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Probing Elongating and Branching β-D-Galactosyltransferase Activities in *Leishmania* Parasites by Making Use of Synthetic Phosphoglycans

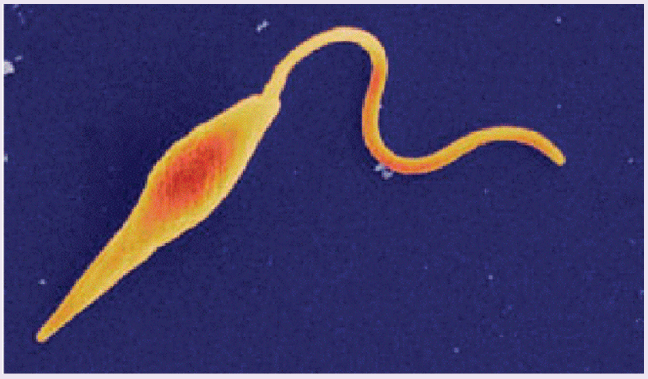
Olga V. Sizova,[‡] Andrew J. Ross,[†] Irina A. Ivanova,[†] Vladimir S. Borodkin,[†] Michael A. J. Ferguson,[†] and Andrei V. Nikolaev[†,*]

[†]College of Life Sciences, Division of Biological Chemistry and Drug Discovery, University of Dundee, Dundee DD1 5EH, U.K.

[‡]N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow, Russia

Supporting Information

ABSTRACT: Protozoan parasites of the genus *Leishmania* synthesize lipophosphoglycans (LPGs), phosphoglycans and proteophosphoglycans that contain phosphosaccharide repeat units of [-6)Gal(β1-4)Man(α1-OPO_3H-]. The repeat structures are assembled by sequential addition of Manα1-OPO_3H and β-Gal. In this study, an UDP-Gal-dependent activity was detected in *L. donovani* and *L. major* membranes using synthetic phospho-oligosaccharide fragments of lipophosphoglycan as acceptor substrates. Incubation of a microsomal preparation from *L. donovani* or *L. major* parasites with synthetic substrates and UDP-[6-^{3}H]Gal resulted in incorporation of radiolabel into these exogenous acceptors. The [^{3}H]galactose-labeled products were characterized by degradation into radioactive, low molecular mass fragments upon hydrolysis with mild acid and treatment with β-galactosidases. We showed that the activity detected with *L. donovani* membranes is the elongating β-D-galactosyltransferase associated with LPG phosphosaccharide backbone biosynthesis (eGalT). The eGalT activity showed a requirement for the presence of at least one phosphodiester group in the substrate and it was enhanced dramatically when two or three phosphodiester groups were present. Using the same substrates we detected two types of galactosyltransferase activity in *L. major* membranes: the elongating β-D-galactosyltransferase and a branching β-D-galactosyltransferase (bGalT). Both *L. major* enzymes required a minimum of one phosphodiester group present in the substrate, but acceptors with two or three phosphodiester groups were found to be superior.



The trypanosomatid protozoan parasite *Leishmania* infects over 12 million people worldwide, causing a variety of diseases that range from mild cutaneous lesions to fatal visceral infections. General aspects of leishmaniasis and overall control strategies have been reviewed.[1,2] The parasite exists in two forms: the flagellated promastigote in the female sand fly vector and the amastigote in the mammalian host. Amastigotes are obligate intracellular parasites of macrophages, where they survive and multiply. A key step of the infectious cycle is the ability of the parasite to be transmitted to new hosts by the insect vector. In the recent years, visceral leishmaniasis is reported to be rapidly emerging as an opportunistic infection in HIV patients,[3,4] in pregnant females, and in organ transplant patients.[5] Globalization and consequent travel of people across the world has increased the chances of spreading the infection.[6]

Pentavalent antimonials were brought into use against leishmaniasis more than 50 years ago. Along with Pentostam and Glucantime, non-antimonial polyene antibiotic Amphotericin B, as well as Paromomicin and Miltefosine have also proved to be successful for the treatment of *L. donovani* induced visceral leishmaniasis. However, these have the disadvantage of high toxicity.[1−4] Since the available treatment for leishmaniasis poses many problems, researchers are looking at novel biochemical targets in order to develop new drugs.

Leishmania produce characteristic glycoconjugates: LPGs, secreted phosphoglycans, and secreted and membrane-bound proteophosphoglycans. Several studies have emphasized the importance of LPG, the most abundant molecules on the parasite cell surface.[7−12] The basic LPG structure in all *Leishmania* consists of a 1-*O*-alkyl-2-*lyso*-phosphatidylinositol lipid anchor and heptasaccharide core, to which is joined a long phosphoglycan polymer composed of 15−30 repeating units with the

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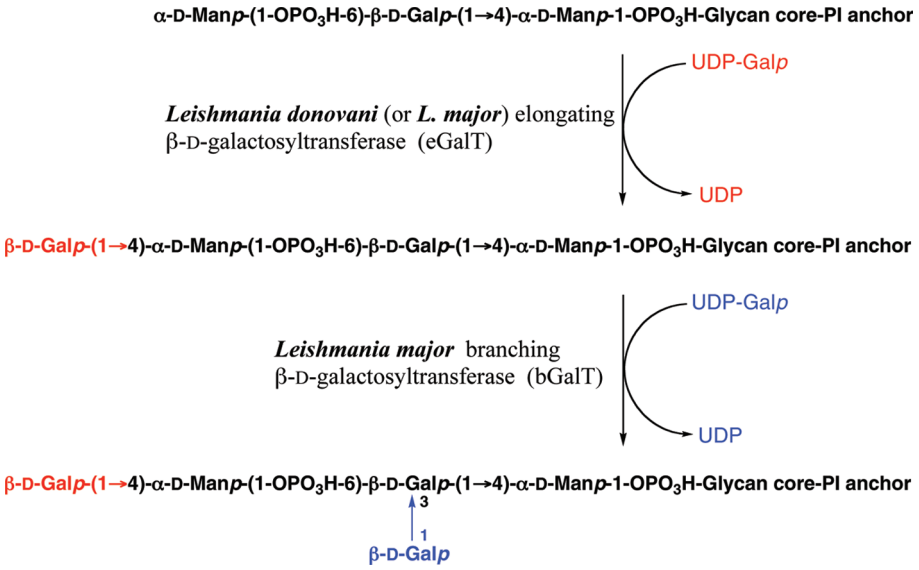
Scheme 1. Enzymatic Galactosylation in the Growing LPG Chain: Elongation of LPG Repeats for *L. donovani* and *L. major* (Red) and Side Chain Formation for *L. major* (Blue)



backbone structure of [-6)Gal(β1-4)Man(α1-OPO_3H-]. LPG is terminated at the non-reducing end with a capping oligosaccharide.[13] For *L. donovani* the phosphoglycan polymer is a linear chain,[16] but in *L. major* the C(O)3 positions of galactose residues in the phosphorylated disaccharide repeats are highly substituted with Gal(β1−3) side chains,[14] which are required for binding to insect midgut lectins and survival.[15] There is variability in the type and degree of substitution of the backbone repeat units at different developmental stages and during parasite transformation from promastigotes to amastigotes.[8,17,20] The LPGs are common to all *Leishmania* promastigotes [18,19] and *L. major* amastigotes.[20]

In order to elucidate and understand the control of LPG biosynthesis it is necessary to isolate and characterize glycosyltransferases involved in the process of construction of the repeat units. Galactosyltransferase (GalT) activity involved in the synthesis of the backbone repeat units of *L. donovani* LPG was first identified by Carver and Turco.[21] Subsequently, the *L. major* side chain galactose transfer was characterized by Bacic and colleagues.[22,23] These studies indicated that the addition of the (β1−3)-linked galactose branches is independent of the synthesis of the repeating phosphodisaccharide units.[7,12] The addition of the (β1−3)-linked galactose residues is catalyzed by more than one (β1−3)-galactosyltransferase. A family of genes encoding the LPG side chain (β1−3)-GalTs was identified by Beverley and colleagues.[24] Moreover, it was shown that deficiency in side chain galactosyltransferases influences the *Leishmania*−sand fly interactions.[25] For *L. donovani* promastigotes it was found that the cap terminal (β1-4)-GalT enzyme is developmentally regulated and expressed only in the attenuated parasites.[26]

The importance of the lipophosphoglycan for parasite infectivity and survival makes the enzymes responsible for its biosynthesis of great interest. We have examined the cell-free membrane-bound β-galactosyltransferase activities from *L. donovani* and *L. major*, using synthetic phospho-oligosaccharide acceptors and UDP-Gal as the sugar donor and have established *in vitro* assays for the elongating GalT (for both *L. donovani* and *L. major*) and branching GalT (for *L. major* only) activities involved in the biosynthesis of the LPG.

RESULTS AND DISCUSSION

Assay Conditions. The great deal of information about the LPG repeat domain biosynthesis has come from *in vitro* cell-free system studies using *Leishmania* membranes. A cell-free system assay using synthetic exogenous acceptor substrates was developed for characterization of the elongating mannosylphosphate transferase (eMPT) activity from three species of *Leishmania*.[27,28] In common with other studies of glycosyltransferases involved in the repeat domain biosynthesis,[27−29] we used washed parasite membranes as an enzyme source, as originally described by Carver and Turco.[21] UDP-[^{3}H]Gal was used as a donor of [^{3}H]galactose residues. Enzymatic galactosylation of the growing LPG chain is shown in Scheme 1. It involves elongation of the LPG repeats for both *L. donovani* and *L. major* with the action of the elongating (β1-4)-GalT[21] and the side chain formation for *L. major* only with the action of the branching (β1−3)-GalT.[22,23] In the present study we established an assay for galactose transfer to chemically prepared acceptors. The compounds **1**−**8** (Figure 1A), tested as acceptor substrates, are LPG components and their structural analogues, contain D-mannose residue at the non-reducing end, and are expected to work as potential acceptors for *Leishmania* elongating galactosyltransferases (Scheme 1). The compounds tested were different in the chain length and the number of phosphodiester groups. All the compounds contain a hydrophobic dec-9-enyl or *n*-decyl aglycone moiety to assist biochemical asays.

It was reported that the addition of the zwitterion detergent CHAPS stimulated the activity of the mannosylphosphate transferases but inhibited the activity of the galactosyltransferases involved in the biosynthesis of the LPG repeats.[21] We tried nonionic detergent NOG to stimulate galactosyltransferase activity. The concentration of 0.3% NOG was found to be the best. Under these conditions, transfer of [^{3}H]galactose was linear

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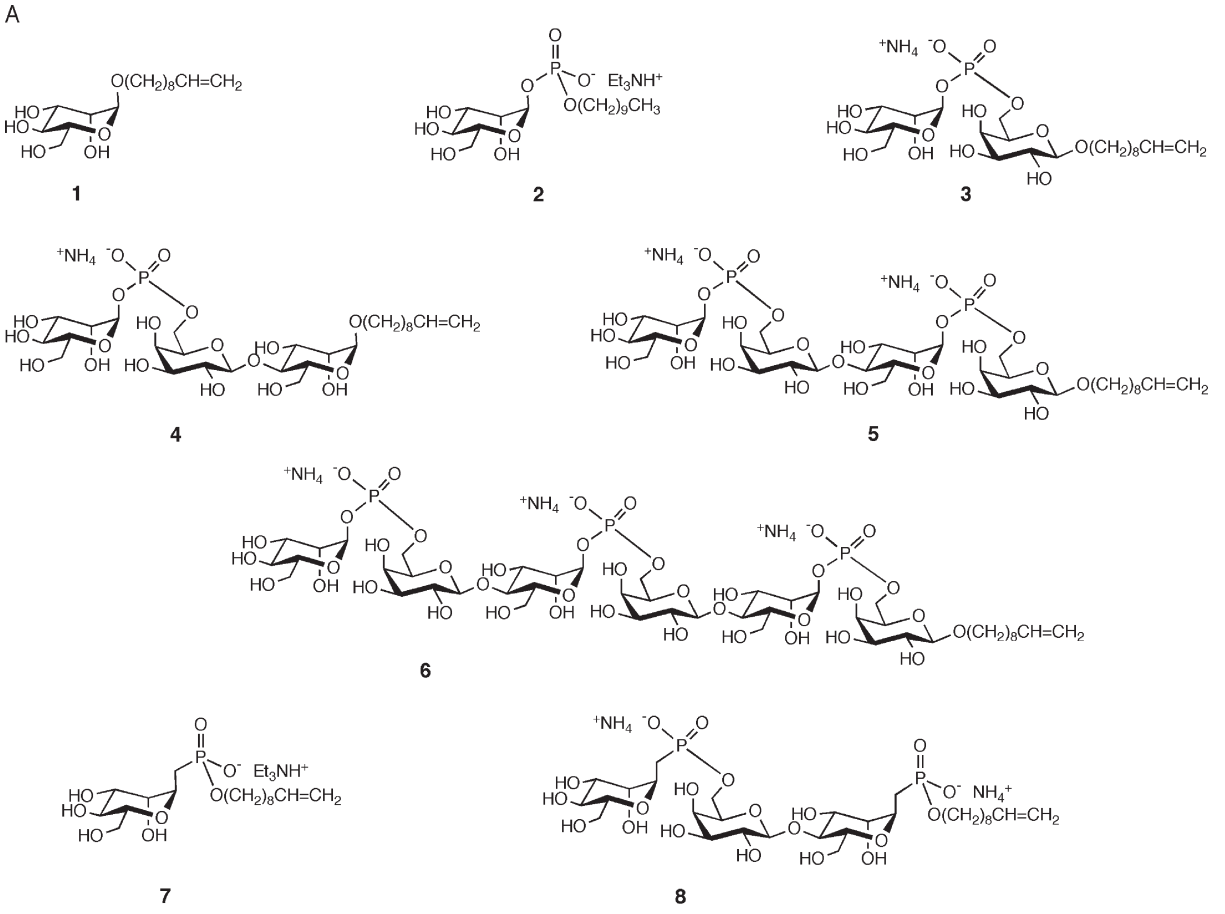
A



B

Compound	*L. donovani*	*L. major*
1	Npd	Npd
2	Npd	Npd
3	3.94 ± 0.17	1.35 ± 0.02
4	6.26 ± 0.62	3.94 ± 0.09
5	82.28 ± 5.76	14.97 ± 1.03
6	95.26 ± 7.34	20.99 ± 1.31
7	Npd	Npd
8	Npd	1.45 ± 0.04

Figure 1. (A) Structures of synthetic compounds **1**−**8** tested as acceptor substrates for β-D-galactosyltransferases from *L. donovani* and *L. major*. Compounds **1** and **2** are derivatives of α-D-mannopyranose with (**2**) or without (**1**) phosphate at O-1. Related compounds **3**, **5**, and **6** are LPG fragments containing one, two, or three Man(α1-OPO_3H-6)Gal disaccharide phosphate repeats, respectively. Compound **4** is an extended analogue of **3** with one extra α-D-Man residue at the reducing end. Compound **7** is a C-phosphonate analogue of the mannosyl phosphate **2**. Compound **8** is a C-phosphonate analogue of the tetrasaccharide diphosphate **5** lacking one β-D-Gal residue at the reducing end of the chain. All of the compounds contain a hydrophobic dec-9-enyl or *n*-decyl aglycone moiety to assist biochemical asays. (B) Activity of *L. donovani* and *L. major* galactosyltransferases (pmol of [^{3}H]galactosylated product formed in the standard assay) using synthetic substrates **1**−**8**. The data represent the average of three independent experiments. The samples were applied to C18 reverse-phase cartridges, and bound radioactivity was eluted and then measured by scintillation counting. There was no detectable [^{3}H]Gal-labeled product in the absence of synthetic acceptor substrate in the incubation mixture. Npd = no product detected.

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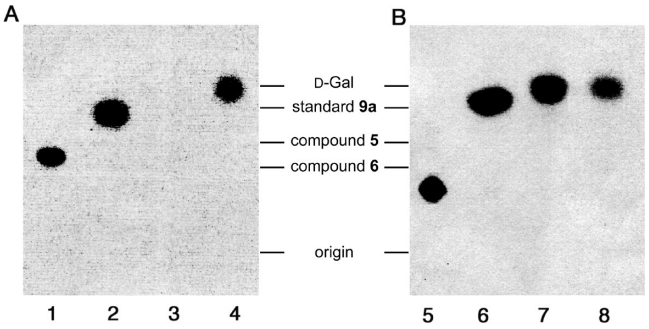


Figure 2. Characterization of the [^{3}H]galactosylated products of synthetic phospho-oligosaccharides **5** and **6** with *L. donovani* microsomes. (A) The [^{3}H]-labeled product formed from compound **5** was analyzed by HPTLC and fluorography before (lane 1) and after (lane 2) mild acid hydrolysis (0.04 M aq TFA, 100 °C; see Scheme 2) and after mild acid hydrolysis followed by incubation with *S. pneumoniae* specific (β1-4)-galactosidase (lane 4). No [^{3}H]-labeled product was detected after incubation of UDP-[^{3}H]Gal with *L. donovani* membrane preparation but without compounds **5** or **6** (lane 3). (B) The [^{3}H]-labeled product formed from compound **6** was analyzed by HPTLC and fluorography before (lane 5) and after (lane 6) mild acid hydrolysis (0.04 M aq TFA, 100 °C; see Scheme 2) and after mild acid hydrolysis followed by incubation with specific (β1-4)-galactosidase (lane 7) or with bovine testes β-galactosidase (lane 8). Samples (2 μg of each) of D-galactose and compounds **5**, **6**, and **9a** (Scheme 2) were run on the same plate and stained with orcinol reagent; their positions are indicated.

over 40 min and reached a maximum after about 1 h (data not shown). Incubation times for quantitative experiments were kept within the linear period (30 min). An acceptor concentration of 200 μM was chosen as a compromise between product yield and synthetic acceptor usage.

***L. donovani* Membranes Catalyze the Transfer of [^{3}H]Gal from UDP-[^{3}H]Gal to Synthetic Phospho-oligosaccharides.** The washed microsomes from *L. donovani* promastigotes were incubated with UDP-[^{3}H]Gal in the presence and absence of the synthetic compounds **5** or **6** (Figure 1A), which are LPG fragments of various length. After removing the membranes, the products binding to a C18 reverse-phase cartridge were subsequently eluted with 80% aq propan-1-ol and analyzed by HPTLC and fluorography. The *L. donovani* LPG phosphosaccharide repeat domain is a linear structure without side chains, and therefore *L. donovani* membranes are expected to contain only elongating GalT activity (Scheme 1). Each of the substrates **5** and **6** is expected to have only one site for enzymatic galactosylation (*i.e.*, the non-reducing α-D-Man residue) using *L. donovani* membranes. Consistent with this, one major radiolabeled product was observed when compound **5** (Figure 2, lane 1) or **6** (Figure 2, lane 5) was incubated with *L. donovani* membranes and UDP-[^{3}H]Gal. In the absence of compound **5** or **6** in the incubation mixture there were no detectable [^{3}H]Gal-labeled products (Figure 2, lane 3). Thus, compounds **5** and **6** appear to act as acceptor substrates for galactosyltransferase activity present in the *L. donovani* membranes.

Acceptor Substrate Specificity of the Galactosyltransferase Activities in *L. donovani* and *L. major* Membranes. Structurally related synthetic compounds **1**−**8** (Figure 1A) were tested as acceptor substrates for galactosyltransferase(s) in *Leishmania*. The washed microsomes from *L. donovani* or *L. major* promastigotes were incubated with UDP-[^{3}H]Gal in the presence and absence of one of the compounds. The products binding to a C18 reverse-phase cartridge were eluted with 80% aq propan-1-ol, and radioactivity was quantified by liquid-scintillation counting (Figure 2B). Compounds **5** (a tetrasaccharide diphosphate) and **6** (a hexasaccharide triphosphate) showed the greatest acceptor activity for both *L. donovani* and *L. major* microsomes. Compounds **3** (a disaccharide monophosphate) and **4** (a trisaccharide monophosphate) seemed to be quite poor acceptor substrates. Both compounds contain only one phosphate group between mannose and galactose residues. Compound **8** resulted in a poor [^{3}H]galactose incorporation after incubation with *L. major* microsomes but did not show any acceptor activity with *L. donovani* membranes. Compound **8** is an analogue of compound **5**, containing two C-phosphonate units instead of phosphates and lacking one D-galactose residue at the reducing end. Monosaccharide compounds **1**, **2**, and **7** did not work as substrates at all.

The inability of compounds **1**, **2**, and **7** to act as acceptors for galactosyltransferases from *L. donovani* and *L. major* indicates that the presence of a mannoside (**1**), a mannosyl phosphate (**2**), or a mannosyl C-phosphonate (**7**) unit is not enough for enzyme recognition and/or catalysis. A phosphodiester group between mannose and galactose residues in the Man(α1-OPO$_3$H-6)Gal terminal block seems to be essential for enzyme activity. Minimal detectable enzyme activity appeared for compounds **3** and **4**, each of them containing one phosphodiester group, and for C-phosphonate analogue of **5** (compound **8**) in the case of *L. major*. Compounds containing two (**5**) or three (**6**) phosphodiester groups were clearly the best substrates for both *L. donovani* and *L. major* galactosyltransferases. These results demonstrate that *Leishmania* membranes catalyze the transfer of [^{3}H]Gal from UDP-[^{3}H]Gal to synthetic phospho-oligosaccharides **3**−**6** and **8** in a structure-specific manner.

It is noteworthy that the acceptor structure for the first β-galactosylation reaction in *Leishmania* LPG (*i.e.*, for action of the "initiating phosphosaccharide backbone" galactosyltransferase), which produces the first Gal(β1-4)Man(α1-OPO$_3$H unit of the phosphoglycan, is a phosphosaccharide Man(α1-OPO$_3$H-6)Gal(α1−6)Gal(α), containing just one phosphodiester group and where a mannosyl 1-phosphate unit is linked to the α-Gal residue of the LPG glycan core. Subsequently, elongating phosphosaccharide backbone β-galactosylation reactions utilize, however, different acceptor structures (Scheme 1), as all are similar to phosphosaccharides **5** and **6** (where a terminal mannosyl 1-phosphate unit linked to the β-Gal residue!!) and containing two (at least), three, and more phosphodiester groups. This probably can explain a great advantage of the acceptor substrates **5** and **6** in the β-galactosyltransferase activity over the monophosphates **3** and **4**. The difference is most spectacular for the *L. donovani* membranes (containing, presumably, the phosphosaccharide backbone associated initiating and elongating β-galactosyltransferases) but is also noticeable for the *L. major* membranes, where in addition to the former enzymes (as in the *L. donovani* preparation), branching side-chain β-galactosyltransferases are also present (see the Results and Discussion below). On the basis of our data, by using the acceptor substrates **5** and **6**, we have revealed the activity of the elongating phosphosaccharide backbone β-GalT in *L. donovani*.

Characterization of [^{3}H]Galactosylated Phospho-oligosaccharides Prepared from Synthetic Substrates 5 and 6 with *L. donovani* Microsomes. In order to characterize the products of the enzymatic reaction, we needed to properly

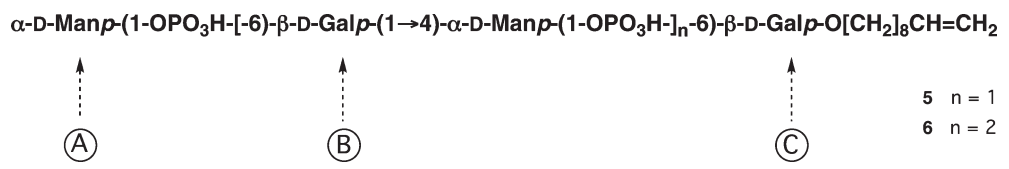


Figure 3. All possible sites of enzymatic galactosylation of acceptor substrates **5** and **6** by the action of β-galactosyltransferases from *L. donovani* (site **A** only) and *L. major* (sites **A**, **B**, and **C**).

identify the site(s) of the galactosylation in compounds **5** and **6** (Figure 3). The site **A** (a terminal mannose residue) was expected to be the only galactosylation site with the action of *L. donovani* microsomes. The prepared [^{3}H]galactosylated phospho-oligosaccharides were subjected to mild acid hydrolysis (0.04 aq TFA, 100 °C) to cleave the acid-labile α-D-mannosyl 1-phosphate bonds,[14] followed by HPTLC analysis. Possible products of the acidic degradation are shown in Scheme 2.

The only radiolabeled acid-generated compound co-migrated with synthetic disaccharide Gal(β1-4)Man **9a** (Scheme 2), thus suggesting that it is the labeled disaccharide [^{3}H]Gal(β1-4)Man **9** (Figure 2, lanes 2 and 6). The disaccharide **9** was further treated with bovine testes β-galactosidase. This enzyme has a broad specificity for any terminal, non-substituted β-galactopyranose residue. HPTLC analysis showed that product of digestion co-migrated with standard galactose (Figure 2, lane 8).

The quantitative release of [^{3}H]Gal from [^{3}H]-labeled fragment **9** with highly specific (β1-4)-galactosidase from *S. pneumoniae* confirmed that the galactosyl residues were (β1-4)-linked (Figure 2, lanes 4 and 7). These results proved that the elongating galactosyltransferase from *L. donovani* membranes catalyzes the transfer of β-[^{3}H]galactose residue from UDP-[^{3}H]Gal to the C(O)4 position of the terminal mannose residue in synthetic substrates **5** and **6** (site **A** in Figure 3).

Mass Spectrometry of Galactosylation Reaction Products of Compound 6 with *L. donovani* Microsomes. Negative ion electrospray ionization mass spectrometry (ES-MS) was performed on the products of the enzymatic galactosylation reaction of compound **6** with UDP-Gal (Figure 4A). The substrate **6** and the galactosylated product both appeared as $[M\text{-}2H]^{2-}$ ions, at m/z 683.213 and 764.239, respectively. The ion corresponding to the galactosylated product (m/z 764) was subjected to tandem mass spectrometry (ES-MS/MS), and the product ion spectrum was recorded (Figure 4B). The spectrum is consistent with the additional Gal residue being present at the non-reducing end of the linear phosphosaccharide chain, as evidenced by the m/z 403, 602 (doubly charged), and 1125 product ions; see Figure 4C for the ion assignments.

Inhibition of the Elongating (β1-4)-Galactosyltransferase from *L. donovani*. The compounds that were not substrates for the elongating (β1-4)-galactosyltransferase from *L. donovani* or revealed only traces of acceptor activity (**1**−**4**, **7**, **8**) were tested for inhibitory capability. They were used at an equimolar concentration (200 μM) to the acceptor substrate **6**. Compounds **1** and **7** (which lacked a phosphodiester group) showed zero inhibition, while compound **2** (with one α-D-mannosyl 1-phosphate unit) showed 8% inhibition (Figure 5A). Compounds **3** and **4** (fragments of the LPG with one phosphodiester group) showed 15% inhibition. Compound **8**, the C-phosphonate analogue with two phosphonic esters in the chain, inhibited the enzyme for about 19%.

We also surveyed a range of synthetic compounds **12**−**21** (Figure 5B),[31−36] which were successful substrates for the eMPT from *L. donovani*,[27,28,34,36] for their capacity to inhibit the eGalT from the same parasite at an equimolar concentration (200 μM) to the acceptor substrate **6**. All of the compounds contained a β-D-galactose residue at the non-reducing termini of the chain that abrogated their ability to work as substrates for the elongating galactosyltransferase. Compounds **12**−**14** (*L. donovani* LPG fragments) and **15** and **16** (*L. major* LPG fragments) showed 25−32% inhibition. Compound **17**, *L. mexicana* LPG fragment containing three disaccharide phosphate repeats and β-D-glucose in the side chain, displayed inhibition of 40%. Compounds **18**−**21** were C-phosphonate analogues of *L. donovani* LPG fragments and inhibited [^{3}H]galactosyl transfer to substrate **6** by 19−20%. Although none of the tested compounds revealed strong inhibitory effect, compounds **12**−**17** of the native LPG structure appeared to be less poor as inhibitors (possibly, thanks to better recognition by the enzyme) compared to the others.

Characterization of [^{3}H]Galactosylated Phospho-oligosaccharides Prepared from Synthetic Substrates 5 and 6 with *L. major* Microsomes. The *L. major* LPG is highly substituted with Gal(β1−3) side chains at the C(O)3 positions of the galactose residues in the phosphorylated disaccharide repeats.[7,8,14,17,20] Thus we had expected two types of galactosyltransferase activity after incubation of synthetic acceptors with UDP-[^{3}H]Gal and *L. major* microsomes (Scheme 1). There are three possible sites for enzymatic galactosylation in the substrates **5** or **6** (Figure 3). There is one terminal D-mannose residue, which could be galactosylated by the elongating GalT (Figure 3, site **A**), and two D-galactose residues for compound **5** (or three galactose residues for **6**) within the substrate linear chain, which could be galactosylated by the branching GalT(s) (Figure 3, sites **B** and **C**). After incubation with the *L. major* membranes, the [^{3}H]galactosylated products derived from compounds **5** and **6** were isolated and analyzed by HPTLC and fluorography (Figure 6).

In the presence of compound **5**, a major radiolabeled band with R_f value just lower than that for **5** was observed (Figure 6, lane 1). In the presence of compound **6**, there was a major radiolabeled band with R_f value lower than that for **6** (Figure 6, lane 4). The assignment of the structures of these radiolabeled compounds was based on the HPTLC analysis of products generated therefrom by acid and enzyme degradation. The analysis was performed using chemically prepared standards **9a**, **10a**, and **11a**.[30] Since the mild acid hydrolysis conditions (0.04 M aq TFA, 100 °C) were selective for the α-D-mannosyl 1-phosphate bonds cleavage,[14] we expected the products to be the [^{3}H]-labeled compounds **9**, **10**, or **11** (or any combination thereof) with a [^{3}H]Gal residue at the non-reducing terminus in each of them (Scheme 2). HPTLC analysis (Figure 6, lanes 2 and 5) showed only two acid-generated fragments: one of them co-migrated with synthetic disaccharide Gal(β1-4)Man **9a**, and another co-migrated with synthetic phosphotrisaccharide **10a**. The absence in the mixture of a radiolabeled analogue of

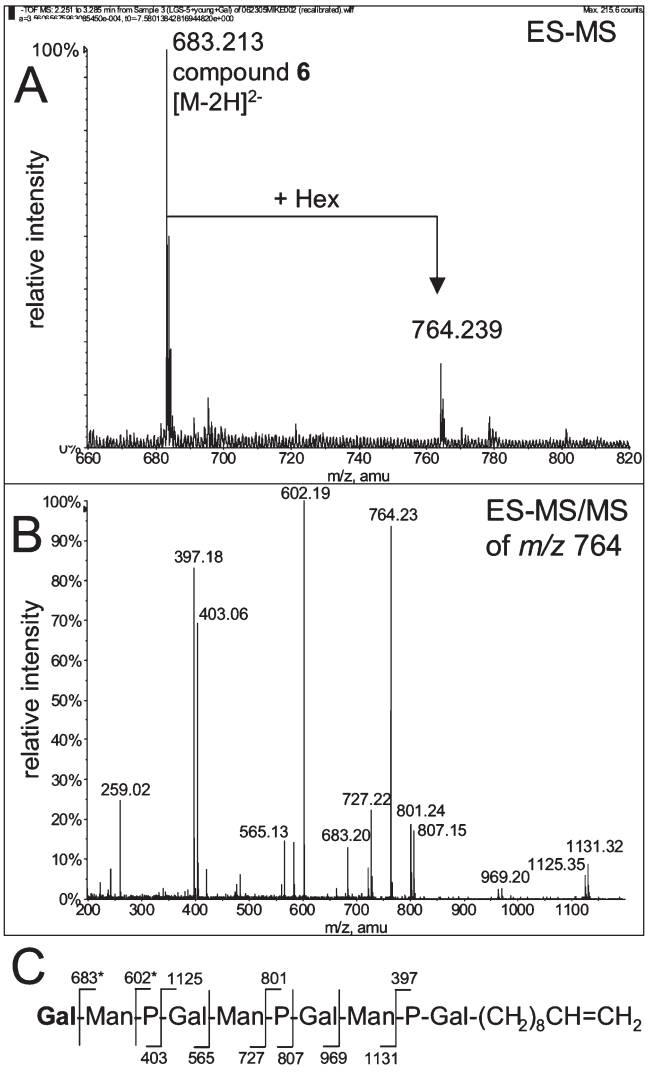


Figure 4. Negative ion mass spectrometry of the reaction products of the substrate **6** and UDP-Gal with *L. donovani* microsomes. The products of the reaction were analyzed by negative ion ES-MS (A), which revealed $[M\text{-}2H]^{2-}$ ions for compound **6** (m/z 683) and its galactosylated product (m/z 764). Mass M corresponds to H^+-form of structure **6** (Figure 1). Tandem mass spectrometry of the m/z 764 ion (B) revealed fragment ions consistent with a linear structure with the additional Gal residue (in bold) at the non-reducing end of the structure (panel C). The ions marked * are doubly charged.

synthetic **11a** (*i.e.*, compound **11**) implies that the Gal residue at the reducing end of substrates **5** and **6** (site **C** in Figure 3) has not been $[^3H]$galactosylated as the branching (β1−3)GalT needs, probably, to recognize a stretch of unsubstituted phospho-oligosaccharide repeats for activity.

Samples of radiolabeled acid-generated products (**9** + **10**), derived from **6** (Figure 6, lane 5), were subjected to HF assisted dephosphorylation (48% aq HF, 0 °C, 60 h). HPTLC analysis of the $[^3H]$galactosylated products showed the new band of the dephosphorylated trisaccharide Gal(β1−3)Gal(β1-4)Man instead of phosphotrisaccharide **10** (data not shown). The mobility of **9** after dephosphorylation procedure was unchanged.

The acid-generated $[^3H]$-labeled products **9** and **10** were also treated with bovine testes β-galactosidase. This enzyme has a broad specificity for any terminal, non-substituted β-galactopyranose residue. There was only one product of digestion, which co-migrated with standard galactose (Figure 6, lanes 3 and 7). Direct treatment of $[^3H]$galactosylated phospho-oligosaccharide(s) derived from substrate **5** with nonspecific β-galactosidase quantitatively released $[^3H]$Gal (Figure 6, lane 6). These data are consistent with all of the radioactive products having terminal non-substituted β-$[^3H]$galactosyl residues in their structures.

Thus, using synthetic phospho-oligosaccharide acceptors **5** and **6** as the substrates, we detected two different galactosyltransferase activities in *L. major* membranes: (i) $[^3H]$Gal transfer to the C(O)4 position of the terminal mannose residue (site **A** in Figure 3) due to the action of the elongating GalT and (ii) transfer of $[^3H]$Gal to the C(O)3 position of middle chain galactose residue(s) (site **B** in Figure 3) due to the action of the branching GalT. The quantitative release of $[^3H]$Gal from $[^3H]$-labeled degradation products **9** and **10**, or directly from $[^3H]$galactosylated phospho-oligosaccharide(s) derived from **5**, by bovine testes β-galactosidase confirmed that all $[^3H]$galactose residues were β-linked. In agreement with Bacic,[22,23] our data would imply that the addition of the side-chain (β1−3)Gal residues was compatible with a mechanism independent of the synthesis of the repeating backbone units.

In the case of *L. major* LPG, branching structures containing two or three (β1−3)Gal units in the side chain are common, but larger structures with up to 11 (β1−3)Gal units in the side chain have been isolated from *L. major* amastigote LPG (and are present in LPGs at very low levels)[15] and from a *L. major* mutant cell line LPG.[37] It was postulated that the rate of addition of the first Gal onto exogenous linear acceptor (initiating side-chain GalT) is much higher than the rate of addition of the second and the third galactose residues (elongating side-chain GalTs).[22] The acceptor structure for the first galactosylation reaction, which produces branching structures, is a C-3 hydroxyl group on a 6-substituted Gal and involves a phosphodisaccharide unit on the acceptor. The subsequent galactosylation reactions, however, utilize different acceptor structures, as all are a C-3 hydroxyl group on a terminal non-substituted Gal. On the basis of our data we have revealed the activity of the initiating side-chain (*i.e.*, branching) GalT in *L. major*.

To summarize, this paper describes the *in vitro* synthesis of the (β1-4)-galactosylated phospho-oligosaccharides by a microsomal membrane preparation from *L. donovani* or *L. major* using synthetic LPG fragments as acceptor substrates and UDP-Gal as the sugar donor. A second finding from this study was that the synthetic substrates could function as acceptors for the initiating side-chain (β1−3)-galactosyltransferase activity from *L. major* microsomal membranes. The simple assay described here, based on synthetic oligosaccharide acceptors that produce easily identifiable products, should be useful for monitoring the solubilization and purification of (β1-4)-galactosyltransferases from *Leishmania*. The information about the substrate specificity of the elongating (β1-4)-galactosyltransferases from *L. donovani* and *L. major* and initiating side-chain (β1−3)-galactosyltransferase from *L. major* will be useful for future design of enzyme inhibitors.

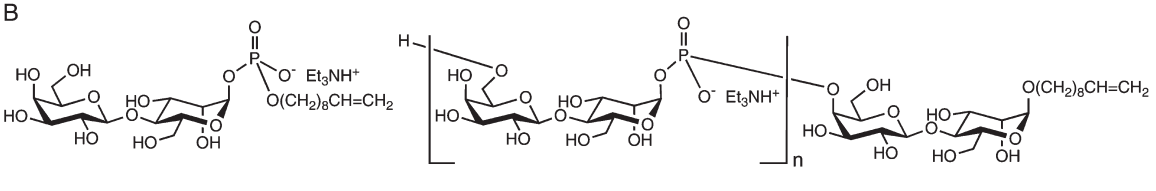
METHODS

Materials. UDP-[6-^{3}H]Gal (20 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. En^{3}Hance spray and

A

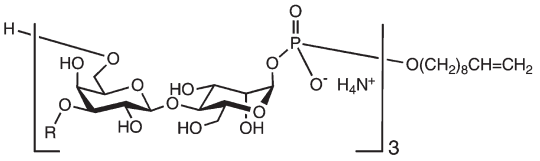
Compound	% inhibition	Compound	% inhibition
1	0	**14**	28.5 ± 4.4
2	8.2 ± 5.0	**15**	25.7 ± 14.8
3	15.3 ± 4.6	**16**	25.4 ± 15.2
4	15.7 ± 9.8	**17**	39.8 ± 11.6
7	0	**18**	18.9 ± 7.9
8	18.8 ± 8.4	**19**	19.0 ± 6.3
12	28.1 ± 15.6	**20**	19.9 ± 13.4
13	32.7 ± 9.0	**21**	19.5 ± 11.3

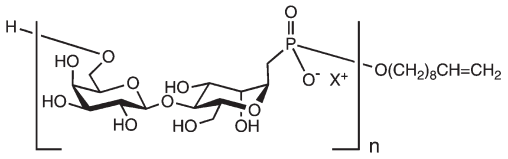
B



12

13 n = 1
14 n = 2





15 R = β-D-Gal*p* for the central Gal-Man-phosphate unit,
R = H for both flanking Gal-Man-phosphate units
16 R = β-D-Gal*p* for the terminal non-reducing Gal-Man-phosphate unit,
R = H for the central unit and for the unit linked to dec-9-en-1-ol
17 R = β-D-Glc*p* for the central Gal-Man-phosphate unit,
R = H for both flanking Gal-Man-phosphate units

18 n = 1, X = Et_3NH
19 n = 2, X = NH_4
20 n = 3, X = NH_4
21 n = 4, X = NH_4

Figure 5. (A) Inhibition of elongating galactosyl transferase from *L. donovani* using compounds **1**−**4**, **7**, **8** and **12**−**21**. Assay was performed at the standard incubation conditions. Radioactivity quantified by scintillation counting. The data represent the average of three independent experiments. (B) Structures of synthetic compounds **12**−**21** tested as inhibitors for the elongating β-D-galactosyltransferase from *L. donovani*. Compounds **12**−**14** are LPG fragments from *L. donovani* containing one or two disaccharide phosphate repeats and (for **13** and **14**) an extra Gal(β1-4)Man fragment at the reducing end. Compounds **15**−**17** are *L. major* (**15** and **16**) and *L. mexicana* (**17**) LPG fragments, each containing three disaccharide phosphate repeats and either β-D-Gal (for **15** and **16**) or β-D-Glc (for **17**) residue in the side chain. Compounds **18**−**21** are C-phosphonate analogues of the *L. donovani* LPG fragments containing from 1-4 disaccharide C-phosphonate repeats, respectively.

universal high flash-point LSC-cocktail (Formula-989) were obtained from PerkinElmer Life and Analytical Science. Bovine testes β-galactosidase was obtained from Sigma-Aldrich. *Streptococcus pneumoniae* (recombinant *E. coli*) specific (β1-4)-galactosidase and *n*-octyl β-D-glucopyranoside (NOG) were from Calbiochem. Aluminum-backed silica gel 60 HPTLC plates were purchased from Merck and C18 reverse-phase cartridges (100 mg) were from International Sorbent Technology (IST).

Preparation of Synthetic Compounds. Compounds **1**, **3**−**6**, **9a**−**11a** and **12**−**21** were synthesized as described in references 30 (for **9a**−**11a**), 31 (for **1**, **13** and **14**), 32 (for **4** and **17**), 33 (for **12**), 34 (for **15** and **16**), 35 (for **18**), 36 (for **19**−**21**), and 38 (for **3**, **5**, and **6**). The synthesis of compounds **2**, **7** and **8** is described in Supporting Information.

Analytical Procedures. Concentrations of synthetic compound stock solutions were determined by triplicate phenol-sulphuric acid assays.[39] Protein concentrations were determined using the bicinchoninic acid assay from Pierce (the BCA Protein Assay).[40]

Preparation of *Leishmania* Cell Lysate. *L. donovani* (LV9) promastigotes were cultured in Grace's Insect Medium supplemented with haemin (4 mg/mL) to a density of (1.25−1.65) × 10^7 cells/mL. *L. major* (V121) promastigotes were grown to (1.25−1.65) × 10^7 cells/mL in Schneider's *Drosophila* medium at 26 °C. Both media were supplemented with 10% heat-inactivated fetal calf serum and penicillin

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G sodium/streptomycin sulfate (100 units/0.1 mg/mL). Cells were pelleted, washed, and disrupted in a nitrogen cavitation bomb (Kontes) pressurized to 2.8 MPa as described in ref 27 and 28. Aliquots of 2.5 × 10^8 cell equiv in storage buffer (50 mM Hepes pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mM TosLysCH$_2$Cl, 1 μg/mL leupeptin), supplemented with 20% glycerol were snap-frozen in liquid nitrogen and stored at −80 °C.

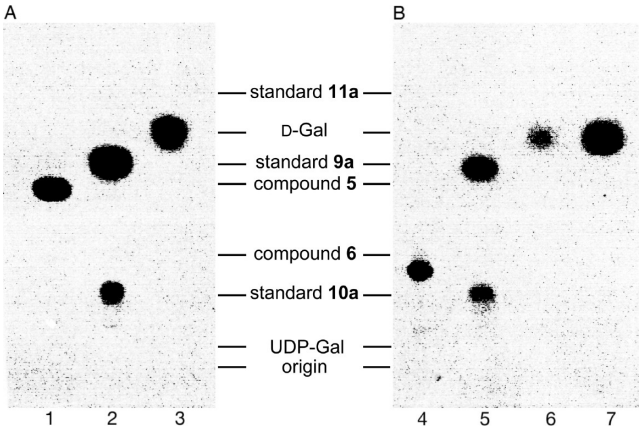
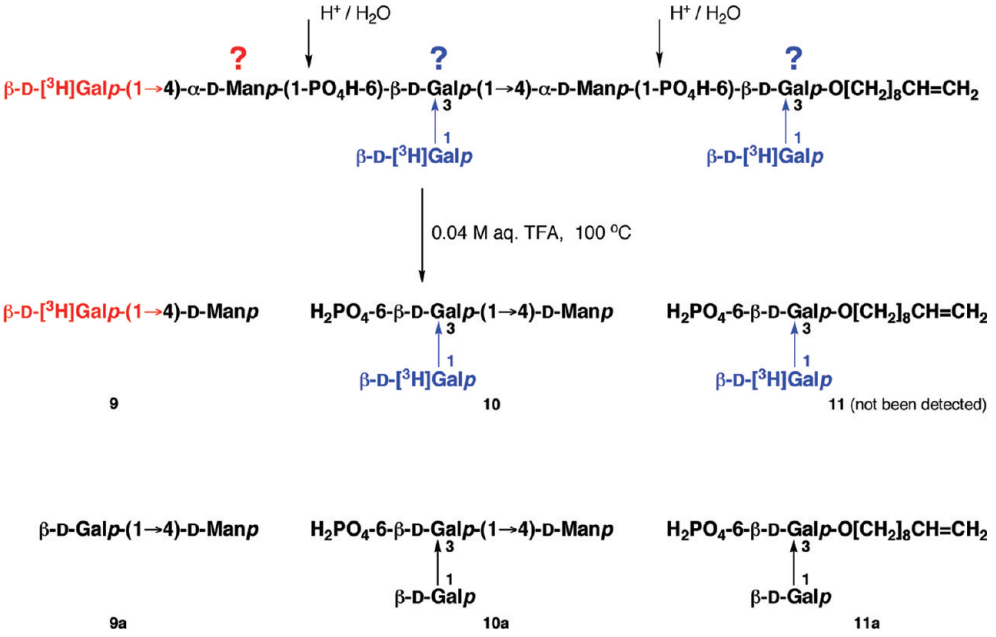


Figure 6. Characterization of the [^{3}H]galactosylated products of synthetic phospho-oligosaccharides **5** and **6** with *L. major* microsomes. (A) The [^{3}H]-labeled product(s) formed from compound **5** were analyzed by HPTLC and fluorography before (lane 1) and after (lane 2) mild acid hydrolysis (see Scheme 2) and after mild acid hydrolysis followed by incubation with bovine testes β-galactosidase (lane 3). (B) The [^{3}H]-labeled product(s) formed from compound **6** were analyzed by HPTLC and fluorography before (lane 4) and after (lane 5) mild acid hydrolysis and after mild acid hydrolysis followed by incubation with bovine testes β-galactosidase (lane 7). The [^{3}H]-labeled product(s) formed from compound **5** were analyzed by HPTLC and fluorography after incubation with bovine testes β-galactosidase (lane 6). Samples (2 μg of each) of D-galactose, UDP-Gal, and compounds **5**, **6**, **9a**, **10a**, and **11a** (Scheme 2) were run on the same plate and stained with orcinol reagent; their positions are indicated.

***L. donovani* and *L. major* Galactosyltransferase Assay.** The assay was performed as described for the elongating mannosylphosphate transferase from *Leishmania*.[27,28] Cell lysate aliquots were thawed on ice. The membranes were washed with storage buffer (see above) and pelleted in a Beckman JS13-1 rotor (7700 × *g*) for 10 min at 4 °C. The procedure was repeated. Washed membranes were resuspended in 2x concentrated storage buffer supplemented with 2 mM ATP, 1 mM dithiothreitol (DTT), 0.8 μg/mL tunicamycin, and 0.6% NOG, and protein concentration was determined. Aliquots of the membrane suspension (50 μL = 1.25 × 10^8 cell equiv) were added to 50 μL of water containing 0.25 μCi UDP-[6-^{3}H]Gal, 23 μM UDP-Gal and 400 μM synthetic acceptor. Assay tubes were incubated at 28 °C for 30 min and cooled to 0 °C, and the membranes were pelleted in a microfuge for 5 min at 4 °C (10000 × *g*). The [^{3}H]galactosylated products were recovered on C18 columns, as described.[27,28] The supernatant was mixed with 1 mL of 1 M NH_4OAc and applied to a C18 reverse-phase cartridge that was previously washed successively with 5 mL pure propan-1-ol, 5 mL 80% aq propan-1-ol (solvent A) and 10 mL of 1 M ammonium acetate. The cartridges were washed with 20 mL 1 M ammonium acetate, 1 mM galactose, 0.05 mM UDP-Gal, after which the bound material was eluted with 4 mL of solvent A. An aliquot of the product was taken for scintillation counting and the remainder was evaporated by nitrogen to dryness and then dissolved in solvent A for further HPTLC analysis. Radioactivity was quantified by liquid-scintillation counting using a Beckman LS 6000SE scintillation counter.

Scheme 2. Possible Products of [^{3}H]Galactosylation of Synthetic Acceptor 5 by the Action of *L. donovani* (Red) or *L. major* (Red and Blue) β-Galactosyltransferases (Top Structure(s); Sites of Acidic Cleavage H^+/H_2O Shown with Arrows) and Products of Their Mild Acid Hydrolysis (Structures 9−11)[a]



[a] Structures of relevant synthetic compounds **9a**, **10a**, and **11a** prepared[30] and used as HPTLC standards in order to analyse the hydrolysis products are also shown. The disaccharide **9** and the phosphosaccharides **10** and **11** are possible products of the hydrolysis, but only compound **9** (for *L. donovani* product) and compounds **9** and **10** (for *L. major* galactosylation products) were detected in the hydrolysates. These results indicate that the galactosylation takes place at the sites **A** (Figure 3; action of the elongating GalT) and **B** (action of the branching GalT) but does not happen at the site **C**.

β-Galactosidase Digestion of [^{3}H]Galactosylated Products. Aliquots of [^{3}H]galactosylated oligosaccharides formed from **5** and **6** (or their mild acid-generated products) were dried and incubated (16 h at 37 °C) with either 2 mU (β1-4)-specific galactosidase in 20 μL of 50 mM phosphate buffer (pH 5.6), or 20 mU of bovine testes β-galactosidase in 20 μL of 0.1 M citrate-phosphate buffer (McIlvaine's, pH 4.4). The digested samples were heated for 5 min at 100 °C, and precipitated proteins were pelleted in microfuge (18400 × *g*, 5 min). The supernatants were desalted on a column of 0.1 mL Dowex AG50x12(H^+) over 0.2 mL Dowex AG3x4(OH^-), eluted with 1.5 mL water, and dried in a Speedvac concentrator. The products were analyzed by HPTLC.

Mild Acid Hydrolysis of [^{3}H]Galactosylated Products. Selective cleavage of the mannosyl 1-phosphate bonds was achieved by hydrolysis of the [^{3}H]galactosylated phospho-oligosaccharides with 50 μL of 40 mM TFA for 10 min at 100 °C. The TFA-treated samples were dried, resuspended in 50 μL of water, and dried again. The procedure was repeated three times to eliminate the acid. The radiolabeled products were analyzed by HPTLC.

High-Performance Thin-Layer Chromatography (HPTLC). Each sample was dissolved in 5 μL of solvent A and applied onto an aluminum-backed silica gel 60 HPTLC plate that was developed with chloroform/methanol/0.25% KCl (10:10:3, v/v/v). The plate was subsequently fluorographed by spraying with En^{3}Hance and exposed to Kodak X-Omat XAR-5 film at −80 °C using an intensifying screen. Lanes containing nonradioactive samples were cut out after development of the plate, sprayed with orcinol reagent (180 mg of orcinol monohydrate dissolved in 90 mL of a mixture ethanol/H_2SO_4 conc/water, 75:10:5, v/v/v; stored at 4 °C) and heated for 5 min at 110 °C.

Mass Spectrometry of Galactosylation Reaction Products. Mass spectrometric analysis of the transfer of galactose residues to acceptor **6** by *L. donovani* membranes was performed with 0.6 mM acceptor substrate in the presence of 5 mM UDP-Gal. After the standard purification of the products on C18 cartridges, small aliquots were transferred to Waters nanotips (Millipore, Milford, MA; type F) and analyzed by electrospray ionization-mass spectrometry (ES-MS) in negative ion mode on an ABI Q-StarXL mass spectrometer. The product ion spectra were used to identify the galactosylation reaction products.

Inhibition Assay for *L. donovani* Elongating Galactosyltransferase. The transfer of [^{3}H]galactose residue to acceptor **6** by *L. donovani* membranes was measured with 0.2 mM substrate **6** in the presence and absence of 0.2 mM potential inhibitors **1**−**4**, **7**, **8**, and **12**−**21**.

For kinetic parameters and properties of the elongating (β1-4)-galactosyltransferase from *L. donovani*, see the Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge *via* the Internet.

■ AUTHOR INFORMATION

Corresponding Author
*a.v.nikolaev@dundee.ac.uk

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