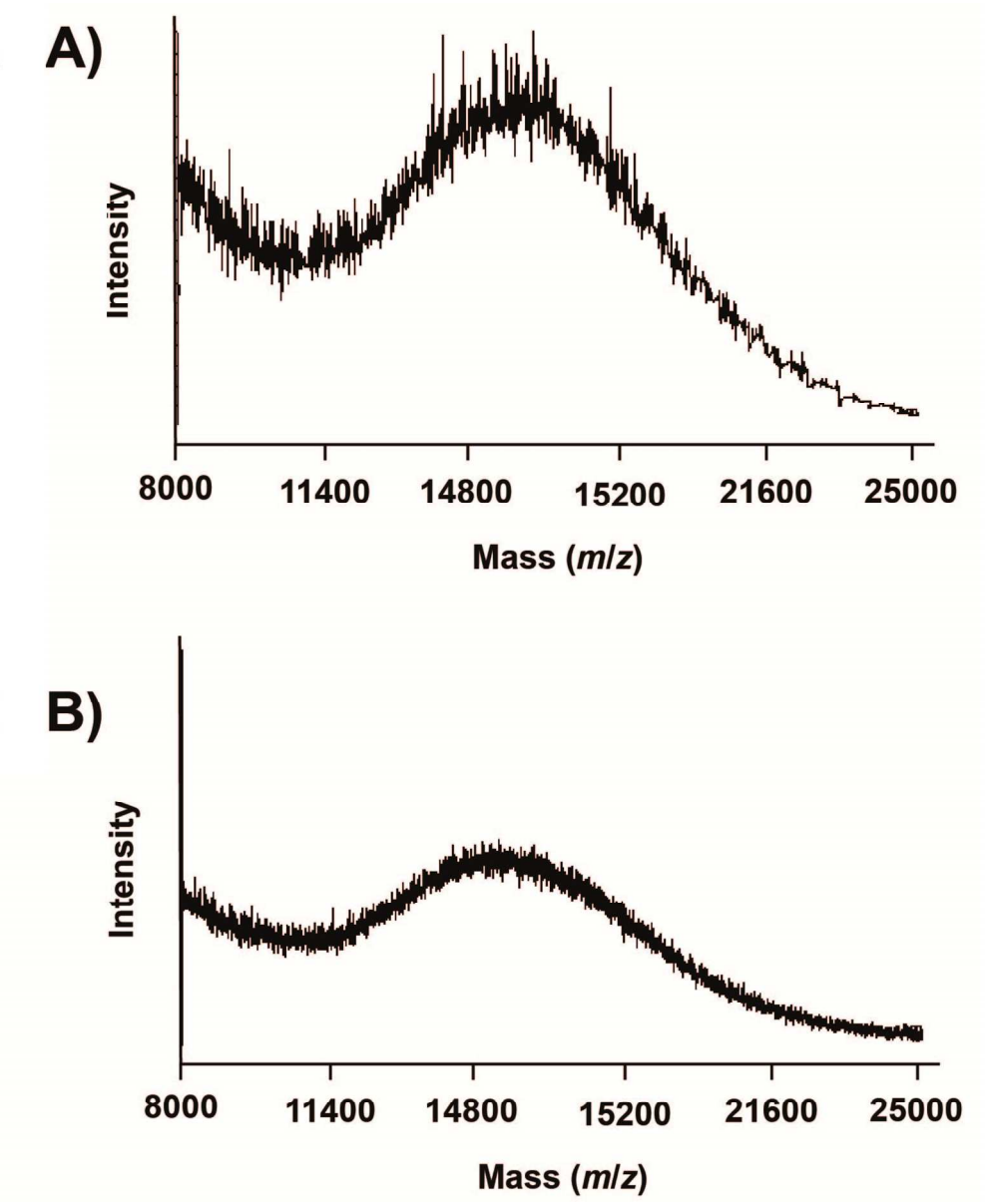
5 µg of native (lane 1) and LY-labelled (lane 2) LAM were submitted to DOC-PAGE migration. The same gel was revealed first by zinc sulphate negative staining (A) and then by UV (B). std, protein ladder.

69x43mm (600 x 600 DPI)



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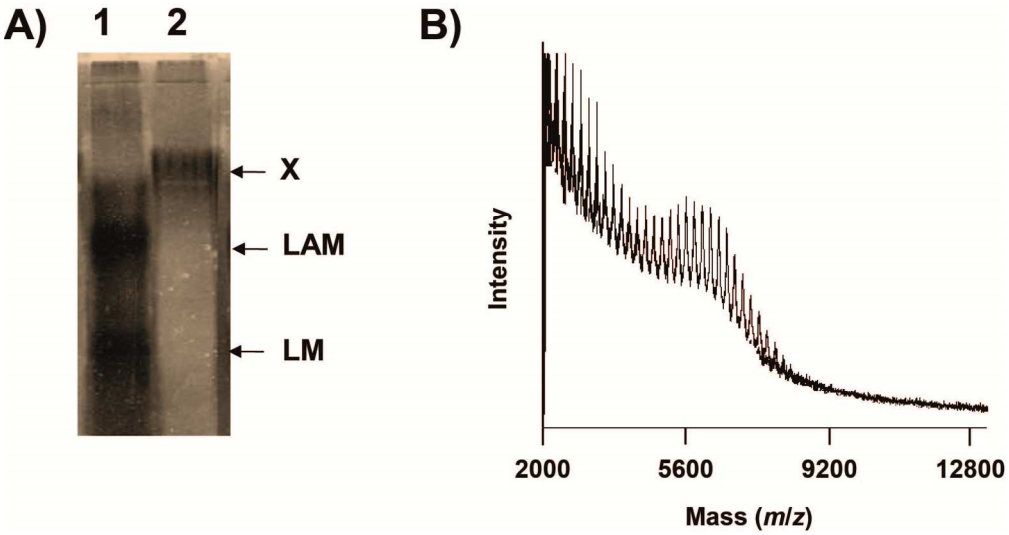
124x151mm (300 x 300 DPI)



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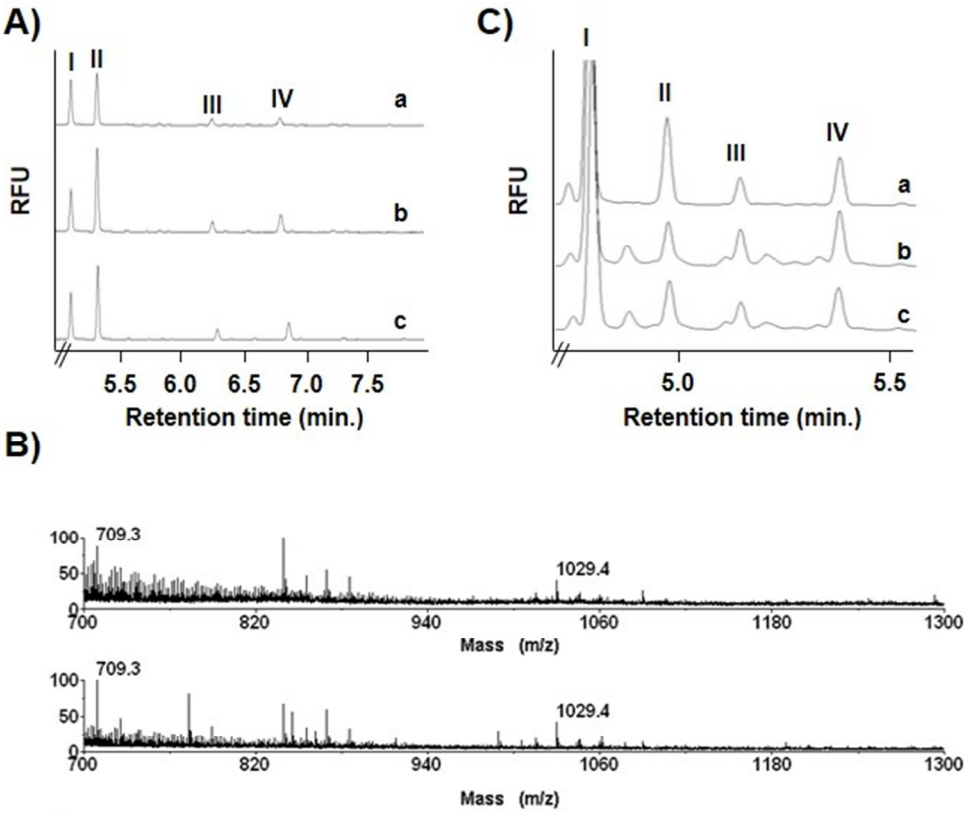

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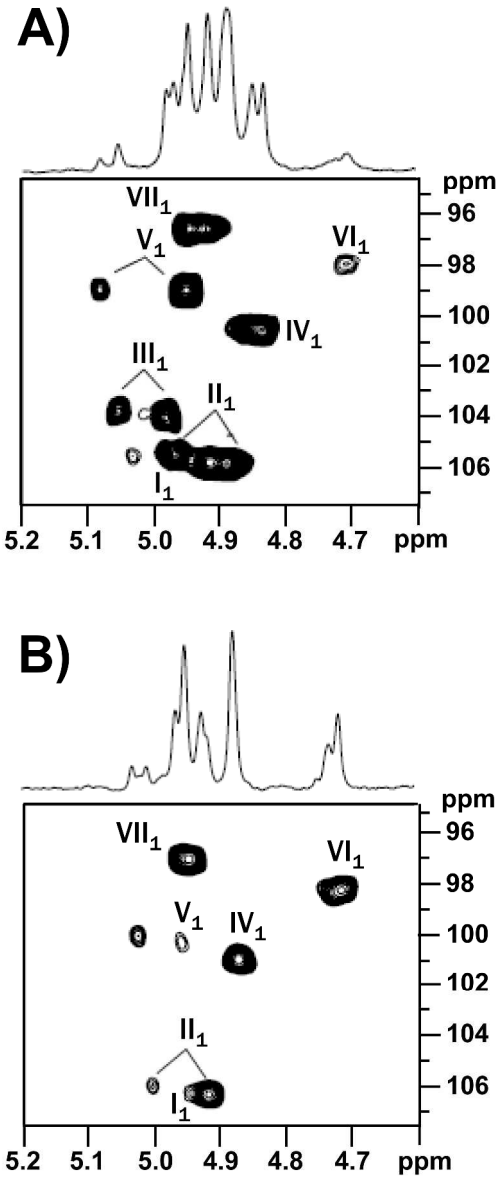

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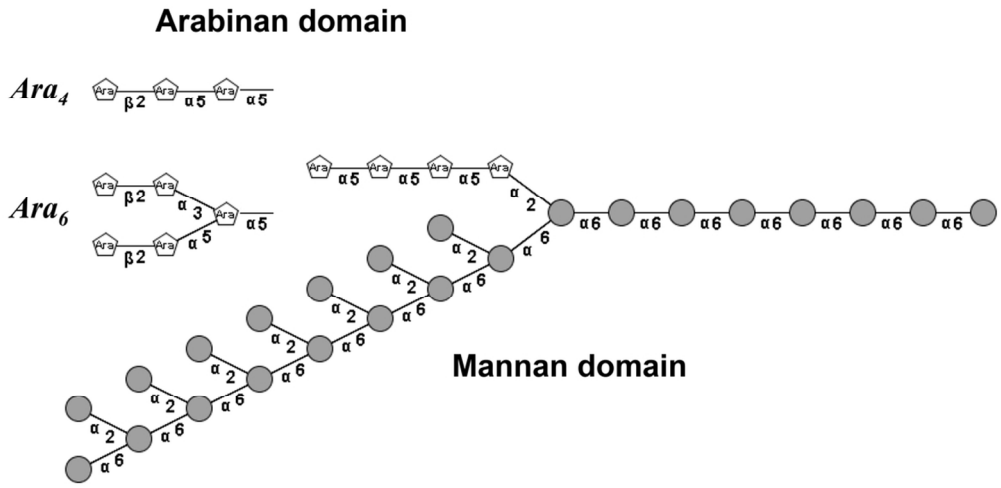

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99x49mm (300 x 300 DPI)