Characterization of the Elongating α-D-Mannosyl Phosphate Transferase from Three Species of *Leishmania* Using Synthetic Acceptor Substrate Analogues†

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ABSTRACT: *Leishmania* express lipophosphoglycans and proteophosphoglycans that contain Galβ1-4Manα1-P phosphosaccharide repeat structures assembled by the sequential addition of Manα1-P and βGal. The synthetic acceptor substrate Galβ1-4Manα1-P-decenyl and a series of analogues were used to probe *Leishmania* α-D-mannosyl phosphate transferase activity. We show that the activity detected with Galβ1-4Manα1-P-decenyl is the elongating α-D-mannosyl phosphate transferase associated with lipophosphoglycan biosynthesis (eMPT^{LPG}). Differences in the apparent K_m values for the donor and acceptor substrates were found using *L. major*, *L. mexicana*, and *L. donovani* promastigote membranes, but total activity correlated with the number of lipophosphoglycan repeats. Further comparisons showed that lesion-derived *L. mexicana* amastigotes, that do not express lipophosphoglycan, lack eMPT^{LPG} and that nondividing *L. major* metacyclic promastigotes contain 5-fold less eMPT^{LPG} activity than dividing procyclic promastigotes. The fine specificity of promastigote eMPT^{LPG} activity was determined using 24 synthetic analogues of Galβ1-4Manα1-P-decenyl. The three species gave similar results: the negative charge of the phosphodiester and the C-6 hydroxyl of the αMan residue are essential for substrate recognition, the latter most likely acting as a hydrogen bond acceptor. The C-6′ hydroxyl of the βGal residue is required for substrate recognition as well as for catalysis. The rate of Manα1-P transfer declines with increasing acceptor substrate chain length. The presence of a monosaccharide substituent at the C-3 position of the terminal βGal residue abrogates Man-P transfer, showing that chain elongation must precede side chain modification during lipophosphoglycan biosynthesis. In contrast, substitution of the penultimate phosphosaccharide repeat does not abrogate transfer but is slightly stimulatory in *L. mexicana* and inhibitory in *L. major*.

The sandfly transmitted protozoan parasites of the genus *Leishmania* cause a variety of diseases throughout the tropics and subtropics, ranging from relatively mild cutaneous lesions (*L. major*) to diffuse cutaneous lesions (*L. mexicana*) and lethal visceral infections (*L. donovani*). The parasites divide in the midgut of the sandfly vector as flagellated procyclic promastigote forms that, following transformation into nondividing metacyclic promastigote forms, make their way to the mouth-parts of the vector. The metacyclic promastigotes that are injected into the mammalian host with the insect saliva rapidly attach to and invade host macrophages. Within the macrophage phagolysosome, the metacyclic promastigotes differentiate into small round amastigote forms that divide until the host cell is ruptured. The infection is then propagated by amastigote invasion of other macrophages and the life-cycle is completed when a sandfly ingests infected macrophages. The procyclic promastigote stages of most *Leishmania* species can be grown in liquid culture, and these can produce metacyclic promastigotes when the cultures become stationary (*1*). The amastigote forms of *L. mexicana* can be prepared from the skin lesions of infected mice (lesion-amastigotes), and amastigote-like forms can be cultured axenically (*2*, *3*).

Leishmania produce characteristic glycoconjugates that contain phosphosaccharide repeat units of (-6Galβ1-4Manα1-P-)$_n$ (*4*). Such molecules have been found in both the promastigote and the amastigote stages of all species of the *Leishmania* analyzed so far and include lipophosphoglycan (LPG),[1] secreted phosphoglycan (PG), and secreted and membrane-bound proteophosphoglycans (PPGs) (*5*−*7*).

Four types of secreted PPG have been described in detail (see refs *5* and *7* for recent reviews): (i) a polymeric secreted acid phosphatase (sAP) from *L. mexicana* promastigotes (*8*),

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[1] Abbreviations: LPG, lipophosphoglycan; PG, phosphoglycan; PPG proteophosphoglycan; aPPG, amastigote PPG; pPPG, promastigote PPG; sAP, secreted acid phosphatase; MPT, α-D-mannosyl phosphate transferase; iMPT^{LPG}, initiating MPT of LPG biosynthesis; iMPT^{PPG}, initiating MPT of PPG biosynthesis; eMPT, elongating MPT; eMPT^{LPG}, eMPT of LPG biosynthesis; eMPT^{PPG}, eMPT of PPG biosynthesis.

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(ii) a monomeric sAP from *L. donovani* (*9*), (iii) a filamentous mucin-like proteophosphoglycan secreted by *L. major* promastigotes (pPPG) (*10*), and (iv) a secreted nonfilamentous mucin-like proteophosphoglycan from *L. mexicana* amastigotes (aPPG) (*11*). All *Leishmania* species, except *L. major*, appear to secrete sAPs (*12*), although not always in the beadlike polymers found for *L. mexicana*. The promastigotes of most, possibly all, species of *Leishmania* secrete pPPG and the distribution of aPPG synthesis has yet to be determined. A GPI-anchored membrane-bound PPG (mPPG) has also been described in *L. major* promastigotes (*7*).

LPGs are common to all *Leishmania* promastigotes (*13*−*18*) and *L. major* amastigotes (*19*), and they are absent in *L. donovani* and *L. mexicana* amastigotes (*20*, *21*). LPGs have the following general structure: Manα1-2(±Galβ1-4)-Manα1-P-[6(±R-3)Galβ1-4(±R′-2)Manα1-P-]$_n$-6Galα1-6Galα1-3Galfβ1-3(±Glcα1-P-6)Manα1-3Manα1-4GlcNα1-6*myo*-inositol-1-P-3(*sn*-1-*O*-alkylglycerol). Species- and stage-specific differences are seen in (i) the structure of the nonreducing terminal oligosaccharide, (ii) the nature of the saccharides (R and R′) that can substitute the basic phosphosaccharide repeat, (iii) the average number (*n*) of repeats, and (iv) the proportion of structures containing a Glcα1-P- substituent on the glycoinositol−phospholipid core. For a comprehensive review of LPG structures, see refs *17* and *18*.

The secreted PGs of *L. major* (*22*) and *L. mexicana* (*23*) have been described and that of *L. donovani* (*24*) has been characterized as mainly Manα1-2(±Galβ1-4)Manα1-P-(6Galβ1-4Manα1-P-)$_{10}$-6Galα1-4Man. Thus, PGs are essentially LPG molecules without the glycoinositol−phospholipid core.

The importance of LPG for promastigote infectivity in both the insect vector (*25*−*27*) and the mammalian host (*28*), as well as the possible roles of pPPG in parasite transmission from the insect vector (*5*, *7*) and of aPPG in amastigote survival in the phagolysosome (*29*) and following macrophage rupture (*30*), make the biosynthetic enzymes responsible for the formation of these *Leishmania*-specific glycoconjugates of interest.

The in vitro synthesis of LPG by *L. donovani* membranes, using endogenous LPG intermediates as acceptors and GDP-Man and UDP-Gal as donors, has been described (*31*), and an *L. donovani* mannosyl phosphate transferase (MPT) activity has been partially characterized using the exogenous acceptor substrate Galα1-6Galα1-3Galfβ1-3Manα1-3Manα1-4GlcNα1-6*myo*-inositol-1-P-3(*sn*-1-*O*-alkylglycerol), obtained by mild acid and alkaline phosphatase treatments of purified LPG (*32*). This study showed that Man-P is transferred en bloc from GDP-Man. More recently, the activity was assayed using stachyose (Galα1-6Galα1-6Glcβ1-2Fru) as an acceptor analogue (*33*). The activity detected in this way is the initiating MPT that adds the first Man-P group to the LPG glycoinositol−phospholipid core (referred to here as iMPT^{LPG}) (*34*). Recently, assays for the initiating MPTs (both GDP-Man:Ser-protein MPTs) that add the first Man-P group to *L. mexicana* (*35*) and *L. donovani* sAP (*9*) have been described. These PPG initiating MPT activities are referred to here as iMPT^{PPG}.

We previously reported that the synthetic phosphosaccharide Galβ1-4Manα1-P-decenyl (compound **1**; Figure 1) acts as an acceptor substrate for MPT activity in *L. major* promastigote membranes (*36*). We further concluded that the activity acting upon it was an elongating MPT (eMPT) because an analogue lacking the phosphodiester group was not an acceptor. In this paper, we (i) confirm that compound **1** is not an acceptor for iMPT activities and is specific for the eMPT associated with LPG biosynthesis, (ii) compare the eMPT activities of *L. major*, *L. mexicana*, and *L. donovani* procyclic promastigotes; *L. mexicana* axenic and lesion-derived amastigotes; and *L. major* metacylic promastigotes, (iii) probe the fine specificities of *L. major*, *L. mexicana*, and *L. donovani* promastigote eMPTs, and (iv) assess the effects of substrate chain length and side chain substitution on eMPT activity.

EXPERIMENTAL PROCEDURES

Materials. GDP-(2-^{3}H)Man and En^{3}Hance spray were obtained from NEN Life Science Products. Aluminum-backed silica gel 60 HPTLC plates were purchased from BDH-Merck and C18 Sep-pak cartridges from Waters Associates. Synthetic peptides based on *L. major* pPPG (APSASSSSAPSSSSSAPSAK) (*7*, *37*) and *L. mexicana* aPPG (GSSSFAATSESSAVEGSSSFHSGSK) (*38*) were custom-synthesized by MWG-Biotech. The synthetic compounds **1**−**25** used in this study were synthesized as described in refs *39*−*42*.

Analytical Procedures. Concentrations of synthetic compound stock solutions were determined by triplicate phenol−sulfuric assays. Protein concentrations were determined using the bicinchoninic acid assay from Pierce.

Preparation of Leishmania Cell Lysates. L. major (V121), *L. mexicana* (M379), and *L. donovani* (LV9) were grown to 1.25×10^7 cells/mL in Schneider's *Drosophila* medium supplemented with 10% heat-inactivated fetal calf serum and 50 μg/mL gentamycin sulfate at 24 °C. *L. major* infective metacyclic promastigotes were prepared by depletion of stationary culture with peanut agglutinin as described by da Silva and Sacks (*43*). *L. mexicana* axenic amastigotes were cultivated as described by Bates et al. (*2*, *3*). *L. mexicana* lesion amastigotes were isolated from tail lesions in BALB/c mice and were generously provided by Thomas Ilg (Max Plank Institute für Biologie, Tübingen). Lysates were obtained by disruption of the cells in a nitrogen cavitation bomb pressurized to 2.8 MPa as described in ref *36*. Aliquots of 2.5×10^8 cell equiv in storage buffer (50 mM Hepes, pH 7.4, 25 mM KCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 0.1 mM TosLysCH_2Cl, and 1 μg/mL leupeptin) supplemented with 20% glycerol were snap frozen in liquid nitrogen and stored at −70 °C.

Elongating Mannosyl Phosphate Transferase (eMPT) Assay. Unless indicated otherwise, the following standard assay was used to test the acceptor activity of the synthetic compounds. Cell lysates were thawed on ice, and the membranes were washed twice with storage buffer. Washed membranes were resuspended at 5×10^9 cell equiv/mL in 2× concentrated storage buffer supplemented with 2 mM ATP, 1 mM DTT, 0.8 μg/mL tunicamycin, and 0.05% Chaps. Protein concentration was determined on an aliquot of the membrane suspension using the bicinchoninic acid assay. A total of 50 μL of membrane suspension was immediately added to 50 μL of water containing 1 μCi of GDP-[2-^{3}H]-Man, 23 μM GDP-Man, 0.05% Chaps, and 0.4 mM synthetic

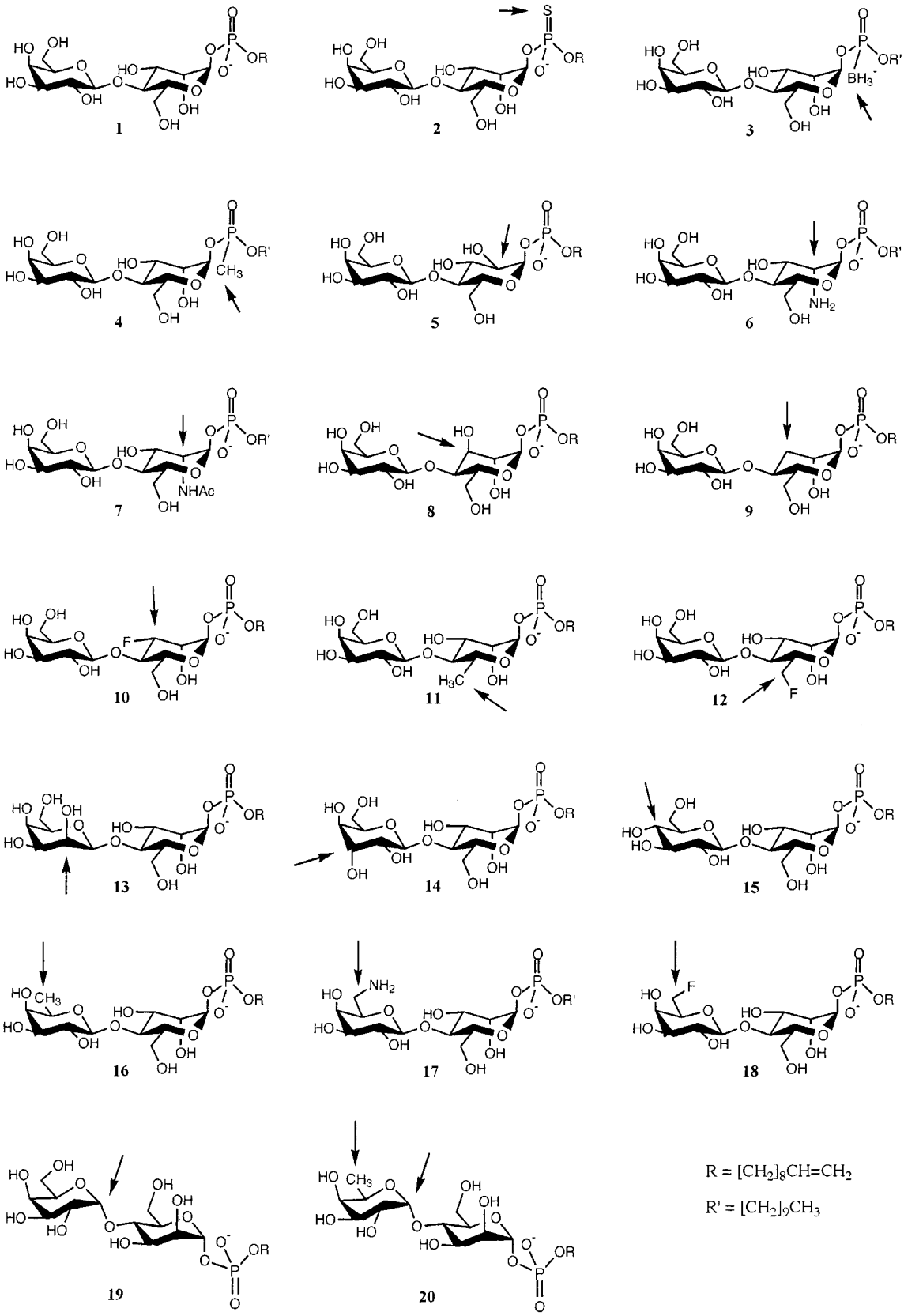

FIGURE 1: Structures of compounds used in this study. Arrows indicate the sites of modification relative to compound **1,** which is a single Galβ1−4Manα1-P phosphosaccharide repeat linked to a decenyl group (R). Compounds are analogues of **1** in which the phosphate group is replaced by a thiophosphate (**2**), boranophosphate (**3**), and methylphosphonate (**4**); analogues of **1** in which the mannose residue is replace by glucose (**5**), mannosamine (**6**), *N*-acetylmannosamine (**7**), altrose (**8**), 3-deoxymannose (**9**), 3-fluoromannose (**10**), rhamnose (**11**), and 6-fluoromannose (**12**); analogues of **1** in which the galactose residue is replaced by talose (**13**), gulose (**14**), glucose (**15**), fucose (**16**), 6-aminogalactose (**17**), and 6-fluorogalactose (**18**); and α-galactose (**19**) and 6-deoxy-α-galactose (**20**). All monosaccharides have D-configuration.

phosphodisaccharide. The tubes were incubated at 28 °C for 20 min, cooled to 0 °C and the membranes were pelleted in a microfuge for 5 min at 4 °C. The phosphomannosylated products recovered in the supernatant were purified on C18 Sep-pak cartridges as previously described (*36*). One-tenth of the products were taken for scintillation counting.

Measuring eMPT Kinetic Parameters. K_m for substrate **1** and GDP-Man were determined using the standard assay described above but with 2 μCi of GDP-[2-^{3}H]Man and varying concentrations of GDP-Man and substrate **1**: 0.3 mM GDP-Man and 0.2, 0.4, 0.8, 1.6, 2.4, 3.2, 4.0, or 4.6 mM substrate **1** were used to determine the K_m for synthetic substrate **1** and 2 mM substrate **1**, and 0.1, 0.2, 0.3, 0.4, 0.5, or 0.6 mM GDP-Man were used to determine the K_m for GDP-Man. K_m and V_{max} were obtained with a nonlinear regression program using the software GrafFit.

Substrate Specificity of eMPT. The substrate specificity of eMPT was tested using a competition assay. *L. major* promastigotes membranes were incubated with 0.2 mM substrate **1** using the standard assay with or without 0.2, 0.5, or 2 mM synthetic pPPG peptide acceptor for iMPT^{PPG} and with and without 2 mM stachyose (iMPT^{LPG} acceptor). Controls without substrate **1** and with 0.2, 0.5, or 2 mM peptide or 2 mM stachyose were also performed.

Determination of the eMPT Temperature Optimum. A total of 1.25×10^8 cell equiv of promastigote and 2.5×10^8 cell equiv of axenic amastigote membranes (containing similar amounts of protein) were incubated for 20 min with 0.8 mM substrate **1** and 0.1 mM GDP-Man at 15, 20, 25, 28, 30, 35, or 40 °C.

Comparison of eMPT Activities in Different Life-Cycle Stages of Leishmania. eMPT activities in *L. major* promastigote and metacyclic promastigotes membranes were tested using the standard assay. *L. mexicana* promastigotes, axenic amastigotes, and lesion amastigotes were compared using the standard assay except that the concentrations of substrate **1** and GDP-Man were 1.6 mM and 0.1 mM, respectively, and half the amount of promastigote membranes was used.

aPPG Initiating Mannosyl Phosphate Transferase (iMPT^{aPPG}) Assay. The assay is similar to that of the standard eMPT assay, described above, except that 1 mM aPPG synthetic peptide was used as the acceptor substrate, and 2 μCi of GDP-[^{3}H]Man and 5×10^8 cell equiv of membranes (from *L. mexicana* procyclic promastigotes, axenic amastigotes, and lesion-derived amastigotes) were used, and propan-1-ol was replaced by acetonitrile for the Sep-pak steps.

eMPT Inhibition Assays. The transfer of [^{3}H]mannosyl phosphate to acceptor **1** by *L. major* membranes was measured with 0.2 mM substrate **1** in the presence and absence of 0.2 mM compounds **4**, **11**, **16**, **17**, **18**, **19**, and **20** and with 0.2 mM acceptor **1** in the presence and absence of 0.2, 0.4, 0.8, 2, and 4 mM compounds **16** and **17**.

Enzyme and Chemical Analyses of Phosphomannosylated Compounds. Phosphomannosylated products were digested with jack bean α-mannosidase (30 unit/mL) in 0.1 M sodium acetate buffer, pH 5.0, and 0.1% sodium taurodeoxycholate for 18 h at 37 °C and desalted on a column of Dowex AG50X12(H^+) over Dowex AG3X4(OH^-) over QAE-SephadexA25(OH^-). Selective cleavage of the phosphodiester bond was achieved by mild acid hydrolysis of the (^{3}H)mannosylated products with 200 μL of 40 mM trifluoroacetic acid for 8 min at 100 °C. Samples were dried, resuspended in water, and dried again (three times) to eliminate the acid. The products of jack bean α-mannosidase treatment and mild acid hydrolysis were analyzed by HPTLC.

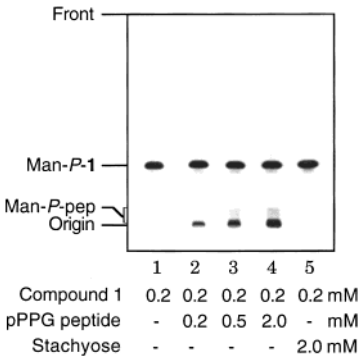

FIGURE 2: Acceptor substrate **1** is specific for eMPT activity. Substrate specificity of eMPT was tested using a competition assay. *L. major* promastigote membranes were incubated with GDP-[^{3}H]-Man and substrate **1** (lane 1) or both substrate **1** and synthetic peptide acceptor for iMPT^{PPG} (lanes 2, 3, and 4) or stachyose, an acceptor for iMPT^{LPG}, (lane 5) at the concentrations indicated. After incubation, the products were purified on a Sep-Pak C18 cartridge and analyzed by HPTLC and fluorography. The level of eMPT activity was measured by counting the radioactivity associated with the scraped Man-P-**1** band. Note: Transfer of Man-P to the pPPG synthetic peptide (Figure 2, lanes 2−4) shows that this compound is a substrate for the iMPT^{PPG}.

HPTLC. Samples were applied in 5 μL of 40% propan-1-ol to aluminum-backed silica gel 60 HPTLC plates that were developed with chloroform/methanol/0.25% KCl 10/10/3 (v/v/v). The plates were subsequently fluorographed by spraying with En^{3}Hance and exposing to Kodak X-Omat XAR-5 film at −70 °C using an intensifying screen. After exposure, bands were scraped and taken for scintillation counting.

RESULTS AND DISCUSSION

Leishmania iMPT^{LPG} and iMPT^{PPG} activities have been solubilized with β-dodecylmaltoside and Triton-X100, respectively (*33*, *35*), although partial purification of these activities has not been reported. Despite considerable efforts, we were unable to produce a stable detergent-solubilized preparation of *Leishmania* eMPT. Thus, in common with all other studies on this enzyme (*31*, *32*, *34*−*36*), we used washed parasite membranes as an enzyme source, as originally described by Carver and Turco (*31*). The structures of the synthetic compounds used in this study are shown in Figure 1 (*39*−*42*). In all cases where transfer of [^{3}H]Man to acceptor substrates was observed, product characterization similar to that described in ref *36* was performed (data not shown). In all cases, the transferred [^{3}H]Man was shown to be mild acid- and jack bean α-mannosidase-sensitive, consistent with Man-P transfer.

*Compound **1** Acceptor Substrate is Specific for Elongating MPT (eMPT) Activity.* As previously described, washed *L. major* procyclic promastigote membranes transfer Man-P from GDP-Man to the synthetic substrate **1** (Figure 2, lane 1). The dependence of this activity on the phosphodiester group of **1** was interpreted to mean that **1** was acting as an acceptor for elongating MPT activity (*36*). To assess whether **1** might also act as an acceptor for the LPG and the PPG initiating MPTs (iMPT^{LPG} and iMPT^{PPG}), synthetic substrates for those activities (stachyose (*33*, *34*) and pPPG synthetic

Table 1: eMPT Kinetic Parameters

kinetic parameters[a]	*L. major* promastigote	*L. mexicana* promastigote	*L. mexicana* ax. amastigote[b]	*L. donovani* promastigote
apparent V_{max} [pmol min^{-1} (mg of protein)$^{-1}$]	600 ± 40	970 ± 70	475 ± 10	ND[c]
apparent K_m for compound **1** (mM)[d]	1.9 ± 0.3	2.0 ± 0.3	3.2 ± 0.2	>3.0
apparent K_m for GDP-Man (mM)[e]	0.26 ± 0.03	0.20 ± 0.03	ND	ND

[a] K_m and V_{max} were obtained by fitting the data to a nonlinear regression program using GrafFit. [b] ax. amastigote = axenic amastigote. [c] ND = Not determined. [d] K_m for substrate **1** was determined using a concentration of 0.3 mM GDP-Man. [e] K_m for GDP-Man was determined using a concentration of 2 mM substrate **1**.

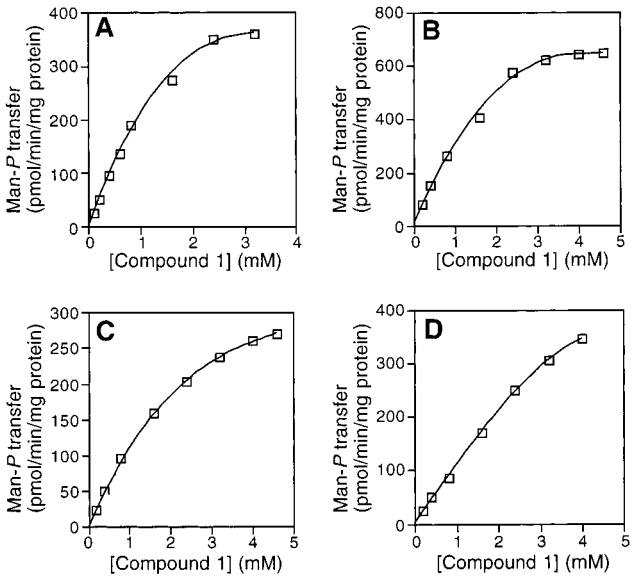

FIGURE 3: Determination of the Michaelis constant for substrate **1** in different species of *Leishmania*. Membranes from *L. major* promastigotes (A), *L. mexicana* promastigotes (B), *L. mexicana* axenic amastigotes (C), and *L. donovani* promastigotes (D) were incubated with 0.3 mM GDP-[^{3}H]Man and increasing concentrations of substrate **1**. Products were analyzed by HPTLC and fluorography. The level of eMPT activity was measured by counting the radioactivity associated with the scraped Man-P-**1** band.

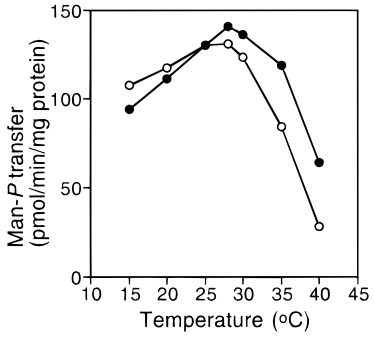

FIGURE 4: Effect of temperature on eMPT activity in *L. mexicana* promastigote and axenic amastigote membranes. eMPT activities were determined at 15, 20, 25, 28, 30, 35, and 40 °C using membranes of *L. mexicana* promastigotes (●) and *L. mexicana* axenic amastigotes (○).

peptide, respectively) were added to the assay mixture together with **1**. Neither compound inhibited the transfer of Man-P to **1** (Figure 2, lanes 2−5), even at concentrations 10-fold higher than that of **1**, showing that **1** is a specific acceptor for eMPT activity. A similar result was recently reported using *L. mexicana* membranes and sAP synthetic peptide (*32*).

eMPT Kinetic Parameters. The apparent K_m and V_{max} values for the eMPT activities in the washed membranes of

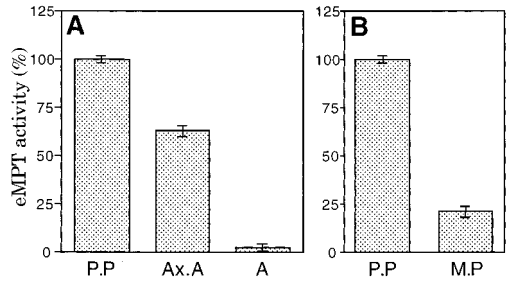

FIGURE 5: eMPT activity in different life-cycle stages of *Leishmania*. eMPT activity was measured in procyclic promastigote (P.P), axenic amastigote (Ax.A), and lesion-derived amastigote (A) forms of *L. mexicana* (panel A) and procyclic promastigote (P.P) and metacyclic promastigote (M.P) forms of *L. major* (panel B). The standard assay was used in panel B, whereas in panel A, 1.6 mM compound **1** and 0.1 mM GDP-Man was used to detect the trace activity in lesion amastigotes.

Table 2: Relative Activity of eMPT Using Different Analogues of Compound **1**[a]

compound	*Leishmania* species: *L. major* (%)	*L. mexicana* (%)	*L. donovani* (%)
1	100	100	100
2	75 ± 4	75 ± 7	75 ± 4
3	52 ± 4	51 ± 2	53 ± 9
4	Npd[b]	Npd	Npd
5	9 ± 1	5 ± 2	7 ± 2
6	26 ± 3	27 ± 1	30 ± 3
7	7 ± 1	6 ± 1	5 ± 1
8	tr[c]	tr	tr
9	131 ± 7	131 ± 8	139 ± 17
10	138 ± 5	140 ± 13	146 ± 10
11	tr	tr	tr
12	27 ± 3	17 ± 1	22 ± 2
13	28 ± 2	18 ± 3	21 ± 2
14	9 ± 2	10 ± 3	9 ± 2
15	14 ± 1	6 ± 3	12 ± 1
16	Npd	Npd	Npd
17	Npd	Npd	Npd
18	Npd	Npd	Npd
19	Npd	Npd	Npd
20	Npd	Npd	Npd

[a] The figures represent the average of three independent experiments (±1 SD) and were determined by counting the radioactivity associated with the scraped band from HPTLC or by counting one-tenth of the product when the fluorograph indicated that all the radioactivity was associated with Man-P-substrate. [b] Npd = no product detected. [c] tr = trace (<5%).

the procyclic promastigote stages of three *Leishmania* species (*L. major*, *L. mexicana*, and *L. donovani*) and of the axenic amastigotes of *L. mexicana* were measured using compound **1** as the acceptor substrate and GDP-Man as the donor substrate. Representative data are shown in Figure 3 and the complete results are summarized in Table 1. The sensitivity of the eMPT activity in the washed membranes to concentra-

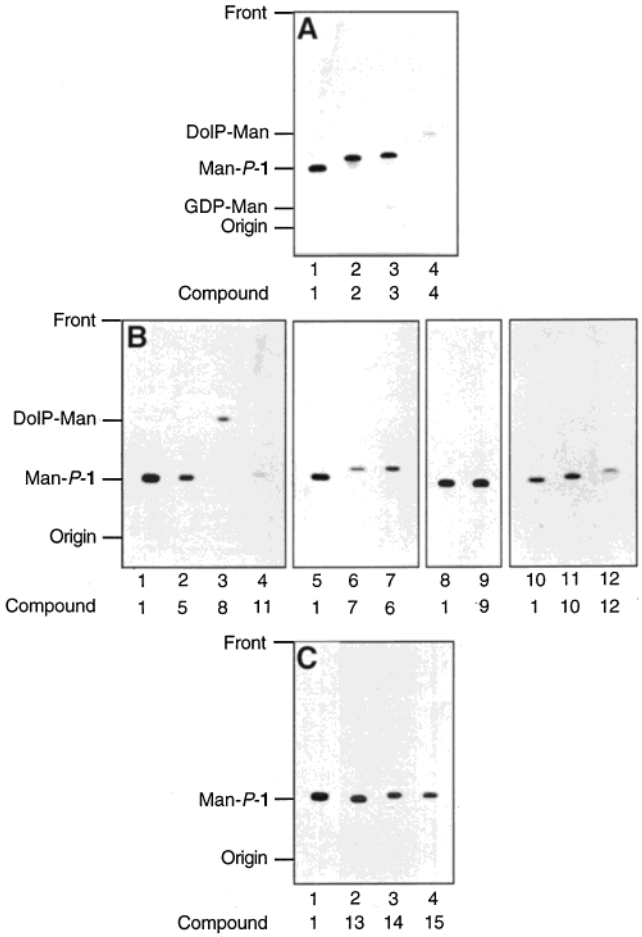

FIGURE 6: Substrate specificity of *Leishmania* eMPT activity. Analogues of acceptor substrate **1** in which the phosphate group (panel A), the mannose residue (panel B), or the galactose residue (panel C) is modified were incubated with *L. major* membranes using the standard assay. Sep-Pak purified products were analyzed by HPTLC and fluorography. The level of eMPT activity was measured by counting the radioactivity associated with the Man-P-substrate band.

tions of compound **1** above 5 mM, due to the detergent-like properties of this amphiphilic substrate, prevented an accurate estimate of the apparent K_m and V_{max} values for the *L. donovani* activity. However, it appears that while the *L. major* and *L. mexicana* promastigote MPT activities have similar apparent K_m values for the acceptor and the donor substrates, the *L. donovani* activity has a significantly lower affinity for the acceptor substrate. The higher apparent V_{max} value for the *L. mexicana* promastigote activity as compared to that of the *L. major* promastigotes may simply reflect higher specific activity of a similar enzyme in *L. mexicana*. In any case, in terms of total promastigote eMPT activity measured with compound **1**, *L. mexicana* membranes are more active than *L. major* membranes, which are more active than *L. donovani* membranes. This relative order correlates with that for the average numbers of phosphosaccharide repeats in the promastigote for these same parasites (*17*), suggesting that average LPG chain length may simply reflect eMPT specific activity.

Different eMPT Activities May Be Associated with LPG Biosynthesis and Lesion-Derived Amastigote PPG Biosynthesis. eMPT activity was assayed with washed membranes from *L. mexicana* procyclic promastigotes and axenic amastigotes. Although there was a small difference in the apparent K_m values for the acceptor substrate (Table 1), no significant differences were observed in the temperature optima (25−30 °C) (Figure 4) or substrate specificities of

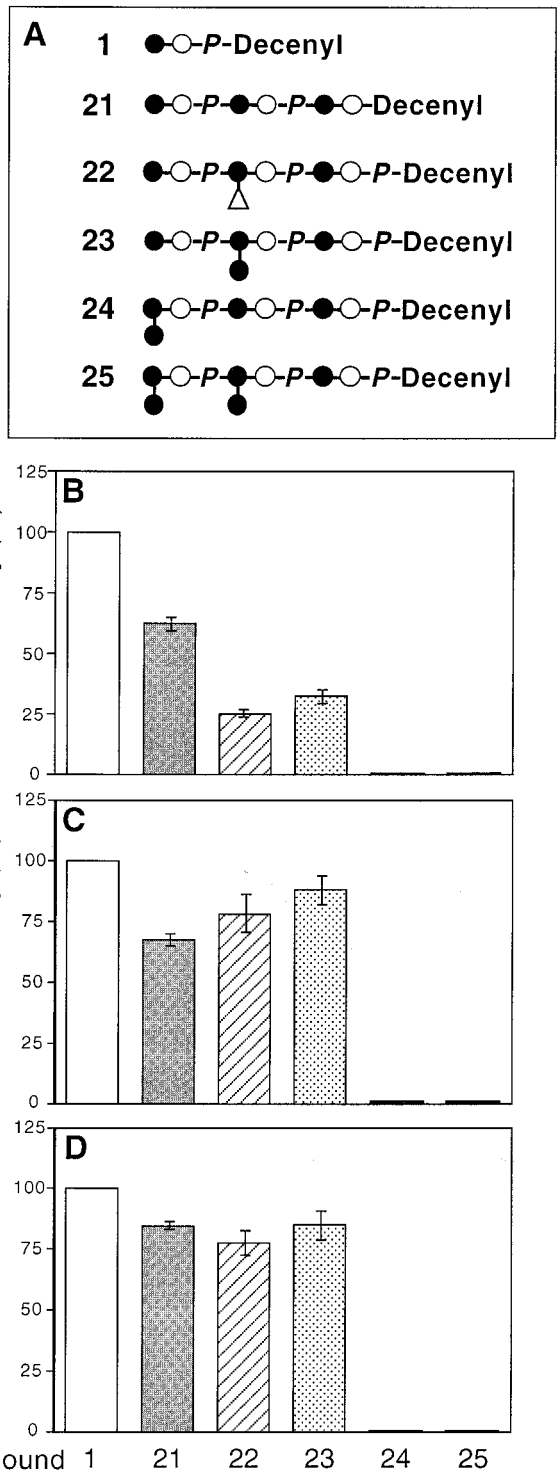

FIGURE 7: Effect of substrate length and substitution on eMPT activity in *L. major*, *L. mexicana*, and *L. donovani* promastigotes. Panel A: schematic representations of the compounds used. Compound **1** is Galβ1-4Manα1-P-decenyl; compound **22** contains a βGlc residue (△) in 1→3 linkage and mimicks *L. mexicana* LPG; compounds **23**, **24**, and **25** contain βGal residue(s) (●) in 1→3 linkage and mimic *L. major* LPG. Panels B, C, and D show the results using these compounds with promastigote membranes of *L. major* (panel B), *L. mexicana* (panel C), and *L. donovani* (panel D). The $eMPT^{LPG}$ activities are shown relative to those for compound **1** (100%).

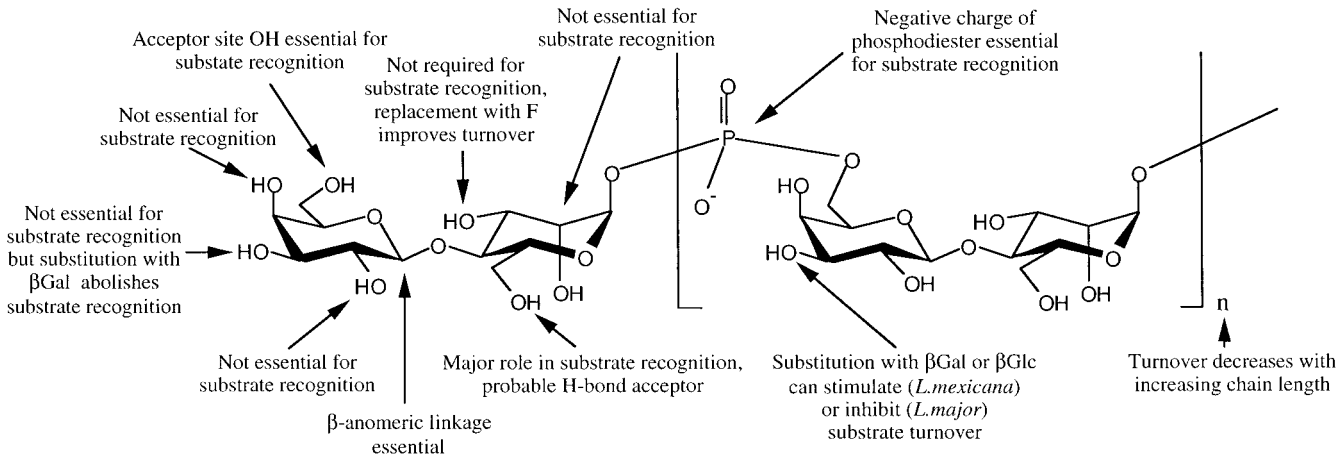

FIGURE 8: Summary of the eMPT^{LPG} substrate specificity data presented in this paper.

the promastigote and axenic amastigote enzymes,[2] suggesting that similar or identical eMPT enzymes are present in procyclic promastigotes and axenic amastigotes. The specific activity of the axenic amastigote membranes was about 65% of that of the procyclic promastigote membranes (Figure 5A).

In contrast, only trace eMPT activity (approximately 1% as compared with procyclic promastigotes) could be detected using lesion-derived *L. mexicana* amastigote membranes and compound **1** as the acceptor substrate (Figure 5A). Since lesion-derived *L. mexicana* amastigotes express aPPG (*11*) but not LPG (*20*), this result is consistent with the idea that LPG and aPPG biosynthesis might utilize different eMPT activities, with compound **1** being a specific substrate for eMPT^{LPG}. The same lesion-derived amastigote membranes showed iMPT^{aPPG} activity, i.e., they successfully catalyzed the transfer of Man-P to a synthetic peptide based on the sequence of aPPG (data not shown), suggesting that these membranes might also contain active eMPT^{PPG} activity. However, in the absence of a specific substrate for eMPT^{aPPG}, the possibility that eMPT^{LPG} and eMPT^{PPG} are the same cannot be formally excluded.

In summary, these data suggest (i) that axenic amastigotes may not be representative of the naturally occurring lesion-derived amastigotes with respect to phosphoglycan biosynthesis [consistent with the report that axenic amastigotes do not secrete aPPG (*11*)] and (ii) that the eMPT activity needed in lesion amastigotes for aPPG biosynthesis may be different from that required for LPG biosynthesis. For these reasons, eMPT activity detected with compound **1** as the acceptor substrate will be referred to as eMPT^{LPG}.

eMPT^{LPG} Activity is Down-Regulated in Metacyclic Promastigotes. To assess the activity of the eMPT activity in procyclic versus metacyclic promastigotes, *L. major* procyclic promastigotes were harvested from the logarithmic phase of growth, and *L. major* metacyclic promastigotes were harvested from stationary phase cultures after depletion with peanut agglutinin (*43*). The specific activity of eMPT was 5-fold lower in metacyclic promastigotes as compared to procyclic promastigotes (Figure 5B). A similar reduction in specific activity for the iMPT^{PPG} of *L. mexicana* has also been reported (*35*). These data suggest that although LPG increases in length during metacyclogenesis (*17*, *18*, *44*, *45*) the lack of cell division of the metacyclic form results in an overall down regulation of the eMPT activity. Whether this is at the level of eMPT^{LPG} expression or eMPT^{LPG} modulation is unknown.

Negative Charge of the Phosphate Group is Essential for Acceptor Activity. Three analogues of compound **1** were synthesized with modifications to the phosphodiester group (Figure 1). Two of these, the thiophosphate- and the boranophosphate-containing analogues **2** and **3**, were good *L. major*, *L. mexicana*, and *L. donovani* promastigote eMPT^{LPG} acceptor substrates, whereas the methylphosphonate-containing analogue **4** was inactive (Figure 6A; Table 2). The thiophosphate and the boranophosphate compounds are both negatively charged analogues, whereas the methylphosphonate compound is neutral. These data demonstrate that the negative charge of the phosphodiester group in **1** is essential for enzyme recognition by eMPT^{LPG} in all three *Leishmania* species.

Influence of the Mannose Residue on Substrate Recognition. A series of analogues containing specific modifications to the α-D-Man residue of **1** were prepared (Figure 1, compounds **5**−**12**). One of these, compound **8**, was no longer an acceptor substrate for *L. major*, *L. mexicana*, and *L. donovani* procyclic promastigote eMPT^{LPG} (Figure 6B, lane 3; Table 2), showing that epimerization of the C-3 hydroxyl of the Man residue was not tolerated by the enzyme. To assess whether the C-3 hydroxyl of the Man residue forms an essential hydrogen bond to the enzyme, or whether the effect was due to a steric clash between the enzyme and the epimerized hydroxyl group, we prepared the C-3 deoxygenated and C-3 fluorinated compounds **9** and **10**, respectively. The enzyme showed increased activity with both analogues relative to **1** (Figure 6B, lanes 9 and 11; Table 2), supporting the latter conclusion and showing that the affinity of the substrate for eMPT^{LPG} can be improved by manipulating the C-3 position of the Man residue.

In contrast to the results with the C-3 deoxygenated compound, deoxygenation of the C-6 position of the Man residue (compound **11**) essentially abolished substrate recognition (Figure 6B, lane 4; Table 2), suggesting that the enzyme makes an essential hydrogen bond to the C-6

[2] The activity found in the axenic amastigote membranes showed the same properties as *L. mexicana* promastigote eMPT^{LPG} with respect to dependence on the negative charge of the phosphodiester group of compound **1** and the effects of acceptor substrate chain length and side chain substitution.

hydroxyl group. Replacement of the C-6 hydoxyl group with fluorine (**12**) reduced (to about 30%), but did not abolish, Man-P transfer (Figure 6B, lane 12; Table 2), suggesting that the C-6 hydroxyl of **1** acts principally as a hydrogen bond acceptor.

Epimerization of the C-2 hydroxyl group produced a compound (**5**) that acted as a substrate for eMPT but with reduced kinetics (Figure 6B, lane 2; Table 2). Two other compounds with modifications at the C-2 position, i.e., those containing mannosamine (**6**) or *N*-acetylmannosamine (**7**), were also tested and found to be substrates but with reduced kinetics relative to **1** (Figure 6B, lanes 6 and 7; Table 2).

In summary, these data suggest (i) that only the C-6 hydroxyl group of the Man residue is essential for substrate recognition as a hydrogen bond acceptor; (ii) that the C-3 hydroxyl makes no contribution to substrate recognition and that substrate recognition can be improved by its removal or replacement with fluorine; and (iii) that the C-2 hydroxyl makes a small, but significant, contribution to enzyme recognition and/or that, when the substrate is bound, the C-2 position is close to the enzyme surface such that epimerization causes a minor steric clash.

Influence of the Galactose Residue on Substrate Recognition. A series of analogues containing specific modifications to the β-D-Gal residue of **1** were prepared (Figure 1, compounds **13**−**20**). The analogue lacking the C-6′ acceptor-site hydroxyl group of the Gal residue (**16**) was not a substrate for *L. major*, *L. mexicana*, and *L. donovani* procyclic promastigote eMPT^{LPG}. Similarly, neither the 6′-deoxy-6′-amino analogue of **1** (**17**), nor the 6′-deoxy-6′-fluoro analogue (**18**), the α-anomer (**19**), nor the 6′-deoxy α-anomer (**20**) were substrates (Table 2). These results with substrate analogues lacking the acceptor site hydroxyl group were as expected and show that there is no MPT activity that can act at sites other than the C-6′ hydroxyl of the β-D-Gal residue. In contrast, the C-2′, C-3′, and C-4′ epimers of **1** (**13**, **14**, and **15**) were all substrates for eMPT^{LPG} but with reduced kinetics relative to **1**. Epimerization of the C-2′ hydroxyl had the smallest effect on substrate turnover and epimerization of the C-3′ and C-4′ hydroxyls had similar effects (Figure 6C, lanes 2−4; Table 2).

These data suggest (i) that, as expected, the C-6′ hydroxyl group acceptor site of the Gal residue is essential for substrate turnover and (ii) that the C-2′, C-3′, and C-4′ hydroxyl groups are not essential for substrate recognition. With respect to (ii), the impairment of substrate recognition by epimerization of these hydroxyls may be due to the introduction of minor steric clashes with the enzyme rather than the disruption of hydrogen bonds.

Inhibition of eMPT. The analogues that were not substrates for eMPT^{LPG} were tested for inhibitory activity using *L. major* procyclic promastigote membranes. None of the compounds were inhibitory when used at an equimolar concentration (200 μM) to the acceptor substrate **1**. For several of these compounds, this was not surprising. For example, the C-6 deoxy compound (**11**), the α-anomer (**19**), the 6′-deoxy α-anomer (**20**), and the methylphosphonate (**4**) presumably fail as substrates because they are not recognized by the enzyme, and, therefore, they are unlikely to act as inhibitors. On the other hand, the lack of inhibition with the C-6′-deoxy (**16**), C-6′-amino (**17**), and C-6′-fluoro (**18**) compounds suggest that the C-6′ hydroxyl group is essential for enzyme recognition as well as for catalysis.

eMPT Activity is Dependent on Acceptor Chain Length and Side-Chain Substitution. Transfer of Man-P to a series of acceptors differing in chain length (compounds **1** and **21**) and in side chain substitution (**22**−**25**) (Figure 7A) was measured with *L. major* (Figure 7B), *L. mexicana* (Figure 7C), and *L. donovani* (Figure 7D) promastigote membranes. The results show that, for all three *Leishmania* species, eMPT^{LPG} activity decreased when the chain length was increased from one to three repeats, although this effect was less significant for *L. donovani* than for *L. major* and *L. mexicana*. The significance of this is unclear, but it might suggest that there are multiple eMPT^{LPG} activities in promastigotes with overlapping, but distinct, specificities for the acceptor phosphosaccharide chain length (with **1** detecting the activity that prefers shorter phosphosaccharide chains). Alternatively, if there is only one eMPT^{LPG} activity, LPG polymerization may naturally attenuate itself with increasing phosphosaccharide chain length.

In all cases, the presence of a substituent on the terminal Gal residue of the acceptor (as in compounds **24** and **25**) prevented the action of eMPT^{LPG} (Figure 7A), demonstrating that phosphosaccharide repeat formation must precede side chain modification and that, unlike epimerization, substitution of the βGal 3-OH with a monosaccharide abrogates substrate recognition by eMPT^{LPG}.

No statistically significant differences were observed whether the internal substituent was βGlc (compound **22**) or βGal (compound **23**). However, the effects of internal side chain substituents were different in the three species. The *L. donovani* eMPT^{LPG} did not discriminate between unsubstituted and substituted phosphoglycan acceptors, whereas *L. mexicana* eMPT^{LPG} showed slightly increased transfer to the substituted acceptors, and *L. major* eMPT^{LPG} was significantly inhibited by the same substituents. These results are somewhat counterintuitive since *L. major* LPG is the most heavily substituted, with side chains present on >85% of the phosphosaccharide repeats, whereas *L. mexicana* LPG is substituted on <30% of the repeats (*14*, *16*, *46*). These results may suggest that, at least for *L. major*, phosphosaccharide backbone synthesis occurs either before side chain addition or proceeds ahead of side chain addition. The latter model is reminiscent of that proposed for the biosynthesis and modification of glycosaminoglycan chains (*47*).

Summary. A summary of much of the data described in this paper on the substrate specificities of the *L. major*, *L. mexicana*, and *L. donovani* eMPT^{LPG} activities is presented in Figure 8. This information will be useful for future enzyme inhibitor design.

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