Posttranslational Modifications

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Chemical Biology of Glycosylphosphatidylinositol Anchors

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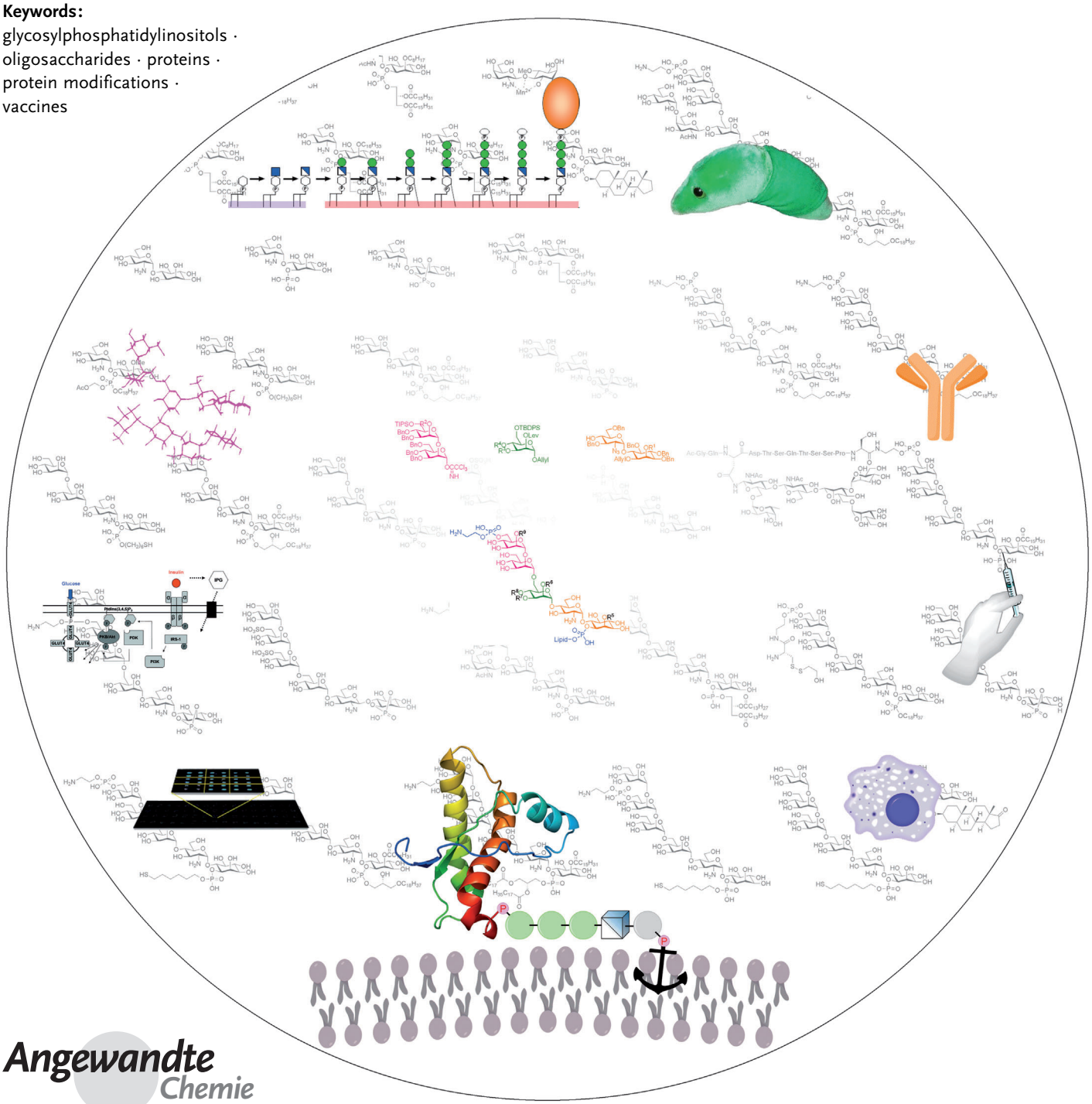
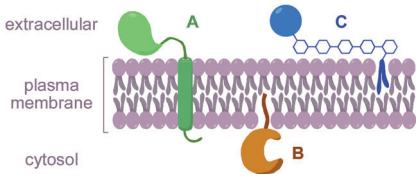


Glycosylphosphatidylinositols (GPIs) are complex glycolipids that are covalently linked to the C-terminus of proteins as a posttranslational modification. They anchor the attached protein to the cell membrane and are essential for normal functioning of eukaryotic cells. GPI-anchored proteins are structurally and functionally diverse. Many GPIs have been structurally characterized but comprehension of their biological functions, beyond the simple physical anchoring, remains largely speculative. Work on functional elucidation at a molecular level is still limited. This Review focuses on the roles of GPI unraveled by using synthetic molecules and summarizes the structural diversity of GPIs, as well as their biological and chemical syntheses.

From the Contents

1. Discovery of GPIs

Membrane-bound proteins are essential for proper cell function.[1] It was originally thought that the membrane-bound proteins can be associated with the lipid bilayer only through a hydrophobic peptide sequence embedded in the membrane (A in Scheme 1). Later it was found that proteins containing free amino or thiol groups could be acylated with long-chain fatty acids providing a lipophilic tail to anchor proteins in the membrane (B in Scheme 1). In the late 1970s a novel phospholipase, phosphatidylinositol phospholipase C (PI-PLC), was purified from various bacteria.[2] This enzyme was found to release proteins, such as alkaline phosphatase (AP), 5′-nucleotidase and acetylcholinesterase (AChE) from various tissues.[3] Based on these experiments, the proteins were suggested to be covalently attached to the cell membrane via a PI-containing anchor (C in Scheme 1).[4] Further structural analysis revealed that the anchor contains a glycan chain in addition to the inositol unit,[5] which led to the establishment of a general structure for the glycosylphosphatidylinositol (GPI) class of glycolipids. In addition to GPIs that serve as protein anchors, non-protein-linked free GPIs have also been found in abundance on the surface of several protozoa.[6]

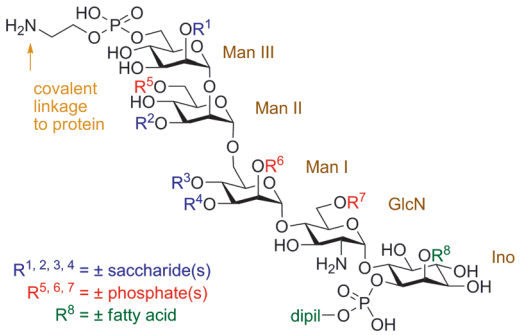


Scheme 1. Classification of integral membrane proteins. A) transmembrane protein, B) prenylated or fatty-acylated protein, C) GPI-anchored protein.

2. Occurrence and Structure of GPIs

Hundreds of GPI-anchored proteins have been identified in various organisms, including protozoa, fungi, yeast, plants, mollusks, insects, and vertebrates, thus indicating that this mode of protein modification is common in eukaryotes.[7] GPI-anchored proteins have also been indirectly detected in archaea,[8] but not in bacteria so far.[9] GPI-anchored proteins are functionally diverse and play vital roles in signal transduction, immune response, cancer cell invasion and metastasis as well as the pathophysiology of parasites.[10] In some cases, the presence of GPI anchors is essential for proper protein function.[11]

All GPIs identified to date, with one exception,[12] share a common core structure, $H_2N(CH_2)_2OPO_3H$-6Manα1→2Manα1→6Manα1→4GlcNα1→6*myo*-Ino1-phospholipid (Scheme 2).[13] The presence of a free amine in the glucos-



Scheme 2. Conserved core GPI structure and possible modifications.

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Table 1: Structures of selected GPIs from various organisms and tissues.[a]

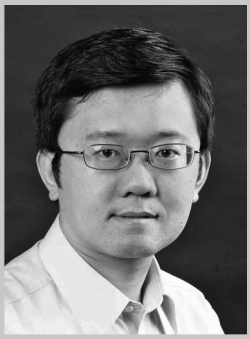
Entry	Origin	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	Lipid
1	*T. brucei* VSG 117[25]	H	H	H	$Gal_{2-4}\alpha$	H	H	H	H	DAG
2	*T. brucei* VSG 121[26]	H	Galβ	H	$Gal_{2-4}\alpha$	H	H	H	H	DAG
3	*T. brucei* VSG 221[27]	±Galα	±Galβ	H	$Gal_{2-4}\alpha$	H	H	H	H	DAG
4	*T. congolense* VSG[28]	H	H	Gal-GlcNAcβ	H	H	H	H	H	DAG
5	*T. cruzi* 1G7[29]	Manα	H	H	H	H	H	H	H	AAG
6	*T. cruzi* NETNES[30]	Manα	H	H	H	H	H	AEP	H	AAG
7	*T. cruzi* epimastigote[31]	Manα	H	H	H	H	H	AEP	H	AAG
8	*A. fumigatus*[32]	$Man_{1-2}\alpha$	H	H	H	H	H	H	H	ceramide
9	*L. major* PSP[33]	H	H	H	H	H	H	H	H	AAG
10	*P. falciparum*[34]	±Manα	H	H	H	H	H	H	acyl	DAG
11	*T. gondii*[35]	H	H	±Glc-GalNAcβ	H	H	H	H	H	DAG
12	*S. cerevisiae*[36]	$Man_{1-3}\alpha$	H	H	H	H	H	H	H	ceramide
13	*P. communis* AGP[37]	H	H	±Galβ	H	H	H	H	H	ceramide
14	rat brain Thy-1[38]	±Manα	H	GalNAcβ	H	H	PEtN	H	H	n.d.
15	hamster brain PrP^{Sc}[39]	±Manα	H	±Sia-±Gal-GalNAcβ	H	H	PEtN	H	H	n.d.
16	mouse muscle NCAM[40]	±Manα	H	±GalNAcβ	H	H	PEtN	H	H	n.d.
17	human kidney MDP[41]	±Manα	H	±Gal±GalNAcβ	H	n.d.	PEtN	H	H	n.d.
18	porcine kidney MDP[41]	H	H	±Gal/Sia-±GalNAcβ	H	±PEtN	PEtN	H	H	DAG
19	*Torpedo* AChE[42]	Glcα	H	±GalNAcβ	H	±PEtN	PEtN	H	H	DAG
20	human erythrocyte AChE[43]	H	H	H	H	±PEtN	PEtN	H	palmitoyl	AAG
21	human CD52[44]	±Manα	H	H	H	H	PEtN	H	±palmitoyl	DAG
22	human sperm CD52[45]	H	H	H	H	H	PEtN	H	palmitoyl	AG
23	human erythrocyte CD59[46]	H	H	±GalNAcβ	H	PEtN	PEtN	H	palmitoyl	AAG
24	human urine CD59[47]	±Manα	H	±GalNAcβ	H	H	PEtN	H	n.d.	n.d.
25	human placental AP[16]	H	H	H	H	GlcNAcβ-P	PEtN	H	H	AAG

[a] The positions of the residues R^1–R^8 and of the lipid mirror those in Scheme 2. AAG = 1-alkyl-2-acylglycerol; AEP = aminoethylphosphonate; AG = 1-alkyl-2-lysoglycerol; DAG = diacylglycerol; Gal = galactose; GalNAc = *N*-acetylgalactosamine; Glc = glucose; GlcNAc = *N*-acetylglucosamine; Man = mannose; n.d. = not determined; Sia = sialic acid; PEtN = phosphoethanolamine.

amine unit of GPIs is unusual, since glucosamines are *N*-acetylated or -sulfated in most naturally occurring glycoconjugates.[14] The common modifications of the core structure include the addition of a fourth mannose residue to the ManIII, branching at the C3 or C4 position of the ManI and additional phosphorylations (Table 1). All known variations of the GPI phospholipid, including (*lyso*)-diacylglycerol, (*lyso*)-alkylacylglycerol, and ceramide, feature hydrocarbon chains of differing lengths and varying degrees of unsaturation. Additionally, a fatty acid ester may be present at the C2 position of *myo*-inositol, which renders the GPI resistant to the hydrolytic cleavage by PI-PLC.[15] It is clear that significant structural differences exist among the GPIs from different species (Table 1, entries 17–20) or among GPIs from different tissues of the same species (Table 1, entries 21–24). Nevertheless, the functional significance of these variations remains largely unknown.

It must be kept in mind that the restricted variations observed in GPIs characterized to date may be a function of the current state of the technology used for the isolation, analysis, and structural elucidation of GPIs. For example, a recent report revealed the presence of a β-GlcNAc phosphate diester residue attached to the GPI of human placental alkaline phosphatase (Table 1, entry 25)[16] that had not been detected previously.[17] However, problems associated with the isolation of pure GPI samples due to their amphiphilic and heterogeneous character always render structural determination by NMR and X-ray crystallography difficult. As a result, many structures were proposed from indirect evidence, such as: mass spectrometry, enzymatic and lectin assays.

Yu-Hsuan Tsai completed his undergraduate study at the National Taiwan University before joining the group of Peter H. Seeberger in 2007. He obtained a master's degree from the ETH Zurich in 2008. From 2009 until 2012 he performed his doctoral work at the Max Planck Institute of Colloids and Interfaces focusing on the chemical synthesis of GPIs. Currently he works as a post-doctoral research associate in the group of Jason W. Chin on genetic incorporation of unnatural amino acids into proteins.

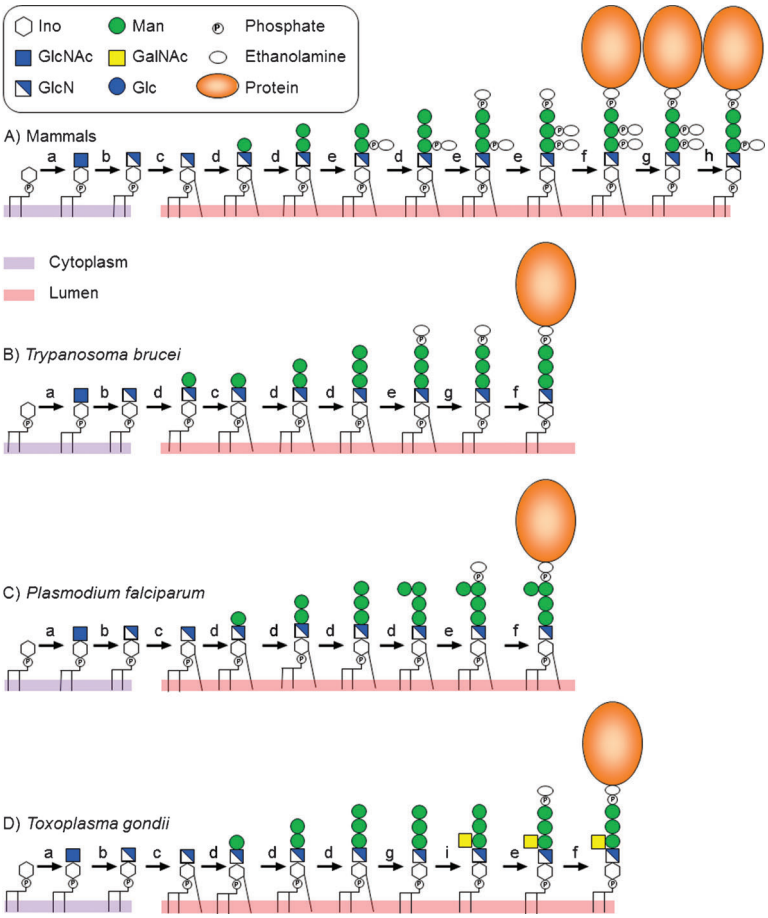


Xinyu Liu received a BE in 2002 from Kyoto University and a doctoral degree from the ETH Zurich under the tutelage of Peter H. Seeberger in 2007, followed by postdoctoral research with Christopher T. Walsh at Harvard Medical School. Since 2010 he is an assistant professor of chemistry at the University of Pittsburgh.

3. GPI Biosynthesis

Biosynthesis of GPI anchors is essential for homeostatic function of organisms from fungi[18] to animals.[19] Malfunction of GPI biosynthesis in human red blood cells can lead to paroxysmal nocturnal hemoglobinuria, an acquired disorder causing anemia due to the impaired expression of CD59, a GPI-anchored protein.[19] Inherited mutation of genes for GPI biosynthesis in humans can also lead to non-regulated homeostasis of blood coagulation[20] or mental retardation.[21] Hence, the understanding of GPI biosynthesis in mammalian cells is of great importance.

The biosynthetic pathways of GPI in mammalian cells and several protozoan parasites are well studied, and genes coding for enzymes that account for most transformations in GPI biosynthesis have been characterized.[22] We discuss first the GPI biosynthesis in mammals and highlight differences in the parasites *Trypanosoma brucei*, *Plasmodium falciparum*, and *Toxoplasma gondii*. Since GPI biosynthesis is vital for the growth of many parasites in their mammalian hosts,[23] the differences in GPI biosynthetic enzymes and pathways between mammals and parasites provide potential targets for the development of antiparasitic therapeutics.[24] Development of antimicrobial agents targeting GPI biosynthetic enzymes of *T. brucei* will be discussed in more detail in Section 5.4.



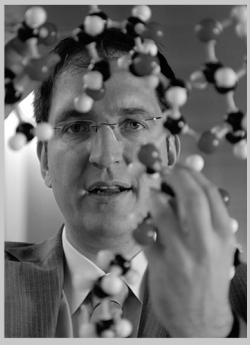
Scheme 3. Biosynthesis of GPI-anchored proteins on the endoplasmic reticulum (ER) membrane in A) mammals, B) *T. brucei*, C) *P. falciparum*, and D) *T. gondii*. Reactions: a) transfer of GlcNAc; b) *N*-deacetylation, followed by flipping of GlcN-PI into the luminal side of ER; c) acylation of inositol; d) mannosylation using dolichol-phosphate-mannose as donor; e) addition of PEtN; f) attachment of GPI to protein by GPI transamidase; g) inositol deacylation; h) removal of the second PEtN; i) addition of GalNAc.

In mammalian cells, GPIs are synthesized by the sequential addition of monosaccharides, acyl chains, and phosphoethanolamine (PEtN) residues to phosphatidylinositol in the endoplasmic reticulum (ER).[22a] The biosynthetic pathway is initiated on the cytoplasmic side of the ER membrane by the transfer of GlcNAc to diacyl-PI (Scheme 3 A). The resulting GlcNAc-PI is *N*-deacetylated to generate GlcN-PI that subsequently flips to the luminal side of the ER. The C2 position of inositol is subsequently palmitoylated by mammalian PIG-W. This biosynthetic step is crucial since the amount of GPI-anchored proteins decreases dramatically in PIG-W-deficient cells.[48] It is believed that the lipid portion of GlcN-PI is then exchanged from diacyl- to alkylacyl-glycerol, since most mammalian GPIs and their intermediates bear 1-alkyl-2-acylglycerol.[49] Mannose and PEtN residues are added sequentially prior to attachment of the GPI anchor to the protein.

After attachment of the GPI anchor to protein, the palmitate on inositol and the second PEtN are often removed from the GPI-anchored protein. The molecule is then transported to the Golgi apparatus. Both hydrolysis reactions are important for efficient transport of GPI-anchored proteins from the ER to the Golgi apparatus.[50] Nevertheless, the presence of palmitate and additional PEtN residue in many mammalian GPIs (Table 1, entries 18–23) indicates that these two steps are not obligatory.

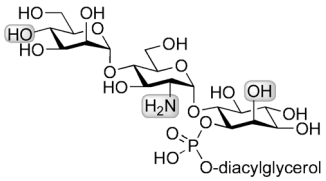
In the Golgi apparatus, the unsaturated acyl chain of the *sn-2* position is first removed,[51] and a saturated acyl chain, usually a stearic acid, is transferred to the *lyso*-GPI.[52] This lipid structure of GPI anchors is critical for association to membrane microdomains.[53] GPI-anchored proteins bearing two saturated lipids are then transported to the cell membrane.

The GPI biosynthesis in *T. brucei* exhibits several distinct features when compared to the biosynthesis in mammalian



Peter H. Seeberger (BS University Erlangen-Nürnberg, Ph.D. University of Colorado) was a postdoctoral fellow at Sloan-Kettering Institute in New York (1995–1997) and an assistant and tenured associate professor at MIT from 1998 to 2003. After a professorship at the ETH Zurich from 2003 to 2009, he became the director at the Max Planck Institute of Colloids and Interfaces and professor at the Free Univeristy of Berlin, honorary professor at the University of Potsdam, and affiliated Professor at the Sanford-Burnham Medical research institute.

cells.[24] Inositol acylation occurs after transfer of the first mannose to GlcN-PI, and no PEtN is added to the ManI or ManII during the biosynthesis (Scheme 3B). Recent studies with synthetic Man_2-GlcN-PI analogues showed that the presence of the C4 hydroxyl group of ManI is essential for inositol acylation (Scheme 4), which explains why mannosylation occurs prior to inositol acylation in the trypanosomal pathway.[54] Fatty acid remodeling in trypanosomes occurs before the GPI is attached to the protein, whereas mammalian and yeast lipid remodeling occurs after the attachment of GPI to the protein.



Scheme 4. Summary of the features recognized by the inositol acyl transferase.[54] Functional groups required for inositol acylation are highlighted.

In the protozoan parasite *P. falciparum*, inositol acylation of GlcN-PI precedes mannosylation. This is also the case in mammalian cells (Scheme 3C), albeit with only one PEtN group addition to ManIII before protein attachment. One distinct feature of this pathway lies in the dolichol phosphate mannose synthase (DPM) that catalyzes the synthesis of the mannose donor for GPI synthesis and *N*-glycosylation. *P. falciparum* DPM is neither able to complement a *Saccharomyces cervisiae* mutant nor a mouse mutant deficient in this enzyme, indicating the presence of significant difference between these genes.[55]

GPI biosynthesis in *T. gondii* is distinct from other organisms in several aspects (Scheme 3D). In mammalian cells, the transfer of GlcNAc to diacyl-PI is catalyzed by GPI-GlcNAc transferase, a multi-subunit enzyme with seven components (PIG-A, PIG-C, PIG-H, PIG-P, PIG-Q, PIG-Y, and DPM2). However, after searching the *Toxoplasma* genome for homologues of known *P. falciparum*, human and yeast GPI biosynthetic genes, no homologue of mammalian PIG-H, PIG-P, and DPM2 genes was found in *T. gondii*.[56]

Additional saccharide(s) that constitute the branch at C3 or C4 position of the ManI, such as the GalNAc residue in GPI anchors of Thy-1 and CD59 or α-galactosides in GPI anchors of the variant surface glycoprotein (VSG) of *T. brucei* are thought to be added after transfer of GPI to protein.[24] However, the GalNAc residue in *T. gondii* GPIs is added before transfer of GPI to protein.[56] In fact, addition of the GalNAc residue here is believed to precede the addition of PEtN, since the PEtN group is not present in all isolated *T. gondii* GPIs.[35]

4. Chemical Synthesis of GPIs

GPIs are among the most complex classes of natural products as they combine lipids, carbohydrates, and phosphate groups. This challenge, coupled with the ever-increasing biological importance of this class of glycoconjugates constitutes the main driving force for developing strategies toward efficient syntheses of GPI molecules. To date, a number of GPIs has been prepared by using various glycosylation and protecting group strategies (Table 2). Most of the syntheses began with glycan assembly, which were followed by the installation of phosphate groups and final deprotections. Here we summarize the progress in the chemical synthesis of GPIs, particularly in the context of fundamental challenges such as synthetic convergency, the incorporation of unsaturated lipids, and the synthesis of GPI-anchored proteins. Interested readers are also referred to comprehensive reviews focusing on chemical synthesis of GPIs.[57]

4.1. Synthesis of Native Lipidated GPIs

In the context of multistep synthesis, convergent strategies are more efficient than linear syntheses in which the overall

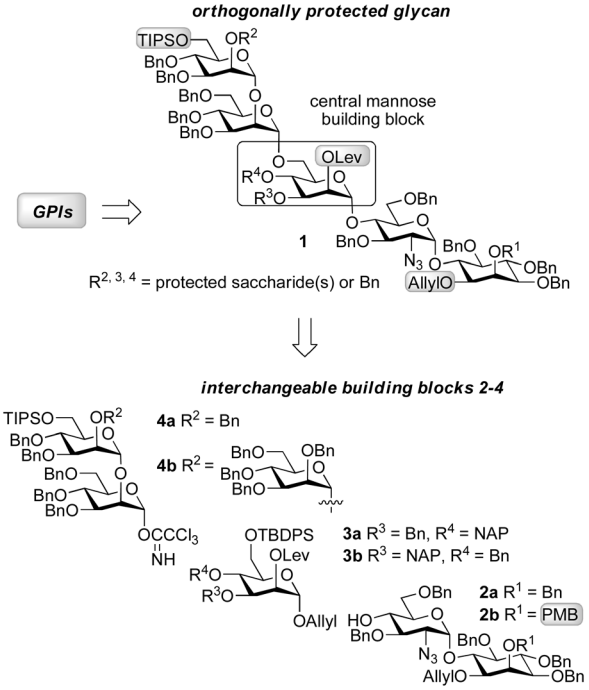
Table 2: Total syntheses of various GPIs.[a]

Entry	Origin	R^1	R^3	R^4	R^6	R^7	R^8	Lipid	Group [Year]
1	*T. brucei* VSG	H	H	$Gal_2\alpha$	H	H	H	DAG	Ogawa (1991)[59] Ley (1998)[60]
2	*T. cruzi* 1G7	Manα	H	H	H	H	H	AAG	Vishwakarma (2005)[61]
3	*T. cruzi* trypomastigote	Manα	H	H	H	AEP	H	AAG	Nikolaev (2006)[62]
4	*T. gondii*	H	GalNAcβ	H	H	H	H	DAG	Seeberger (2005)[63]
5	*T. gondii*	H	Glcα1-4GalNAcβ	H	H	H	H	DAG	Seeberger (2011)[58]
6	*P. falciparum*	H	H	H	H	H	myristoyl	DAG	Fraser-Reid (2004)[64]
7	*P. falciparum*	Manα	H	H	H	H	palmitoyl	DAG	Seeberger (2005)[65]
8	*P. falciparum*	Manα	H	H	H	H	palmitoyl	DAG	Vishwakarma (2010)[66]
9	yeast	Manα	H	H	H	H	H	ceramide	Schmidt (1994)[67]
10	rat brain Thy-1	Manα	GalNAcβ	H	PEtN	H	H	AAG	Fraser-Reid (1995)[68] Schmidt (2003)[69]
11	human CD52	H	H	H	PEtN	H	palmitoyl	AG	Guo (2007)[70]
12	human lymphocyte CD52	Manα	H	H	PEtN	H	H	DAG	Guo (2012)[71]

[a] The positions of the residues R^1–R^8 and of the lipid in which $R^2 = R^5 = H$, mirror those in Scheme 2. Abbreviations are in accordance with those in Table 1.

yield quickly drops with each reaction step. Most GPI syntheses reported to date are linear and target-oriented. As a result, these syntheses are not able to easily accommodate modifications that would produce analogues or other GPI anchors. To unravel the functional significance of different GPI modifications, a general unifying route that enables efficient access to a wide range of GPI anchors is essential. This goal has been accomplished recently.[58]

This general approach is based on the construction of orthogonally protected glycans **1** from a group of common building blocks **2**–**4** (Scheme 5) and additional carbohydrates

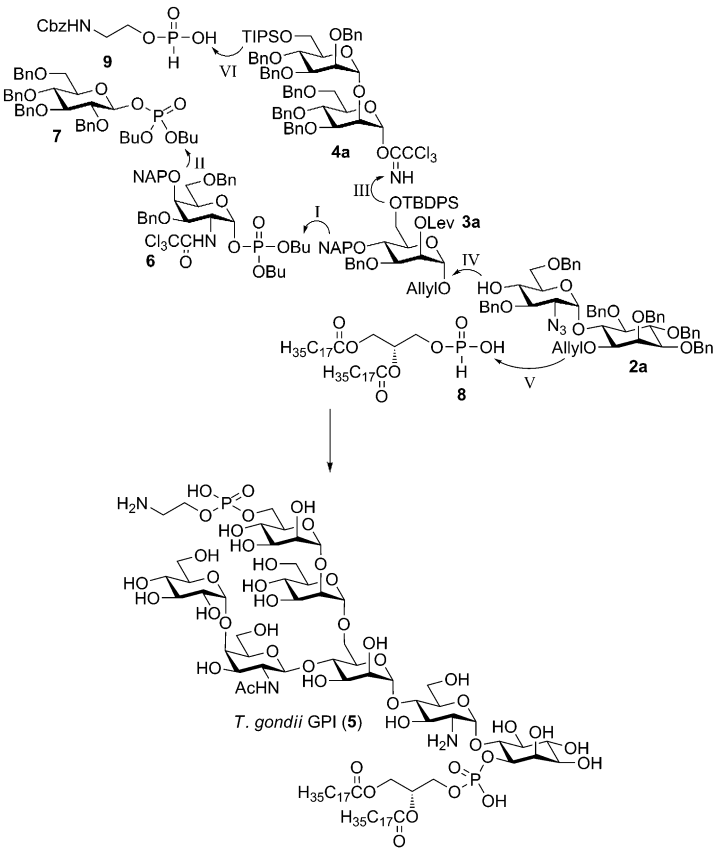


Scheme 5. General retrosynthetic analysis for GPI anchors designed by Seeberger and colleagues.[58] Orthogonal protecting groups for late-stage modifications are highlighted. Bn = benzyl, Lev = levulinoyl, NAP = 2-naphthylmethyl, PMB = *p*-methoxybenzyl, TIPS = triisopropylsilyl.

which are necessary for the formation of branched structures. Since the preparation of building blocks is considered to be the most labor intensive and time-consuming phase in oligosaccharide synthesis, applying a set of interchangeable building blocks saves time and increases synthetic throughput.

The protecting group pattern in glycan **1** allows for rapid installation of different modifications. Structures with acylated inositol and different phosphorylation patterns are readily accessible using this strategy. Different GPI anchors and derivatives suitable for biological assays and physical measurements can be efficiently prepared in this fashion.

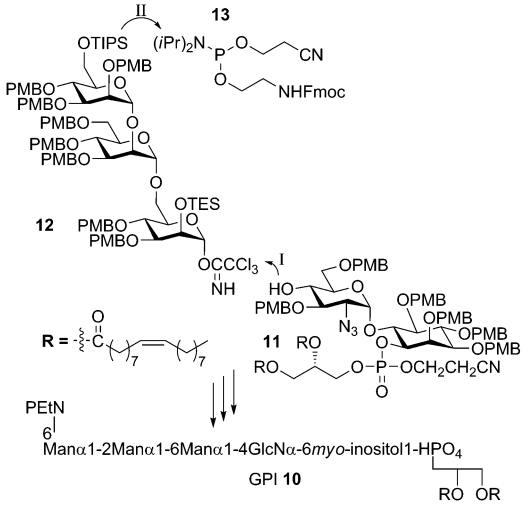
This strategy served for the synthesis of the low molecular weight antigen of *T. gondii* (Scheme 6).[58] A trisaccharide subunit was first assembled from building blocks **3a**, **6**, and **7** (via glycosylations I and II, Scheme 6). Further coupling with dimannosyl imidate **4a** and pseudodisaccharide **2a** in a [2+3+2] glycosylation sequence furnished the full glycan.



Scheme 6. Retrosynthetic analysis of the low-molecular-weight antigen of *T. gondii*.[58] Roman numerals indicate the assembly sequence. Cbz = benzyloxycarbonyl, TBDPS = *tert*-butyldiphenylsilyl.

Phosphorylations with H-phosphonates **8** and **9**, followed by global deprotection afforded the low molecular weight antigen of *T. gondii* (**5**).

Benzyl groups are commonly employed for permanent hydroxyl protection in oligosaccharide and GPI syntheses, thereby precluding the incorporation of unsaturated fatty acids and other reductive-sensitive functional groups, such as azides and alkynes, as chemical handles. It was proposed that unsaturated fatty acids were responsible for potent proinflammatory responses of isolated *Trypanosoma cruzi* GPI, a molecule that is as active as a bacterial lipopolysaccharide (LPS).[73] To address this issue, Nikolaev and co-workers developed synthetic routes for the synthesis of *T. cruzi* GPIs bearing an unsaturated fatty acid using either base-labile or acid-labile permanent protecting groups.[57a] In a similar effort, the Guo group used *p*-methoxybenzyl (PMB) ethers for permanent hydroxyl protection and accomplished the synthesis of GPIs bearing unsaturated fatty acids (**10**, Scheme 7).[72] One of the key features in this synthesis is the use of phosphorylated pseudodisaccharide **11** to minimize late-stage protecting group manipulations, since phosphate groups are normally installed at a late stage, before global deprotection. Here, removal of PMB groups was achieved with 10% trifluoroacetic acid in dichloromethane, instead of oxidative conditions. GPIs bearing an azide or alkyne handle[74] as well as the GPI of human lymphocyte CD52 were prepared by using this strategy.[71]



Scheme 7. Synthesis of a GPI anchor bearing unsaturated lipids.[72] Roman numerals indicate the assembly sequence. Fmoc = 9-fluorenylmethoxycarbonyl, TES = triethylsilyl.

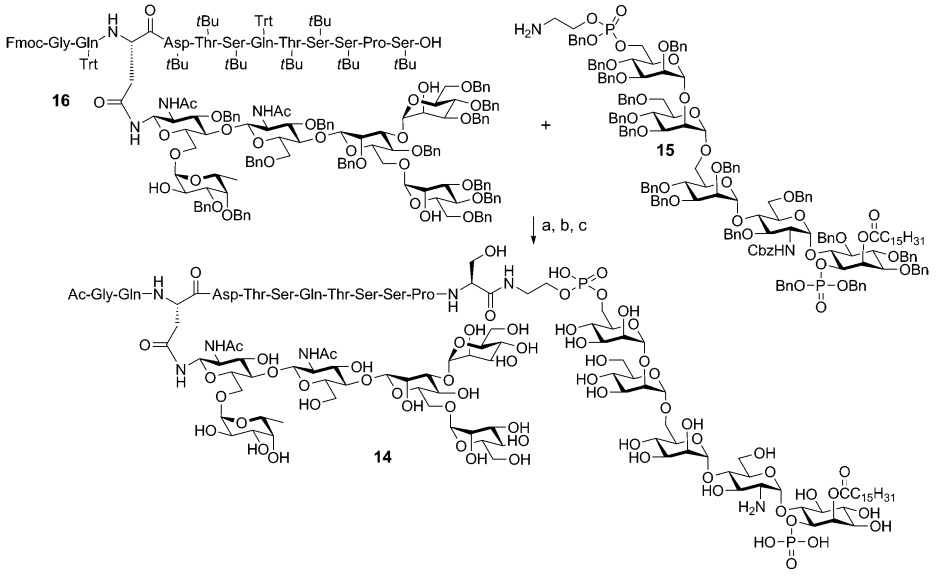
4.2. Synthesis of GPI-Anchored Proteins

Anchoring proteins to the plasma membrane is one of the primary functions of GPIs. This post-translational modification has profound effects on protein function.[75] In contrast to the numerous reports on the synthesis of GPI glycans and native lipidated GPIs, the synthesis of a GPI-anchored protein is much more challenging. One has to not only incorporate a peptide chain in the glycan backbone, but also ensure the synthetic GPI-anchored protein is properly folded to retain the function of its natural counterpart.

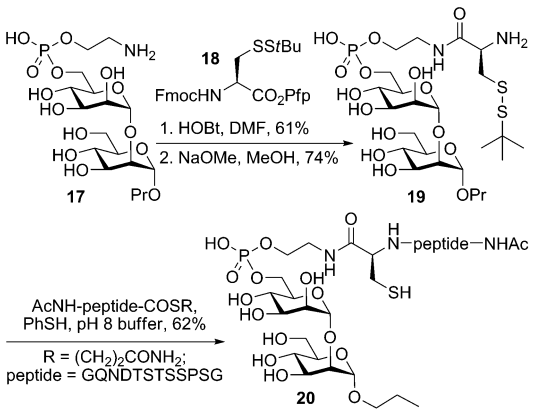
The Guo group reported a chemical synthesis of a skeleton structure of sperm CD52 **14**,[76] a GPI-anchored glycopeptide containing 12 amino acids and an *N*-glycan (Scheme 8). Protected GPI glycan **15** and protected glycopeptide **16**prepared by solid-phase peptide synthesis, were directly coupled. Following deprotection, the synthetic molecule **14** lacks only a phosphoethanolamine group and the phospholipid when compared to native CD52. However, since most proteins consist of more than 12 amino acids, this approach may not be applicable for other, larger GPI-protein targets. On the other hand, coupling of a protected GPI to a native protein may be difficult due to solubility issues and conditions for deprotection that might denature or otherwise harm the protein.

Direct coupling of unprotected peptides and GPIs was first explored by the Nakahara group, which reported the ligation of amino acids and peptides to an unprotected dimannoside GPI fragment **17** (Scheme 9).[77] Taking advantage of the higher reactivity of the amino group in **17** towards acylation, treatment of **17** with amino acid pentafluorophenyl (Pfp) esters **18** resulted in the desired *N*-acylated product **19** in 60% yield with some *O*-acylated byproducts. A model dodecapeptide with the *C*-terminal thioester was coupled to **19** via the native chemical ligation (NCL) protocol. Treatment of **19** with dodecapeptide thioester in the presence of thiophenol gave **20** in 62% yield after ten days. Bertozzi and colleagues also applied NCL to synthesize analogues of GPI-anchored green fluorescent protein (GFP).[78] GPI analogues bearing a cysteine residue and a phospholipid were coupled to recombinant GFP (Scheme 10). These results indicate that the NCL is potentially useful for addressing the challenge of GPI-anchored protein synthesis.

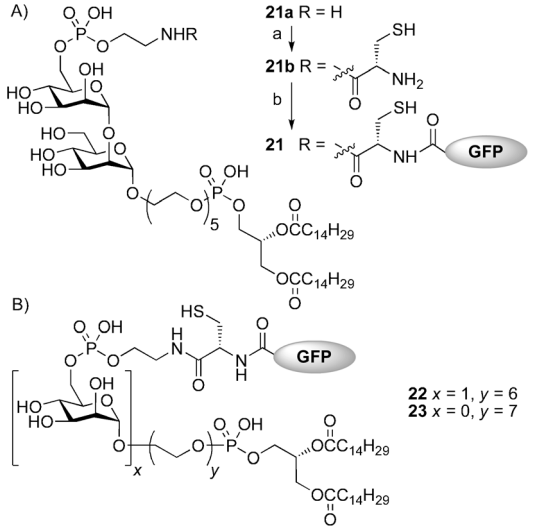
Recently, synthesis of a GPI-anchored prion protein was achieved relying on late stage NCL (Scheme 11).[79] The judicious choice of a protecting group strategy to incorporate cysteine into the GPI glycan was essential, since thiols and thioethers, in general, can poison heterogeneous palladium catalysts necessary for global hydrogenolytic removal of benzyl ethers.[80] A GPI anchor bearing a cysteine moiety (**24**) was synthesized from a per-*O*-benzylated precursor after palladium-catalyzed hydrogenation. Ligation of a recombinant prion protein (rPrP) containing a *C*-terminal thioester with **24** afforded GPI-anchored PrP **25**, the most complex GPI-anchored protein synthesized to date. The GPI-anchored PrP **25** was recognized by a PrP-specific antibody when folded properly in aqueous solution, and was incorporated into liposomes via its phospholipid anchor.[79]



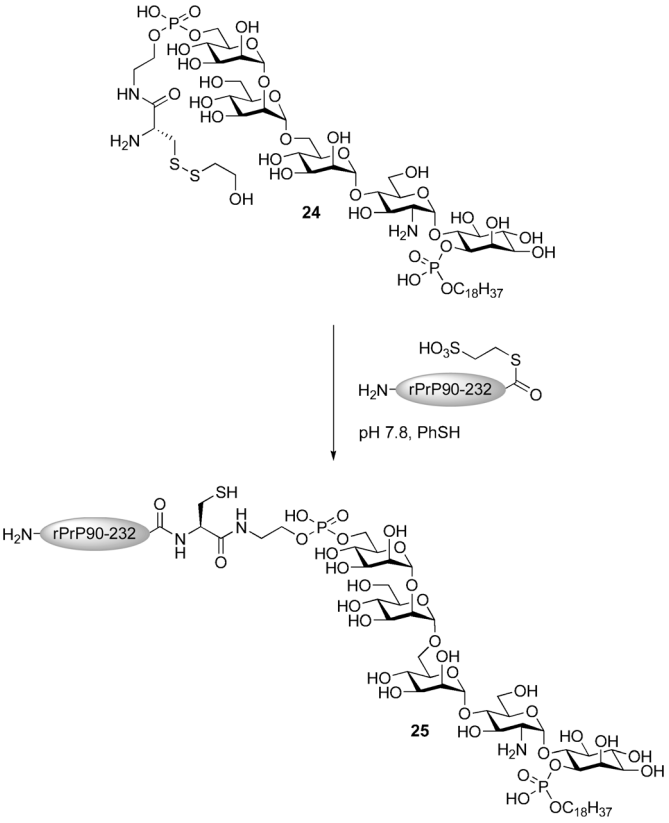
Scheme 8. Synthesis of a GPI-anchored CD52.[76] Reagents and conditions: a) HOBt, EDC, CH_2Cl_2/NMP (2:1), 70%; b) 10% Pd/C, H_2, $CHCl_3$/MeOH/H_2O (10:10:3); c) TFA/Et_3SiH/CH_2Cl_2 (2:3:15), 85% over two steps. *t*Bu = *tert*-butyl, EDC = *N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride, HOBt = 1-hydroxybenzotriazole, Me = methyl, NMP = *N*-methylpyrrolidinone, TFA = trifluoroacetic acid, Trt = trityl.



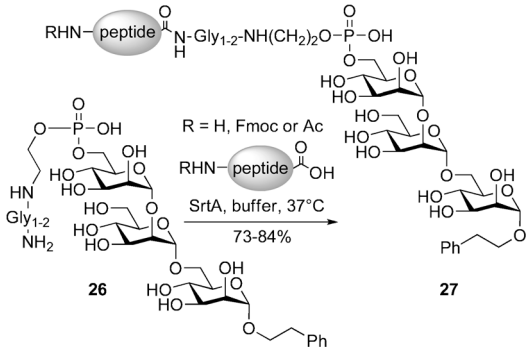
Scheme 9. Synthesis of a GPI-anchored peptide.[77] DMF = *N*,*N*-dimethylformamide, Pfp = pentafluorophenyl, Ph = phenyl, Pr = propyl.



Scheme 10. Structure and synthesis of GPI-anchored GFPs **21**-**23**.[78] A) Synthesis of GFP analogue **21**: a) 1. Boc-Cys(Trt)-OPfp, DIPEA, CH_2Cl_2, MeOH; 2. 3:3:15:79 TIS:EDT:TFA: CH_2Cl_2, 66%; b) GFP-MESNa, octyl β-D-glucopyranoside, MESNa, 59%. Boc = *tert*-butoxycarbonyl, EDT = 1,2-ethanedithiol, DIPEA = diisopropylethylamine, MES = 2-mercaptoethanesulfonate, TIS = triisopropylsilane. B) Structure of GFP analogues **22** and **23**.



Scheme 11. Synthesis of a GPI-anchored prion protein.[79]



Scheme 12. SrtA-catalyzed GPI-peptide ligation.[81b,c]

Covalent coupling of peptides and GPI glycans can also be performed enzymatically.[81] Sortase A (SrtA), a transpeptidase with broad substrate tolerance, has been reported to catalyze ligation of different glycopeptides with a ManI-ManIII GPI fragment **26** in good yields (Scheme 12). Although promising, this approach may suffer from regioselectivity issues when native GPI anchors that bear two to four amino groups are used.

4.3. Challenges and Perspectives in GPI Synthesis

With current state-of-the-art techniques, the chemical synthesis of GPIs is largely feasible, as demonstrated by a recently developed general strategy.[58] Nevertheless, a typical biomedical laboratory in need of synthetic GPIs normally falls short of the non-trivial synthetic skills and technical setup required to access these molecules. Automated solid-phase synthesis of oligosaccharides provides rapid access of divergent chemical tools[82] and is an ideal solution to this problem. GPIs, however, have not yet been prepared by automated solid-phase synthesis.[83]

Despite numerous syntheses of lipidated GPIs, only a few incorporate unsaturated lipids into synthetic material. Preparative HPLC is often used for purification at several stages and the final transformations were performed on less than one milligram of material.[71,72,74] These facts highlight the experimental difficulties associated with the synthesis of GPI anchors with functional groups sensitive to hydrogenolysis.

Therefore, a scalable synthesis of GPI anchors containing unsaturated lipids remains a challenge.

The synthesis of GPI-anchored proteins can provide tools to understand many complex biological problems. Although GPIs have been implicated in the development and propagation of prion diseases, their specific role remains elusive.[84] A synthetic, homogeneous GPI-anchored PrP would provide insight into this controversial field. However, synthesis of a homogeneous GPI-anchored protein in its natural state remains an unsolved problem.

5. Synthetic GPIs as Tools in Chemical Biology

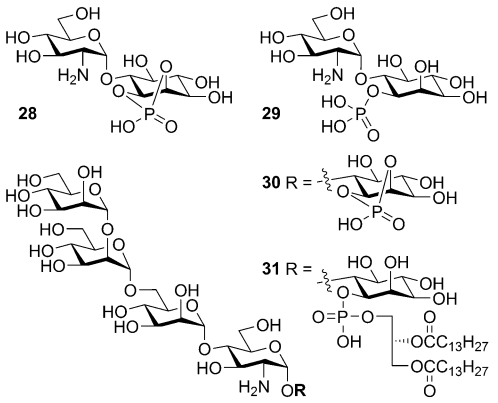
The aim of chemical syntheses of GPIs is not only to showcase the power of organic synthesis, but also to provide defined chemical tools to unravel the biological functions of this class of molecules. Besides several total syntheses of native GPI anchors, numerous smaller, truncated versions of GPIs have been prepared and used to address biological questions.

5.1. Conformation of GPIs

Insights into the three-dimensional structure of a carbohydrate molecule are indispensable to obtain full understanding of the molecular recognition processes involving carbohydrates, such as protein–carbohydrate interactions. A detailed understanding of GPI structures and dynamics in solution is expected to unravel certain unexpected functions of this class of molecules, especially the roles of different modifications on the core pseudopentasaccharide that is otherwise difficult to observe in biological assays.

A combination of computational methods and other techniques has been used to determine the conformation and dynamics of several GPI-anchored proteins. The GPI anchor was found to affect the conformation of Thy-1,[85] but has little influence on that of PrP.[86] In trypanosome VSG, the GPI glycan containing a galactoside side chain closely associates with the protein and fills the space between the protein and the membrane,[87] indicating that the GPI glycan is an integral part of GPI-anchored proteins.[88] However, such a function of the side chain may not be ubiquitous. For example, 30% of the PrP GPI contains a terminal sialic acid residue,[39] which is unlikely to be in direct contact with the negatively charged plasma membrane as in the case of VSG.

A detailed investigation of GPI conformation relies on the availability of GPI glycan fragments. Three-dimensional structures of synthetic GPI glycans ranging from di- to pentasaccharides were investigated by using X-ray crystallography and NMR spectroscopy (Scheme 13).[89] The dynamic properties of these molecules in solution were studied by molecular dynamics calculations. Pseudodisaccharides **28** and **29** exist in one major conformer, in which the charged ammonium and phosphate groups appear close in space.[89b] However, it was not possible to define a unique conformation for **30**, which exists in an extended conformation that undergoes large torsional oscillation about the Manα1→6Man linkage with fluctuations of hydroxyl groups on a picosecond timescale.[89c] Studies of lipidated GPI anchor **31** incorporated into micelles revealed that the structural properties of the glycan are similar to those of **30**,[89d] in addition to the complex dynamic behavior with a faster relative motion at the terminal mannose residue and decreased mobility close to the micelle.[89e] This result implies that the intrinsic behavior of GPI anchors does not change through the vicinity of the lipid–water interface, and molecular dynamics might be a suitable tool for the prediction of the conformational behavior of GPIs in solution.



Scheme 13. Synthetic GPIs for conformational studies.

So far, the effect of phosphorylations on GPI conformation has not been addressed. All mammalian GPIs bear an additional PEtN residue at the C2 position of the ManI. It is likely that phosphorylation imposes certain constraints on the glycan conformation and results in a different conformation in mammalian GPIs, although such a hypothesis has not been tested yet.

5.2. GPIs as Immunomodulators

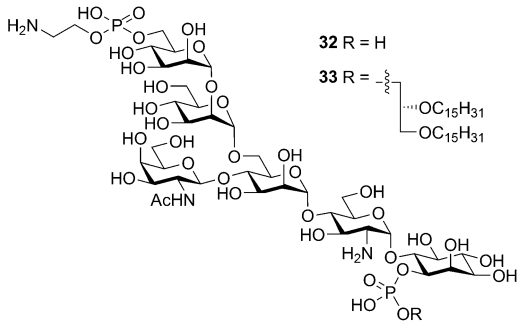
Immunomodulators are becoming increasingly popular in pharmaceutical companies for the treatment of various diseases, and new drugs of this class have been developed.[90] An immunomodulator can alter the immune response by augmenting or reducing the ability of the immune system to produce antibodies or sensitized cells, such as natural killer T (NKT) cells and macrophages that recognize and react with appropriate antigens.[91]

GPIs are recognized by NKT cells.[92] The resulting stimulation provides a rapid immune response to invasive parasitic pathogens. A synthetic Thy-1 GPI anchor[68b] can trigger an immune response in a non-MHC-restricted pathway (MHC = major histocompatibility complex).[92] This *CD1d*-mediated immune response in NKT cells was confirmed in a GPI-based vaccination against *T. brucei*, in which the protective effect was abrogated in $CD1d^{-/-}$ mice.[93] However, the stimulation of NKT cells by synthetic Thy-1 GPI suggests that GPI-mediated NKT cell activation may play a significant role in autoimmune diseases.[92]

GPIs can also trigger macrophages to produce cytokines,[94] as investigated in the context of *T. gondii* patho-

genesis. Infection with *T. gondii* is estimated to affect almost two billion people worldwide.[95] Initial infection with *T. gondii* during pregnancy causes congenital infectious diseases that might lead to fetal death.[96] *T. gondii* is also responsible for severe encephalitis, a major cause of death among immunodeficient people, such as AIDS patients.[97]

Studies using isolated and synthetic *T. gondii* GPIs have revealed the detailed signaling pathway in the GPI activation of macrophages. Synthetic GPIs **32** and **33** share the same glycan structure (Scheme 14),[98] but differ in the lipid moiety.



Scheme 14. Synthetic *T. gondii* GPI analogues **32** and **33** for TNF-α production in macrophages. GPIs purified from *T. gondii* tachyzoites exist as a mixture bearing diacylglycerol as the lipid moiety, which is predominantly palmitic and stearic acids. Additionally, a αGlc residue can be found at the C4 position of GalNAc, while in some structures the PEtN group is absent.

Whereas GPI **33** bears a non-natural dialkylglycerol, GPI **32** is not lipidated. Both isolated GPI and **32** induce TNF-α production in macrophages by activation of the transcription factor NF-κB.[99] These compounds act via a toll-like receptor (TLR), and the protein MyD88 is involved in this process.[100] In addition, galectin-3 binds strongly to **32** and is critical for the production of TNF-α induced by *T. gondii* GPIs in macrophages.[101] Interestingly, the lipidated synthetic GPI **33** does not induce but inhibits production of TNF-α in macrophages and activation of NF-κB induced by isolated *T. gondii* GPIs.[99]

GPIs can act on both NKT cells and macrophages to trigger an immune response.[102] The GPI glycan without the lipid domain is not sufficient for generating *CD1d*-restricted immunoglobulin G (IgG) responses by NKT cells.[92] In contrast, the GPI glycan is sufficient to induce TNF-α production in macrophages. Therefore, it would be of great interest to determine the minimum oligosaccharide construct required to induce an immune response in macrophages and test whether mammalian GPIs, namely GPIs with an extra PEtN on ManI, have similar effects.

5.3. The Malaria Toxin—A GPI

Malaria is a devastating parasitic disease that affects 40% of the world's population and claims more than two million lives each year; mostly young children in developing countries.[103] Transmitted by female *Anopheles* mosquitoes, the disease is caused by the protozoan parasite of the genus *Plasmodium*. Of the four known species of *Plasmodium* that infect humans, *P. falciparum* accounts for the majority of infections and leads to the most severe forms of the disease.

Significant amounts of GPIs are present on the cell surface of *P. falciparum*, both in the protein-linked and protein-free form. In fact, GPIs are the main carbohydrate components on the surface of *P. falciparum*, whereas *N*- and *O*-linked glycans or other glycolipids are present at very low levels.[104] Schofield and Hackett proposed a GPI-related structure as the toxin of *P. falciparum* based on the fact that administration of isolated *P. falciparum* GPIs to mice induces syndromes similar to acute malaria, including the release of pro-inflammatory cytokines, pyrexia, hypoglycemia, and eventually caused death.[105] The initial skepticism concerning the GPI-based malarial toxin theory was dismissed by immunological studies involving synthetic GPI glycans and the success of a GPI-based vaccine.[106]

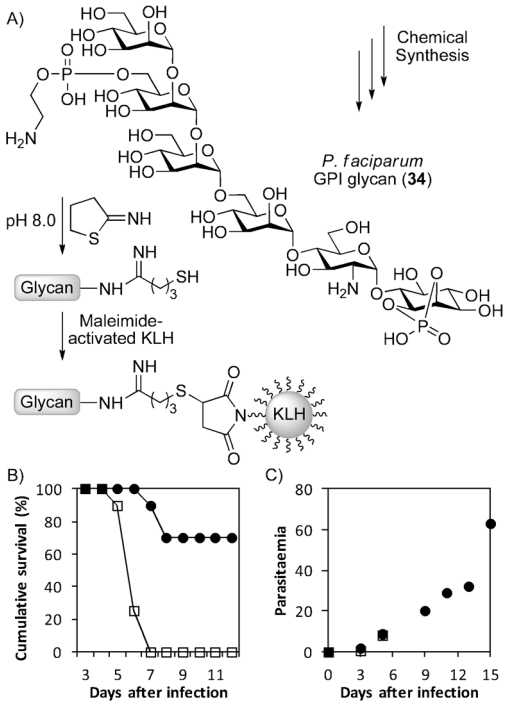
5.3.1. A Synthetic GPI as Antitoxic Malarial Vaccine Candidate

Vaccination is the ideal solution for reducing death due to malarial infections, however, a therapeutically useful malaria vaccine remains elusive despite intensive work in the field. Most of the vaccine candidates employ antigenic proteins or peptides present on the parasite surface as antigens.[107] These strategies, however, suffer from problems in generating appropriate protection. The anti-toxin vaccine represents an alternative approach that targets the main cause of host pathology.[108] Anti-toxin vaccines against tetanus and diphtheria have been highly successful in humans.

A consensus *P. falciparum* GPI glycan (**34**), derived from chemical and enzymatic hydrolysis of the native GPI and non-toxic itself, was synthesized.[108,109] The synthetic GPI glycan was further modified with 2-iminothiolane and conjugated to a maleimide-activated keyhole limpet hemocyanin (KLH) that acts as a carrier protein (Scheme 15A). The GPI glycan–KLH conjugate is immunogenic in rodents.[108] Antibodies from KLH–glycan immunized animals gave positive IgG titers and selectively recognized the intact parasite, but not uninfected erythrocytes, due to the structural differences between human and parasitic GPIs. Furthermore, mice treated with the synthetic GPI–KLH conjugate had much lower mortality rates, and 75% of the vaccinated mice ($n=16$) survived for two weeks after the parasitic challenge, whereas the sham-immunized mice all died within seven days (Scheme 15B). Interestingly, immunization of mice did not alter infection rates of the animals and overall parasitaemia, indicating that the anti-GPI antibody neutralized toxicity without killing the parasites (Scheme 15C).

These studies provide convincing evidence that the GPI glycan is a viable target for malaria vaccine development. Ancora Pharmaceuticals (Woburn, MA, USA) has developed synthetic strategies to access large quantities of the GPI glycan **34** and set the stage for clinical studies.[110]

GPI antigens were also used for vaccination against *T. brucei*.[93] Isolated GPIs incorporated into liposomes were injected into mice, and reduction of the infection-associated pathology was observed in the immunized group.[93] Besides *P.*



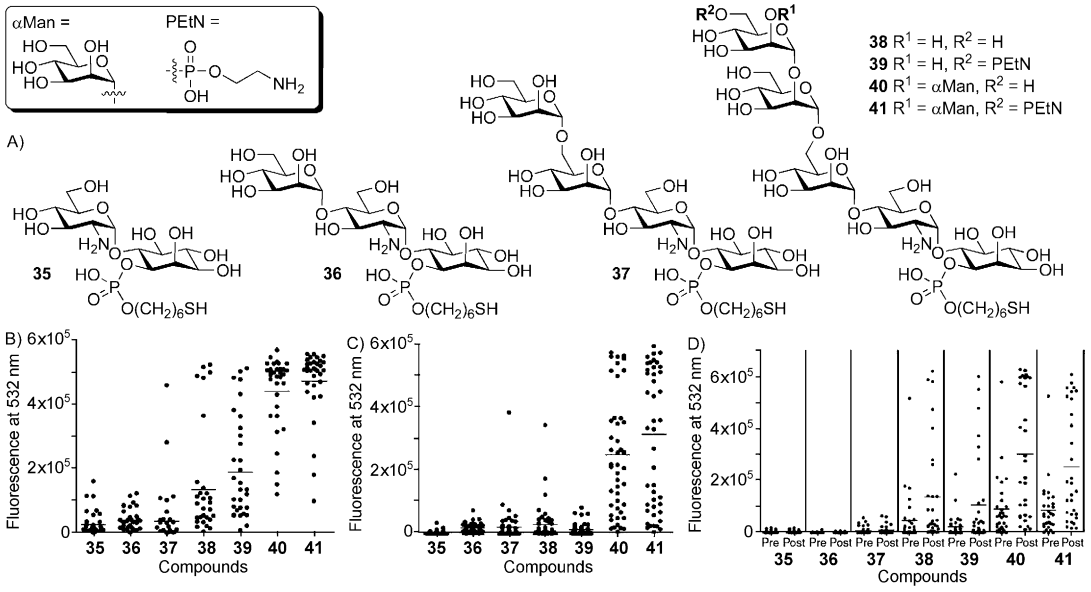
Scheme 15. Synthetic GPI glycan–KLH conjugate protects against murine cerebral malaria.[108] A) Synthesis of non-toxic *P. falciparum* GPI glycan and its KLH conjugate. B) Kaplan–Meier survival plots, and C) parasitaemias, up to two weeks post-infection, of KLH–glycan-immunized (closed circles) and sham-immunized (open squares) mice challenged with malaria parasite.

falciparum and *T. brucei*, many other parasitic protozoa also express large quantities of GPIs in comparison to mammals. Further synthetic work is needed to prepare the molecules found in these pathogens and probe the effectiveness of GPI-based vaccines for prevention of these infections.

5.3.2. *Synthetic GPI Microarrays To Define Antimalarial Antibody Response*

Immunization studies provided convincing evidence to corroborate that the GPI is the dominant toxin of *P. falciparum*. However, a central question concerning the association of altered risk of malaria in humans with anti-GPI antibodies remains to be clarified. Isolated *P. falciparum* GPI was employed to study acquired antibody responses to GPI in humans by enzyme-linked immunosorbent assays (ELISAs).[111] In some studies, a statistically significant association of an anti-GPI IgG response with protection against symptoms of severe malaria was reported,[111a,f] and in others no such relationship was observed.[111b,e] To precisely address this issue, a series of synthetic GPI glycans that are structurally related to *P. falciparum* GPI was prepared.[112] GPIs **35**–**41** (Scheme 16 A) contain a unique thiol linker that enables chemoselective functionalization.

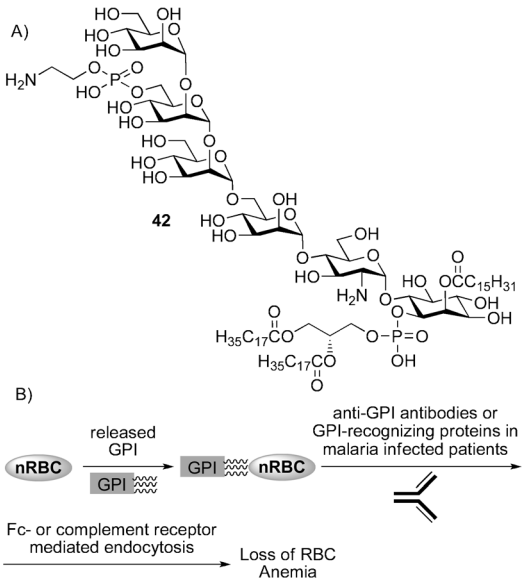
To study the anti-GPI antibody response in a high-throughput fashion, a GPI-based microarray was designed.[113] This microarray enables the screening of several thousand binding events in parallel with a minimal amount of sera and ligand required.[114] To define the minimal length of the glycan structure required to induce an immune response as well as a specific anti-GPI response strictly related to malarial infection, synthetic GPI fragments **35**–**41** were covalently attached to maleimide-activated microarray slides.[113] It was initially observed that the sera from malaria-exposed African



Scheme 16. Synthetic GPI microarrays for studying antitoxic malarial responses. A) Chemically synthesized *P. falciparum* GPI-related glycans **35**–**41** immobilized on microarrays for studying anti-GPI antibody response. B) IgG levels against synthetic GPI **35**–**41** in sera from cohorts of African donors living in a malaria endemic area, and C) in Europeans not exposed to malaria. Each data point represents one serum; bars indicate the mean antibody level. D) Increase in mean serum IgG levels against synthetic GPI compounds after experimental *P. falciparum* sporozoite challenge of Europeans not exposed to malaria. Sera were taken prior to (pre) or after experimental challenge (post). Each data point represents one serum; bars indicate the mean level.

adults had high-IgG levels against GPIs **38**–**40** (Scheme 16B). Surprisingly, most sera of malaria-unexposed European adults also contained antibodies that bind to **40** and **41** (Scheme 16C) indicating that the Man_3-GPI (**38** and **39**) response might represent a more specific antimalarial GPI response. To further confirm that the anti-Man_3-GPI responses are primarily elicited by malarial parasites, sera from European volunteers involved in a sporozoite challenge vaccine trial were examined. Indeed, the development of a significant anti-Man_3-GPI antibody response was observed. A follow-up study using this GPI microarray showed that anti-GPI antibodies are rarely found in children under 18 months. This result indicates the vulnerability of infants to severe malaria and suggests that a GPI-based vaccine will be clinically important for children.[115]

The approach using synthetic GPI-based microarrays provided more detailed insights than those obtained from isolated material because well-defined glycans were used as probes together with fragments that are not naturally occurring on the cell surface. Anti-GPI antibody levels measured with isolated GPI from *P. falciparum*, critically depend on the ratio of Man_3- and Man_4-GPI in the antigen preparations.



Scheme 17. Involvement of GPIs in malaria-associated anemia. A) Chemically synthesized *P. falciparum* GPI anchor **42**. B) Freely circulating or released *P. falciparum* GPI inserts into nRBCs and results in the recognition by anti-GPI antibodies that may contribute subsequent RBC eliminations.

5.3.3. Synthetic GPIs as Tools To Study Malaria-Associated Anemia

Anemia is one of the world's leading causes of disability and represents a serious global public health problem.[116] One major cause of anemia is the infection with *P. falciparum*.[116] Severe anemia is the most prevalent complication of malarial infections and contributes substantially to morbidity and mortality.[116] In malaria-infected patients the rate of infection of red blood cells (RBCs) is normally lower than 5%, whereas the degree of RBC loss rises between 25% and 50%, indicating the destruction of a great number of non-parasitized RBCs (nRBCs) during malarial infection.[117]

T. brucei VSG with an intact GPI anchor can transfer from parasite to erythrocytes in vivo,[118] and human GPI-anchored CD55 can transfer from erythrocytes onto parasites in vivo.[119] On the other hand, GPI-anchored proteins can be found under physiological conditions in solution with intact lipid where they exchange among cells.[120] These observations led to a hypothesis that *P. falciparum* GPIs may insert into the RBC membranes leading to attack of those cells by the immune system and lysis of the altered cells (Scheme 17).

A synthetic lipidated *P. falciparum* GPI (**42**)[65] was used to explore the association with naïve nRBCs. The detection of GPI insertion into RBC was monitored by anti-GPI antibodies present in a malaria patient, followed by flow-cytometric analysis. The results strongly support the notion that released *P. falciparum* GPI can insert into nRBC membranes which results in recognition by circulating anti-GPI antibodies and possible elimination.[121] This process might contribute to malaria-associated anemia. However, more detailed investigations are required before a definitive conclusion can be drawn. For example, in vivo administration of GPI-incorporated liposomes may provide more insights into this field. Since anemia is also one of the major symptoms in African trypanosomiasis, an improved knowledge of malaria-associated anemia may provide novel tools to combat trypanosomiasis as well.

5.3.4. Perspectives of Synthetic GPIs in Parasitology

Many questions concerning the malarial GPI remain open, such as the molecular receptor of parasitic GPIs. Toll-like receptors have been often implicated in this process, but it is unknown if other receptors are involved as well. It is also unclear if all parasitic GPIs act in the same manner. Galectin-3, for instance, was considered crucial for the recognition of *T. gondii* GPIs by macrophages,[101] but it is unlikely that galectins play any significant role in the recognition of *P. falciparum* GPIs that bear no galactoside. Synthetically well-defined GPI molecules are equipped to shed light on such uncertainties.

Synthetic GPIs with unique chemical tags allow facile conjugation to solid supports, such as affinity chromatography resins. A couple of proteins involved in GPI biosynthesis were identified by passing a crude parasite protein extract through a column with GPI **38** functionalized resins.[122] On the other hand, conjugation of synthetic GPIs to different crosslinking and fluorescent probes may also provide useful tools for biological research with respect to identification of interacting partners and visualization of molecules in cells.

5.4. GPI Biosynthesis as a Drug Target to Combat Trypanosoma brucei

As discussed in the Section 3, the GPI biosyntheses of protozoa and mammalian cells are different. Malfunction of the GPI biosynthetic pathway can be lethal for protozoa in

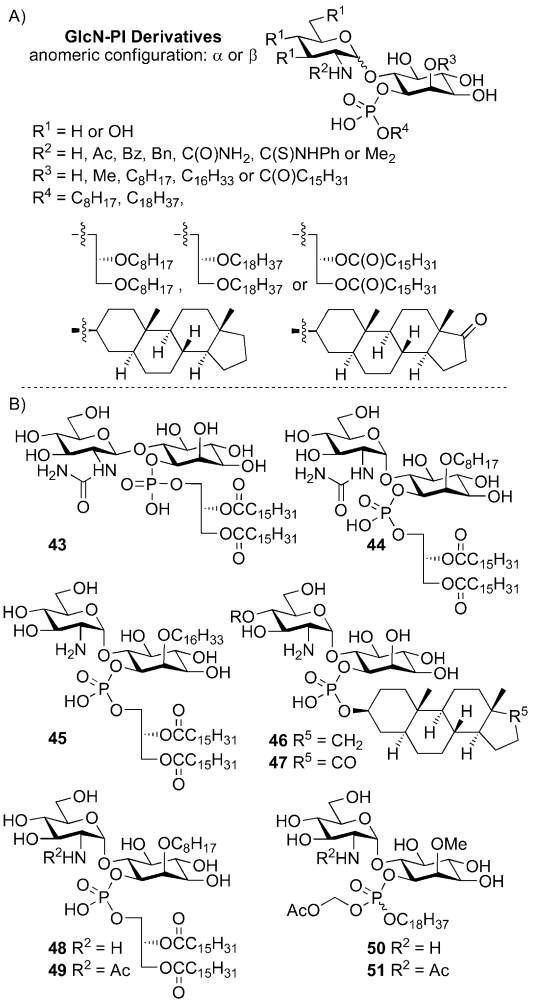
mammalian hosts.[23] Therefore, the selective inhibition of GPI biosynthetic pathways in protozoa may constitute an effective chemotherapeutic approach to treat parasitic infections.[24] Inhibitors for GPI biosynthesis in *T. brucei* have been most widely studied.

T. brucei causes African trypanosomiasis, more commonly known as the sleeping sickness in humans and nagana in animals. Sleeping sickness threatens millions of people in 36 countries in sub-Saharan Africa and it is fatal if untreated.[123] Animal trypanosomiasis causes huge economic losses in Africa, and limits the livestock production on the continent.[124]

Ferguson and colleagues have investigated the substrate specificities of *T. brucei* and human GPI biosynthetic enzymes using a set of GlcN-PI analogues.[125] The GlcN-PI analogues differ in the anomeric configuration, amino substituent, the absolute configuration, and substituent of the *myo*-inositol residue, as well as the nature of the lipid moiety (Scheme 18 A). These diligent efforts identified several compounds that specifically inhibit the *T. brucei* GPI biosynthetic pathway in vitro. Disaccharides **43** and **44** are two potent (IC_{50} = 8 nM) *T. brucei* specific GlcNAc-PI *N*-deacetylase suicide substrate inhibitors (Scheme 18 B),[125c] **45** is an inhibitor of mannosyltransferase 1 (MT-1), whereas **48** and **49** are for inhibitors of inositol acyltransferase.[125b] Interestingly, steroidal GlcN-PI analogues **46** and **47** were efficiently mannosylated by the trypanosomal α-mannosyl transferases, but did not undergo inositol acylation or the addition of phosphoethanolamine.[125f] However, due to the negative charge on the phosphodiester group of the PI moiety, the aforementioned inhibitors are unable to penetrate the cell membrane to act on the living parasites. To solve this issue, two cell-permeable analogues (**50** and **51**) were designed.[125e] The negative charges in **50** and **51** are blocked with acetoxymethyl groups to give a neutral and esterase-labile phosphotriester. These molecules were shown to kill all trypanosomes in 6 h at 40 μM, whereas they have no noticeable effect on HeLa cell cultures at 100 μM over 72 h.[125d]

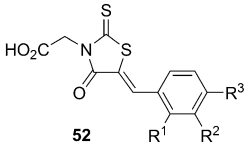
One major drawback of using carbohydrates as drug candidates is their poor pharmacokinetics, such as low oral availability and fast renal excretion.[126] To this end, drug-like small molecules, such as thiazolidinones, as inhibitors of dolichol phosphate mannose synthase and inositol-3-phosphate synthase have been studied (Table 3).[127] Both enzymes play crucial roles in *T. brucei* GPI biosynthesis. However, the best trypanocidal activities (ED_{50}) are only in the μM range. In addition, thiazolidinones have been shown to inhibit other enzymes.[128] Therefore, such inhibitors may not be selective to specific enzymes and are of limited use as drug leads.

Since the GPI biosynthetic machinery differs enough between humans and a wide range of eukaryotic pathogens, it represents a potential therapeutic target. Further in depth analysis of the specificity of the biosynthetic enzymes in humans and pathogens will allow the design of novel inhibitors. Lead inhibitors provide the opportunity to develop cheap, effective drugs for some of the most neglected diseases on the planet.

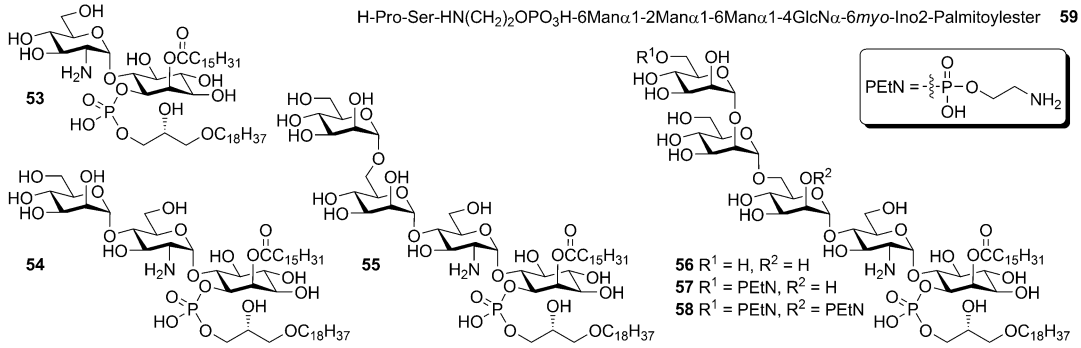


Scheme 18. Synthetic GlcN-PI library for studying the *T. brucei* GPI biosynthetic pathway. A) Structural variations in the synthetic GlcN-PI libraries. Bz = benzoyl. B) **43** and **44**: *T. brucei* GPI *N*-deacetylase inhibitors; **45**: *T. brucei* GPI MT-1 inhibitor; **46** and **47**: steroidal GlcN-PI analogues that inhibit the normal processing of GlcNAc-PI; **48** and **49**: *T. brucei* GPI inositolacyltrasferase inhibitors; **50** and **51**: cell-permeable GPI analogues that kill *T. brucei* parasites.

Table 3: Thiazolidinone inhibitors of *T. brucei* DPM.



Compound	R^1	R^2	R^3	Trypanocidal activity [ED_{50}, μM]
52a	H	H	H	232
52b	H	H	OBn	338
52c	H	OBn	H	96
52d	H	OBn	OBn	492
52e	OH	H	H	107
52f	H	H	OH	427
52g	H	OH	H	345
52h	H	H	CN	244
52i	H	H	Cl	398
52j	H	H	CCH	>1000



Scheme 19. Synthetic GPIs **53**–**59** for binding studies of the CAMP factor.

5.5. GPIs as Receptors for Bacterial Toxins

Similar to many mammalian cell surface glycoconjugates, GPI-anchored proteins can be exploited by pathogens for host invasion. It has been shown that GPIs are receptors for hemolytic pore-forming toxins like aerolysin,[129] tetanus toxin,[130] *Streptococcus agalactiae* CAMP factor,[131] *Clostridium septicum* α-toxin,[132] *S. cerevisiae* K1 killer toxin,[133] and Cry1A toxin.[134] These toxins create pores in the host cell membrane and kill the host cells by breaching the cell permeability barrier.[135]

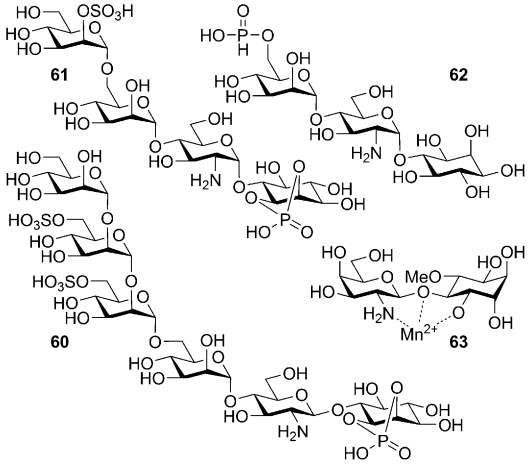
The synthetic GPI molecule **59** (Scheme 19) was shown to inhibit lysis induced by the CAMP factor in sheep erythrocytes.[136] A more detailed study was performed with the set of GPIs **53**–**58**, related to the sperm CD52 GPI anchor. Binding of these GPIs and CAMP factor was studied by using a quartz crystal microbalance.[137] It was observed that the CAMP factor has a high affinity for binding both the intact GPI anchor and its fragments. These experiments support the notion that the CAMP factor has a strong affinity for GPIs and implicates the possibility that a small GPI-like molecule may inhibit these pore-forming toxins.[138]

5.6. GPIs as Insulin Mimetics

Understanding insulin signal transduction is of paramount importance as it may lead to new strategies for the treatment of diabetes, one of the most prevalent metabolic diseases in the world. GPIs have long been considered potential precursors to novel second messengers of hormone action, in particular the effect of insulin.[139] Numerous studies indicate that insulin promotes the hydrolysis of GPIs with release of an inositol-containing phosphoglycan (IPG), which shows insulin-like effects in intact adipocytes and hepatocytes. Even in the absence of insulin, IPGs generated in vitro from *T. brucei* and *S. cerevisiae* by lipolytic and proteolytic degradation of their GPI-anchored proteins were demonstrated to activate the insulin-signaling pathway.[140]

Despite the evidence of the insulin mimetic effects of the GPI-related molecules, their exact chemical structures and mechanisms of action remain elusive. To understand the relationship between structure and biological activity of these molecules one has to resort to synthetic IPGs of defined structure. The intuition that understanding the nature of insulin mimetic action will lead to new anti-diabetic therapeutics has prompted several groups to synthesize IPGs with different structural features and test their insulin mimetic effects.[141] To date, more than 80 different IPGs have been synthesized and tested.

The most comprehensive investigation was reported by the Müller group at Sanofi-Aventis.[142] Synthetic IPGs ranging from pseudotri- to pseudohexasaccharides that vary in the number, position, and nature of substituents, as well as stereochemistry of the anomeric linkage of GlcN were prepared. Among these molecules, IPG **60** (Scheme 20) was



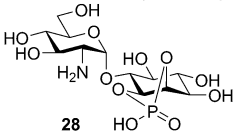
Scheme 20. Selected examples of synthetic IPGs.

identified as the most active insulin-mimetic compound with 96% of the maximal insulin response (MIR) and an EC_{50} of 3 μM in stimulation of lipogenesis in rat adipocytes.[143] This molecule represents the most potent synthetic compound identified to date with insulin-mimetic activity.

Despite these studies, research into the insulin-mimetic effect of smaller IPG analogues has generated conflicting results. For example, IPG **61** was first reported as highly potent (EC_{50} 1.1 μM and 78% MIR in lipogenesis),[144] but failed to show activity when tested by another group.[145] Different groups have investigated IPG **63** using the same assay and reported conflicting results.[146] In contrast, IPG **62**

was first identified as inactive in lipogenesis assays,[142] but a later report from the same group using the same assay showed moderate activity.[143] The most dramatic example in terms of insulin-mimetic activities came from studies of pseudodisaccharide **28** (Table 4). The biological activities of **28** have been investigated by different groups in various assays and produced contradictory results. At the same time, results regarding the ability of IPG **28** to stimulate lipogenesis in rat adipocytes highlight the difficulties in reproducing these data.[142,144–145,147]

Table 4: Insulin-mimetic activities of synthetic pseudodisaccharide **28**.

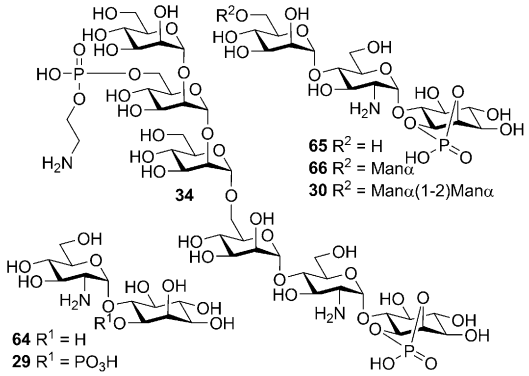


28

Study	Assay	Activity
d'Alarcao (1992)[147a]	gluconeogenesis	no activity
	cAMP phosphodiesterase activity	no activity
	lipogenesis	30–40% MIR at 40 μM
d'Alarcao (2005)[144]	lipogenesis	18% MIR, EC_{50} = 10.5 μM
d'Alarcao (2005)[147b]	lipogenesis	24% MIR, EC_{50} = 15.9 μM
Müller (1998)[142]	lipogenesis	MIR < 20%
Seeberger (2010)[145]	lipogenesis	no activity
	PKB/Akt activation	no activity
	glucose transport	no activity
	insulin tolerance test	no activity
Martin-Lomas (1999)[89a]	cell proliferation of otic vesicles	max. effect at 10 μM
	Fos oncoprotein extression	max. effect at 2.2 μM

The idea that GPIs might be the putative precursors of IPGs originated from generation of IPG from bovine liver after treatment with PI-PLC.[148] It is believed that treatment of GPI anchors with PI-PLC generates molecules bearing an inositol 1,2-cyclic phosphate, because C2 acylation of *myo*-inositol renders the GPIs resistant to hydrolytic cleavage by PI-PLC. A number of IPGs from pseudodi- to pseudohexasaccharides that structurally resemble PI-PLC treated GPIs were synthesized (Scheme 21). None of these molecules showed any insulin-mimetic activity.[89c,141,145,147a,149] These results imply that GPI-derived IPGs alone are not sufficient to trigger insulin-like responses in cells.

In summary, IPGs containing phosphate, inositol, hexosamine, saccharides, and other negatively charged groups have shown insulin-like functions, although the minimum pharmacophore for the required activity remains elusive. Understanding the molecular basis of insulin-like effects of IPGs is the key issue for designing more potent compounds. Previous research showed that IPGs can stimulate lipogenesis without entering the cell,[147b] but a receptor mediating such events has not yet been identified. Identification of the receptor would greatly aid the design of more potent IPGs. Insulin-mimetic effects of current IPGs can only be achieved in the μM range, a concentration that may merely serve as a starting point for drug discovery. A detailed understanding of IPG-induced signaling cascades may reveal proper targets to test IPG activities, and help to resolve current issues regarding data reproducibility.



Scheme 21. Selective examples of GPI-like IPGs, which showed no activity in bioassays.

5.7. Synthetic GPI-Anchored Proteins

GPIs contribute to the topological changes in the plasma membrane and membrane dynamics.[150] GPI-anchored proteins were thought to exhibit greater extracellular mobility than the transmembrane proteins that span the entire membrane and thus exhibit resistance to lateral movement.[151] However, recent studies suggest that the high lateral mobility of GPI-anchored proteins in biological membranes might be due to the interactions with other cell surface components.[152]

The Bertozzi group studied the mobility and trafficking of GPI-anchored proteins using synthetic GPI–protein analogues (**21**–**23**, Scheme 10).[153] These modified proteins integrated into the plasma membranes of a variety of mammalian cells and trafficked to recycling endosomes similarly to GFP bearing a native GPI anchor. The GPI–protein analogues **21**–**23** also diffused freely in cellular membranes. However, changes in glycan structure significantly affected membrane mobility with the loss of monosaccharide units correlating to decreased diffusion. These studies demonstrate that the glycan of a GPI anchor may play an important role in regulating the behavior of the attached protein.

6. Conclusions and Perspectives

More than three decades have passed since the initial discovery of GPIs.[3] The biological significance of GPIs has been widely recognized, even though the knowledge about their functions remains limited. Since pure material cannot be obtained from natural sources, synthetic GPI tools have overcome this shortage. However, the challenges in chemical synthesis of GPIs bearing unsaturated fatty acids and GPI-anchored proteins have hampered our understanding in these areas. Although an unsaturated lipid may result in highly

potent proinflammatory properties of the *T. cruzi* GPI,[73] the functional importance of unsaturated lipids has never been validated. Similarly, the understanding of the role of GPI anchors in GPI-anchored proteins remains limited in part due to the synthetic shortcomings.

Advances in the chemical synthesis of GPIs have enabled the preparation of various GPIs and analogues. A general strategy for GPI assembly has allowed rapid synthesis of different structures using the same strategy and common building blocks.[58] Facile access to synthetic GPIs will broaden the scope of questions that can be tackled, such as the conformation of different branched GPIs and the molecular interactions between GPI and bacterial toxins.

Parasitic GPIs can trigger immune responses in mammalian hosts. Their distinct structures enable the use of these structures as antigens for vaccine development as demonstrated in the case of malaria and African trypanosomiasis. Since GPIs are essential for the growth of many parasites in mammalian hosts, an alternative solution to combat these protozoa is to disturb their GPI biosynthetic pathways. In the case of *T. brucei*, various enzymes have been targeted. A similar approach can be applied to treat malaria, as the distinct feature of *P. falciparum* DPM can be exploited for developing novel drugs.

Ever since their discovery, GPIs have been considered potential precursors to second messengers of hormone action in general and insulin action in particular. However, the insulin-mimetic activity of GPI-like molecules remains highly controversial. The lack of knowledge about the putative IPG receptor has hampered further developments. One possible approach to identify the IPG receptor(s) is by photoaffinity labeling, which employs a potent IPG bearing a photoaffinity tag.

Progress in the chemical synthesis of GPIs has spurred the biological research in this area. The biomedical breakthroughs have, in turn, motivated further development of synthetic methodologies to provide better tools for probing the unanswered biological questions. With an ever increasing repertoire of tools from both fields, the study of GPIs is poised to advance rapidly.

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