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Galactofuranose containing molecules in *Aspergillus fumigatus*

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Galactofuranose is a major carbohydrate in *Aspergillus fumigatus*. It became famous in medical mycology as being part of the galactomannan which was shown 30 years ago to be the major antigen circulating in the body fluid of patients suffering from invasive aspergillosis. Four different molecules contain galactofuranose in *A. fumigatus*: (i) the galactomannan present in the alkali soluble and insoluble fraction of the cell wall (ii) N- and O glycan moieties of secreted glycoproteins (iii) a GPI- anchored lipophosphogalactomannan and (iv) several sphingolipids also anchored to the membrane by an inositol phosphoceramide.

Keywords Aspergillus, aspergillosis, cell wall, glycobiology, genetics

Introduction

Galactofuranose is an unusual 5-membered-ring form of galactose that is present at the surface of many pathogenic pro-and eukaryotes. In prokaryotes, it is a key component of *Mycobacterium* cell wall and LPS O-antigens or extracellular polysaccharides [1,2]. In protozoa, it is a major component of glycosyl phosphoinositol (GPI)-anchored glycoconjugates such as the lipophosphoglycan and glycolipids and glycoproteins of *Leishmania* [3] or mucin, GPI-anchored proteins and lipids in *Trypanosoma* [4]. In fungi, Gal*f* has been found in pathogenic *Penicillium*, *Histoplasma*, *Cryptococcus* and *Aspergillus* species [5–7]. In *A. fumigatus* it has been the basis of the development of a commercial kit that detects its presence in the biological fluid of patients with invasive aspergillosis. One question remained open: what is the source and the chemical nature of the Gal*f* containing molecules circulating in the blood, urine or lung fluid of infected patients? This review will present our current understanding of all the molecules containing Gal*f* that are synthesized by *A.fumigatus*.

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An essential tool for searching Galactofuranose containing molecules

Seven monoclonal antibodies (MAbs) against *A.fumigatus* galactomannan (EB-A1-A7) were characterized [8]. They were all immunoglobulin M antibodies reacting in an indirect enzyme-linked immunosorbent assay with purified *A.fumigatus* galactomannan, with avidity constants in the 10^9/M range. Enzyme-linked immunosorbent assay inhibition experiments with modified galactomannan and synthetic oligomers of β(1,5) galactofuranose demonstrated that the MAbs bound to the β(1,5) galactofuranose-containing side chains of the galactomannan molecule. Using β(1,5) Gal*f* oligomers of increasing length showed that the minimal size of the oligosaccharide recognized was a tetramer. A 50% inhibitory concentration of Gal*f* between 2–10 ng/ml by ELISA inhibition and 1 ng/ml in a sandwich format showed the extreme sensitivity of these MAbs [9] that were used later on in the Platelia® commercial kit used for the detection of circulating antigen in patients with invasive aspergillosis.

Galactofuran is part of the galactomannan bound covalently to the cell wall Polysaccharides

The structural organization of the cell wall of *A. fumigatus* has been studied. Hot alkali treatment of the SDS-Mercaptoethanol treated cell wall released 2 fractions that both contain significant amounts of

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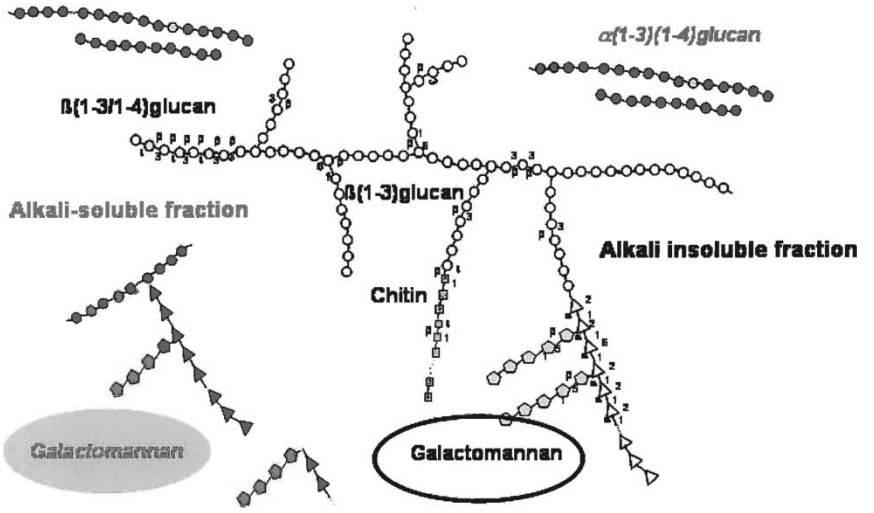

Fig. 1 Occurrence of galactofuranose in the alkali-soluble (grey) and -insoluble (open) fractions of the cell wall polysaccharides of *Aspergillus fumigatus.*

Galactofuran (Fig. 1). In the alkali-insoluble fraction, it was found that Galactofuran constitutes the side chain of a galactomannan molecule that is bound covalently to the branched β(1,3) glucan-chitin core [10]. Galactomannan was also found in the alkali soluble fraction but its association with the α(1,3) glucan that is the major component of the cell wall remains unknown [11].

Secreted molecules also contain Galatofuran epitopes

Fig. 2 shows that anti-Gal*f* positive MAb molecules are continuously and actively secreted during mycelial growth. In the culture filtrate, it was found that both polysaccharides and glycoproteins are recognized by this MAb. The chemical structure of the secreted galactomannan was elucidated [12] and its repeating unit is shown in Fig. 3. This molecule was used for the differential selection of anti- Gal*f* MAb using either the native molecule or a molecule treated with 0.01NHCl at 100°C that will both remove the Gal*f* moiety and the reactivity of specific clones. Several glycoproteins positive with the anti- Gal*f* MAb were also purified from the culture filtrate. They were identified by Mass spectrometry as an α(1,2) mannosidase, a phytase, an alkaline phosphatase and a phospholipase C [13]. The search was not exhaustive and more than these four proteins certainly exist in the culture filtrate. The N-glycan of the two most important glycoproteins was analysed. A common structure was found with an N-glycan containing a single galactofuranose unit linked with a (1,2) linkage in a non-reducing way to the mannan core (Fig. 4). Removal of the Gal*f* moiety by a short acid treatment removes the reactivity of the MAb to the protein. This result showed that the chemical specificity of the anti- Gal*f* MAb is not strict since it is able to both recognize a tetra β(1,5) galactofuran

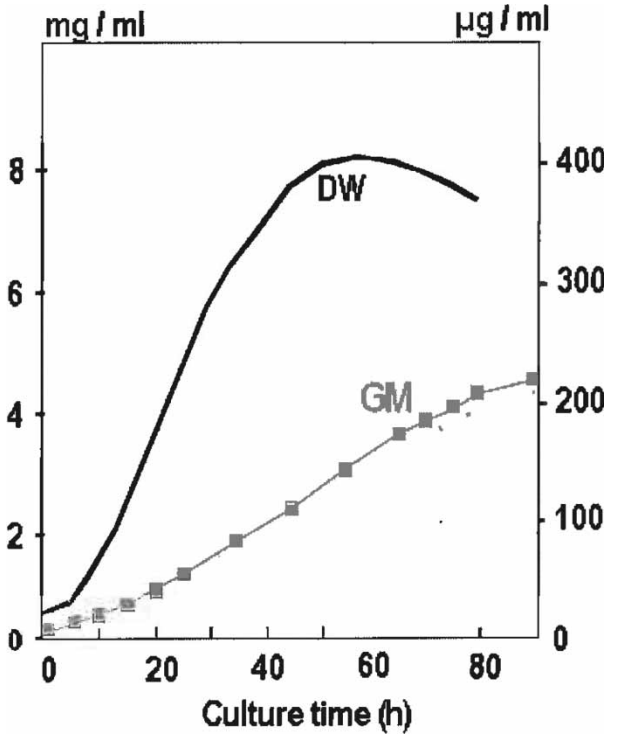

Fig. 2 Synthesis during mycelial growth of molecules that are positive with the anti-Gal*f*- MAb.

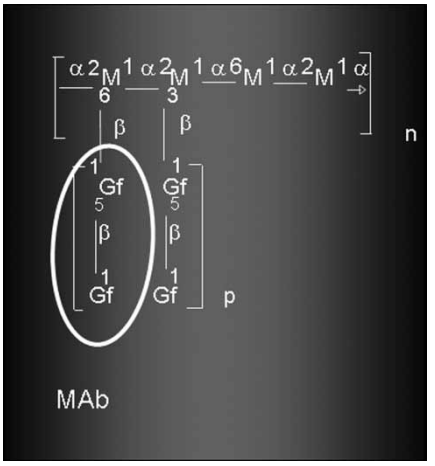

Fig. 3 Chemical structure of the galactomananan of *Aspergillus fumigatus*.

oligosaccharide but also an hybrid galactomannan oligosaccharide, with a single galactofuranose terminal unit 1,2 linked to the manno-oligosaccharide. Deglycosylation of the proteins also abolishes the antigenicity of these antigenic proteins towards human aspergilloma patients sera indicating that the peptide part of these glycoproteins is not antigenic. The galactofuranose can also be O-linked to other proteins [14,15]. In *Penicillium charlesii*, a complex lipo-peptidophosphogalactomannan was isolated [16–18]. This polymer is composed of a peptide part containing 30 to 32 amino acids to which mono-and oligosaccharides were attached through O-glycosidic linkages of the terminal mannosyl residues to the hydroxyl groups of serine and threonine residues. In addition, it contained ethanolamine and choline linked through phosphodiester bonds to C-6 of the mannose residues.

Galactofuranose in membrane-bound molecules

Galactofuranose was found both in the pellet and supernatant of membrane preparation extracted with chloroform/methanol. The pellet was treated with pronase and the soluble material was fractionated by hydrophobic interaction [19]. A major peak bound to the octyl-Sepharose column reacted positively with the EBA1 MAb. This purified fraction had an apparent molecular mass of 30 kDa and represented 0.04% of total mycelium dry weight. It was mainly composed of mannose and galactose residues in a ratio 3:2 and a small amount of glucosamine, phosphate, *myo*-inositol, 2-monohydroxy-$C_{24:0}$ fatty acid, and C_{18}-phytosphingosine suggesting that the GM was anchored to the membrane by a GPI anchor. The carbohydrate structure of the lipogalactomannan was elucidated by GC-MS after different treatments of the molecule: (1) nitrous acid deamination (2) mild acid treatment followed by Jack Bean and αmannosidase (3) aqueous HF treatment (4) acetolysis (5) Phosphatidylinositol-specific Phospholipase C digestion. These specific chemical and enzymatic degradations and mass spectrometry analysis showed that the lipid anchor is a GPI. The lipid part is an inositol phosphoceramide containing mainly C_{18}-phytosphingosine and monohydroxylated lignoceric acid (2OH- $C_{24:0}$ fatty acid). GPI glycan is a tetramannose structure linked to a glucosamine residue: Manα1-2Manα1-2Manα1-6 Manα1-4GlcN. The galactomannan polymer is linked to the GPI structure throught the mannan chain. The GPI structure is closely related to the one previously described for the GPI-anchored proteins of *A. fumigatus*. This is the first time that a fungal polysaccharide is shown to be a GPI-anchored. Because of its very low charge and the presence of lipids, this LPGM could produce the wide smear seen in >100kDa M_r range after electrophoresis of a total culture filtrate probed with the anti-GM MAb by Western blotting.

The glycosphingolipids recovered in the supernatant were identified by mass spectrometry and NMR. The total amount of GSL represents around 0.02% of the total mycelium dry weight. Nine GIPCs of different structures have been identified in *A. fumigatus* [20] (Table 1). All GSLs have the same ceramide moiety composed of a 2-hydroxylated lignoceric acid (2-OH $C_{24:0}$) associated with $C_{18:0}$-phytosphingosine base. This lipid moiety is common to most fungal species. The glycan part has more variability and two types of GPICs have been isolated from *A. fumigatus* mycelium. First, a zwitterionic GSL that is the major GIPC from *A. fumigatus* mycelium contains a glucosamine residue linked in α(1,2) to the inositol ring. Secondly, the other acidic GSL structures contain a common sequence α-Man-(1-3)-α-Man-(1-2)-Inositol. A galactofuranose residue is linked to Man residues found at the terminal position via a β(1,2) linkage in 3 GSLs. However, in

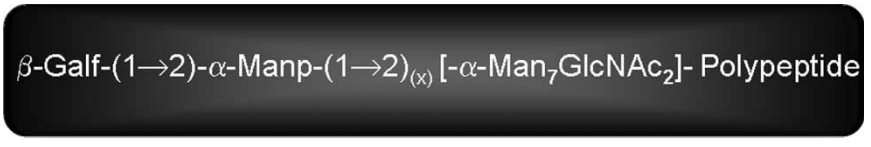

Fig. 4 Structure of the Galf-containing glycoproteins.

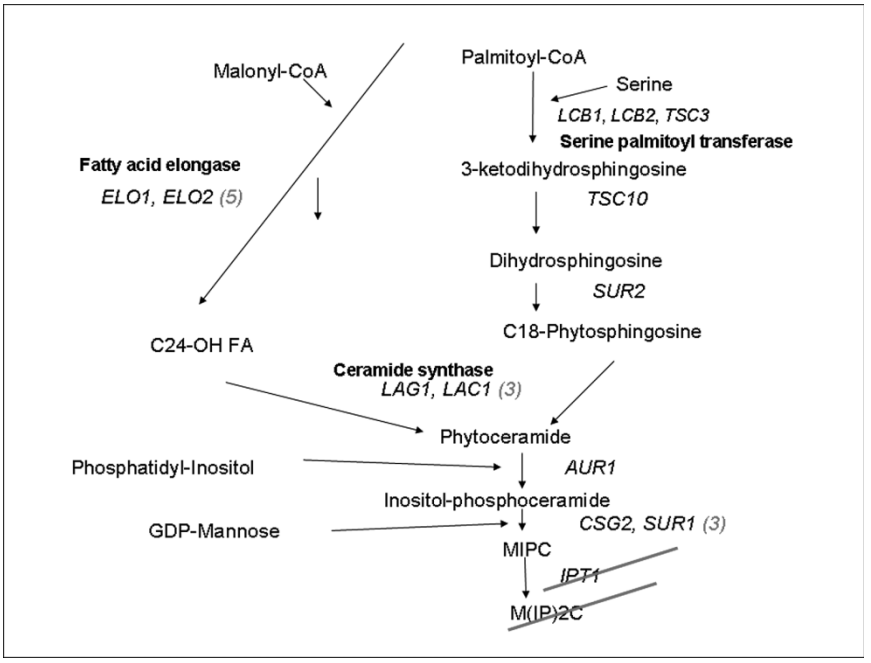

Fig. 5 Putative sphingolipid biosynthesis pathway in *Aspergillus fumigates*. Genes identified by BLAST in the *A. fumigatus* genome server. In brackets are the number of orthologous genes in yeast (http: //www. cadre-genomes.org.uk/Aspergillus_fumigatus/).

contrast to glycoproteins with a Gal*f* at the terminal position of the N-glycan, the linking of β Gal*f* to a Man residue linked to another mannose moiety through a 1,3 linkage is not recognized by the anti-Gal*f* MAb. Six GSLs analyzed contained galactofuranose in *A. fumigatus*. The presence of galactofuranose in GSLs has been reported in other human pathogens [20]. In fungi, sphingolipids are essential for growth. This is also the case in *Aspergillus* since the deletion of the inositol phosphoryl ceramide synthase is lethal. This suggests that the biosynthetic pathway of sphingolipids are very similar in yeast and moulds. Fig. 5 shows that all steps are indeed identical with the exception of the end product of the pathway $M(IP)_2C$ that cannot be produced because *IPT1* is absent from the *A.fumigatus* annotated genome. In *A. fumigatus*, four types of membrane-anchored molecules present the same IPC lipid moiety: GPI-anchored proteins [21], the lipogalactomannan, a GPI-anchored polysaccharide [19], and two types of GIPCs in which the inositol ring of an IPC structure is substituted in position 2 by a mannose or a glucosamine residue (Fig. 6). In these 2 former molecules, the glucosamine residue is linked in α1-6 to the inositol ring in the GPI-anchor structures [21]. Anchoring of the GPI to the protein and polysaccharide is also different; no phosphoethanolamine group has been identified on the LGM anchor and the fifth α(1,3)linked mannose residue of the GPI structure of anchored proteins is replaced by the galactomannan in the LGM structure.

Table 1 GIPC structures described in *Aspergillus fumigatus*.

α -Man-(1-3)- α -Man-(1-6)- α -GlcN-(1-2)-Ins-P-cer
α -Man-(1-3)- α -Man-(1-2)-Ins-P-cer
α -Man-(1-2)- α -Man-(1-3)- α -Man-(1-2)-Ins-P-cer
α -Man-(1-3)- [β-Galf-(1-6)]- α -Man-(1-2)-Ins-P-cer
α -Man-(1-2)- α -Man-(1-3)-[β-Galf-(1-6)]- α -Man-(1-2)-Ins-Pcer
β-Galf-(1-2)- α -Man-(1-3)- α -Man-(1-2)-Ins-P-cer
β-Galf-(1-2)- α -Man-(1-3)-[β-Galf-(1-6)]- α -Man-(1-2)-Ins-P-cer
Choline-P-6-β-Galf-(1-2)- α -Man-(1-3)- α -Man-(1-2)-Ins-P-cer

Discussion

Galactofuranose is a major ubiquitous molecule in *A. fumigatus*. It is a constitutive component of the cell wall as part of the galactomannan present both in the alkali soluble and alkali insoluble fraction of the cell wall. It also terminates the N-glycan of many glycoproteins in a similar way as the α(1,3) Man of yeast glycoproteins [22]. It is also present in the membranes in lipophosphogalactomannan and GIPCs that are anchored to the membrane by a inositol phosphoceramide moiety.

The occurrence of many molecules containing Gal*f* has allowed to revise the specificity of the anti-galactofuran antibody used in the Platelia diagnostic kit of invasive aspergillosis. This MAb recognized β(1,5) linked Galactofuran but also Gal*f*(1,2) Man if the Man residue is not α(1,3) linked to another carbohydrate. Other studies have shown that β(1,6) linked Gal*f* either at the non reducing end of a protein or internally in a

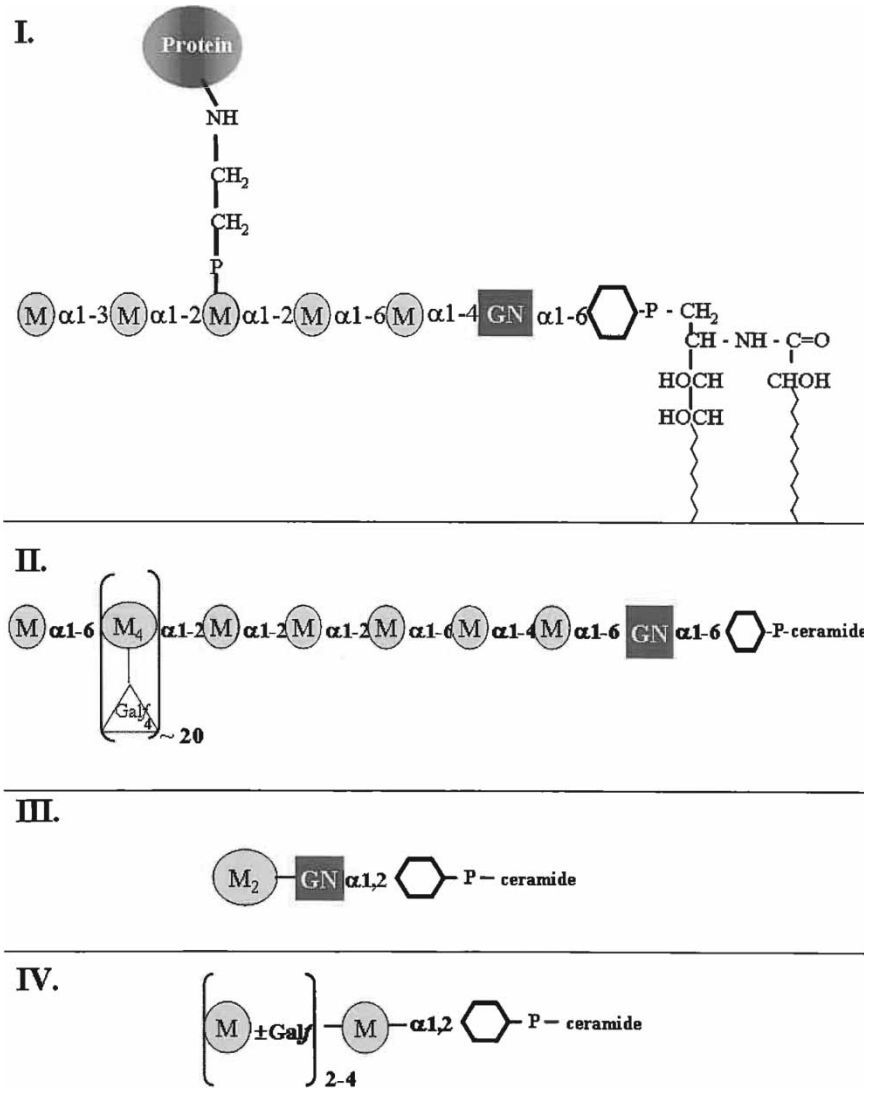

Fig. 6 Four types of molecules bound to membrane by an inositolphosphoceramide anchor. M, mannose; GN, glucosamine.

GPIC are not recognized [20,22]. Internal galactofuran do not seem indeed to be recognized either. For example, although *Fusarium* contain galactofuranose, our MAbs failed to bind *Fusarium.*Analysis of the structures of the extracellular polysaccharides from *Fusarium moniliforme* and *F.solani* demonstrated the presence of D-galactose in the furanose form. However, unlike the galactofuranoside residues of the galactomannan of *A. fumigatus*, the galactofuranoside residues of the polysaccharide of *F. moniliforme* are (1,2) or (1,6) linked, with some branching at C-2 or C-6 [23]. In *F. solani*, only branched galactofuranoside residues could be demonstrated [24]. In neither species were galactofuranosides located at terminal positions.

Up to date, the nature of the molecule(s) circulating in the body fluid of patients with invasive aspergillosis remains unknown. This diversity could however be at the origin of differential clinical false positive data, since the different molecules could display different ability to cross epithelia, especially the ones damaged by the immunosuppressive therapies. These false positive were seen in babies with IA or in patients recently treated with antibacterial drugs [25,26]. However these clinical false positives were true ELISA positive. Isolation of positive molecules from these positive sera through an immunoaffinity column and mass spectrometry show the presence of a m/z ion characteristic of galactofuranose unit in a terminal non-reducing position [Dubeaupuis *et al.*, unpublished].

Biosynthesis of galactofuran remains unknown in *A. fumigatus.* This biosynthetic pathway requires two different steps: the first one is the conversion of a

UDP-Gal*f* from UDP-Gal*p* by a UDP-Gal mutase. A gene coding for this enzyme has been identified in *A. fumigatus* [27,28] but no mutant devoided of this activity has been published yet. The other step is the transfer of Gal*f* to either another Gal*f* or a Man residue. The galactosyltransferases responsible for this reaction has not been identified yet neither has been the putative acceptor. Mutant devoided of this activity have been constructed in my laboratory and showed that the galactofuranose biosynthetic pathway is not essential in *A.fumigatus* and cannot become a drug target as it is in bacteria since galactofuranose is absent from mammals.

Declaration of interest: The author reports no conflicts of interest. The author alone is responsible for the content and writing of the paper.

References

1 Pedersen LL, Turco SJ. Galactofuranose metabolism: a potential target for antimicrobial chemotherapy. *Cell Mol Life Sci* 2003; **60**: 259–266.
2 Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. *Annu Rev Biochem* 2002; **71**: 635–700.
3 Kleczka B, Lamerz AC, van Zandbergen G, *et al*. Targeted gene deletion of *Leishmania major* UDP-galactopyranose mutase leads to attenuated virulence. *J Biol Chem* 2007; **282**: 10498–10505.
4 Previato JO, Wait R, Jones C, *et al*. Glycoinositolphospholipid from *Trypanosoma cruzi*: structure, biosynthesis and immunobiology. *Adv Parasitol* 2004; **56**: 1–41.
5 Barr K, Laine RA, Lester RL. Carbohydrate structures of three novel phosphoinositol-containing sphingolipids from the yeast *Histoplasma capsulatum*. *Biochemistry* 1984; **23**: 5589–5596.
6 Vaishnav VV, Bacon BE, O'Neill M, Cherniak R. Structural characterization of the galactoxylomannan of *Crytococcus neoformans*. *Carbohydr Res* 1998; **306**: 315–330.
7 Latge JP, Kobayashi H, Debeaupuis JP, *et al*. Chemical and immunological characterization of the extracellular galactomannan of *Aspergillus fumigatus*. *Infect Immun* 1994; **62**: 5424–5433.
8 Stynen D, Sarfati J, Goris A, *et al*. Rat monoclonal antibodies against galactomann. *Infect Immun* 1992; **60**: 2237–2245.
9 Stynen D, Goris A, Sarfati J, Latgé JP. A new sensitive sandwich enzyme-linked immunosorbent assay to detect galactofuran in patients with invasive aspergillosis. *J Clin Microbiol* 1995; **33**: 497–500.
10 Fontaine T, Simenel C, Dubreucq G, *et al*. Molecular organization of the alkali-insoluble fraction of *Aspergillus fumigatus* cell wall. *J Biol Chem* 2000; **275**: 27594–27607.
11 Maubon D, Park S, Tanguy M, *et al*. *AGS3*, an α(1-3)glucan synthase gene family member of *Aspergillus fumigatus*, modulates mycelium growth in the lung of experimentally infected mice. *Fungal Genet Biol* 2006; **43**: 366–375.
12 Latgé JP, Kobayashi H, Debeaupuis JP, *et al*. Chemical and immunological characterization of the galactomannan secreted by *Aspergillus fumigatus*. *Infect Immun* 1994; **62**: 5424–5433.
13 Morelle W, Bernard M, Debeaupuis JP, *et al*. Galactomannoproteins of *Aspergillus fumigatus*. *Eukaryotic* 2005; **4**: 1308–1316.
14 Hearn VM, Griffiths BL, Gorin PA. Structural analysis of water-soluble fractions obtained from *Aspergillus fumigatus* mycelium. *Glycoconjugate J* 1989; **6**: 85–100.
15 Leitao EA, Bittencourt VC, Haido RM, *et al*. Beta-galactofuranose-containing O-linked oligosaccharides present in the cell wall peptidogalactomannan of *Aspergillus fumigatus* contain immunodominant epitopes. *Glycobiology* 2003; **13**: 681–692.
16 Gander JE, Jentoft NH, Drewes LR, Rick PD. The 5-*O*-β-D-glactofuranosyl-containing exocellular glycopeptide of *Penicillium charlesii*. Characterization of the phosphogalactomannan. *J Biol Chem* 1974; **249**: 2063–2072.
17 Rick PD, Drewes LR, Gander JE. The 5-*O*-β-D-galactofuranosyl containing exocellular glycopeptide of *Penicillium charlesii*. The occurrence of ethanolamine and partial characterization of the peptide portion and the carbohydrate-peptide linkage. *J Biol Chem* 1974; **252**: 3219–3226.
18 Unfeker CJ, Jackson C, Gander JE. The 5-*O*-β-D-galactofuranosyl containing exocellular glycopeptide of *Penicillium charlesii*. Identification of phsophorylcholine attached to C-6 mannopyranosyl residues of the mannan region. *J Biol Chem* 1982; **257**: 2491–2497.
19 Costachel C, Coddeville B, Latgé JP, Fontaine T. Glycosylphosphatidylinositol-anchored fungal polysaccharide in *Aspergillus fumigatus*. *J Biol Chem* 2005; **280**: 39835–39842.
20 Simenel C, Coddeville B, Delepierre M, *et al*. Glycosylinositol-phosphoceramides in *Aspergillus fumigatus*. *Glycobiology* 2007; **18**: 84–96.
21 Fontaine T, Magnin T, Melhert A, *et al*. Structure of the glycosylphosphatidylinositol membrane anchors from *Aspergillus fumigatus* membrane proteins. *Glycobiology* 2003; **13**: 169–177.
22 Almeida IC, Neville DC, Mehlert A, *et al*. Structure of the N-linked oligosaccharide of the main diagnostic antigen of the pathogenic fungus *Paracoccidioides brasiliensis Glycobiology*. 1996 Jul;6:507–515.
23 Siddiqui S, Adams WA. An extracellular polysaccharide from *Giberella fujikoroi* (*Fusarium moniliform*). *Can J Chem* 1961; **39**: 1683–1694.
24 Miyazaki T, Naoi Y. Chemical structure of main extracellular polysaccharides of *Alternaria solani* and *Fusarium solani*. Studies on fungal polysaccharides. *Chem Pharm Bull* 1975; **23**: 752–1758.
25 Aubry A, Porcher R, Bottero J, *et al*. Occurrence and kinetics of false-positive *Aspergillus* galactomannan test results following treatment with beta-lactam antibiotics in patients with hematological disorders. *J Clin Microbiol* 2006; **44**: 289–294.
26 Sulahian A, Boutboul F, Ribaud P, *et al*. Value of antigen detection using an enzyme immunoassay in the diagnosis and prediction of invasive aspergillosis in two adult and pediatric hematology units during a 4-year prospective study. *Cancer* 2001; **91**: 311–318.
27 Beverley SM, Owens KL, Showalter M, *et al*. Eukaryotic UDP-galactopyranose mutase (*GLF* gene) in microbial and metazoal pathogens. *Eukaryotic Cell* 2005; **4**: 1147–1154.
28 Bakker H, Kleczka B, Gerardy-Schahn R, Routier FH. Identification and partial characterization of two eukaryotic UDP-galactopyranose mutases. *Biol Chem* 2005; **386**: 657–661.

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