Chapter 3: Synthesis of GPI anchor analogues

3.1 GPI-Anchored proteins (GPI-APs):

GPI-APs are produced in all eukaryotic cells ranging in complexity from single cell protozoa to multicellular organisms. The GPI anchor of the GPI-AP is a ubiquitous post-translational glycolipid modification that assists in anchoring the protein to the membrane. GPI-APs are diverse in function (as adhesion molecules, receptor proteins, enzymes and signaling receptors) (Chatterjee and Mayor, 2001). Unlike in mammalian cells, protozoan parasites like Trypanosoma, Leishmania and Plasmodium species, produce GPI-APs in high density to form a glycocalyx that helps to protect the parasite and invade the host system (Ferguson, 1999). A plethora of studies have synthesized parasitic GPI mimics and utilized them to dissect the biosynthetic pathway in the parasites and to generate vaccines against dreadful parasitic infections. However, the study documented in this thesis has synthesized and utilized GPI analogues to unravel its role in the membrane organization of GPI-APs.

3.1.1 GPI-AP discovery:

GPI-AP was discovered in 1976, when a purified bacterial enzyme phosphatidylinositol specific phospholipase-C (PI-PLC) capable of cleaving alkaline phosphatase (AP), acetylcholinesterase (AChE) and 5’-nucleotidase from a diverse set of mammalian tissue samples were identified (Chatterjee and Mayor, 2001). Later studies revealed the existence of functionally distinct GPI-APs in different species and cell types. By 1985, the chemical structure of GPI was derived from independent studies conducted on Trypanosoma brucei variant surface glycoprotein (VSG), Torpedo AChE, human erythrocyte AChE, rat brain and thymocyte Thy-1 protein. The discovery of GPI lipid modifications led to a surge in
the research of its structure, biosynthesis, distribution and function of various GPI-APs. These studies revealed that the GPI-APs form a large class of functionally distinct proteins with a common feature of GPI anchorage. The functional diversity mainly stems from the presence of structurally different protein attached to the GPI anchor. However the basic structure of GPI anchor present in a cell remains constant.

3.1.2 GPI Structure:

The analysis of various GPI-APs from different sources suggests that the GPI anchor is broadly classified into 3 classes, type-1: Man\(\alpha_1\)-6Man\(\alpha_1\)-4GlcN\(\alpha_1\)-6PI, type-2: Man\(\alpha_1\)-3Man\(\alpha_1\)-4GlcN\(\alpha_1\)-6PI and type3 (a hybrid of type1 and 2): Man\(\alpha_1\)-6Man\(\alpha_1\)-3Man\(\alpha_1\)-4GlcN\(\alpha_1\)-6PI. The GPI anchor of GPI-AP belongs to type1 class and contains a common core structure; ethanolamine-PO\(_4\)-6Man\(\alpha_1\)-2Man\(\alpha_1\)-6Man\(\alpha_1\)-4GlcN\(\alpha_1\)-6-D-my-o-inositol-1-PO\(_4\)-lipid (Ferguson, 1995; Ruhela et al., 2012). The protein is covalently bound to the core structure at the terminal mannose via the amino group of ethanolamine whereas the glycerolipid is attached to the 1-O position of inositol. In some cases like in red blood cells (human AChE in erythrocytes, human alkaline phosphatase, T brucei PARP), the GPI anchor carries an extra palmitoyl group at the 2-O position of inositol. This additional lipid makes the GPI anchor insensitive to PI-PLC activity. Additionally, the glycan core can acquire side chain modifications such as an extra ethanolamine (hAChE, gpi10, Thy-1) or a mannose residue in a cell type or tissue or species-specific manner. Therefore glycan substitution forms the basis for the variation in the GPI anchor between different species. GPI anchor can attain either diacyl chains (eg: Torpedo AChE, T brucei VSG) or acyl alkyl chains (DAF, erythrocyte AChE, folate receptor, placental alkaline phosphatase) as lipid chains (Chatterjee and Mayor, 2001).
3.1.3 GPI Biosynthesis:

This is discussed in detail in section 1.2.1 of Chapter 1. Briefly the GPI anchor biosynthesis is initiated in the ER where the GPI anchor is synthesized in a step wise manner starting from phosphatidylinositol (PI) and building it up to ethanolamine-PO₄-6Manα1-2Manα1-6Manα1-4GlcNα1-6-D-my o-inositol(2-O)-palmitoyl-1-PO₄-lipid. The protein destined to be attached to the GPI anchor contains an N-terminal signal sequence for entry into ER and C-terminal hydrophobic residues (12-20) for GPI attachment (Ferguson, 1999). The C-terminal signal sequence is cleaved in the ER and replaced with a pre-assembled GPI anchor to form GPI-AP. This is followed by the de-acylation of the palmitoyl at the 2 position of inositol and the transport of the GPI-AP to Golgi where the lipid chains undergo fatty acid remodeling by specific Golgi resident enzymes (Kinoshita et al., 2008). The completely processed GPI-AP is then trafficked to the plasma membrane by secretory vesicles. However in many cases the cells may also contain protein free GPls, which are end products of GPI biosynthesis both produced excessively and not used up by the protein or precursor end products. Unlike GPI-APs, these free GPls are widely distributed in the cells by mechanisms that are available for trafficking of lipids, which includes lipid transfer proteins, contact mediated and vesicular transport.

3.1.4 Chemical Synthesis of GPI anchor:

Till date, there have been many reports on the chemical synthesis of full length GPI anchors (Becker et al., 2008; Saikam et al., 2011; Guo, 2011). But very few efforts have been made to utilize these structural and functional mimics of GPls in addressing specific questions about their role in GPI-AP membrane organization. Paulick et al ((Paulick et al., 2007) have used expressed protein ligation method (EPL) to attach GFP (green fluorescent
protein) to synthetic GPI analogues with varying number of mannose residues and studied how the GPI anchor sugar residues can modulate the GPI-AP membrane diffusion properties. In a different study, Bhagatji et al (Bhagatji et al., 2009) varied the size of proteins attached via polyethyleneglycol units (PEG) to phosphatidylethanolamine (PE) and investigated the role of bulk protein in the endocytosis of GPI-AP via GEEC. However these synthetic GPI analogues lacked the glucosamine and inositol residues thus making it impossible to map GPI anchor structural contributions to the fullest. An important strategy in one of these studies was the utilization of enzymes to covalently bind synthetic GPI anchors to peptides or proteins (Wu et al., 2010; Paulick and Bertozzi, 2008). This work has invoked a lot of interest lately since the chemically synthesized full length GPI anchors of desired structure can be coupled to a peptide or protein of interest thereby providing the potential to generate the GPI-APs of desired structure in vitro.

Since we are interested in dissecting the individual roles of GPI anchor domains namely the glycan core and glycerolipid moiety in GPI-AP nanoclustering at the plasma membrane, our strategy was to synthesize fluorescently tagged GPI analogues with a systematic increase in glycan structure starting from a minimal disaccharide to a full length pentasaccharide of all possible combinations of fatty acyl chains and saturation. Keeping this in mind, it was essential to design a synthetic strategy that was flexible enough to bring modifications in the various domains (the glycan and fatty acyl chains) of the GPI anchor. Thus, a synthetic strategy to synthesize minimal possible GPI anchor with the potential to convert it into higher order GPI analogues was designed (Fig 3.1 A). This work was done in collaboration with Dr. Ram A Vishwakarma, IIIM, Jammu, India. Additionally, he provided us with the NBD tagged GPI analogues with the desired glycan substitution. The
synthesis of unsaturated fatty acyl chain containing GPI analogues was incompatible with the synthetic strategy designed. Hence we collaborated with Dr. Zhongwe Guo, Wayne state university who provided us with the fluorescently tagged GPI analogues containing unsaturated acyl chains. This was achieved by utilizing paramethoxybenzyl (PMB) groups instead of benzyl (Bn) groups as a major protecting group. The utilization of PMB instead of Bn group allowed the final deprotection of the major protecting groups in mild acidic conditions instead of hydrogenation (hydrogenate unsaturated double bonds) thereby retaining the unsaturated double bonds in the fatty acyl chains. Thus, all the GPI analogues utilized in my study contain a minimal glycan, disaccharide made up of glucosamine and inositol. These GPI analogues were conjugated to a fluorophore either at the head group on the glucosamine or at the fatty acyl chain. Accordingly, the head group and fatty acyl chain tagged GPI analogues were utilized to study the role of fatty acyl chains and glycans respectively.

The GPI analogues are classified into two categories based on the site of attachment of the fluorophore namely head group and fatty acyl chain tagged GPI analogues. The head group tagged GPI analogues are further subdivided into two groups based on the variation in fatty acyl ‘chain length’ or ‘saturation’. The first category comprises GPI analogues tagged to Bodipy$^{\text{TMR}}$ at the 6-OH of glucosamine residue and carrying variations in the acyl chain length either long acyl chains C16:0 (di-palmitoyl, denoted as GPI$_{\text{C16:0/C16:0}}$) or short acyl chains C8:0 (di-octanoyl, denoted as GPI$_{\text{C8:0/C8:0}}$)(Saikam et al., 2011) (Figure. 3.1B). The second category consists of fluorescein tagged GPI analogues carrying either saturated (C18:0) or unsaturated (C18:1) acyl chains (analogues are denoted as GPI$_{\text{C18:0/C18:0}}$ and GPI$_{\text{C18:1/C18:1}}$ respectively) (Johnson and Guo, 2013). The acyl chain tagged GPI analogues
are tagged to NBD fluorophore at the acyl chain of the sn-1 position of glycerol and includes GlcNPI-NBD and GlcNHbocPI-NBD (Vishwakarma et al., 2005; Vishwakarma and Menon, 2005) (Figure 3.1 C& D).

The strategy utilized to synthesize Bodipy^{TMR} tagged GPI analogues is described in this chapter. Briefly, the GPI analogues are synthesized from 3 key building blocks namely suitably protected myo-inositol, glucosamine and glycerolipid which in turn were synthesized from basic sugar molecules (Saikam et al., 2011). The various building blocks are linked together in the right stereochemistry to obtain the GPI analogue of desired structure. The 6-OH of 2-azidoglucose is converted into azido group by Tosyl chemistry, which at the final step of hydrogenation is converted to free amine. This free amine is conjugated to the fluorescent dye (N-hydroxysuccinimide ester) to obtain fluorescently tagged GPI. However, hydrogenation may also convert the azido (N$_3$) group of 2-azidoglucose to an amine group. This requires protecting the second azido group with a tertiary butyloxy carbonyl (Boc) prior to conversion of 6-OH to N$_3$. In the final step, Boc is de-protected using TFA (trifluoroacetic acid) to obtain the desired fluorescently tagged GPI analogue. This synthetic strategy has the flexibility to incorporate variations in the acyl chain length, mannos at 4-OH of glucosamine (retained unreacted for a specific purpose) to obtain higher order glycans and to attach the fluorophore of choice.

3.2 Synthesis of GPI Analogues.

3.2.1 Synthetic strategy (Scheme 6 & 7):

The synthesis of Bodipy^{TMR} tagged GPI analogues were initiated from 3 different building blocks, namely racemic (DL) myo-inositol, glucosamine and glycerolipid (Figure 3.2).
Since the naturally existing GPI anchors carry, pure D-\textit{myo}-inositol, it is essential to optically resolve this compound at an intermediate step of GPI synthesis. Briefly, compound 13 was benzylated at 3-OH group of 2-azidoglucose and converted to 14, after which the compound was subjected to acid hydrolysis to deprotect 4,6-di-O-benzylidene and form the compound 15. The resulting free hydroxyls (4-OH and 6-OH on 2-azidoglucose) were acetylated to give 16, followed by the conversion of azido (N$_3$) to NH-Boc in 17. The 4,6-di-O-acetyl groups were then de-acetylated to give 18 and the 6-OH group on glucosamine residue was converted to azide by tosyl chemistry. Tosyl being a bulky group can be specifically used to protect a primary alcohol in presence of a secondary alcohol. Thus the compound 18 was subjected to tosylation to yield compound 19 followed by heating with NaN$_3$ to convert tosyl to azido. The compound thus obtained 20 was subjected to benzylation of 4-OH group on glucosamine residue in 21. Finally the \textit{p}-methoxybenzyl (PMB) group at 1-OH of inositol was de-protected to obtain 22 followed by coupling to 1,2-isopropylidene-\textit{sn}-glycerol-H-phosphonate to give 23. The 1,2-isopropylidene was subjected to acidic cleavage and the resulting diol 24 were palmitoylated to give rise to the fully protected disaccharide 25, which was then hydrogenated to de-protect all benzyl groups. The resulting free amine (6-azido of glucosamine) in 26 was conjugated to the fluorophore (NHS ester) of choice, followed by Boc deprotection (in TFA) to obtain the desired compound 27. Similar synthetic strategy can also be utilized to synthesize a GPI analogue with short acyl chains (di octanoyl).

\textbf{3.2.2 Synthesis of Building blocks}
The synthesis of various building from basic sugar molecules such as \textit{myo}-inositol, glucose, ethanolamine and glycerol and (Figure 3.2) is described below and elaborated in scheme 1-4.

\textbf{3.2.3 Synthesis of Azido-glucosyl donor}

The glucosyl donor was synthesized from commercially purchased 3,4,6-Tri-\textit{O}-acetyl-D-glucal as reported in Schmidt et al, 1984. This compound was subjected to azidonitration by treating with CAN, NaN$_3$ at -20°C. The nitro (NO$_2$) group in the resulting compound was hydrolysed by heating the reaction mixture in dioxan/water, NaNO$_2$ at 80°C, followed by trichloacetimidation with Na$_2$CO$_3$ and trichloroacetonitrile (Scheme 3).

\textit{2-Azido-2-deoxy-3, 4, 6-tri-\textit{O}-acetyl-D-glucopyranose}

Tri-\textit{O}-acetyl-D-glucal (12 g, 0.04 mmol) in dry acetonitrile (250 mL) was cooled to -20°C, CAN (74 g, 0.17 mmol) and NaN$_3$ (4.36 g, 0.07 mmol) added and stirred for 8 hours at -20°C. This mixture was diluted with 400 mL diethyl ether and the reaction quenched with ice. The organic layer was washed with H$_2$O and saturated NaHCO$_3$, separated and concentrated to obtain a residue. This residue was dissolved in dioxan (80 mL), 10 g NaNO$_2$ in 12 mL H$_2$O and stirred at 80°C for 8 hours, after which the reaction mixture was cooled to room temperature and worked up with 200 mL ether, and then water. The ether layer was collected, concentrated and purified on silica gel column purification to obtain the desired compound 10 g (71%). TLC was monitored in (petroleum ether/ether 1:2). The results obtained here corroborate with the data previously reported in Schmidt et al, 1984.

\textit{2-Azido-2-deoxy-3,4,6-tri-\textit{O}-acetyl-D-glucopyranosyl trichloroacetimidate}
The compound (5 g, 15 mmol) from previous step was dissolved in dry DCM (100 mL) and then added trichloroacetonitrile (5 mL, 49.85 mmol) and freshly dried K$_2$CO$_3$ (2 g, 14.47 mmol). This reaction mixture was stirred at RT for 6 hours. The completion of reaction was detected by TLC followed by filtration of the reaction mixture through celite (to remove K$_2$CO$_3$). The filtrate was concentrated and purified on silica column chromatography to obtain the desired compound 10 (2.4 g, 34%); TLC was detected in $R_f$ 0.40 (pet ether/ether 3:2). The data obtained for this product corroborated with previously described data in Schmidt et al, 1984.

3.2.4 Synthesis of Inositol intermediate (refer scheme 1 & 2)

1, 2: 4, 5-Di-O-cyclohexyldene-D/L-myoinositol (1)

This compound was synthesized as described in scheme 1 (Billington et al., 1989). Briefly a mixture of myo-Inositol (35 g, 195 mmol) in cyclohexanone (205 mL), toluene (50 mL) and $p$TSA (6 mL of 10% solution in DMF) was heated at 160°C for 9 hours. $p$TSA (6 mL 10% in DMF) was replenished every 2 hours in the reaction mixture and the water generated during this process was removed using a Dean-Stark apparatus. After the completion of reaction, the mixture was cooled to room temperature and neutralized with TEA. The solvents were evaporated and the residue obtained was dissolved in CH$_2$Cl$_2$, washed with 5% NaHCO$_3$, water and saturated NaCl. The organic phase was collected, evaporated to dryness to obtain oil like product. This was further dissolved in minimum amount of acetone and petroleum ether and heated to 40-60°C to induce crystallization of 1,2:4,5-bis-ketal. The crystals obtained were filtered and recrystallized from acetone/petroleum ether to give pure compound 1 (8.3 g, 12.5%). The compound was
confirmed on TLC with the standard (previously synthesized and characterized) available in the laboratory. TLC solvent system used was 60% EtOAc/Hexane (Rf 0.27). ^1H NMR (500 MHz, CDCl3): 1.59-1.71 (m, 20H), 3.31 (t, 1H, J = 10 Hz), 3.81 (t, 1H, J = 9.8 Hz), 3.87 (dt, 1H, J = 2.8, 6.4 Hz), 4.01 (dt, 1H, J=4.6, 9.8 Hz), 4.07 (t, 1H, J=6.2 Hz), 4.47 (t, 1H, J = 4.8 Hz); MALDI TOF MS: calculated for C18H28O6 (M+Na)^+ 363.1784, found 363.1785.

3-O-Benzyl-1,2:4,5-di-O-cyclohexylidene-D/L-myo-inositol (2)

To the compound 1 (19 g, 55.8 mmol) in DMF (323 mL) was added barium oxide (16.15, 97 mmol), barium hydroxide octahydrate (2.185 g, 12.8 mmol) and benzyl bromide (7.41 mL, 43 mmol). This mixture was stirred at room temperature for 4 days and then diluted with diethyl ether (260 mL), neutralized with aqueous acetic acid and washed with water (400 mL), NaHCO₃ solution (400 mL, 10%, w/v) and water (400 mL) respectively. The organic layer was collected, filtered over Na₂SO₄ and concentrated in vacuum. The residue obtained after this process was purified using silica column chromatography to give compound 2 (12.4g, 52%). TLC solvent system 60% EtOAc/Hexane (Rf 0.45).^1H NMR(300MHz, CDCl3):1.44-1.76 (m, 20H), 3.28 (t, 1H, J = 9Hz), 3.77 (dd, 1H, J = 4.2, 10.1 Hz), 3.88 (dt, 1H, J = 2.8, 8.6 Hz), 3.94 (t, 1H, J=5.6 Hz), 4.03 (t, 1H, J=9.7 Hz), 4.36 (t, 1H, J = 4.5 Hz), 4.82 (d, 1H, J=12.5 Hz), 4.90 (d, 1H, J = 12.5 Hz), 7.28-7.46 (m, 5H); MALDI TOF MS: calculated for C25H34O6 (M+Na)^+ 453.2248, found 453.2255.

6-O- Allyl-3-O-Benzyl-1,2: 4,5-di-O-cyclohexylidene-D/L-myo-inositol (3)

The compound 2 (3.78 g, 8.79 mmol) was dissolved in DMF 70 mL followed by the addition of allyl bromide (940 mL, 10.89 mmol, 1.2 equivalents). The reaction mixture was
cooled to 0°C after which NaH (939 mg, 39.129 mmol, 4.45 eq) was added in small portions. The low temperature is maintained to prevent the heating up of reaction mixture resulting from NaH reaction. After the addition of NaH, the reaction mixture was cooled to room temperature and stirred for 1.5 hours at RT. The completion of reaction was monitored on TLC, followed by the termination of reaction with MeOH. EtOAc was added to the reaction mixture and washed with brine (NaCl solution). The organic layer was collected, filtered over Na₂SO₄ and concentrated in vacuum. The residue obtained in this process was purified by silica column chromatography to provide compound 3 (3.462 g, 91%). TLC solvent system utilized was 60% EtOAc/Hexane (Rₓ 0.8); ¹H NMR (500MHz, CDCl₃): 1.50-1.71 (m, 20H), 3.28 (t, 1H, J = 9.5 Hz) 3.62 (dd, 1H, J = 6.6, 10.6 Hz), 3.74 (dd, 1H, J = 4.2, 8.6 Hz), 3.99 (t, 1H, J=5.4 Hz), 4.01(t, 1H, J=9.7 Hz), 4.29-4.33 (m, 3H), 4.81 (d, 1H, J=12.5 Hz), 4.88 (d, 1H, J = 12.5 Hz), 5.19 (d, 1H, J=10.4 Hz), 5.34 (d, 1H, J = 17.2 Hz), 5.93 (m, 1H), 7.26-7.43 (m, 5H); MALDI TOF MS: calculated for C₂₈H₃₈O₆ (M+Na)⁺ 493.2561, found 493.2557.

6-O-Allyl-3-O-Benzyl-1,2-O-cyclohexyldiene-D/L-myoinositol (4)

Compound 3 (3.4 g, 7.2 mmol) was dissolved in 70 mL of acetonitrile:CHCl₃ (1:1) and 4.5 mL (80 mmol) ethylene glycol, to which pTSA.H₂O (114 mg) was added. The reaction mixture was stirred for 15 minutes at RT until the reaction was complete and then neutralized with TEA. TLC was monitored in 60% EtOAc/hexane (Rₓ 0.23). 120 mL of DCM was added to the reaction mixture, washed with saturated NaHCO₃ and then with water. The organic layer was collected and concentrated in vacuum to obtain compound 4 (2.8 g, 100%). This compound was directly used up for the next step without purification. ¹H NMR (500MHz, CDCl₃): 1.61-1.72 (m, 10H), 3.28 (t, 1H, J = 9.5 Hz) 3.64 (dd, 1H, J =
6.6, 10.6 Hz), 3.76 (dd, 1H, J = 4.2, 8.6 Hz), 4.00 (t, 1H, J = 5.4 Hz), 4.02 (t, 1H, J = 9.7 Hz), 4.30-4.34 (m, 3H), 4.82 (d, 1H, J = 12.5 Hz), 4.89 (d, 1H, J = 12.5 Hz), 5.20 (d, 1H, J = 10.4 Hz), 5.35 (d, 1H, J = 17.2 Hz), 5.95 (m, 1H), 7.27-7.44 (m, 5H); MALDI TOF MS: calculated for C_{22}H_{30}O_{6}(M+Na)^+ 413.1935, found 413.1953.

6-O-Allyl-3,4,5-tri-O-Benzyl-1,2-O-cyclohexylidene-D/L-myoinositol (5)

Compound 4 (2.8 g, 10.6 mmol) was subjected to benzylation as described previously for compound 5 (4.09 g, 100% yield). TLC was monitored in 60% EtOAc/hexane (R_f 0.69). ^1H NMR (500 MHz, CDCl_3): 1.48-1.76 (m, 10H), 3.27 (t, 1H, J = 9.4 Hz) 3.58-3.62 (m, 2H), 3.82 (t, 1H, J = 8.6 Hz), 3.92 (t, 1H, J = 5.7 Hz), 4.16-4.18 (m, 2H), 4.27-4.28 (m, 1H), 4.67-4.76 (m, 6H), 5.08 (d, 1H, J = 10.4 Hz), 5.20 (d, 1H, J = 17.2 Hz), 5.88 (m, 1H), 7.18-7.29 (m, 15H); MALDI TOF MS: calcld for C_{36}H_{42}O_{6}(M+Na)^+ 593.2874, found 593.2865.

6-O-Allyl-3,4,5-tri-O-Benzyl-D/L-myoinositol (6)

Compound 6 (4 g, 7 mmol) was subjected to pTSA (113 mg) mediated hydrolysis in 68 mL acetonitrile-CHCl_3 (1:1) and 4.4 mL ethylene glycol at 50°C in a water bath and was stirred for 4 hours until the reaction was complete. The reaction mixture was neutralized with TEA, diluted with CHCl_3 and washed with NaHCO_3 and H_2O. The organic layer was collected, filtered over Na_2SO_4 and concentrated to dryness in vacuum. The residue obtained after this process was purified by silica column chromatography to obtain pure compound 6 (2.653 g, 76%). TLC run in 30% EtOAc/hexane (R_f 0.3); ^1H NMR (400 MHz, CDCl_3): δ 2.73-2.70 (m, 2H), 3.44-3.38 (m, 3H), 3.72-3.67 (t, J = 9.6, 1H), 3.95-3.90 (t, J = 9.6, 1H), 4.18-4.17 (t, J = 2.4, 1H), 4.28-4.23 (dt, J = 1.3, 5.6 and 12.6, 1H), 4.43-4.37 (dt, J = 1.3, 5.9 and 12.4, 1H), 4.70-4.69 (m, 2H), 4.93-4.79 (m, 4H), 5.17-5.14 (dd, J =
1.3 and 10.4, 1H), 5.28-5.23 (dd, *J* = 1.6 and 17.1, 1H), 5.98-5.91 (m, 1H), 7.34-7.22 (m, 15H). $^{13}$C NMR (100 MHz, CDCl$_3$): 69.25, 71.69, 72.74, 74.33, 75.67, 75.91, 79.96, 80.93, 81.56, 83.19, 117.16, 128.51-127.60 (multiple peaks), 135.02, 137.79, 138.53, 138.66. MALDI TOF MS: calcd for C$_{30}$H$_{34}$O$_6$ (M+Na)$^+$ 513.2253, found 513.2035.

6-**O-**Allyl-1-**O**-(4-methoxy benzyl)-3, 4, 5-**tri-O**-benzyl-D/L-**myo**-inositol (7)

Compound 6 (5.11 g, 10.4 mmol) was dissolved in anhydrous MeOH (281 mL), mixed with dibutyltin oxide (1.997 mL, 15.5 mmols) and refluxed in MeOH for 2 hours. MeOH was removed by evaporation and the reaction mixture was dried by repeated evaporation with toluene. To this mixture were added CsF (2.236 g), KI (2.34 g) and PMBCl (1.997 mL in 300 mL dry DMF) and stirred under argon gas at RT for an overnight. The solvent was evaporated and the residue obtained was purified by silica column chromatography to provide compound 7 (6.54 g, 95%). TLC was monitored in 30% EtOAc/hexane (*R*$_f$ 0.61); $^1$H NMR (500 MHz, CDCl$_3$): 2.42 (s, 1H), 3.30-3.29 (dd, *J* = 2.0 and 9.8, 1H), 3.41-3.35 (m, 2H), 3.81-3.77 (m, 4H), 3.91-3.90 (t, *J* = 9.6, 1H), 4.15-4.14 (t, *J* = 2.3, 1H), 4.37-4.30 (dtdt, *J* = 1.7, 5.9 and 12.3, 1H), 4.45-4.38 (dtdt, *J* = 1.6, 5.6 and 12.2, 1H), 4.69-4.60 (m, 4H), 4.89-4.81 (m, 4H), 5.15-5.13 (dd, *J* = 1.6 and 11.74, 1H), 5.20-5.17 (dd, *J* = 1.2 and 15.57, 1H), 6.03-5.93 (m, 1H), 6.88-6.86 (d, *J* = 8.59, 2H), δ 7.33-7.25 (m, 17H); $^{13}$C NMR (100 MHz, CDCl$_3$): 55.29, 67.71, 72.47, 72.70, 74.56, 75.91, 75.96, 79.27, 79.75, 80.89, 81.11, 83.16, 113.86, 113.96, 116.58, 128.63-127.54 (multiple peaks), 129.47, 130.14, 135.35, 137.99, 138.72, 138.80, 159.36. MALDI TOF MS: calculated for C$_{38}$H$_{42}$O$_7$ (M+Na)$^+$ 633.2828 found, 633.2833.

6-**O-**Allyl-1-**O**-(4-methoxy benzyl)-2,3,4,5-tetra-**O**-Benzyl-D/L-**myo**-inositol (8)
Compound 7 (1.96 g, 3.2 mmol) in anhydrous DMF (31 mL) was cooled to 0°C and then added BnBr (1.066 mL, 8.97 mmol, 2.8 eq) and NaH (627 mg, 26.4 mmol, 8.25 eq) in small batches for 30 minutes. The mixture was cooled to room temperature and stirred for additional 4 hours. The reaction was quenched with MeOH, diluted in water and the organic compound was extracted in EtOAc followed by normal work up. EtOAc layer was collected, concentrated to obtain 8 and proceeded directly to the next step. TLC was monitored in 60% EtOAc/hexane (Rf 0.61); \(^1\)H NMR (300MHz, CDCl\(_3\)): 3.24 (dd, J = 2.2 and 9.7 Hz, 1H), 3.31 (dd, J = 2.2 and 9.7, 1H), 3.39 (dd, J = 9.1 Hz, 1H), 3.82 (s, 3H, OMe), 3.89-4.04 (m, 3H), 4.24-4.41 (m, 2H), 4.52-4.65 (m, 4H), 4.77-4.90 (m, 6H), 5.15 (m, 2H), 5.85-6.10 (m, 1H,allyl), 6.84-6.87 (m, 2H), 7.22-7.42 (m, 22H, Ph); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): 55.30, 67.72,72.71, 72.48, 74.57, 75.98, 77.24, 77.76, 79.29, 79.76, 80.91, 81.12, 83.17, 113.86, 116.59, 128.65-128.64 (multiple peaks), 129.48, 130.14, 135.36, 137.99, 138.72, 138.81, 159.36. MALDI TOF MS: calcd for C\(_{43}\)H\(_{48}\)O\(_7\) (M+Na)\(^+\) 723.3298, found 723.3182.

\(1\)-O-(4-methoxy benzyl)-2,3,4,5-tetra-O-Benzyl-D/L-myoinositol (9)

To convert the allyl to vinyl, residue 8 (2.25 g, 3.2 mmols) was dissolved in in DMSO (32.6 mL, 458 mmols) and heated to 80°C after which t-BuOK (5.1 g) was added and stirred for 3 hours. The completion of reaction was monitored using TLC, followed by quenching the reaction with ice. The organic product was extracted with EtOAc, washed with water to remove DMSO completely. The reaction had proceeded to 100% and so proceeded to next step with out purification. Conversion of allyl to vinyl is confirmed by \(^1\)H NMR as indicated by disappearance of a multiplet at (~ 5.8) and the appearance of a doublet at (~ 6.2). \(^1\)H NMR (300MHz, CDCl\(_3\)): 2.1 (d, J = 9.3 Hz, 3H), 3.24 (dd, J = 2.2
and 9.7 Hz, 1H), 3.31 (dd, J = 2.2 and 9.7, 1H), 3.39 (dd, J = 9.1 Hz, 1H), 3.82 (s, 3H, OMe), 3.92 (m, 1H), 4.24-4.41 (m, 2H), 4.52-4.65 (m, 4H), 4.77-4.90 (m, 6H), 5.15 (m, 1H), 6.86 (doublet, 1H, vinyl), 6.84-6.87 (m, 2H), 7.22-7.42 (m, 22H, Ph). TLC system used was 30% EtOAc/hexane (rf 0.6).

The residue (2.25 g, 3.4 mmol) obtained in the previous step was dissolved in 81 mL HCl-Acetone (1:9) and heated to 50°C to enhance the hydrolysis of vinyl group. TLC in 30% EtOAc/hexane (Rf 0.29) indicated the completion of reaction. The reaction mixture was then cooled to RT and quenched with TEA. The solvent was concentrated and the residue obtained was washed with water and purified by silica column chromatography to obtain compound 9 (1.6g, 70%). \(^1\)H NMR (300 MHz, CD\(_3\)OD): 2.45 (s, 1H, OH), 3.17 (dd, J = 1.8 and 9.8 Hz, 1H, 3-H), 3.36 (dd, J = 9.8 Hz, 1H, 5-H), 3.39 (dd, J = 2.0 and 9.6 Hz, 1H, 1-H), 3.80 (s, 3H, OMe), 4.10 (bs, 1H), 4.04 (dd, J = 9.8 Hz, 1H), 4.14 (m, 1H, 6-H), 4.46-4.88 (m, 10H, CH\(_2\)Ph), 6.84-6.87 (m, 2H, Ph), 7.20-7.36 (m, 22H, Ph); \(^13\)C NMR (100 MHz, CDCl\(_3\)): 55.29, 71.92, 72.74, 72.86, 73.66, 74.06, 75.33, 75.33, 75.79, 79.75, 81.11, 81.41, 83.47, 113.92, 128.40-127.38 (multiple peaks), 129.39, 129.97, 138.34, 138.81, 138.87, 159.35. MALDI TOF MS: calcd for C\(_{42}\)H\(_{44}\)O\(_7\) (M+Na)\(^+\) 683.2985, found 683.2974.

3.2.5 Synthesis of isopropylidene glycerol H-phosphonate (39, scheme 4)

The glycerolipid intermediate was synthesized from commercially available 1,2-isopropylidene-sn-glycerol. Imidazole (1.36 g, 20 mmol) was evaporated with toluene several times to remove traces of water and then dissolved in 15 mL dry acetonitrile and stirred at 0°C under argon. To this mixture was added PCl\(_3\) (400 \(\mu\)L, 4.4 mmol in 4 mL dry acetonitrile) followed by TEA (1.58 mL, 11.4 mmol, in 4 mL dry acetonitrile) drop wise.
After 15 minutes of stirring the reaction mixture, 1,2-isopropylidene-\textit{sn}-glycerol (200 mg, 1.5 mmol) in dry acetonitrile (15 mL) was added drop by drop in 30 minutes. The reaction was continued for 2-3 hours until the reaction was complete and then quenched with 4:1 pyridine/H\textsubscript{2}O (30-40 mL). The organic layer was extensively extracted with CHCl\textsubscript{3}, collected and washed with TEAB buffer, dried, concentrated to obtain an organic residue, which was purified by silica gel column chromatography to obtain pure 42. TLC run in 25% MeOH/DCM in 0.1% TEA (R\textsubscript{f} 0.28).\textsuperscript{1}H NMR (300MHz, CDCl\textsubscript{3}): 1.3-1.4 (m, 6H, 2 CH\textsubscript{3}), 3.05-3.12 (t, 1H), 3.89 (dd, 2H), 4.05 (m, 1H), 4.33 (dd, 1H, J = 5.7), 5.8 (d, 1H, J\textsubscript{PH}= 625 Hz).

\textbf{3.2.6 Coupling reactions}

(3,4,6-Tri-\textit{O}-acetyl-2-azido-2-deoxy-\textit{\alpha}-D-glucopyranosyl)-(1\textright{}6)-2,3,4,5-tetra-\textit{O}-benzyl-1-\textit{O}-(4-methoxybenzyl)-(D/L)-myo-inositol (11, scheme 5)

The acceptor 9 (3 g, 4.5 mmol) and donor 10 (1.7 g, 3.5 mmol) along with a magnetic bead was toluene dried (to remove traces of water/moisture in the mixture), dissolved in anhydrous DCM (28 mL) with freshly activated molecular sieves and stirred at 0\textdegree{}C under argon. To this mixture was added TMSOTf (788 µL, 0.2 M) in a drop wise manner with continuous stirring for approximately 30 minutes until TLC (30% EtOAc/hexane) indicated completion of the reaction. The reaction was quenched with TEA, filtered through celite to remove the molecular sieves, dried and purified by silica column chromatography to obtain pure compound 11 (3.36 g, 76%).\textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}): 1.83-2.07 (3x s, 9H, OAc), 3.11- 3.15 (m, 1H), 3.39-3.42 (m, 1H), 3.44-3.46 (dd, J = 9.6 Hz, 1H), 3.47-3.50 (dd, J = 1.8 and 9.5 Hz, 1H), 3.60-3.63 (m, 2H), 3.82 (s, 3H, OCH\textsubscript{3}),
4.06 (s, 1H), 4.13-4.14 (dd, J = 9.5 Hz, 1H), 4.20-4.33 (m, 2H), 4.40-5.18 (m, 10H, CH₂Ph), 4.92 (m, 1H), 5.40-5.43 (m, 1H), 5.64 (d, J = 3.6 Hz, 0.5H), 5.79 (d, J = 3.6 Hz, 0.5H), 6.84-6.87 (m, 2H, Ph), 7.21-7.39 (m, 22H, Ph); ¹³C NMR (75 MHz, CDCl₃): 20.54, 20.58, 20.63, 55.19, 60.89, 61.17, 66.73, 67.99, 68.99, 68.99, 70.57, 71.62, 71.66, 71.84, 72.77, 72.85, 73.61, 73.97, 75.04, 75.23, 75.69, 79.69, 80.74, 81.04, 81.33, 81.96, 83.41, 83.97, 97.35, 97.16, 113.83, 127.72-129.29 (multiple peaks), 138.09-138.81 (multiple peaks), 159.37, 169.48, 169.98, 170.64; HRMS (ESMS, M+Na⁺) calcd for C₅₄H₅₉O₁₄N₃Na 996.3895, found 996.3885.

(2-Azido-4,6-di-O-benzylidene-2-deoxy-α-D-glucopyranosyl)-(1→6)-2,3,4,5-tetra-O-benzyl-1-O-(4-methoxybenzyl)-D-myoinositol (13, scheme 5)

The compound 11 (3.36 g, 3.4 mmol) was dissolved in anhydrous DCM:MeOH (194 mL, 1:3) and treated with saturated solution of sodium methoxide (2.9 g) for 2 hours at RT. The reaction mixture was neutralized with cation exchange resin (Amberlite IR 120H⁺), filtered and concentrated to give the desired product 12 (1.29 g). This intermediate 12 (1.29 g) was dissolved in anhydrous acetonitrile (1.80 mL in 5.45 mL) followed by the addition of camphor sulfonic acid (CSA, 240 mg) and benzaldehyde dimethalacetal. The reaction mixture was stirred for 24 hours (TLC in 30% EtOAc/Hexane), quenched with TEA (to neutralize the acid) and concentrated. The organic residue was diluted in EtOAc and worked up with NaHCO₃, saturated NaCl and water. The organic layer was collected and concentrated to dryness. This organic residue was purified on silica gel chromatography isolating the D and L isomers in purity. It is important to note that only 280 mg (15%) of D isomer 13 was obtained from 1.29 g of starting material. [α]D = +57 (c = 1.0, CHCl₃), ¹H NMR (300 MHz, CDCl₃): 3.24 (dd, J = 3.8 and 10.0 Hz, 1H, 2b-H), 3.38 (dd, J = 2.2 and
9.8, 1H, 1a-H or 3a-H), 3.45 (dd, J = 2.2 and 9.8, 1H, 1a-H or 3a-H), 3.46 (dd, J = 9.3 Hz, 1H), 3.52 (m, 2H), 3.80 (s, 3H, OCH₃), 3.96-4.04 (m, 2H, 2a-H, 3a-H), 4.06-4.29 (m, 4H), 4.57-4.96 (m, 10H, CH₂Ph), 5.47 (s, 1H), 5.72 (d, J = 3.8 Hz, 1H, 1b-H), 6.83- 6.89 (m, 2H, Ph), 7.08-7.13 (m, 2H, Ph), 7.17-7.45 (m, 25H, Ph); ¹³C NMR (75 MHz, CDCl₃): 55.20, 61.92, 62.90, 68.53, 71.74, 72.73, 73.51, 74.20, 75.04, 75.35, 75.65, 80.79, 81.27, 81.84, 81.91, 97.70, 101.85, 113.81, 126.38, 127.12, 127.38, 127.58, 127.65, 127.87, 128.04, 128.14, 128.16, 128.28, 128.33, 129.04, 129.55, 129.66, 136.99, 138.16, 138.50, 138.80, 159.31; HRMS (ESMS, M+Na⁺) calcd for C₅₅H₅₇O₁₁N₃Na 958.3891, found 958.3885.

**(2-Azido-3,6-di-O-benzyl-2-deoxy-α-D-glucopyranosyl)-(1→6)-2,3,4,5-tetra-O-benzyl-1-O-(4-methoxybenzyl)-D-myo-inositol 29 (refer scheme 8):** Compound 13 (280 mg) was subjected to benzylation to convert glucosyl 3-OH to 3-O-Benzyl. The reaction was performed exactly as described previously to obtain 300 mg (100%) of the desired compound. The 4,6-benzylidene on azidoglucosamine was subjected to preferential opening to give 6-O benzyl azido glucosylinositol. This was obtained by treating the starting material 14 (10 mg, 0.01 mmol) with sodium cyanoborohydride (2.52 mg, 0.0401 mmol) in freshly activated molecular sieves, dry THF 150 µL and TsOH 2.6 µL (0.04 mmol). The reaction mixture was stirred vigorously till pH 2.5-3 is obtained. The reaction was completed in 30 minutes (as detected on TLC), cooled on ice and quenched with aq. NaHCO₃. One has to keep in mind that acidic pH can cause the hydrolysis of p-methoxybenzyl (PMB). The reaction mixture was washed with NaHCO₃ and H₂O. The organic layer was collected, concentrated and purified to obtain pure 29 (80%). ¹H NMR (300 MHz, CDCl₃): 3.27 (dd, J = 3.8 and 10.0 Hz, 1H, 2b-H), 3.39 (dd, J = 2.2 and 9.8,
1H, 1a-H or 3a-H), 3.45 (dd, J = 2.2 and 9.8, 1H, 1a-H or 3a-H), 3.46 (dd, J = 9.3 Hz, 1H),
3.52 (m, 2H), 3.82 (s, 3H, OCH₃), 3.97-4.04 (m, 2H, 2a-H, 3a-H), 4.04-4.29 (m, 4H), 4.44
(s, 2H, CH₂Ph), 4.51-4.96 (m, 12H, CH₂Ph), 5.47 (s, 1H), 5.73 (d, J = 3.8 Hz, 1H, 1b-H),
6.83-6.89 (m, 2H, Ph), 7.08-7.13 (m, 2H, Ph), 7.17-7.45 (m, 30H, Ph); ¹³C NMR (75
MHz, CDCl₃): 55.20, 62.66, 69.00, 69.30, 71.78, 72.18, 72.64, 73.32, 73.45, 74.16, 74.21,
74.68, 74.76, 75.49, 75.60, 79.27, 80.80, 81.54, 81.84, 81.90, 84.35, 97.30, 113.78, 127.49-
129.96 (multiple peaks), 137.92, 138.17, 138.33, 138.43, 138.57, 138.83, 159.28; HRMS
(ESMS, M+Na⁺) calcd for C₆₂H₆₅O₁₁N₃Na 1050.4517, found 1050.4521.

3.2.7 Synthesis of GPI analogue (refer scheme 6 and 7):

(2-Azido-2-deoxy-α-D-glucopyranosyl)-(1→6)-2,3,4,5-tetra-O-benzyl-O-(4-
methoxybenzyl)-D-myo-inositol (15): 4-O-benzyl protected benzylidene disaccharide 14
(164 mg, 0.159 mmol) in (1:9) 1M HCl-Acetone (50 mL) was heated to 40°C. To this
mixture was added ρTSA (50 mg) to mediate hydrolysis and removal of benzylidene
protection. The reaction was completed in 4 hours as indicated by TLC (60%
EtOAc/Hexane, Rf 0.43), washed with H₂O, extracted in EtOAc and purified on silica gel
column chromatography to obtain compound 15 (146 mg, 98%), which was used up for the
next step.

The compound 15 (20 mg, 0.21 mmol) in 500 μL dry pyridine was cooled to 0°C followed
by the addition of acetic anhydride (500 μL). After this addition, the reaction mixture was
brought to room temperature and stirred overnight. The reaction was quenched with ice.
DCM was added and washed with H₂O, concentrated and purified on silica gel column
chromatography to obtain desired compound 16 (21 mg, 100%). TLC was detected in 40%
EtOAc/Hexane (Rf 0.75). A mixture of compound 16 (140 mg, 0.137 mmol) in pyridine (15 mL), H₂O (2.6 mL), propane dithiol (520 µL) and TEA (978 µL) was stirred for 16 hours, concentrated with toluene:ethanol (5:1) to remove traces of water. To this was added dry toluene (12 mL), (Boc)₂O (1.257 mL), dry pyridine (5 µL) and stirred for 3 hours until the reaction was complete. The organic solvent was removed by evaporation and purified by silica gel column chromatography to obtain compound 17 (149 mg, 99%). TLC in 50% EtOAc/Hexane (Rf 0.66)

The compound 17 (150 mg, 0.137 mmol) in anhydrous DCM:MeOH (1:3, 8 mL) was added catalytic amounts of sodium methoxide and stirred for 1 hour. The reaction mixture was concentrated and purified on silica gel column chromatography to obtain compound 18 (93%). TLC was detected in 50% EtOAc/Hexane (Rf 0.33).

(2-N-(tert-butyloxy carbonyl)-3,6-di-O-benzyl-2-deoxy-α-D-glucopyranosyl)-(1→6)-2,3,4,5-tetra-O-benzyl-1-O-(2,3-isopropylidene-(sn-glyceryl-phosphonato)-D-myoinositol (23): To the compound 18 (10 mg, 0.0125 mmol) in dry pyridine (200 µL), was added freshly activated molecular sieves and subjected to tosylation with TsCl (5.8 mg, 0.03 mmol, 1.1eq) to specifically tosylate the 6-OH of glucosamine residue. This reaction mixture was stirred at room temperature for 17 hours until the reaction was complete and then concentrated, added DCM and filtered through celite. The filtrate was concentrated and purified by silica gel column chromatography to obtain desired 19 (7 mg, 63%). TLC was detected in 40% EtOAc/Hexane (Rf 0.56). ESMS: (M+Cl-) calculated for C₆₇H₇₅O₁₅NSCl 1165.4, found 1200.4.
The tosyl group was converted to azido group by treating the compound 19 (14 mg, 0.011 mmol) in 200 µL dry DMF and NaN₃ (1.45 mg, 0.022 mmol). The reaction mixture was stirred at 85°C for 2 hours, followed by the addition of H₂O. The reaction mixture was cooled to RT and the compound was extracted in DCM. The organic layer collected was concentrated to give an organic residue, which was purified by silica gel chromatography to obtain pure compound 20 (11mg, 87%). TLC was detected in 40% EtOAc/Hexane (Rₜ 0.63). The compound 20 was subjected to benzylation at 4-OH position of glucosamine to obtain compound 21. However in this case the major product obtained was also benzylated at the NHBoc group of glucosamine. TLC was detected in 30% EtOAc/Hexane (Rₜ 0.75). ESMS: (M+H₂O) calculated 1036.53 for C₆₇H₇₅O₁₃N₄, found 1144.25 and proceeded to next step.

In the next step, the compound 21 was subjected to oxidation by DDQ to cleave PMB group. Briefly, the compound 21 (11 mg, 0.0098 mmol) dissolved in 1.5 mL DCM-H₂O (100:1) was added DDQ (8.9 mg, 0.04 mmol) and stirred for 3 hours at RT. The reaction mixture was extracted in DCM and worked up with NaHCO₃, dried and purified to obtain compound 22 (9 mg, 92%). TLC was detected in 30% EtOAc/Hexane (Rₜ 0.22). ESMS: (M+K) calculated 1006.4 for C₅₉H₆₆O₁₁N₄K, found 1045.5.

The next step involved the coupling of compound 22 with isopropylidene-sn-glycerol H-phosphonate. For this, compound 22 (19 mg, 0.019 mmol) and H-phosphonate (37 mg, 0.19 mmol, 10 equivalents) was mixed in 700 µL dry pyridine and then treated with pivaloyl chloride (40 µL, 0.019mmol, 10 equivalents). The reaction was stirred for 6 hours at RT, followed by the addition of iodine (63 mg, in 600 µL of 19:1 pyridine/H₂O) and
continuation of the reaction for additional 6 hours. The reaction mixture was diluted with CHCl₃ and washed with 5% Na₂S₂O₇ to remove iodine. The organic layer was collected, concentrated and purified by silica gel chromatography to obtain the pure compound 23 (20 mg, 88%). TLC system was 15% MeOH/DCM in 0.1% TEA (R_f 0.63). ESMS: (M+2HCOO⁻) calculated 1290.5 for C₆₇H₇₉O₂₀N₄P, found 1290.5

The compound 23 was subjected to pTSA-mediated hydrolysis to remove the isopropylidene protection. Briefly, to the starting material 23 (20 mg, 0.017 mmol) dissolved in 1 mL MeOH was added pTSA (3.5 mg/mL) and stirred for 45 minutes. The reaction was quenched with TEA, followed by normal workup. The organic layer was concentrated and purified to give compound 24 (14 mg, 74%). 15% MeOH/DCM in 0.1% TEA (R_f 0.29). ESMS: (M+2HCOO-) calculated 1250.4 for C₆₄H₇₅O₂₀N₄P, found 1250.5

(2-N-(tert-butyloxy carbonyl)-3,6-di-O-benzyl-2-deoxy-α-D-glucopyranosyl)-(1→6)-2,3,4,5-tetra-O-benzyl-1-O-(1,2-di-hexadecanoyl-sn-glyceryl-phosphonato)-D-myoinositol (25): The starting material 24 (25 mg, 0.0216 mg) was dissolved in dry DCM (3 mL) and treated with palmitic acid (27 mg, 0.1 mmol, 5 eq) in presence of DCC (22 mg, 0.108 mmol, 5 equivalents) and DMAP (2.6 mg, 0.0216 mmol, 1 equivalents) for 24 hours. The reaction mixture was filtered to remove unreacted palmitic acid and the byproduct DCU. The filtrate was concentrated and purified to give compound 25 (6 mg, 17%).; ¹H NMR (500 MHz, CDCl₃): δ 0.89-0.86 (t, J = 7.0, 6H), 1.23-1.19 (m, 57H), 1.42-1.35 (t, J = 6.6, 9H), 1.58-1.54 (m, 4H), 2.30-2.20 (m, 4H), 1H), 3.00-2.95 (q, J = 7.3, 6H), 3.45-3.40 (dd, J = 3.6 and 10.6), 3.56-3.54 (dd, J = 3.2 and 9.6, 1H), 3.65-3.60 (t, J = 8.6, 1H), 4.25-3.85 (m, 10H), 4.44-4.36 (t, J = 9.6, 1H), 4.56-4.52 (d, J = 11.8, 1H), 4.62-4.59 (d, J = 11.7, 1H), 4.99-4.69 (m, 10H), 5.30-5.23 (m, 2H), 5.51-5.50 (d, J = 3.6, 1H), 6.12-6.09 (m,
1H), 7.40-7.14 (m, 30H). $^{13}$C NMR (125 MHz, CDCl$_3$): 8.43, 14.12, 22.69, 24.85, 28.49, 29.71-29.16 (multiple peaks), 31.93, 34.03, 34.29, 45.46, 51.23, 54.88, 62.64, 63.79, 70.33, 62.96, 70.88, 72.27, 73.97, 74.54, 74.78, 75.70, 75.78, 76.43, 77.23, 78.29, 80.78, 82.08, 82.48, 80.34, 98.66, 128.26-127.08 (multiple peaks), 136.90, 138.25, 138.33, 138.43, 138.71, 138.79, 154.90, 156.38; 10% MeOH/DCM in 0.1% TEA ($R_f$ 0.63). ESMS: (M-H+) calculated 1637.9 for C$_{94}$H$_{132}$O$_{18}$N$_4$P, found 1637.2

(2-N-(tert-butyloxy carbonyl)-2-deoxy-α-D-glucopyranosyl)-(1→6)-1-O-(1,2-dihexadecanoyl-sn-glyceryl-phosphonato)-D-myoinositol (26): The compound 25a (3 mg, 0.0018 mmol) in 500 µL MeOH, 500 µL DCM and 25 µL H$_2$O added Pd(OH)$_2$ (8 mg) and stirred under hydrogen gas (H$_2$) overnight. The reaction mixture was evacuated and filled with hydrogen and stirred overnight. The reaction mixture was diluted in MeOH/H$_2$O (8:2, 20 mL) and filtered through celite to remove palladium. The filtrate was concentrated to obtain the desired compound 26 (1.19 mg, 61%). ESMS: (M+H+) calculated 1071.66 for C$_{52}$H$_{100}$O$_{18}$N$_2$P, found 1071.2

The compound was then conjugated to commercially purchased Bodipy$^{\text{TMR}}$ NHS ester (Invitrogen). Starting material 26 (1.19 mg, 0.0017 mmol) was dissolved in 500 µL DMF and reacted to Bodipy$^{\text{TMR}}$ NHS ester (1.15 mg, 0.0017 mmol, 1 eq) in presence of TEA (7.3 µL) under argon for 2-3 hrs. After the completion of the reaction, the mixture was concentrated and taken to the next step. This material (0.5 mg, 0.3 ūmols) was dissolved in 50 µL of DCM: CH$_3$CN: TFA (2:2:1) and stirred for 2 hours to remove the Boc protection at glucosamine. NaHCO$_3$ was added to neutralize the acidic pH and concentrated the reaction mixture, which was then subjected to mass spectrometry for the detection of
compound 27. However the compound detected in the mass spectra contained an acyl chain cleaved (possibly at sn-2 position due to the basic conditions availed during dye coupling reactions). Therefore, three steps of designed synthetic strategy namely, 1) Loss of more than 50% of the total product in reaction 13 due to purification and separation of D and L isomers, 2) Low yield for product 13 and 25, and 3) Loss of desired product due to benzylation of NHBoc at 21 were modified to obtain GPI analogues in better yield. The modifications include camphanate based isolation of optically pure D isomer of myo-inositol for synthesis of the inositol intermediate, conversion of 6-OH of glucosamine to azido group by mesyl instead of tosyl chemistry and coupling of suitably protected disaccharide to 1,2-di-hexadecanoyl-sn-glycerol-H-phosphonate in acetonitrile gave fully protected disaccharide with higher yields (90%). The fully protected disaccharide was subjected to hydrogenation and conjugation to Bodipy™ fluorophore. Preparative TLC and reverse phase HPLC were utilized to purify the resultant product. This purified compound was subjected to TFA treatment to remove the Boc protection. This reaction yielded the desired compound in 60% yield. The modified synthetic strategy is detailed in tetrahedron letters 2011 (Saikam et al., 2011). A di-octanoyl (C8:0) version of the same disaccharide was also synthesized by this modified synthetic strategy.

3.3 Non-hydrolysable GPI analogues:

GPI-APs in some cases are subjected to phospholipase activity, namely GPI or phosphatidylinositol specific phospholipase C (GPI-PLC) or (PI-PLC). In this case the P-O bond linked to the glycerol is subjected to cleavage with the help of the nucleophilic 2-OH group of inositol(Saikam et al., 2011). This leads to the formation of second messengers like DAG (diaclylglycerol) or inositol 1,4,5-tri-phosphate (IP3) which in turn can induce or
activate several responses like cell proliferation, DNA synthesis etc. In order to understand
the influence of this signaling events in the organization of the GPI-AP at the plasma
membrane, non-hydrolysable GPI analogues were synthesized by replacing the PI-PLC
sensitive P-O bond within GPI to a P-C bond (Saikam et al., 2011). To test if these non-
hydrolysable GPI analogues are indeed PI-PLC resistant, an in vitro based PI-PLC assay in
comparison to NBD tagged PI analogue was performed. Further, the PI-PLC inhibition
activity of these non-hydrolysable GPI analogues was measured using readily available
GPI-NBD analogues in the laboratory.

The chemical structure of the first generation non-hydrolysable GPI analogues is depicted
in the (Figure 3.7) and the synthesis of these analogues have been reported in (Saikam et
al., 2011).

3.3.1 Method for PI-PLC reaction:

The GPI non-hydrolysable analogues were dried thoroughly and re-suspended in H₂O by
bath sonication for 5 minutes such that the final concentration of the sample was 10 mM.
To 5 µL of the sample (10 mM) was added 5 µL of sodium deoxycholate (0.8%, w/v) and
10 µL borate buffer (0.1 M sodium borate/hydrochloric acid, pH 7.5) and 4 µL of PI-PLC
enzyme. The reaction mixture was incubated at 37°C for 20 minutes and quenched with
200 µL CHCl₃/MeOH/concentrated HCl (66:33:1). TLC and mass spectrometry of CHCl₃
layer provided information on the progress of the reaction (Figure 3.7 B).

NBD-GPI is added to borate buffer pH-7.5 containing deoxycholate at 2 mM (above
critical micelle concentration). To this, PI-PLC (0.0013 mg/mL) enzyme is added and
warmed to 37°C to initiate the reaction. The reaction is continued for 10 minutes for 50%
of GPI-NBD to undergo PI-PLC cleavage. The reaction vial was placed on ice to terminate
the reaction. The reaction mixture was spotted on TLC plate and run in 7:1:1:1 (EtOAc:
MeOH: AC₂CO: H₂O) solvent system. This TLC was imaged in UV to find the
concentration of product formed in each case (Figure 3.8 A & B). GPI-NBD on PI-PLC
cleavage gives rise to two products namely the glycan and the NBD-glycerolipid, of which
the latter fluoresces in UV. Thus from the fluorescent bands of the reactants (R) and the
product (P) the percentage of reaction (P/R x 100) was calculated. Both the reactant band
and the product band were normalized to the loading control in each lane.

In the case of inhibition reactions, to the above GPI-NBD reactions, varying concentrations
of inhibitor (ie, 7.5 - 0.1 mM in case of 28 and 20 - 0.1 mM in case of 29) was added and
the reaction carried out at 37°C for 10 minutes. For each concentration of the inhibitor
added, corresponding percentage of the reaction occurred was calculated and this was
subtracted from that obtained for a control NBD-GPI reaction (without inhibitor) to derive
the percentage of inhibition.

3.4 Summary and Conclusions

1. A synthetic strategy to obtain fluorescently tagged GPI analogues was designed and
executed. Modification of this synthetic strategy at 3 different steps provided desired GPI
analogues in good yield.

2. Designed and performed an in vitro based PI-PLC assay, to determine the PI-PLC
sensitivity of synthetic GPI analogues. The non-hydrolysable GPI analogues were verified
to be PI-PLC resistant and the IC50 values were determined to be 0.39 mM for compound
28 and 1.6 mM for compound 29.
Figure 3.1

A

Full length GPI

B

Btmr-GlcNPI 1. R=C16:0  
2. R = C8:0

C

FL-GlcNPI  1. R=C18:0  
2. R=C18:1

D

Chemical structure of A) full length GPI anchor: basic back bone of GPI anchor consists of a glycan head group, glycerolipid anchor and ethanolamine phosphate that links the glycan head group to the protein via amide linkage. The glycan group mainly consists of five glycan residues in the order 3Man-GlcN-Ins, where as the glycerolipid contain long and saturated lipid tails either C16:0 or C18:0 via diacyl or alkyl acyl linkages. B) Bodipy-TMR tagged GPI analogues C) Fluorescein tagged GPI analogues and D) NBD tagged GPI analogues.
Figure 3.2

Building blocks

A

B

C

D

E

Chemical structure of building blocks A) glycosyl donor B) myo-inositol acceptor C) mannosyl donor D) glycerol-H-phosphonate and E) ethanol amine -H-phosphonate.
Figure 3.3

A) Chemical structure of non-hydrolysable GPI analogues

B) PI-PLC reaction on non-hydrolysable GPI analogues and PI-NBD and confirmation by mass spectrometry.
IC50 curve for non-hydrolysable GPI analogue. A) \( n=0 \) and B) \( 2; n=1 \) is plotted. UV images of TLC run for the respective inhibition reactions are given below each graph.
Scheme :1

\[
\begin{align*}
\text{myo-inositol} & \xrightarrow{a} \text{Product} & 26\% &+\ & 38\% &+\ & 19\% \\
\end{align*}
\]

Reagent and conditions: a) cycloheanone, toluene, \(p\)TSA, DMF

Scheme :2

Reagent & conditions: b) BaO-Ba(OH)\(_2\), DMF; c) allylbromide, NaH, DMF, rt; d) \(p\)TSA, ethylene glycol e) BnBr, NaH, DMF, rt, 3h; f) \(p\)TSA, ethylene glycol, 50 °C, 3h; g) 1). (Bu)\(_2\)SnO, MeOH, reflux, 2h 2). PMBCl, (Bu)\(_4\)NBr, MeOH, reflux; h) BnBr, NaH, DMF; i) t-BuOK, DMSO, 80 °C, j) 1M HCl:Acetone (1:9), 50 °C.
Scheme 3

Reagent and conditions: a) CAN, NaN₃, -20°C; b) dioan/H₂O, NaNO₂, 80°C; c) CCl₃CN, K₂CO₃

Scheme 4

Reagents & conditions: a) imidazole, PCl₃, TEA, 0 °C
Scheme 5

Reagents & conditions: a) TMSOTf, dry DCM, 0 °C, 30min; b) MeON, MeOH; Benzaldehydedimethacetal, CSA, CH₃CN.

Scheme 6

Reagents & conditions: d) BnBr, NaH, DMF; e) HCl/Acetone, 48 oC; f) (CH₃CO)₂O, pyridine; g) i) HSCH₂CH₂SH, pyridine, H₂O, TEA; ii) Boc₂O, Dry Toluene; h) MeONa, MeOH/DCM i) TsCl, Dry Pyridine, 0 °C; j) NaN₃, DMF, 80 °C.
Reagent and conditions: k) BnBr, DMF, NaNH \[\text{NaH}\]; l) DDQ, DCM, H\(_2\)O; m) pivaloyl chloride, anhy. pyridine; ii) iodoine; n) \(p\)-TSA, MeOH/DCM; o) palmitic acid, DCC, DMAP; p) Pd/C, 30 atm, 3hrs; q) i. Bodipy\textsuperscript{TMR} STP ester, DMF, TEA; ii) TFA