Chemistry and biology of glycosylphosphatidylinositol molecules

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Discovery of glycosylphosphatidylinositol (GPIs) as an alternative mode of anchoring of cell-surface proteins to the plasma membrane has led to significant advances in our understanding of cell and membrane biology in last two decades, providing a new view of the eukaryotic plasma membrane organization and function. Since the first reports of GPI anchors from the African trypanosomes and mammalian brain, a number of GPIs have been isolated across the eukaryotic species, particularly from the parasitic protozoa and humans, representing the most complex and biosynthetically expensive post-translational modification in the cell. In this article, we describe our contributions on chemistry and biology of GPI molecules, particularly on lipophosphoglycan, in the context of the fascinating field of GPI biology.

**Keywords:** Glycosylphosphatidylinositol, GPI anchors, lipophosphoglycan, phosphatidylinositol.

**Introduction**

The discovery of glycosylphosphatidylinositol (GPIs) as a unique class of complex glycolipids, which anchor proteins and glycans to the plasma membrane of eukaryotic cells was a landmark in modern biology as it unravelled an alternative mode for the membrane anchoring of surface macromolecules, a mechanism quite distinct from that of the well-known hydrophobic transmembrane polypeptide domains. The full chemical structure of the GPI anchor was revealed in 1988; first for the variant surface protein (VSG) of *Trypanosoma brucei*\textsuperscript{1} and second for Thy-1 glycoprotein of rat brain\textsuperscript{2}. Subsequently, a number of GPI-anchors and protein-free GPIs have been isolated all across the eukaryotic species, including humans. Examples of GPI-anchored proteins include cell-surface receptors (folate receptor, CD14), neutral cell-adhesion molecules (NCAM), surface hydrolases (acetylcholinesterase, alkaline phosphatase, 5'-nucleotidase), the scrapie-prion protein, protozoan-coat proteins and virulence factors of *Trypanosoma, Leishmania* and malaria species. Overall close to 10–20% membrane proteins entering the secretory pathway are linked to the GPI anchor by a post-translational trans-amidation in the endoplasmic reticulum (ER). The key topological feature of the GPI motif of protein-anchoring, unlike classical hydrophobic transmembrane peptide domains spanning the membrane bilayer, is that the GPI anchor associates only with the outer single membrane leaflet, a feature critical for the clustering of GPI-anchored cell-surface molecules into the lipid-rafts. The biochemistry and cell biology of GPI anchors have been periodically reviewed\textsuperscript{3–6}.

The detailed biochemical and structural analysis of GPIs isolated from mammals, protozoa and yeast has revealed a common core structure: 6-\textit{O}-aminoethylphosphoryl-Man-\textalpha\textsubscript{1-2}-Man-\textalpha\textsubscript{1-6}-Man-\textalpha\textsubscript{1-4}-GlcNH\textalpha\textsubscript{1-6}-D-myoinositol-1-\textit{O}-phospholipid (Figure 1), conserved across the species during evolution.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure1.png}
\caption{A conserved structure of glycosylphosphatidylinositol (GPI) anchor.}
\end{figure}

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However, there are a number of species-specific alterations at various branching points of this GPI core, for example, the presence of additional fatty acid, mannose, galactose, galactosamine and ethanolamine-phosphate residues. Significant micro-heterogeneity has also been found in the phospholipid domain: sn-1,2-dimyrstoylglycerol in T. brucei, sn-1-O-alkyl-2-O-acylglycerol in human acetylcholine-esterase and folate receptor, and the inositol palmitoylated at 2-O-position in the malarial GPI anchors. In addition, in yeast for example, a ceramide-containing sphingosine occurs in the GPI anchor in place of the glycerolipids (Figure 2).

The biosynthesis of the GPI molecules takes place in a stepwise manner (Figure 3) in the ER membrane, initiated by the transfer of N-acetylglucosamine (GlcNAc) from UDP-GlcNAc to phosphatidylinositol (PI) yielding GlcNAC-PI, which is then de-N-acetylated to yield GlcNH2-PI. The next step is the addition of three mannoses from Dol-P-Man via the action of three distinct mannosyltransferases to form the Man3–GlcNH2–PI intermediate, followed by the addition of ethanolamine phosphate to the 6 position of the third mannose residue.

In the final step, the GPI anchor is then attached to nascent proteins by a trans-amidation during which a C-terminal GPI attachment signal is released. The GPI-anchored proteins are finally transported to the plasma membrane by the secretory pathway. By means of complementation, about 20 genes involved in the GPI biosynthesis have been identified. The GPI anchors along with cholesterol and sphingolipids create functional ordered domains (lipid-rafts) as signalling platforms in the biological membranes. Since the discovery of GPI molecules in 1988, the biochemistry and cell biology of these complex glycolipids have remained in focus worldwide. Interestingly, the GPIs are produced in abundance by the protozoan parasites (Trypanosoma, Leishmania and Plasmodium species), compared to that in higher organisms, where they serve as essential virulence factors that allow the parasites to infect, proliferate and subvert the host immunity. Marked differences in the structure, function and biosynthesis of GPIs from the parasites and human cells have been identified providing new targets for therapeutics. Even among the parasites, various species express GPIs with subtle structural differences that manifest in remarkable and, at times, opposing biological functions in the host.

In addition to anchoring proteins to plasma membrane, the GPI mode is also used to express complex phosphoglycans (PGs) at the cell surface by pathogenic protozoans, for example, Leishmania parasite expresses a unique class of GPI-anchored glycoconjugates named lipophosphoglycan (LPG), arguably one of the most complex glycans in nature. The intriguing structure of LPG of
Leishmania (Figure 4) consists of four distinct domains: (i) alkyl-lyso-GPI anchor; (ii) the conserved glycan core with an internal galactofuranose residue; (iii) variable PG repeats, and (iv) a neutral oligomannoside cap.

The most distinct feature of the LPG structure is the variable PG repeat domain, unique amongst all the eukaryotic carbohydrates, made of phosphodisaccharide [6Galp-β-1,4-Manp-α1-phosphate]n repeats linked to each other through a phosphodiester group between the anomeric-OH of mannose of one repeat and 6-OH of galactose of the adjoining repeat. Distinct biological roles have been attributed to each of the LPG structural domains, e.g. the PG repeats form a spring-like helical supra-molecular assembly around the parasite providing resistance to host hydrolytic enzymes and antibodies, and constitute functional epitopes for recognition by macrophage receptors; the GPI core serves as an anchor to attach LPG to the outer leaflet of the plasma membrane, and the neutral oligomannose cap provides biosynthetic termination signal. The dynamic structure of LPG and its role in host–parasite interaction has generated significant interest and its biosynthetic pathway has emerged as a novel therapeutic target.

The unique chemical structure, biosynthesis, cell biology and membrane biology of GPI molecules and their immunological role in the infectivity, survival and proliferation of the protozoan parasites such as Leishmania and Trypanosoma, and malaria attracted our attention in 1995–1996 while setting up a new laboratory at the National Institute of Immunology, New Delhi. In this article, we describe some of our contributions in organic synthesis, biosynthesis and chemical biology of GPI molecules, particularly LPG, in the context of the fascinating field of GPI biology.

Chemical synthesis of GPI molecules

Chemo-enzymatic synthesis of early biosynthetic precursors

Isolation, structure and synthesis of the full-length GPI anchors and LPG present substantial challenges requiring combined expertise of biochemistry, and carbohydrate, lipid, protein and phosphorus chemistries. Our studies to probe the form and function of GPls, particularly LPG required efficient methods of their chemical synthesis. It is a formidable task to isolate natural GPls from biological cells, e.g. 10 litre of Leishmania promastigote culture yields 1 mg of LPG; moreover the product is always a mixture of sub-populations of LPG due to micro-heterogeneity in the lipid and glycan domains. Besides, the highly labile anomeric PG repeats fall apart during the isolation. Therefore, the progress in the biology of GPls required methods for the synthesis of biosynthetic labelled precursors and intermediates in laboratory.

To identify the distinct substrates and enzymes of GPI/LPG biosynthesis in L. donovani, we initiated efforts towards studying the mechanism of lyso-alkyl-phosphatidylinositol (lyso-alkyl-PI), the first lipid precursor of GPI biosynthesis. It was postulated that its biosynthesis is initiated by C-1 acylation of dihydroxyacetone phosphate (DHAP) by DHAP-acyltransferase; the acyl group is then replaced by an alkyl group by a putative alkyl-DHAP-synthase, where a long-chain alcohol nucleophile replaces the fatty acyl moiety by a hitherto unknown mechanism. This is followed by NADPH-mediated reduction of carbonyl group and C-2 syn-acylation leading to a 1-O-alkyl-2-O-acylphosphatic acid, which is then transferred to myo-inositol to form lysoalkyl-PI. For our biosynthetic studies, we required enantiomerically pure and radiolabelled (R)-1-O-alkyl-phosphatic acid, (R)-1-O-alkyl-2-O-acyl-phosphatic acid and lyso-1-O-alkyl-PI precursors and substrates specifically labelled on the glycerol backbone. We designed a new chemo-enzymatic synthesis by the application of phospholipase A2 enzyme from Naja N. mocambique leading to radiolabelled (14C) and chirally pure precursors of GPI biosynthesis (Scheme 1).

Among the prominent biological activities displayed by major Leishmania GPls (LPG and protein-free GPls) is the inhibition of macrophage function such as the protein kinase C (PKC)-dependent signal pathway, and this bioactivity of Leishmania GPls is in contrast to the GPls of T. brucei and Plasmodium falciparum which activate...
pro-inflammatory cytokine function in macrophages. To address the question as to which structural domain of *Leishmania* GPIs is responsible for dramatic down-regulation of PKC-dependent transient *c-fos* gene expression, the chemically synthesized alkylacyl-glycerolipids, as described above, were evaluated for inhibition of PKC and *c-fos* expression in the peritoneal macrophages. The result clearly demonstrated \(^9\) that the unusual alkylacyl-phosphatidic acid domain was primarily responsible for the bioactivity.

In continuation with the above, we discovered a novel enzymatic approach\(^10\) to synthesize a key biosynthetic intermediate D-*myo*-inositol 1,2-cyclic monophosphate. For this a whole cell culture of recombinant bacteria *Bacillus subtilis*, over-expressing phosphatidylinositol-specific phospholipase C (PI-PLC) gene of *Bacillus thuringiensis* was used as an efficient catalyst for practical multi-gram scale synthesis of D-*myo*-inositol 1,2-cyclic monophosphate (12, Scheme 2), directly from the phospholipids mixture (soyabean lecithin) containing PI. This synthesis did not require protein purification and growing bacterial culture was directly used, and the progress of reaction was monitored by *in situ* \(^31\)P NMR. The bacterial PI-PLC combines two activities: a phosphotransferase activity producing 1,2 : cyc-PI (12) and diacylglycerol (13) with overall retention of configuration at the phosphorus atom, and the phosphodiesterase producing D-*myo*-inositol-1-phosphate (14) through slow hydrolysis of 12. Interestingly, the bacterial PI-PLC, and not its mammalian counterpart, can cleave GPI-anchored proteins and carbohydrates from the outer leaflet of the plasma membrane. In fact, this was the seminal observation that led to the discovery of GPI anchors\(^5\).

### Chemical synthesis of PG repeats of LPG

A unique feature of the LPG structure is the PG repeating domain of \([\text{Gal}_\beta 1,4-\text{Man}_\alpha 1-\text{phosphate}]_n\) linked to each other through a phosphodiester group between anomic-OH of the mannose of one repeat and 6-OH of the galactose of the adjoining repeat; the 1,4-\(\beta\) stereochemistry between Gal and Man reside being unique among the eukaryotic glycoconjugates. Since the PGs are extremely labile due to anomic phosphodiester linkages between the PG repeats, their chemical synthesis is challenging. Synthesis of the carbohydrate anomic phosphodiester is complicated, compared to that of non-anomic types (oligonucleotides), by the requirement of stereochemical control at the anomic centre and the instability of the phosphodiesters, primarily due to the propensity of the glycosyl ring to form a stabilized anomic carbocation by expulsion of anomic phosphomonooester leaving group. For this reason, only a few syntheses of anomerically linked oligomers of carbohydrates are reported. The first synthesis of *Leishmania* PGs was reported by Nicolaev *et al.*\(^11\) from suitably protected galactose donor and mannose acceptors. However, this elegant but laborious approach required multiple protection/deprotection, glycosylation and phosphorylation steps, even before the PG assembly could begin.

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**Scheme 1.** (a) Acetone, pTSA; (b) (i) Br(CH\(_2\))\(_7\)CH\(_3\), NaH, (ii) 10% HCl; (c) TBDMSCl, imidazole; (d) palmitoyl chloride, pyridine; (e) TBAF, AcOH; (f) diphenyl phosphochloridate, py; (g) PO\(_4\), H\(_2\), MeOH and (h) phospholipase A\(_2\), CaCl\(_2\), Palitzsch buffer, pH 7.5.

**Scheme 2.** Synthesis of D-*myo*-inositol 1,2-cyclic monophosphate.
Synthesis of PG repeats

We designed a new, glycosylation-free approach to construct PGs, from readily available disaccharide lactose, suitable for solution, solid-phase and polycondensation modalities, without involving any anomeric glycosylation (Scheme 3).

Moreover, the PG chain could be extended towards the non-reducing (6'-OH) as well as the reducing (1-OH) end in iterative steps. Key elements of this approach included: (i) a gluco → manno transformation by glycal chemistry and regioselective 6'-protection to convert lactose (Galβ1,4Glu) into the central Galβ1,4Man building-block; (ii) ability to use it as a PG-phosphate donor or acceptor; (iii) extension of PG repeats in either direction, non-reducing or reducing end, followed by α-phosphitylation, and (iv) iterative PG coupling (Scheme 4).

Solid phase synthesis of PG

Encouraged by the success, the method was attempted for solid-phase synthesis of PGs, which presented a unique problem; the presence of labile anomeric phosphodiesters between the PG repeat units. If we were to use single PG H-phosphonate donor (19) in iterative coupling cycles, removal of the final product from the solid support would necessitate selective hydrolysis of first terminal anomeric phosphodiester without affecting the other internal ones. This requirement of cleaving one specific phosphodiester was a formidable obstacle, without any literature precedent. We reasoned that by placing a cis allyloxy linker group adjacent to the first anomeric phosphodiester, it should be possible to make it marginally susceptible to cleavage under mild acidic condition, whereas the other anomeric phosphodiester would not hydrolyse. To examine this proposition, we designed a new allyloxyphosphoryl linker, from cis-2-butene-1,4-diol by first blocking one of the hydroxyls by dimethoxytritylation to get 4-(4,4'-dimethoxytrityl)-cis-2-buteno (Scheme 5). The functionalized resin 32 was prepared by coupling this to Merrifield solid support, unreacted sites capped by methylation followed by removal of DMTr to obtain ready-to-couple resin.

Now the linker-functionalized resin 32 was coupled to PG H-phosphonate 19 using pivaloyl chloride followed...
by oxidation to afford single PG repeat linked by anomeric phosphodiester to the linker-resin (Scheme 6). To test our hypothesis, a small portion of PG-linker-resin 33 was treated with 0.1 N HCl at 100°C for 1 min; the condition known to cleave phosphodiester at anomeric position. The cleaved product on global deprotection provided Gal-1,4β-Man (34) identical to the authentic compound. However, when the resin 33 was treated with tris-(triphenylphosphine)-rhodium (I) chloride (Wilkinson’s catalyst) in 0.1 N HCl at rt, the product that was cleaved off was 2,3,6-tri-O-acetyl-4-O-[2,3,4-tri-O-acetyl-6-O-(t-butyl-dimethylsilyl)]-β-D-galactopyranosyl]-α-D-mannopyranosyl-phosphate, which on deprotection provided Gal1-4β-Man-1α-phosphate (35). This validated the hypothesis that under milder Wilkinson’s condition, the anomeric phosphodiester from the resin was selectively cleaved towards the allylic side with anomeric phosphate intact, and not towards the anomeric side losing the phosphate.

With the validity of our linker design proven, we decided to extend the PG synthesis to the solid support as illustrated in Scheme 7. Two consecutive cycles on the solid support provided the free phosphotetrasaccharide 39 with two PG repeats. The third coupling and cleavage cycle provided phosphohexasaccharide with three PG repeats. The coupling efficiency of each iterative cycle was more than 90% as determined by cleavage after each cycle and analysis of the deprotected product. The progress of these PG coupling cycles could be easily monitored by taking a small aliquot of the reaction mixture and treating it with the cleavage reagent for negative-ion electrospray MS analysis.

Synthesis of PGs by polycondensation

With the method established for synthesis of PGs by solution and solid-phase, we explored the possibility of
assembling linear PGs by one-pot polycondensation; the rationale (Scheme 8) being that selectively i-butyl-dimethylsilyl de-blocked H-phosphonate 40 can serve as a bifunctional monomer for such a polycondensation. Indeed the polycondensation of 40 by pivaloyl chloride in pyridine-Et3N (10 : 1), but a high concentration of both the monomer and the coupling reagent to avoid formation of cyclic products, followed by oxidation gave protected PGs (41). Final deprotection with 0.1 M NaOMe followed by filtration through Dowex X8 (H+) resin afforded free PGs (42). The size of the PGs was determined by negative ion ESMS and 31P NMR analysis, indicating a sub-population of 19–22 PG repeats. Although this approach did not give a homogeneous PG, it led to a mixture containing larger PGs (42) closer to the biological LPG. Analysis of the CD data of this PG showed it to contain significant amount of helicity.

**Synthesis of branched PG repeats**

One remarkable interspecies difference in the structure of LPG of *L. donovani* and *L. major* is the presence of an additional branching point (1,3β-galactosylation) in the PG domain, which controls the metacyclogenesis (transformation of noninfective procyclic form to the infective form) and the attachment of infective form to the sandfly midgut and human macrophages. While working on synthesis of PG of *L. donovani*, we discovered an interesting method for regioselective alkylation at position 3 (among six free hydroxyls) of the lactal (15), paving the way to a key branched intermediate 43 (Scheme 9).

This was achieved by first placing a p-methoxybenzyl group on the 3′-OH of 15 by dibutyltin chemistry followed by selective protection of the 6′-OH of 43 with TBDMS, NaH and catalytic amounts of 18-crown-6. This reaction showed remarkable regioselectivity in favour of the 6-OH of the Gal residue over the 6-OH of the glucal residue (product ratio 85 : 15) providing the desired 3′-O-PMB-6-O-TBS-d-lactal (44). The gluco → manno transformation via sodium bicarbonate-catalysed m-CPBA reaction, followed by per-acetylation led to the key intermediate 45 as the major isomer. Now the PMB group was removed by DDQ to yield the acceptor 46.

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**Scheme 8.** (a) (1) pivaloyl chloride, pyridine–Et3N (10 : 1), rt, 3 h; (2) I2 in 95% aq pyridine, 2 h, TEAB and (b) 4.6 M NaOMe in MeOH, 23 h, 4°C.

**Scheme 9.** (a) Bu₂SnO, MeOH, reflux, 4 h; PMBCl, CsF, KI, DMF, rt, 48 h; (b) NaH, 18-crown-6, TBSCI, THF, 0°C, 0.5 h; (c) mCPBA, ether–bicarbonate buffer, 0°C, 3 h; Ac₂O, Py, rt, 16 h; (d) DDQ, DCM–H₂O, rt, 12 h and (e) TMSOTf, –20°C, 4 h.

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Scheme 10. (a) BnBr, NaH, TBAI, DMF, rt, 3 h; (b) DMD, DCM, 0°C, 2 h; (c) MeOH, rt, 4 h; (d) (COCl)₂, DMSO, DCM, –78°C; NaBH₄, rt, 4 h; (e) TMSOTf, DCM, 45 min, –30°C; (f) Me₂NH, CH₃CN, –20°C, 5.5 h; CCl₃CN, DBU, DCM; (g) TMSOTf, DCM, 45 min, –30°C; (h) Pd(OH)₂, H₂, 4 h; (i) NaOMe, MeOH and (j) Ac₂O/AcOH/H₂SO₄, rt; Na₂CO₃, MeOH, rt.

which was coupled with the glycosyl donor 47 (Scheme 9) to make the central intermediate 48, which was then elaborated to the PG of L. major₁₅, as described in the previous section.

Synthesis of tetrasaccharide cap domain

The neutral oligosaccharide cap contains a signal for termination of PG assembly and helps the parasite to attach to digestive tract of the sandfly and human macrophage. We designed a new synthesis of this tetramannoside cap from lactose and mannose starting materials (Scheme 10), and it also enabled the preparation of the radiolabelled cap of LPG₁₆.

The important features of our approach included glucosidomanno transformation via glycal and dimethyl-dioxirane chemistry to convert protected lactose (Galβ₁→4-Glu) into a suitably protected Galβ₁→4-Man intermediate 52 with a free C₂-OH group available for stereoselective coupling with a mannobiose donor 56. The synthesis started with hexa-O-benzyl-D-lactal (49); stereoselective α-epoxidation with 2,2-dimethyldioxirane to the corresponding 1α,2α-oxirane (50), followed by methanolysis to open the epoxide ring gave the β-glucoside (51). Swern oxidation followed by reduction gave the intermediate with manno configuration (52) with free C-2 hydroxyl available for glycosylation with mannobiosyl donor 56. The donor 56 was synthesized by glycosylation of 1,3,4,5,6-tetra-O-acetylmannose (53) with 2,3,4,6-tetra-O-acetylmannosyl-1-O-trichloroacetimidate (54) to give 55, followed by conversion to its corresponding anomic trichloroacetimidate 56. Coupling of 52 and 56 followed by a three-step global deprotection yielded the neutral cap domain (60) of LPG.

Synthesis of GPI core domain of LPG

The key feature of the GPI glycan core of Leishmania LPG is the internal galactofuranose residue. It has been postulated that during the biosynthesis of LPG, this internal Gal₃ residue is derived from its donor UDP-Gal₃, which in turn must be synthesized from UDP-Gal₂ by a mutase reaction. Since this mutase reaction occurs in the cytosol, there must be a unique Golgi-specific UDP-Gal₂-transporter in the parasite. To address these biosynthetic questions, we ventured into the synthesis of the glycan core. The overall synthetic strategy towards the glycan core (71) is described in Scheme 11. We made three key suitably protected intermediates; Gal₃(1-6α)-Gal₃(1-3α)-Gal₃ (68), Man₃(1-3α)-Man₃ (69) and GlcN-(1-6α)-inositol (70). These advanced intermediates were in turn synthesized from the monosaccharide building blocks (61–67), the key reaction being the application of substituted galactonolactone donor (63) to place the internal Gal₃ residue, followed by isoamylborane reduction, activation as trichloroacetimidate donor (68) and coupling...
with the mannobiose acceptor (69, unpublished results). The synthesis of the glucosamine-inositol 70 is described in the next section. In addition, we synthesized UDP-Galp and UDP-Galα and established methods of their enzymatic inter-conversion using recombinant UDP-Gal mutase and HPLC (unpublished results).

**Synthesis of full-length GPI anchors**

In addition to our focus on *Leishmania* LPG, we became interested in the biology of GPI anchors, particularly from the point of view of their biosynthesis and cell biology, and designed new approaches for synthesis of full-length GPI anchors. The structural complexity and biological function of GPIs inspired widespread interest, and a number of approaches towards the GPI anchors have been reported, including that of yeast17, rat brain Thy-1 (ref. 18), T. brucei19, sperm CD-52 (ref. 20), T. cruzi21 and *P. falciparum*22 (reviewed in ref. 22). However, despite the concerted efforts, construction of full-length GPI anchor still remains a daunting task due to: (i) structural and functional differences within the species, and (ii) significant microheterogeneity in the lipid and glycan domains. Arguably, the most demanding aspect of GPI synthesis is to make glucosamine-inositol motif requiring optically pure D-myoinositol acceptor and azidoglucosyl donor. This was done either by resolution of bis-cyclohexylidene-myoinositol using camphanate auxiliares and enzymes, or through a synthesis from D-glucose by Ferrier reaction. We discovered an interesting solution to this problem23. Instead of a priori resolution of myoinositol, we reasoned, based on structural modelling, that if sufficient strain was built through a cyclic group, the azidoglycosyl unit itself could function as a chiral auxiliary. To test this proposition, the racemic 1-O-PMB-2,3,4,5-tetra-O-benzyl-myoinositol 73 was glycosylated with 2-azidoglycosyl donor 72 to get pseudosaccharide 74 (Scheme 12), which on deacylation (75) and benzylation gave 4,6-cyclic-acetal. To our surprise, this led to a clean separation of the two enantiomers, 76 and 77. The next two steps, benzylation at 3-OH and regioselective opening of benzylidene acetal by NaCNBH3, provided key building block 78 and unnatural isomer 79.

After the efficient access to glucosamine-inositol 78, we designed a new and convergent [2 + 2] approach for tetramannose building block 80 (Scheme 13 for retro-synthetic plan and key intermediates). Compound 80 was prepared from two protected mannobiosides, the activated donor 81 and acceptor 82. The donor 81 was prepared by coupling of allyl-3-O-benzyl-4,6-O-benzylidene-α-D-mannoside with 2,3,4,6-tetra-O-benzyl-α-D-mannosyl trichloroacetimide (prepared from mannose). The glyco-
Sylation went smoothly and the product was subjected to simultaneous removal of anomeric allyl and 4,6-benzylidene groups (KOTBu, DMSO, 80°C; 1M HCl-acetone, 1:9, 60°C). The peri-acetylation of resultant triol, selective removal of anomeric acetyl (Me₂NH, MeCN, –20°C), and activation (CCl₃CN, DBU) provided the desired mannobiase. It needs mention that the two acelyls at position 4 and 6-OH were deliberately placed keeping in view our future target, the [4-deoxy-Man-III]-GPI analogue.

Lower mannobiase was prepared by glycosylation (TSTf, NIS) of allyl-2,3,4-tri-O-benzyl-α-D-manno-pyranoside (from mannose) with 3,4,6-tri-O-benzyl-β-D-man-1,2-pent-4-enyl orthobenzoate, followed by removal of benzoyl from position 2. Having access to both mannobiase donor and acceptor, further glycosylation provided a fully protected tetramannose. This, after anomeric allyl-removal (KOTBu, DMSO, 80°C; 1M HCl-acetone, 1:9, 60°C) and activation (CCl₃CN, DBU), afforded the desired tetramannose. The next step of the [4 + 2] glycosylation of glucosamine-inositol with the tetramannose went smoothly (TMSOTf, DCN, 0°C, 70%) to provide a pseudohexasaccharide as the central point for both the GPI anchor. For the synthesis of the GPI anchor, two acetyls were first removed and primary 6-OH of the diol was silylated (TBDDPSCI, imidazole) to get, followed by benzylolation of the 4-OH (BnBr, NaH) and TBDPS removal (TBAF, THF) to obtain the pseudohexasaccharide ready for phosphorylation with ethanolamine. The coupling of with NHCbz-ethanolamine phosphoramidite was carried out with 1H-tetrazole followed by mCPBA oxidation. Now the PMB group from position 1 of the myo-inositol residue was removed and the product was phospholipidated with 1-O-alkyl-2-O-acyl-sn-glycero-H-phosphonate to provide fully-protected GPI anchor. The final step involved global deprotection and azide reduction by hydrogenolysis to the target GPI anchor.

**Synthesis of GPI anchor of Plasmodium falciparum**

Two key structural features that distinguish malarial GPIs from those of other parasitic species include: the presence of an extra fatty acid at position-2 of the myo-inositol residue rendering Pf-GPIs resistant to the host PI-PLC-mediated hydrolysis, and an additional fourth mannose at the non-reducing end of the glycan domain. The GPI anchor of the malarial parasite presents increasing challenges due to the presence of a third fatty acid group at position-2 of the myo-d-inositol residue. For this reason, so far only one total synthesis of fully lipidated Pf-GPI has been reported. In addition, synthesis of a model GPI (lacking fourth mannose and with short-chain fatty acid lipids) has been reported. We built upon our experience with the synthesis of T. cruzi GPI anchor and addressed the issue of placing a third fatty acid; essentially the same method was used except an additional protecting allyl group was placed at position-2 of inositol. For this the racemic 1-O-PMB-2-O-allyl-3,4,5-tri-O-benzyl-(DL)-myo-inositol (from 1,2-4,5-bis-cyclohexylidene-(DL)-myo-inositol) was coupled with azido-deoxyglycosyl donor, and the desired optically pure glucosamine-inostiol intermediate was prepared. The entire synthetic sequence followed in our synthesis of Pf-GPI (93) is summarized in Scheme 14.

**Chemical biology of GPI molecules**

**Trans-bilayer flip-flop of GPIs across endoplasmic reticulum**

Flip-flop of lipids across biological membranes is a fundamental feature of membrane biogenesis. Phospholipids, the building blocks of membrane bilayers, are made on the cytoplasmic face of biogenic (self-synthesizing)
membranes like the ER and must be flipped to the opposite face (lumen) for bilayer expansion. Flipping does not occur spontaneously and requires specific transporter proteins or ‘flippases’ that facilitate ATP-independent, bi-directional movement of lipids across the membrane. Phospholipid flippases, and not the ABC transporters of the plasma membrane, are yet to be identified in biogenic membranes and the mechanism of lipid flipping remains a mystery.\textsuperscript{3,6} The issue of lipid flipping acquires an additional dimension in the case of ER-localized biosynthesis of GPI-anchored proteins. GPI synthesis begins on the cytoplasmic side of the ER, while the addition to proteins occurs in the lumen (Figure 5), implying that a GPI intermediate must flip across the ER during the GPI assembly.

It is also unclear whether GPI flipping occurs via an ATP-independent process and is mediated by specific proteins. As part of the efforts to elucidate the molecular details of GPI biosynthesis, we designed a chemical synthesis of novel, functional GPI probes and used them to demonstrate, ATP-independent, protein-mediated flip-flop of GPI lipids. For this, a biochemical reconstitution assay using proteoliposomes enriched with flippase-rich ER fraction was set up to show that phospholipid flipping in the ER requires specific proteins. In this simple method, trace amounts of fluorescent acyl-NBD-labelled

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**Scheme 13.** Synthesis of full-length GPI anchor of Trypanosoma cruzi.

**Scheme 14.** Synthesis of fully lipidated GPI anchor of Plasmodium falciparum.
phospholipids were added during reconstitution and flipping of the NBD-lipids was assayed with dithionite, a reagent that quenches the NBD fluorophore at the outer leaflet of vesicles. For this study, two NBD-labelled GPI probes, NBD-GlcNAc-PI and GlcN-PI were synthesized (Scheme 15). Although approaches for GPI synthesis were reported, including the one for placing a label in the glycan domain27, synthesis of fluorescent GPI probes labelled in the glycerolipid domain required a new strategy (Scheme 15).

Our synthetic design28 involved three chiral building blocks: (a) 1-allyl-2,3,4,5-tetra-O-benzyl-D-myo-inositol 94 made in eight steps starting from inositol; (b) the 3,4,6-tri-O-acetyl-2-azido-2-deoxy-β-D-glucosyl donor 72 prepared from tri-O-acetyl-d-glucal by azidonitration, and (c) the phosphatidyl donor 99 with a protected terminal amine in the sn-1 acyl chain prepared from 1,2-isopropylidene-sn-glycerol. Glycosylation of 94 with 72 gave the α-glucosaminyl(1 → 6) inositol 95 followed by deprotection of the allyl group and replacement of the azido group with NHBoc to enable the selective coupling with the NBD probe. Phospholipidation of 98 with 99 using H-phosphonate chemistry gave the protected GPI 100. The next three steps, i.e. removal of benzyls, coupling with the NBD probe, and deprotection of the NHBoc provided NBD-GlcN-PI (102), which on N-acetylation gave NBD-GlcNAc-PI (103).

We made proteoliposomes from TE, egg PC and trace amounts of NBD-GPI (102 or 103). Protein-free liposomes were prepared by omitting the TE component. Since the GPI probes are presumed to be symmetrically distributed in the membrane of the reconstituted vesicles, treating liposomes with dithionite should cause ~50% fluorescence loss; treatment of flippase-active protopigm probes should yield ~100% loss, since NBD-lipids in the inner leaflet will flip out and be exposed to dithionite (Figure 6).

Fluorescence dropped rapidly by ~40–45% when dithionite was added to the liposome samples (Figure 6b, green traces), and was eliminated when the vesicles were detergent-permeabilized indicating that dithionite was sufficient to reduce all NBD present. For protopigm s, dithionite caused a similarly rapid, yet greater fluorescence loss (Figure 6h, purple traces) that: was (a) reduced by protease treatment, (b) did not require ATP and (c) depended on the amount of TE used for reconstitution (Figure 6c). The protein-dependence profile was identical for both GPI probes (Figure 6c). Interestingly, the protein-dependent profile obtained in assays of NBD-PC flipping was identical to that obtained for the GPI probes. Although our assay did not provide data on flipping kinetics, it is clear that both GPI probes are transported rapidly, on a timescale of ~1 min (Figure 6a), similar to that measured for glycerophospholipid flipping in the ER. Thus the flipase(s) appears not to be able to distinguish between GlcN-PI, an intermediate that is not consumed in the ER lumen, and GlcN-PI, a substrate for luminal mannosyl transferases (Figure 5).

In continuation with our collaborative work showing that nanoclusters of GPI-anchored proteins are formed by cortical-actin driven activity29, and to elucidate the chemical basis and the stereochemical role of GPIs in clustering of cholesterol-rich ordered domains, we have recently synthesized30 a new BODIPY-labelled fluorescent GPI probe and its antipode, with the label in the head group, for membrane and lipid-raft experiments.

Trans-bilayer distribution of PI in ER does not depend on stereochemistry

The key glycerophospholipids (PC, PE, PS and PI) are synthesized on the cytoplasmic side of the ER and then flipped to the exoplasmic layer for uniform expansion of the bilayer. This transbilayer movement requires specific membrane proteins called flipases. It is an accepted wisdom in the field that flipase-mediated flip-flop across the ER is controlled by the stereochemistry of the glycolipid31; however this issue has not been resolved. This question assumes greater importance in the case of PI lipids, which are key signalling molecules. The importance of PIs in membrane biology is profound, as close to 100 isoforms of kinases (e.g. PI3K) and phosphatases...
Figure 6. Flipping of NBD-GPIs: a. Predicted 50% fluorescence loss on adding dithionite in liposomes, compared to 100% loss in proteoliposomes due to the flipping of NBD-GPIs. b. Fluorescence traces of assays with liposomes and proteoliposomes. c. Protein dependence of the extent of dithionite reduction of NBD-GPIs.

Figure 7. Structures of diastereoisomers of acyl-NBD-labelled phosphoinositides (PI). Compound 104 corresponds to the stereochemistry of PI that occurs naturally in mammalian cells, whereas compounds 105–107 represent non-natural stereoisomers of PI.

These included one ‘natural’ with D-myoinositol and sn-1,2-glycerol and three ‘non-natural’ diastereoisomers (D-inositol with 2,3-sn-glycerol, L-inositol with sn-1,2-glycerol, and L-inositol with sn-2,3-glycerol). The four new isomers (Figure 7, compounds 104–107) were prepared with a fluorescent NBD appended to the sn-1-linked acyl chain. The second acyl chain in each case was stearate (C18:0) to mimic the acyl chain of naturally occurring mammalian PI. The synthetic strategy and methods for these PI probes are described in Schemes 16–18. All of the synthetic targets (104–107) required D and L enantiomers of suitably protected 2,3,4,5,6-penta-O-benzyl-myoinositol intermediates 112 and 113 (Scheme 16).

The starting material, racemic 3,4,5,6-tetra-O-benzyl-myoinositol (108), was prepared in two steps, and then converted to racemic 2,3,4,5,6-penta-O-benzyl-myoinositol (109) in three high-yielding steps (regioselective 1-O-allylation, 2-O-benzylolation and allyl removal from the 1-position). The racemate 109 was resolved into its enantiomers 110 and 111 via the diastereoisomeric pair 112 and 113 of (–)-camphanic chloride followed by alkaline hydrolysis.

We employed two chiral glycerol building blocks: 2-O-octadecanoyl-1-O-[6-(N-carbobenzyloxy amino)-hexanoyl]-sn-glyceryl-H-phosphonate (118) (Scheme 17A) and 2-O-octadecanoyl-3-31 O-[6-(N-carbobenzyloxyamino)-hexanoyl]-sn-glyceryl-H-phosphonate (123) (Scheme 17B). These were prepared from 1,2-isopropylidene-sn-glycerol (114) and 2,3-isopropylidene-sn-glycerol (119) respectively, by a multi-step synthesis as shown in Scheme 17.

Coupling of the D-myoinositol 112 and 1,2-sn-glycerol-H-phosphonate 118 in the presence of pivaloyl chloride followed by in situ iodine oxidation led to fully protected PI 124 (Scheme 18). Deprotection of all benzylos by hydrogenolysis and installation of the NBD at the terminal amine at the sn-1 position with NBD-amino-caproic NHS ester yielded the first fluorescent-PI, 1D-myoinositol 1-O-[1′6′[[6-[(7-nitro-2-oxa-1,3-diazolobenz-4-yl)amino]-hexa-
noyl[amino]-hexanoyl]-2 \(O\)-stearoyl-sn-glycer-3-yl]-phosphate (104). The other three isomers (105, 106 and 107) were synthesized using same strategy from the respective chiral myo-inositol and lipid intermediates.

The PI probes were evaluated for their flipping in rat liver ER vesicles, as well as in flippase-containing proteoliposomes reconstituted from a detergent extract of ER (as described earlier)\(^\text{28}\). Our results showed that the ER flippase-mediated flip-flop of PIs does not depend on the stereochemistry of the lipid, and all isomers flip-flop with rates similar to that for fluorescence-labelled PC used as a control. Our data have implications for recent hypotheses concerning the evolution of distinct homochiral glycerophospholipid membranes during the speciation of archaea and bacteria/eukarya from a common cellular ancestor\(^\text{32}\).

**De novo biosynthesis of myo-inositol in Leishmania parasite**

The pathogenic protozoan parasites such as *Leishmania* express GPIs in high abundance and would therefore require large quantities of myo-inositol precursor. The question of whether the parasite sequesters myo-inositol from its human host or it has a biosynthetic machinery of its own is of interest. The biosynthesis of myo-inositol in yeast and bacteria is mediated by a unique enzyme myo-inositol 1-phosphate synthase (MIP synthase), which catalyses oxidation, enolization, intramolecular aldol condensation and carbonyl reduction steps involved in the transformation of glucose 6-phosphate into inositol 1-phosphate (Scheme 19). The precise mechanism of this remarkable transformation has not been elucidated, but 5-keto-glucose 6-phosphate and myo-2-inosose 1-phosphate have been proposed as intermediates\(^\text{33}\).

Interestingly, in humans MIP synthase is expressed only in the brain and testes, and is not expressed in the cells infected by the parasites. Therefore, we decided to study inositol biosynthesis in *Leishmania* using stable \(^{13}\text{C}\) isotope labelling and electrospray ionization mass spectrometry (ESI-MS)\(^\text{34}\). This approach using \(^{13}\text{C}\) labelling was adopted for the following reasons: (a) in a cell culture parasites take up inositol from the medium (radio-labelling experiments) to incorporate into PI/GPI molecules and (b) if the parasite was also making its own inositol (MIP synthase) from glucose, the \(^{13}\text{C}\) enrichment level would get diluted in PI/GPI products. We evolved two routes for enantioselective synthesis of \(1\text{D}-\text{myo-[1-}\text{13\text{C}]\text{-inositol}}\) from \(\text{D-}[6-\text{13\text{C}]\text{-glucose}}\). In the first enzymatic approach we designed an *in vitro* synthesis using a

\[\text{Scheme 16. Synthesis of the optical antipodes of protected myo-inositols: (a) Bu₂SnO, toluene, reflux, 4 h; allyl bromide, DMF, 80°C, 4 h; NaH, BnBr, DMF; 5% Pd/C, EtOH, pTSA, H₂O; (b) (–)-Camphoric acid chloride, pyridine and (c) 1% NaOH in MeOH, reflux, 30 min.}\]
mutant strain of yeast (*Saccharomyces cerevisiae*) over-expressing MIP synthase (Scheme 20).

In an alternative chemical synthesis strategy D-[6-13C]-glucose was used as a starting material for the preparation of D-myo-[1-13C]-inositol in an eight-step method as described in Scheme 21; the key step being the Ferrier rearrangement of the enol-acetate 133 to the inosose intermediate 134. For larger-scale synthesis, we found the chemical route to be more convenient as it involved less cumbersome purification. These were the first reported methods for synthesis of 13C-labelled myo-inositol. The D-myo-[1-13C]-inositol and D-[6-13C]-glucose precursors were then biosynthetically incorporated into *L. donovani* promastigote culture, and biosynthetic PI and its hydrolysis products glyceo-PI and inositol were isolated and analysed by negative-ion ESMS, and the isotopomeric ratio was determined. These incorporation experiments clearly showed substantial isotopic dilution34, indicating the presence of MIP synthase and biosynthetic machinery in the parasite, which was further confirmed by the direct incorporation of D-[6-13C]-glucose in the parasitic PI/GPIs.

**Inhibition of eMPT – a key enzyme involved in the iterative assembly of LPG**

The putative mannose phosphate synthase (MPTs) are unique enzyme that transfers an intact α-D-mannose 1-phosphate moiety from the nucleotide sugar donor GDP-Man to the GPI-glycan core of LPG (Figure 8)35. It should be mentioned here that in normal biology a mannose is transferred from GDP-Man with a release of a GDP unit, whereas in *Leishmania* intact α-D-mannose 1-phosphate is transferred with the release of GMP. Since there are no MPTs in human biology, the *Leishmania* MPTs present opportunities for new drug design. Considering this, we first established an *in vitro* eMPT assay using microsomal membrane preparation of *Leishmania* promastigotes36, a lipid-linked PG as a substrate mimic, and radio-labelled GDP-[3H]-Man as the donor. Based on a transition state model, we designed a new generation of 1-oxabicyclic β-lactam analogues showing interesting inhibition of eMPT activity37, the key synthetic step being a [2 + 2] heteroatom cycloaddition on a suitable disaccharide–glycal scaffold with trichloroacetylisocyanate. These molecules were tested *in vitro* in the LPG biosynthetic assay and exhibited good (micromolar range)

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**Scheme 18.** (a) Pivaloyl chloride, pyridine, rt, 1 h; 12 in 95% aq pyridine, 30 min; (b) Pd(OH), MeOH–CH2Cl2–H2O, H2, 12 h; (c) NBD-X, SE, DMF, Et3N, 2 h, rt.

**Scheme 19.** Proposed mechanism of MIP synthase.

**Scheme 20.** Enzymatic synthesis of myo-[1-13C]-inositol using recombinant MIP synthase.
inhibition of MPT activity. Since most of the enzymes involved in LPG biosynthesis, particularly the eMPTs are unique to the parasites, they present new opportunities for new drug discovery.

Cell surface GPIs of Entamoeba histolytica

The intestinal protozoan parasite E. histolytica is a causative agent of invasive amoebiasis and glycoconjugates are involved in disease pathology. The glycocalyx layer on the surface of the pathogenic strain is predominantly made of GPI-anchored proteophosphoglycan (PPG). We isolated two novel ‘protein-free’ glycosylated inositol phospholipids (GIPLs) from E. histolytica and determined their structure by chemical analysis and metabolic labelling experiments, and showed that these GPIs inhibited PKC in macrophages. A new in vitro biosynthetic method for PPG was established and a number of genes involved in GPI pathway identified by bioinformatic
tools applied to the *E. histolytica* genome. One of the key genes PIG-L for GlcNAc-PI deacetylase was characterized, and an anti-sense RNA-mediated inhibition of GPI biosynthetic enzymes as an approach to decreasing the amount of GPI conjugates in *E. histolytica* was demonstrated.

### Summary

Significant progress has been made in our understanding of GPI anchors in biology and a new view of the organization of plasma membrane has emerged. The knowledge on structure, biosynthesis, cell biology and membrane biology of GPI-anchored proteins and phosphoglycans has provided a large number of unique biosynthetic pathways and cellular events for new enquires and opportunities for interventions. As always in biology, the synthetic chemistry has played a major role in the field of GPI molecules. The access to the full-length GPI anchors, and their biosynthetic substrates and labelled probes through chemical synthesis in laboratory have provided enabling tools to ask even more challenging questions pertaining to cell, membrane and glyco-biology. The stage is also now set to use this knowledge in designing new therapeutic approaches.

27. Mayer, T. G., Weigart, R., Munstermann, F., Kawai, T., Kurczalia, T. and Schmidt, R. R., Synthesis of labeled glycosyl phos-


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