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Probing Enzymes Late in the Trypanosomal Glycosylphosphatidylinositol Biosynthetic Pathway with Synthetic Glycosylphosphatidylinositol Analogues

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ABSTRACT Glycosylphosphatidylinositol (GPI)-anchored proteins are abundant in the protozoan parasite *Trypanosoma brucei*, the causative agent of African sleeping sickness in humans and the related disease Nagana in cattle, and disruption of GPI biosynthesis is genetically and chemically validated as a drug target. Here, we examine the ability of enzymes of the trypanosomal GPI biosynthetic pathway to recognize and process a series of synthetic dimannosyl-glucosaminylphosphatidylinositol analogues containing systematic modifications on the mannose residues. The data reveal which portions of the natural substrate are important for recognition, explain why mannosylation occurs prior to inositol acylation in the trypanosomal pathway, and identify the first inhibitor of the third α-mannosyltransferase of the GPI biosynthetic pathway.

Glycosylphosphatidylinositol (GPI) acts as a membrane anchor for a small but significant proportion of eukaryotic cell-surface glycoproteins and is particularly abundant in protozoan parasites such as *Trypanosoma brucei*, the causative agent of African sleeping sickness in humans and the related disease Nagana in cattle (*1*). African sleeping sickness, also known as human African trypanosomiasis, is invariably fatal if untreated and kills upward of 50,000 people each year. Current treatments are expensive, toxic, and difficult to administer, leaving an urgent need for new therapeutic agents (*2*). While drug discovery programs for African sleeping sickness have recently started in academia, there remains a need to identify and characterize new drug targets to feed this effort (*3*).

Disruption of GPI biosynthesis in the clinically relevant bloodstream form of *T. brucei* has been genetically (*4–6*) and chemically (*7*) validated as a drug target. Bloodstream form *T. brucei* express $\sim 5 \times 10^6$ GPI-anchored variant surface glycoprotein homodimers per cell, forming a dense surface coat that protects the parasite from the complement pathway of the host. The exposed variant surface glycoprotein undergoes antigenic variation to evade specific immune responses (*8, 9*), leaving little hope of an effective vaccine, but the core GPI structure is invariant. The less abundant ($\sim 3 \times 10^4$ copies per cell) but equally essential parasite transferrin receptor is also GPI-anchored (*10–13*).

The structure, biosynthesis, and function of GPI anchors and related molecules have been extensively reviewed (*1, 14–16*). The basic GPI core, which is con-

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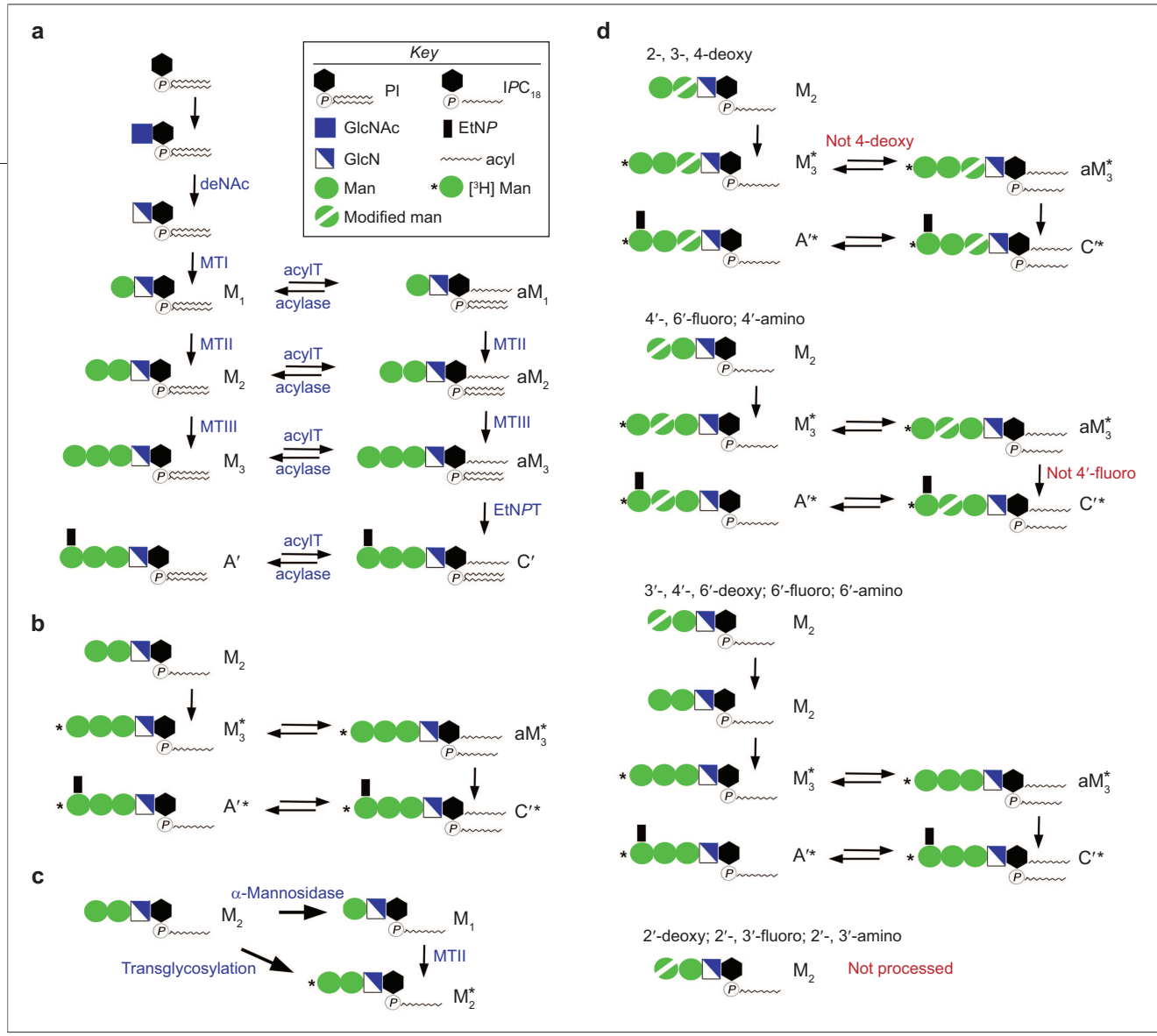
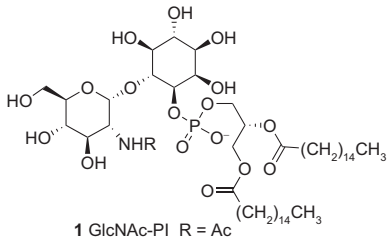
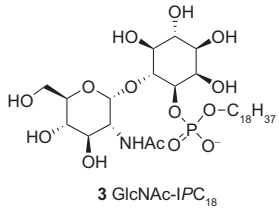


Figure 1. GPI biosynthetic pathway of bloodstream form *T. brucei* and models of the processing of synthetic GPI analogues by the *T. brucei* cell-free system. a) Representation of the complete GPI biosynthetic pathway of bloodstream form *T. brucei*. b) Processing of Man_2GlcN-*IPC*$_{18}$ by the trypanosome cell-free system. c) Alternative mechanisms for the production of [^{3}H]-Man_2GlcN-*IPC*$_{18}$. d) Processing of Man_2GlcN-*IPC*$_{18}$ analogues by the trypanosome cell-free system. Enzymes abbreviations: deNAc, GlcNAc-PI de-*N*-acetylase; MTI-III, mannosyltransferase I—III; acylT, inositol acyltransferase; acylase, inositol deacylase; EtN*PT*, ethanolamine phosphate transferase. Asterisks are used to indicate [^{3}H] mannose-containing species. For structures of the Man_2GlcN-*IPC*$_{18}$ analogues, see Figure 2.

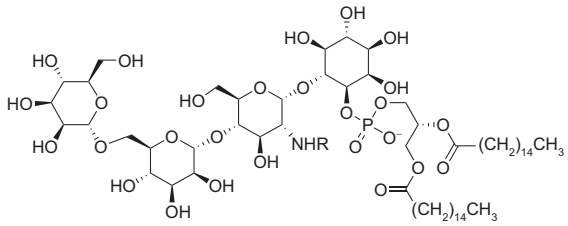
served between eukaryotes, consists of $NH_2CH_2CH_2$-PO_4H-6Manα1-2Manα1-6Manα1-4GlcNα1-6-D-*myo*-inositol-1-HPO_4-lipid, where the lipid can be diacylglycerol, *lyso*-acylglycerol, alkylacylglycerol, or ceramide, and is often further decorated with additional ethanolamine phosphate and/or carbohydrate groups and/or fatty acid attached to the inositol residue (inositol acylation) in a species- and tissue-specific manner. Biosynthesis of GPI in *T. brucei* (Figure 1, panel a), which occurs in the endoplasmic reticulum, is initiated by the transfer of GlcNAc from UDP-GlcNAc to phosphatidylinositol (PI) to generate *N*-acetyl-glucosaminylphosphatidylinositol (**1**, GlcNAc-PI), which is de-*N*-acetylated to produce glucosaminylphosphatidylinositol (**2**, GlcN-PI) (*17*). De-*N*-acetylation is a prerequisite for the subsequent mannosylation of GlcN-PI (*18*), which requires the sequential action of three distinct mannosyltransferases (MTI, MTII, and MTIII) to form trimannosyl-glucosaminyl-phosphatidylinositol (Man_3GlcN-PI). MTIII, a Dol-P-Man: Man_2GlcN-PI α(1-2) mannosyltransferase, has been shown genetically to be essential in bloodstream form *T. brucei* (*4*). From GlcN-PI onward there are significant differences in the GPI biosynthetic pathways of *T. brucei* and mammalian cells. In *T. brucei*, inositol acylation occurs only after addition of the first mannose and is only essential for the addition of ethanolamine phos-



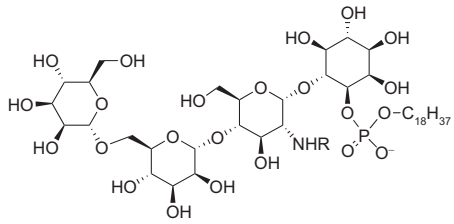
1 GlcNAc-PI R = Ac
2 GlcN-PI R = H



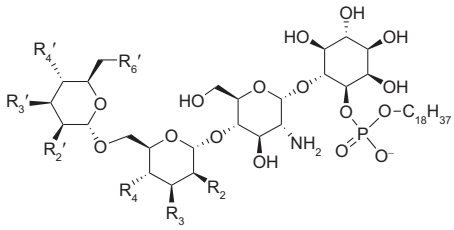
3 GlcNAc-I*PC*$_{18}$



4 Man$_2$GlcN-PI R = H
21 Man$_2$GlcNAc-PI R = Ac



5 Man$_2$GlcN-I*PC*$_{18}$ R = H
22 Man$_2$GlcNAc-I*PC*$_{18}$ R = Ac



Man$_2$GlcN-I*PC*$_{18}$ analogues

	Name	R_2	R_3	R_4	R_2'	R_3'	R_4'	R_6'
5	Man$_2$GlcN-I*PC*$_{18}$	OH	OH	OH	OH	OH	OH	OH
6	2-deoxyMan$_2$GlcN-I*PC*$_{18}$	H	OH	OH	OH	OH	OH	OH
7	3-deoxyMan$_2$GlcN-I*PC*$_{18}$	OH	H	OH	OH	OH	OH	OH
8	4-deoxyMan$_2$GlcN-I*PC*$_{18}$	OH	OH	H	OH	OH	OH	OH
9	2′-deoxyMan$_2$GlcN-I*PC*$_{18}$	OH	OH	OH	H	OH	OH	OH
10	3′-deoxyMan$_2$GlcN-I*PC*$_{18}$	OH	OH	OH	OH	H	OH	OH
11	4′-deoxyMan$_2$GlcN-I*PC*$_{18}$	OH	OH	OH	OH	OH	H	OH
12	6′-deoxyMan$_2$GlcN-I*PC*$_{18}$	OH	OH	OH	OH	OH	OH	H
13	2′-fluoroMan$_2$GlcN-I*PC*$_{18}$	OH	OH	OH	F	OH	OH	OH
14	3′-fluoroMan$_2$GlcN-I*PC*$_{18}$	OH	OH	OH	OH	F	OH	OH
15	4′-fluoroMan$_2$GlcN-I*PC*$_{18}$	OH	OH	OH	OH	OH	F	OH
16	6′-fluoroMan$_2$GlcN-I*PC*$_{18}$	OH	OH	OH	OH	OH	OH	F
17	2′-aminoMan$_2$GlcN-I*PC*$_{18}$	OH	OH	OH	NH_2	OH	OH	OH
18	3′-aminoMan$_2$GlcN-I*PC*$_{18}$	OH	OH	OH	OH	NH_2	OH	OH
19	4′-aminoMan$_2$GlcN-I*PC*$_{18}$	OH	OH	OH	OH	OH	NH_2	OH
20	6′-aminoMan$_2$GlcN-I*PC*$_{18}$	OH	OH	OH	OH	OH	OH	NH_2

Figure 2. Structures of the GPI precursors and precursor analogues used in this study. The diacylglycerol-containing synthetic substrates, GlcNAc-PI and Man$_2$GlcN-PI, differ from the natural substrates only in that the acylation of positions 1 and 2 of the *sn*-glycerol moiety is hexadecanoyl (palmitoyl) rather than octadecanoyl (stearoyl) at position 1 and other long-chain fatty acids at position 2.

phate to the third mannose, whereas in mammalian systems GlcN-PI undergoes inositol acylation before mannosylation occurs (*19, 20*).

No high resolution structural data exists for any of the enzymes of the GPI biosynthetic pathway, and given that the enzymes contain between 1 and 13 predicted transmembrane domains and/or are components of multiprotein complexes, such structural data may prove difficult to obtain. The substrate specificity of the early enzymes of the *T. brucei* and HeLa GPI biosynthetic pathways have been examined *in vitro* using synthetic substrate analogues (*7, 18, 21–27*). The *T. brucei* de-*N*-acetylase and MTI enzymes have less stringent substrate recognition compared with those of the mammalian pathway, enabling substrate-based species-specific inhibitors to be designed (*23, 25, 26*). However, little is known about the substrate specificity of enzymes late in the GPI biosynthetic pathway. Here, we use a chemical biological approach, analogous to site-directed mutagenesis of a protein, to characterize substrate recognition by essential enzymes late in the trypanosomal GPI biosynthetic pathway.

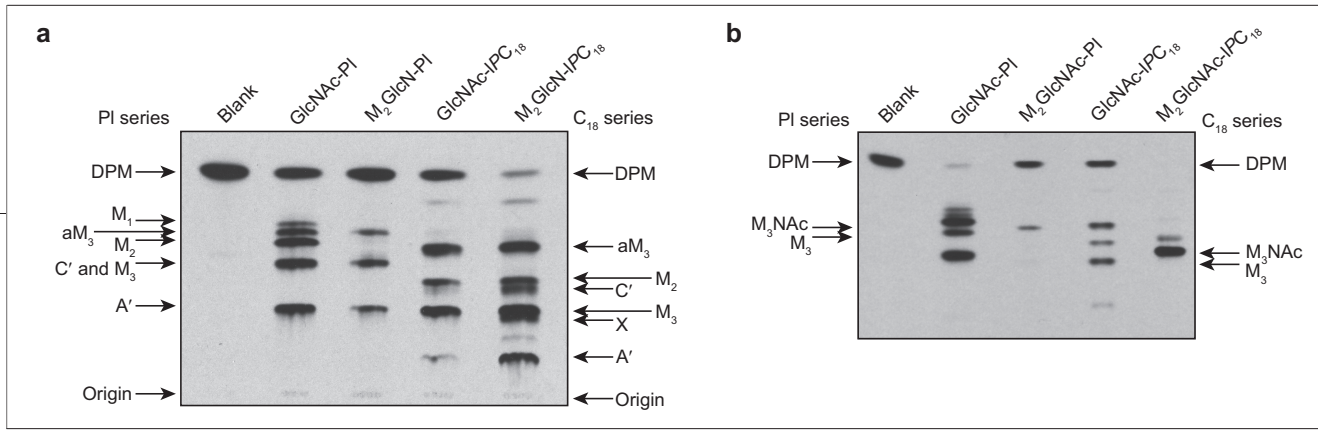


Figure 3. Priming *T. brucei* GPI biosynthesis with synthetic GPI precursors. Synthetic GPI precursors, as indicated, were incubated with GDP-[^{3}H]Man in the *T. brucei* cell-free system, and the radiolabeled GPI intermediates were extracted, separated by hptlc, and visualized by fluorography. Radiolabeled glycolipids: DPM, dolichol-phosphate-mannose; M_n, Man_nGlcN-ln*o*P-lipid; aM3, Man_3GlcN-(acyl)ln*o*P-lipid; M_3NAc, $Man_3GlcNAc$-ln*o*P-lipid; C′, Et*N*PMan_3GlcN-(acyl)ln*o*P-lipid; A′, Et*N*PMan_3GlcN-ln*o*P-lipid; X, novel glycolipid. The lipid component is either dipalmitoylglycerol (for PI) or octadecanol (C_{18}). Notes: PI-containing aM_3 and C′ migrate very close together; *N*-acetylated glycolipids migrate faster than their non-*N*-acetylated counterparts. For clarity, only DPM, M_3, and M_3NAc are labeled in panel b.

RESULTS AND DISCUSSION

Synthetic Analogues of Man_2GlcN-PI. To examine the substrate specificity of the MTIII of trypanosomal GPI biosynthesis, a series of pseudotetrasaccharide analogues of the natural substrate Man_2GlcN-PI were designed and synthesized (Figure 2). We reasoned that any analogues processed by MTIII might also become substrates for subsequent enzymes in the GPI biosynthetic pathway and so yield additional information. Previous studies examining the substrate specificity of the early enzymes of the *T. brucei* GPI biosynthetic pathway have shown that the diacylglycerol portion of GlcNAc-PI (**1**) may be replaced by the simple C_{18} alkyl chain of GlcNAc-I*P*C_{18} (**3**) (*23, 24*). Thus, we hypothesized that replacement of the diacylglycerol portion of Man_2GlcN-PI (**4**) by a simple C_{18} alkyl chain, producing Man_2GlcN-I*P*C_{18} (**5**), would not significantly affect recognition of the analogues while simplifying synthesis and making the compounds less susceptible to the action of phospholipases and esterases. A series of 15 analogues of Man_2GlcN-I*P*C_{18} (**6**−**20**) were synthesized, containing systematic modifications where each of the hydroxyl groups of the two mannose residues were replaced in turn, using hydrogen at each position on the first (**6**−**8**) and non-reducing terminal (second) mannose (**9**−**12**), and fluorine (**13**−**16**) or an amine group (**17**−**20**) at each position on the second mannose residue. The ability of the trypanosomal GPI pathway to recognize and

TABLE 1. Sensitivity of radiolabeled glycolipids to enzymatic and chemical treatments[a]

		Sensitivity to treatment					
ID	Structure	JBAM	PLC	PLD	Acid	Base	ASAM
DPM	Dolichol-P-Man	−	−	−	−	+	−
M_1	Man_1GlcN-PI	+	+	+	+	+	ND
aM_3	Man_3GlcN-(acyl)PI	+	−	+	+	+	ND
M_2	Man_2GlcN-PI	+	+	+	+	+	ND
M_3	Man_3GlcN-PI	+	+	+	+	+	ND
C′	Et*N*PMan_3GlcN-(acyl)PI	−	−	+	+	+	ND
A′	Et*N*PMan_3GlcN-PI	−	+	+	+	+	ND
M_1	Man_1GlcN-I*P*C_{18}	+	+	+	−	−	−
aM_3	Man_3GlcN-I(acyl)*P*C_{18}	+	-	+	−	+[b]	+[c]
M_2	Man_2GlcN-I*P*C_{18}	+	+	+	−	−	−
C′	Et*N*PMan_3GlcN-I(acyl)*P*C_{18}	−	−	+	−	+[b]	−
M_3	Man_3GlcN-I*P*C_{18}	+	+	+	−	−	+[c]
X	Unassigned	+	−	+	−	−	−
A′	Et*N*PMan_3GlcN-I*P*C_{18}	−	+	+	−	−	−

[a]The order of the glycolipids in the table reflects their relative migration on hptlc (highest migrating/most hydrophobic first) and for clarity are grouped according to their lipid moiety (Dolichol, PI or I*P*C_{18}). ND = not determined. [b]Results in species with lower migration, [c]Results in species with higher migration.

process these compounds *in vitro* is summarized in Figure 1, panels b–d and described in detail below.

Synthetic Man_2GlcN-PI and Man_2GlcN-IPC_{18} Efficiently Prime GPI Biosynthesis. To determine whether synthetic Man_2GlcN-PI and analogues thereof can prime the trypanosomal GPI pathway *in vitro*, the cell-free system (i.e., washed trypanosome membranes) was incubated with GDP-[^{3}H]Man in the presence of the compounds to see if they would prime the production of radiolabeled GPI intermediates. The production of endogenous radiolabeled GPI intermediates was prevented by inclusion of *N*-ethylmaleimide, which inhibits the UDP-GlcNAc:PI α1-6 GlcNAc transferase but not the downstream enzymes of GPI biosynthesis (*28*), and the production of dolichol cycle intermediates was prevented by the addition of tunicamycin. As a positive control, the production of radiolabeled GPI intermediates was stimulated by the addition of 10 μM of synthetic GlcNAc-PI or GlcNAc-IPC_{18} (*22–24*). The radiolabeled glycolipids were separated by hptlc and visualized by fluorography (Figure 3, panel a), and their identities were determined by chemical and enzymatic treatments (Table 1). As described previously (*22–24*), radiolabeled glycolipids produced from GlcNAc-IPC_{18} migrate more slowly on hptlc than the corresponding products of GlcNAc-PI as a result of the lower hydrophobicity of their lipid component. The addition of 10 μM synthetic Man_2GlcN-PI produced four radiolabeled downstream products (Figure 3, panel a) that were identified as Man_3GlcN-PI (M_3), Man_3GlcN-(acyl)PI (aM_3), EtN*P*Man_3-GlcN-(acyl)PI (glycolipid C′), and EtN*P*Man_3GlcN-PI (glycolipid A′) (Supplementary Figure S1). The addition of 10 μM synthetic $Man_2GlcNAc$-IPC_{18} also resulted in the production of the expected products (Figure 3, panel a), *i.e.*, those corresponding to M_3, aM_3, glycolipid C′ and glycolipid A′ (Figure 1, panel b), and two additional products (Supplementary Table S2 and Supplementary Figure S2). One of these, labeled X, is a GPI of unknown structure (Table 1; Supplementary Table S2 and Supplementary Figure S3). However, the other, migrating between M_3 and aM_3, was shown to be [^{3}H]-$Man_2GlcNAc$-IPC_{18} (Supplementary Figure S3). The production of a radiolabeled M_2 species occurred only when priming GPI biosynthesis with Man_2GlcN-IPC_{18} and not when priming with Man_2GlcN-PI. We considered the possibility that the generation of the [^{3}H]-M_2 species could have arisen from the partial decomposition of $Man_2GlcNAc$-IPC_{18} to $Man_1GlcNAc$-IPC_{18}, but none was detected.

Next, we investigated whether $Man_2GlcNAc$-IPC_{18} might be trimmed back to $Man_1GlcNAc$-IPC_{18} *via* α-mannosidase activity in the cell-free system. However, the inclusion of the α-mannosidase inhibitors swainsonine and kifunensine had no effect (Supplementary Figure S4). Thus, we favor a transglycosylation mechanism (Figure 1, panel c), and this is discussed later.

Priming GPI Biosynthesis with $Man_2GlcNAc$-PI and $Man_2GlcNAc$-IPC_{18}. GPI biosynthesis in *T. brucei* proceeds *via* the de-*N*-acetylation of GlcNAc-PI to produce GlcN-PI (*17*), which is strictly required for mannosylation by MTI to form Man_1GlcN-PI (*18*). It has been shown previously that the de-*N*-acetylase is unable to act on Man_1GlcN**Ac**-PI, confirming this sequence of events (*18*). However, it is not known if the subsequent mannosyltransferases, MTII and MTIII, require the presence of a free amine group to elaborate Man_1GlcN-PI to Man_2GlcN-PI and Man_2GlcN-PI to Man_3GlcN-PI, respectively. To clarify this situation, we chemically *N*-acetylated the M_2 analogues, to form Man_2GlcN**Ac**-PI and Man_2GlcN**Ac**-IPC_{18}, and tested the ability of the cell-free system to process them. Both *N*-acetylated M_2 analogues were processed to a single prominent product with hydrophobicity slightly higher than the corresponding M_3 analogue (Figure 3, panel b). The identity of the products as *N*-acetylated M_3 species was confirmed *via* chemical *N*-acetylation, which showed that the products contained no free amine and co-migrated with chemically *N*-acetylated M_3 standards (Supplementary Figure S5). Interestingly, although MTIII can act upon the *N*-acetylated M_2 analogues, neither the de-*N*-acetylase nor inositol acyltransferase are able to act upon them or their *N*-acetylated M_3 products. The additional faint band seen with Man_2GlcN**Ac**-IPC_{18} is probably [^{3}H]-Man_2GlcN**Ac**-IPC_{18} produced by the same putative transglycosylation mechanism described above.

Effects of Systematic Deoxygenation of Man_2GlcN-IPC_{18}. To assess which of the mannose hydroxyl groups of Man_2GlcN-IPC_{18} are important for substrate recognition, we tested the ability of the cell-free system to process a series of analogues where each OH group was replaced by H in turn (compounds **6**–**12**). Removal of hydroxyl groups from the first mannose (attached directly to GlcN), as in compounds **6**–**8**, did not prevent recognition and processing by MTIII, such that [^{3}H]Man-Man-deoxyMan-GlcN-IPC_{18} (migrating slightly faster than fully hydroxylated Man_3GlcN-IPC_{18}) was produced in all cases (Figure 4). However, although Man-(4-

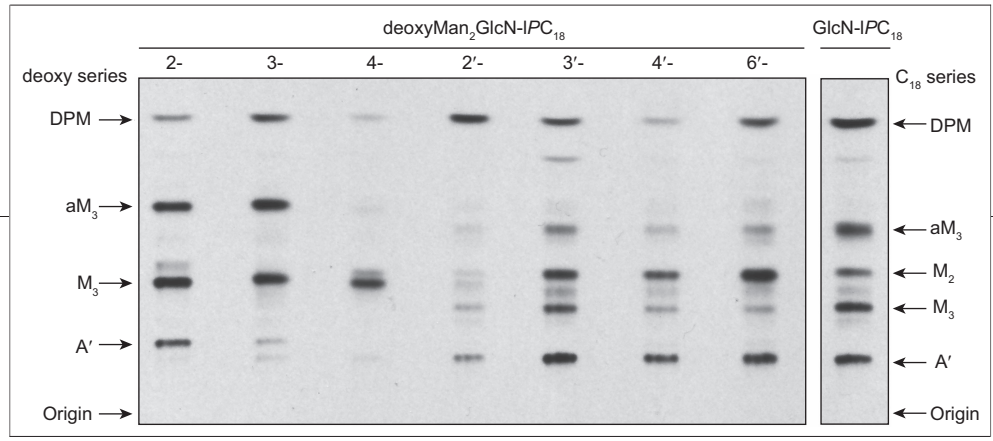


Figure 4. Priming *T. brucei* GPI biosynthesis with deoxyMan$_2$GlcN-I*PC*$_{18}$ GPI-precursor analogues. Sample processing and radiolabeled glycolipid labeling are as for Figure 2. The radiolabeled glycolipids produced by the deoxy series have higher migration than the equivalent fully hydroxylated compounds due to their reduced polarity. The Man-deoxyManGlcN-I*PC*$_{18}$ species are processed to the deoxy series, whereas the deoxyMan-ManGlcN-I*PC*$_{18}$ species are processed *via* [^{3}H]-M$_2$ to the fully hydroxylated C$_{18}$ series.

deoxyMan)-GlcN-I*PC*$_{18}$ was processed to the corresponding M$_3$ product, it did not undergo inositol acylation to form aM$_3$, nor did the addition of ethanolamine phosphate occur. This observation reveals that the 4-hydroxyl of the first mannose is important for substrate recognition by the inositol acyltransferase and nicely explains why mannosylation must precede inositol acylation in the *T. brucei* GPI pathway (*19*). The lack of ethanolamine phosphate addition is consistent with previous work showing that prior inositol acylation is necessary for ethanolamine phosphate addition (*19*).

Removal of hydroxyl groups from the second mannose residue resulted in the production of radiolabeled glycolipids with migration identical to that of the equivalent fully hydroxylated species from Man$_2$GlcN-I*PC*$_{18}$ onward (Figure 4). This strongly suggests that the processing of these compounds proceeds *via* the aforementioned transglycosylation mechanism, whereby the terminal deoxymannose is replaced by [^{3}H]-mannose prior to subsequent elaboration. The production of [^{3}H]-M$_2$ with the deoxyMan-Man-GlcN-I*PC*$_{18}$ species and not the Man-deoxyMan-GlcN-I*PC*$_{18}$ species suggests that the underlying ManGlcN-I*PC*$_{18}$ structure and not the terminal residue is important in determining whether formation of [^{3}H]-M$_2$ occurs. The importance of the aglycone and not the terminal residue for this exchange reaction suggests that the proposed transglycosylation could be mediated by MTII, as glycosyltransferases have previously been observed to mediate transglycosylation (*29*). Transglycosylation was not observed when priming with Man$_2$GlcNAc-PI, and it is unclear whether the observation of *in vitro* transglycosylation with Man$_2$GlcNAc-I*PC*$_{18}$ has relevance to the situation *in vivo*, although such activity could have a potential function in the recycling of GPI anchors and/or regulating flux through the GPI pathway.

Effect of Fluoro and Amino Substitution of Man$_2$GlcN-IPC$_{18}$. The ability of the cell-free system to recognize and process the Man$_2$GlcN-I*PC*$_{18}$ analogues containing fluoro or amino substitutions on the second

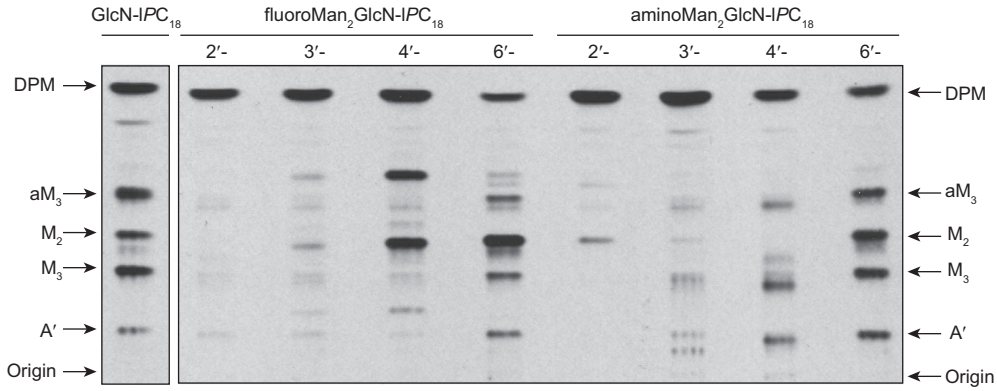


Figure 5. Priming *T. brucei* GPI biosynthesis with fluoro- and amino-substituted Man$_2$GlcN-I*PC*$_{18}$ GPI-precursor analogues. Sample processing and radiolabeled glycolipid labeling are as for Figure 2. The positions of the fully hydroxylated radiolabeled glycolipids are indicated. The 2′- and 3′-substituted compounds are not processed, the 4′-substituted compounds are processed without [^{3}H]-M$_2$ production, the 6′-F compound is processed as is and *via* [^{3}H]-M$_2$, and the 6′-NH$_2$ compound is processed only *via* [^{3}H]-M$_2$.

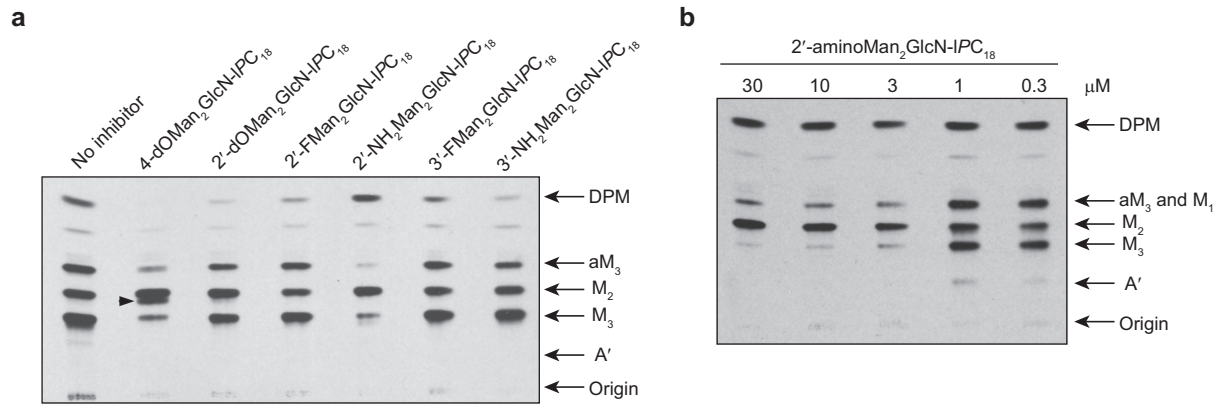


Figure 6. Man_2GlcN-I*PC*$_{18}$ analogues as inhibitors of trypanosomal GPI biosynthesis. a) Potential inhibitors, as indicated, were incubated with the *T. brucei* cell-free system for 5 min prior to the addition of GlcNAc-I*PC*$_{18}$ and GDP-[^{3}H]Man to prime the system. Samples were processed as for Figure 2. With the 4-deoxy$Man_2GlcNAc$-I*PC*$_{18}$ analogue the production of the corresponding deoxy-M_3 analogue was observed (arrowhead), which runs slightly lower than the fully hydroxylated M_2. b) A titration with 0.3−30 μM 2′-aminoMan2GlcN-I*PC*$_{18}$ was performed to assess its potency to inhibit the production of M_3 from M_2.

mannose (compounds **13**−**20**) was determined (Figure 5). Analogues substituted at the 2′- or 3′-positions with either group were neither processed to M_3 nor [^{3}H]-M_2. The inability of MTIII to utilize 2′-substituted analogues is expected, since the 2′-OH is the acceptor site for the third mannose. However, the inability of MTIII to utilize 3′-substituted Man_2GlcN-I*PC*$_{18}$ analogues suggests that this position is also important for MTIII substrate recognition. The 4′-substituted analogues were processed to M_3 and beyond, without the generation of [^{3}H]-M_2, whereas the processing of the 6′-substituted analogues proceeds *via* [^{3}H]-M_2. The lack of [^{3}H]-M_2 production with the 3′- and 4′-substituted analogues is in contrast to the situation observed with the 3′- and 4′-deoxy analogues. Interestingly, the processing of the 4′-fluoro analogue does not proceed past the generation of aM_3, whereas the 4′-amino analogue is processed to glycolipid A′, implying that the 4′-position may be recognized as a hydrogen bond donor site by the ethanolamine phosphate transferase.

Man_2GlcN-PI Analogues As Inhibitors of GPI Biosynthesis. The 2′- and 3′-substituted $Man_2GlcNAc$-I*PC*$_{18}$ analogues (compounds **13**, **14**, **17** and **18**) and the 4-deoxy analogue (compound **8**) were tested for their ability to act as inhibitors of GPI biosynthesis *in vitro*. The trypanosome cell-free system was preincubated with 100 μM $Man_2GlcNAc$-I*PC*$_{18}$ analogues for 5 min prior to the addition of 10 μM synthetic GlcNAc-I*PC*$_{18}$ and GDP-[^{3}H]Man to prime GPI production. Only the 4-deoxy and 2′-amino analogues significantly inhibited the production of M_3 and downstream GPI intermediates (Figure 6, panel a). The 4-deoxy$Man_2GlcNAc$-I*PC*$_{18}$ analogue was processed to the corresponding deoxy-M_3 analogue, as observed previously, but surprisingly, the production of fully hydroxylated M_3 as well as aM_3 was inhibited (Figure 6, panel a). However, it is not clear if the 4-deoxy$Man_2GlcNAc$-I*PC*$_{18}$ analogue or its deoxy-M_3 product is responsible for the observed inhibition, and the exact molecular target is unclear. Radiometric analysis of the hptlc was unable to resolve deoxy-M_3 and M_2 bands, preventing the accurate determination of the apparent IC_{50}, which is estimated as >10 μM (Supplementary Figure S6).

The 2′-amino analogue prevented the production of M_3 and led to an accumulation of M_2, demonstrating that inhibition of the MTIII was occurring (Figure 6, panel b). Quantitative radiometric analysis of the hptlc showed that MTIII was inhibited with an IC_{50} of 1.7 ± 0.2 μM (Supplementary Figure S7). The inhibition of *T. brucei* GPI biosynthesis by mannosamine *in vivo*, which also leads to the accumulation of M_2 species, has previously been proposed to occur *via* the production of ManN-ManGlcN-PI (*30*). The synthetic 2′-aminoMan_2-GlcN-I*PC*$_{18}$ inhibitor, described here, is identical to this species apart from the structure of the lipid moiety. Therefore, our results support the proposed mechanism of inhibition (*30, 31*) whereby interaction of the acceptor 2′-hydroxyl group with a basic group of the glyco-

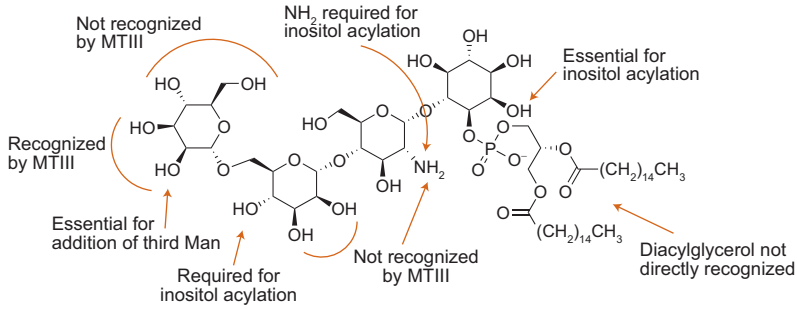


Figure 7. Summary of the features of Man_2GlcN-PI recognized by MTIII and the inositol acyl transferase. Evidence for the essentiality of the inositol 2-OH has been reported previously (*27*).

syltransferase is replaced by a charge−charge interaction with the amino group, which would be protonated under assay conditions.

Summary of Substrate Recognition for Enzyme Late in the Trypanosomal GPI Biosynthetic Pathway. The results we have obtained using a series of analogues of $Man_2GlcNAc$-PI and a cell-free system to examine the substrate recognition of enzymes late in the trypanosomal GPI pathway are summarized in Figure 7. The replacement of the natural diacylglycerol lipid moiety with a simple C_{18} alkyl chain did not affect the recognition and processing of the analogues by the GPI pathway, in agreement with previous studies using GlcNAc-PI analogues (*23, 24*). Substrate recognition by MTIII requires the presence of the hydroxyl groups at the 2- and 3-positions of the reducing terminal mannose, and in contrast to MTI, MTIII does not recognize the amine of the glucosamine residue. This is consistent with earlier work by Brown *et al.* suggesting that MTIII can act on simple hydrophobic dimannosyl acceptors, implying a lack of recognition of both the glucosamine and inositol moiety (*32*). The inositol acyltransferase requires the presence of a hydroxyl group at the 4-position on the first mannose and the presence of a free amine on the glucosamine residue, explaining for the first time the molecular basis behind the requirement for mannosylation prior to inositol acylation in the trypanosomal GPI pathway (*19*).

It is known that the structure of the substrate for mammalian MTIII is more complex than that for the corresponding trypanosomal enzyme. The mammalian MTIII substrate both is acylated on the inositol ring and contains EtN*P* attached to the 2-position of the first mannose residue. Inhibition of EtN*P* addition to the first mannose by the terpenoid lactone YW3548 leads to accumulation of Man_2GlcN-(acyl)PI in mammalian cells (*33*), strongly suggesting that mammalian MTIII requires the presence of the EtN*P* to the first mannose for substrate recognition (*34*). In contrast, the work presented here shows that substrate recognition by trypanosomal MTIII requires relatively few features of the natural substrate. Combining this information with the knowledge that MTIII may be readily inhibited by the introduction of a positive charge at the position of the acceptor hydroxyl group, we aim to produce more drug-like small molecule inhibitors. Differences in the structures of the substrates for mammalian and trypanosomal MTIII suggest that species-specific inhibition may be an achievable goal. Despite the lack of structural and mechanistic data for MTIII, the chemical biology approach applied here has revealed that the MTIII of GPI biosynthesis is an attractive therapeutic against African sleeping sickness.

METHODS

Materials. GDP-[2-^{3}H]-Mannose (GDP-[^{3}H]Man, 20 Ci/mmol) was purchased from American Radiochemicals, En^{3}Hance was purchased from Perkin-Elmer NEN. *Aspergillus saitoi* α(1,2)-mannosidase was purchased from Glyko, serum GPI-specific phospholipase D (GPI-PLD) was used unpurified from human serum, hplc grade solvents were purchased from VWR international Ltd., and all other reagents were purchased from Sigma.

Substrates and Substrate Analogues. The syntheses of D-GlcNα(1-6)-D-myo-inositol-1-HPO_4-dipalmitoylglycerol (GlcNAc-PI) (*35*) and D-GlcN-α(1-6)-D-myo-inositol-1-octadecyl phosphate (GlcNAc-C_{18}) (*36*) have been described previously. The details of the synthesis of Manα(1-6)-Manα(1-4)-D-GlcNα(1-6)-D-myo-inositol-1-HPO_4-dipalmitoylglycerol ($Man_2GlcNAc$-PI), Manα(1-6)-Manα(1-4)-D-GlcNα(1-6)-D-myo-inositol-1-octadecyl phosphate ($Man_2GlcNAc$-C_{18}), and analogues of $Man_2GlcNAc$-C_{18} containing systematic modifications at positions on the first or second mannose (Figure 2) will be reported elsewhere (*37, 38*). High resolution mass spectrometric data and ^{1}H NMR spectra for these compounds can be found in Supplementary Table S3 and Supplementary Figure S8.

The corresponding *N*-acetyl derivates GlcNAc-PI, GlcNAc-I*PC*$_{18}$, Man_2GlcN**Ac**-PI, and Man_2GlcN**Ac**-I*PC*$_{18}$ were prepared by treatment with acetic anhydride (*22*). The identity and purity of the synthetic substrates was assessed by negative ion electrospray-mass spectrometry (ES-MS), and the concentration of stock solutions determined by measurement of the inositol content by selected ion-monitoring GC-MS (*39*).

Trypanosome Cell-Free System Assays. Bloodstream form *T. brucei* (variant MITat1.4) were isolated, and membranes (cell-free system) were prepared as described previously and stored at −80 °C (*40*). Trypanosome membranes (2×10^7 cell equiv per assay) were washed, supplemented with fresh *N*-ethylmaleimide (0.2 M) and GDP-[^{3}H]Man (0.5 μCi per assay), mixed with substrate or substrate analogues (10 μM), sonicated briefly, and incubated at 30 °C for 15 min as described previously (*22*). Glycolipid products were recovered by extraction into a chloroform/methanol/water mixture (10/10/3, v/v), evaporated to dryness, partitioned between butan-1-ol and water (*41*), and analyzed by hptlc.

Inhibition studies were performed as above, except that the cell-free system was incubated with the potential inhibitor for 5 min at 30 °C prior to priming with GDP-[^{3}H]Man (0.5 μCi per assay) and 10 μM GlcNAc-IPC_{18}. For IC_{50} calculation, the production of [^{3}H]-M_3 was quantified by radiographic analysis of the hptlc, calculated as $cpm(M_3)/(cpm(M_3) + cpm(M_2))$, and analyzed by nonlinear regression (GraFit).

High Performance Thin Layer Chromatography. Glycolipid standards and samples were applied to 10-cm aluminum-backed silica gel 60 and developed with chloroform/methanol/13 M ammonia/1 M ammonium acetate/water (180/140/9/9/23, v/v). Dried hptlc plates were analyzed by a radiometric scanner (Bioscan AR2000) and/or sprayed with En^{3}Hance and radiolabeled components visualized by fluorography at −80 °C using Kodak XAR-5 film with an intensifying screen.

Enzymatic and Chemical Treatments of Radiolabeled Glycolipids. Enzymatic digestion with jack bean α-mannosidase (JBαM), *Aspergillus saitoi* α-mannosidase (ASαM), GPI-specific phospholipase D (PLD), and PI-specific phospholipase C (PLC); chemical digestion using acid and base hydrolysis; and chemical *N*-acetlyation were performed as described previously (*18, 22, 41*). The specificity of the treatments is described in Supplementary Table S1.

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

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