Supplementary Information for:

Flip-flop of glycosylphosphatidylinositols (GPI's) across the ER

Ram A. Vishwakarma*[a] and Anant K. Menon*[b]

[a] *Bio-Organic Chemistry Laboratory, National Institute of Immunology, New Delhi 110067, India. Fax: +91-11-26162125; Tel: +91-26717178; E-mail: ram@nii.res.in;*
[b] *Department of Biochemistry, University of Wisconsin-Madison, 433 Babcock Drive, Madison, WI 53706, U.S.A. Fax: +1-608-262-3453; Tel: +1-608-262-2913; E-mail: menon@biochem.wisc.edu*

[A] Biological Membrane Reconstitution of GPI flip-flop

NOTE: Unless stated otherwise, figure numbers refer to figures in 'Supporting Information'.

General Experimental Procedures:

Egg phosphatidylcholine (ePC) and routine chemicals were obtained from Sigma Chemical Co. SM-2 Bio-beads were purchased from Bio-Rad Laboratories. ULTROL™ grade Triton X-100 was from Roche Molecular Biochemicals. 1-acyl-2-C_6-NBD-PC (NBD-PC) was from Avanti Polar Lipids. Salt-washed endoplasmic reticulum (SWER) was prepared from a rat liver homogenate as previously described.[1,2] Triton X-100, phospholipid, and protein were quantitated according to published procedures.[3-6]

Preparation of a Triton X-100 extract (TE) from SWER:

The procedure has been described elsewhere[6] and is summarized here. SWER (~20 mg/ml) was mixed with an equal volume of 10 mM HEPES/NaOH, pH 7.5, 100 mM NaCl, 0.1% (w/v) Triton X-100, left on ice for 30 min, and then ultracentrifuged (TLA 100.3 rotor, 70,000 rpm, 30

min, 4ºC). The supernatant was removed and the pellet was resuspended to the original volume of SWER in 10 mM HEPES/NaOH, pH 7.5, 100 mM NaCl, 1% (w/v) Triton X-100. The sample was mixed by gentle vortexing, and left on ice for 30 min before being ultracentrifuged as above. The resulting clear supernatant, or Triton extract (TE)) was snap-frozen and stored at -80ºC.

Reconstitution of liposomes and proteoliposomes:

A mixture of egg PC (4 μmol) and a trace amount (~0.3 mol %) of NBD-GlcNH_2-PI (compound **1**), NBD-GlcNAc-PI (compound **2**) or NBD-PC was dried under a stream of N_2, then dissolved in 10 mM HEPES/NaOH, pH 7.5, 100 mM NaCl, 1% (w/v) Triton X-100, and combined with TE (typically 5-30 μl of a ~2.6 mg/ml solution) in a total volume of 1 ml. The sample was treated in two stages with 300 mg washed SM2 Bio-Beads to generate proteoliposomes as described previously.[1-3,7] Liposomes were prepared similarly except that TE was omitted. This procedure has been shown to yields single, unilamellar vesicles with a diameter in the range 180-250 nm as determined by electron microscopy and light scattering.[1-3,7]

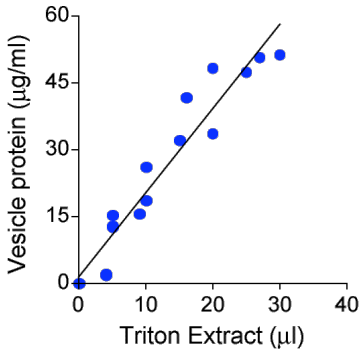

Figure 1. Protein amount recovered in reconstituted vesicles as a function of the amount of TE used per 1 ml reconstitution.
Data are compiled from samples generated in different experiments. Protein measurement was done according to the method of Kaplan and Pedersen.[5] The line represents the best fit of the data. Protein concentration in the TE was ~2.6 mg/ml. Approximately 1 ml of vesicles were obtained per reconstitution giving a protein recovery of ~72-78%.

Vesicle samples prepared from a number of different experiments were characterized. Residual Triton X-100 in the sample after Bio-Bead treatment was determined to be 0.010 $\pm$ 0.002 % (w/v), indicating that 99.9% of the detergent was removed by the beads during vesicle

preparation. The amount of phospholipid recovered was 2.88 $\pm$ 0.17 μmol (irrespective of the amount of TE used in the reconstitution), corresponding to a recovery of ~72%. Protein recovery was ~72-78% (Figure 1). The inflection point of the protein-dependence plot shown in Figure 3C of the *Communication* corresponds to TE~32 μl; based on Figure 1 and the protein and phospholipid yields stated above, this corresponds to a sample with a protein concentration of ~62 μg/ml, a phospholipid concentration of ~2.9 μmol/ml and a protein:phospholipid ratio of ~21 μg/μmol.

Fluorescence assay for GPI flipping:

Vesicles (50 μl of the 1 ml suspension generated as described above) were diluted to 1.5 ml with 10 mM HEPES/NaOH, pH 7.5, 100 mM NaCl and monitored at room temperature in a Perkin-Elmer LS50B spectrophotometer, using excitation and emission wavelengths of 470 nm and 530 nm, respectively. A stable initial fluorescence intensity, F_0, was noted before adding 9 μl of a 1 M solution of dithionite, prepared freshly in 1 M Tris base. The consequent reduction in fluorescence (due to the conversion of NBD to a non-fluorescent amine by dithionite)[8] was monitored for 6 min, yielding a final fluorescence value of F_6. The 6 min time point was chosen empirically after determining that the fluorescence intensity reached a relatively stable plateau after this time interval. The percent reduction caused by dithionite treatment was calculated as $100(F_0-F_6)/F_0$. The percent reduction increased by ~2-3% if a 10 min time point was chosen instead of a 6 min time point. The pH of the sample remained at 7.5 after dithionite addition and no change in fluorescence was seen on addition of 9 μl of 1 M Tris base alone, without dithionite. The assay is described in detail in the publication by Chang et al.[6]

Discussion of the fluorescence assay for GPI flipping:

A. Validity of NBD-lipid probes as reporters of flippase activity. Encouraged by the ubiquitous and successful use of acyl-NBD-labeled glycerophospholipids as reporters of transbilayer lipid translocation in biological membranes,[10] we developed acyl-NBD derivatives of GPI's as probes of GPI flipping. The NBD fluorophore in our GPI probes, as in most NBD-phospholipid probes used in lipid trafficking studies, modifies one of the glycerol-linked acyl chains. The polar nature of the NBD moiety causes the acyl chain to loop back in the membrane such that NBD is positioned at the membrane-water interface[10,11] making it accessible to topological probes such as dithionite (see Figure 3A of the *Communication* which is reproduced below as Figure 2 of the Supplementary Information). The non-natural configuration of acyl-NBD-phospholipids in the membrane is a potential cause for concern. However, such concerns are mitigated when one notes that the 'loop-back' resembles acyl chain conformations that can be achieved dynamically by natural lipids.[11] Also, where explicit comparisons have been made, NBD-glycerophospholipid probes yield results in transport experiments that are at least qualitatively similar to those obtained with other analogs or natural phospholipids.[1,6,9,12-14]

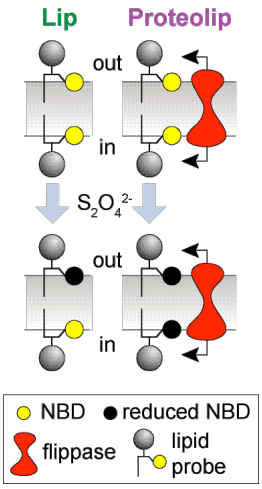

Figure 2: Schematic of flippase assay using acyl-NBD-lipid probes. The figure is identical to Figure 3A of the *Communication* and is reproduced here for convenience. The schematic shows the predicted 50% fluorescence loss on adding dithionite to symmetrically labeled liposomes (or inactive proteoliposomes)(left; labeled "Lip"), compared with 100% loss in flippase-active proteoliposomes (right; labeled "Proteolip") due to flipping of NBD-GPIs. The NBD-bearing acyl-chain is shown looping back to the membrane surface; this has the effect of positioning the polar NBD group at the membrane-water interface where it can react with dithionite. See text for details.

B. Symmetric incorporation of NBD-lipid probes in reconstituted vesicles. The flippase assay relies on incorporation of trace amounts (~0.3 mol%, of a bulk phospholipid (ePC) content of ~4 μmol) of the fluorescent lipid reporter during vesicle reconstitution. The NBD-lipids are expected to distribute roughly symmetrically across the membrane of the reconstituted vesicles (Figure 2 above). Evidence for this is seen in the ~45% reduction in NBD fluorescence obtained when liposomes are treated with dithionite (Figure 3B of the *Communication*), as well as in preliminary collisional quenching experiments carried out on NBD-PC-containing proteoliposomes. The collisional quenching studies were done as described by Hrafnsdóttir et al.[10] except that iodide ions were used for quenching instead of cobalt ions. The studies suggest that ~50-55% of NBD-PC in proteoliposomes is inaccessible to iodide ions, consistent with the proposal that ~50-55% of the NBD-phospholipid is located in the protected inner leaflet of the proteoliposome membrane (not shown). The NBD-GPI probes would be expected to behave in similar fashion. These data argue against the possibility that the mere presence of membrane proteins forces the acyl-NBD-lipid probes into an asymmetric distribution with more probe residing in the outer leaflet of the vesicles. We conclude that increases in fluorescence reduction seen with 'flippase-active' proteoliposomes (beyond the ~45% value observed for liposome samples) can be attributed to transport of acyl-NBD-lipids from the inner to the outer leaflet (Figure 2) rather than to a pre-determined asymmetric distribution of the probes.

C. Incomplete reduction of NBD-lipid probes in liposomes and proteoliposomes after dithionite treatment. The flippase assay is based on elimination of fluorescence emanating from NBD-lipids in the outer leaflet of the reconstituted vesicles (Figure 2). In liposomes (or proteoliposomes lacking a flippase), only NBD-lipids initially located in the vesicle outer leaflet will be reduced by dithionite, resulting in a 50% loss in fluorescence. In flippase-equipped

proteoliposomes, NBD-lipids from the inner leaflet will flip out and also be reduced, resulting theoretically in a 100% loss in fluorescence (Figure 2). Despite the theoretical expectation of a 100% reduction in fluorescence in flippase-equipped vesicles, in practice fluorescence loss rarely exceeds 80% for reasons that are not clear. A similar situation has been noted with other topological probes in assays with other lipids.[1,3,6,9] A possible explanation is that dithionite reduction has an efficiency of ~80%: thus only ~40% fluorescence loss will be seen with liposome samples and ~80% fluorescence loss should be expected with proteoliposomes equipped with a flippase. In the experiments reported here, the extent of fluorescence loss ranges from ~45-75%, roughly consistent with the explanation offered.

D. Dithionite permeation? A potential concern with the dithionite-based assay used here is that the results could be due to protein-dependent dithionite permeation into the vesicles rather than protein-dependent flipping of lipids from the inside to the vesicle exterior. For a number of reasons, we believe that dithionite permeation into the vesicles is not a complicating factor in the assay. We list two reasons. *First*, two independent assays of glycerophospholipid flip-flop in proteoliposomes derived from a Triton X-100 extract rat liver ER, based on an ultra-short-chain radiolabeled phospholipid[1] or a natural phospholipid in conjunction with a phospholipase probe,[9] gave identical results to those obtained with NBD-phospholipids and dithionite[6] indicating that the assay read-out is not due to dithionite permeation into the vesicles or to the use of dithionite *per se*. *Second*, assays using 1-myristoyl NBD-phospholipids in conjunction with a bovine serum albumin back-exchange procedure[14] yielded identical results to those obtained with dithionite on the *same* samples,[6] indicating again that a flippase assay based on dithionite reduction of NBD-labeled phospholipid analogs does not generate an artifactual read-out. These data also argue

against the possibility that our assay read-out results from dithionite-mediated reduction of proteins in the vesicle preparation.

E. Kinetics of flipping. The rate of dithionite reduction of NBD is slower than the rate of flipping facilitated by even a single flippase in a vesicle.[9] Thus our assay cannot provide a measure of flipping kinetics unless it is coupled with a more elaborate stopped flow approach[12,14] that goes beyond the scope of the present work. Instead, the assay reports on flippase abundance in the mixture of proteins used in the reconstitution (see below).

F. Flippase abundance. The inflection point at which the extent of dithionite-mediated fluorescence reduction reaches a plateau (Figure 3C of the *Communication*) corresponds to TE~32 μl or a protein:phospholipid ratio of ~21 mg protein/mmol phospholipid. The inflection point was determined as follows: a] the data were fit to a one phase exponential (see Figure 4 below) to provide an estimate of the maximum extent of reduction that could be obtained in the system (~74% reduction; see Figure 4); b] the exponential fit was modeled by two line segments (see Figure 3C of the *Communication*), one corresponding to the plateau value of 74% and another derived from linear regression of data points from the rising section of the graph (TE $\leq$ 20 μl); c] the point where the rising linear segment intersects the plateau is the inflection point referred to above.

The inflection point can be used to estimate flippase abundance. Each vesicle of ~250 nm diameter prepared at a protein:phospholipid ratio of ~21 mg/mmol (corresponding to the inflection point) contains ~175 proteins (assumed to be ~50 kDa each, a good estimate for ER membrane proteins[1]). This implies that the flippase represents ~0.6% by weight of ER membrane proteins in the TE.[6] Our interpretation of these data[1,3,6,9] is that increasing the protein/phospholipid ratio from 0 mg/mmol to ~21 mg/mmol results in an increasing number of

vesicles being populated with a flippase. At 0 mg/mmol, none of the vesicles in the population have a flippase whereas at ~21 mg/mmol each vesicle has, on average, 1 flippase. Thus the 0-21 mg/mmol regime for reconstituted TE provides an experimental read-out of flippase abundance.

GPI probes are not metabolized during the vesicle reconstitution process:

Liposomes and proteoliposomes containing the GPI probes were analyzed to determine whether the probes were recovered intact after the vesicle reconstitution procedure. An aliquot of vesicles was dried, dissolved in methanol and analyzed by negative ion ES-MS as well as thin layer chromatography (silica plates developed with *n*-butanol-ethanol-ammonia-water, 4:4:4:1 (by volume)). TLC analyses showed only a single fluorescent spot corresponding to the intact NBD-GPI probe; there was no evidence of free NBD-acyl chains. The ES-MS analyses revealed only molecular ions corresponding to the intact lipid with no evidence of *lyso* lipids or other major fragments. These results indicate that both NBD-GlcNAc-PI and NBD-GlcN-PI remain intact through the reconstitution process.

Effect of proteolysis on GPI flipping:

Proteoliposome samples (prepared using 20 μl TE to generate vesicles corresponding to the linear rise of the protein dependence plot shown in Figure 3C of the *Communication*) were treated with proteinase K for 30 min at room temperature (a mock-treated sample was analyzed in parallel) and assayed to determine the extent of dithionite reduction of the NBD-GPI probes. Fluorescence reduction on dithionite addition was 59.6% for the protease-treated proteoliposomes compared with 64.4% for mock-treated control proteoliposomes and 49.2% for liposomes. The 'flippase signal' (obtained by subtracting the % fluorescence reduction seen with

liposomes from that seen with protease-treated or mock-treated proteoliposomes) from protease-treated proteoliposomes was thus 10.4%, compared with 15.2% from mock-treated proteoliposomes, a ~30% decrease. Since the vesicles were prepared in a regime where each vesicle is expected to have 0 or 1 flippase, this result indicates that proteinase K treatment eliminated ~30% of functional flippases in the TE. The incomplete loss of flippase activity on proteolysis has been noted before[1,3,6,9,15] and is likely due to the fact that the flippase protein can adopt either of two possible orientations when it is reconstituted into vesicles. We propose that in one orientation the protein is functionally susceptible to proteolysis whereas in the opposite orientation it is not.[1] Similar data were obtained in a study of the reconstitution of the mannose-phosphate-dolichol transport activity in the ER: extensive trypsin treatment resulted in ~30% loss in activity.[15]

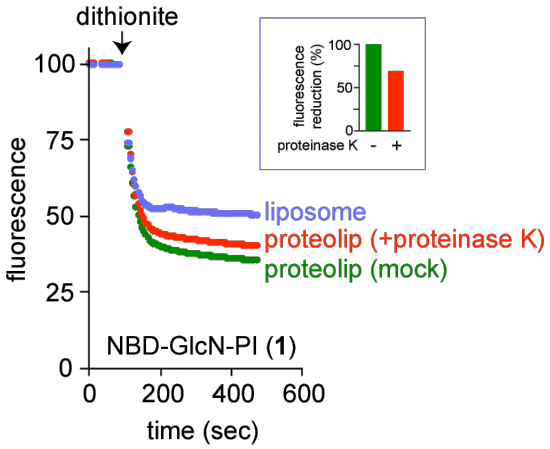

Figure 3. Effect of proteinase K treatment on NBD-GlcN-PI flipping in proteoliposomes. Proteoliposomes containing trace amounts of NBD-GlcN-PI were treated with proteinase K or mock-treated. A liposome sample was analyzed alongside. Fluorescence traces obtained on dithionite addition are shown. The inset shows the fluorescence reduction seen with mock-treated and proteinase K-treated proteoliposomes; the data are determined as fluorescence reduction seen in excess of the value obtained for liposomes with mock-treated proteoliposomes set at 100%.

Flipping of NBD-PC, a fluorescence reporter for bulk membrane phospholipid

We assayed flip-flop of 1-acyl-2-C_6-NBD-PC using the method outlined for the GPI probes above. Raw data were obtained that were essentially identical to the traces shown in Figure 3B of the *Communication*. Figure 4 shows that the extent of dithionite-mediated reduction of NBD-PC (red, filled squares) depends on the amount of TE used for reconstitution. Since the protein-

dependence profile corresponds exactly with the data for the GPI probes (open symbols), it is possible that the ER glycerophospholipid flippase responsible for flipping NBD-PC is also capable of flipping NBD-GlcNAc-PI and NBD-GlcN-PI. Alternatively, as discussed in the *Communication*, the three lipids may be transported by different flippases that are similarly abundant in the TE.

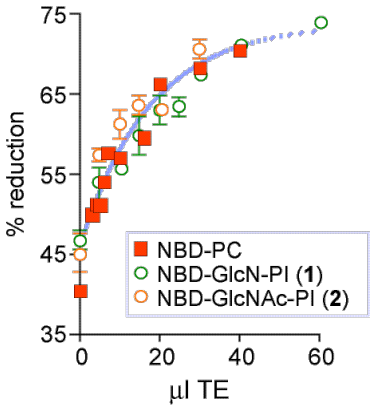

Figure 4. Flipping of 1-acyl-2-C_6-NBD-PC in proteoliposomes reconstituted from a Triton X-100 extract of rat liver ER. The data were obtained in parallel with the data for the GPI probes shown in Figure 3C of the *Communication.* The extent of dithionite-mediated reduction of NBD-PC (red, filled squares) is shown as a function of the amount of TE used for reconstitution. The line through the points is a single-phase exponential fit of the data for the GPI probes (GPI data points shown as open symbols, identical to the data points shown in Figure 3C of the *Communication*), highlighting the fact that the protein-dependence profile is the same for the GPI probes and NBD-PC. The exponential fit has a Ymax at ~74.

References for section [A] of Supporting Information

(Biological Membrane Reconstitution of GPI flip-flop)

1. A.K. Menon, W.E. Watkins and S. Hrafnsdóttir, *Curr. Biol.*, 2000, **10**, 241.
2. S.N. Gummadi, S. Hrafnsdóttir, J. Walent, W.E. Watkins and A.K. Menon, *Meth. Mol. Biol.* 2003, **228**, 271.
3. S. Hrafnsdóttir and A.K. Menon, *J. Bacteriol.* 2000, **182**, 4198.
4. G. Rouser, S. Fleischer and A. Yamamoto, *Lipids*, 1970, **5**, 494.
5. R.S. Kaplan and P.L. Pedersen, *Meth. Enzymol.*, 1989, **172**, 393.
6. Q. Chang, S.N. Gummadi and A.K. Menon, *Biochemistry*, 2004, **43**, 10710.
7. D. Levy, A. Bluzat, M. Seigneuret and J.L. Rigaud, *Biochim Biophys Acta*, 1990, **1025**, 179.
8. J.C. McIntyre and R.G. Sleight, *Biochemistry*, 1991, **30**, 11819.
9. S.N. Gummadi, and A.K. Menon, *J. Biol. Chem.*, 2002, **277**, 25337.
10. A. Chattopadhyaya, *Chem. Phys. Lipids*, 1990, **53**,1.
11. D. Huster, P. Müller, K. Arnold and A. Herrmann, A. *Biophys. J.*, 2001, **80**, 822.
12. S. Hrafnsdóttir, J.W.Nichols and, A.K.Menon, *Biochemistry*, 1997, **36**, 4969.
13. U. Marx, G. Lassmann, H.-G. Holzhütter, D. Wüstner, P. Müller, A. Höhlig, J. Kubelt and A, Herrmann, *Biophys. J.* 2000, **78**, 2628.
14. J. Kubelt, A.K. Menon, P. Müller and A. Herrmann, *Biochemistry* 2002, **41**, 5605.
15. J.S. Rush and C.J. Waechter, *Biochemistry*, 2004, **43**, 7643-7652.

[B] Synthesis of Fluorescent Glycosylphosphatidylinositol probes

General Experimental Procedures:

Solvents were purified according to the standard procedures, and reagents used were of highest purity available. NMR measurements (^{1}H, ^{13}C, ^{31}P, 2D ^{1}H-^{1}H and ^{1}H-^{13}C COSY, HMQC) were recorded on a 300 MHz spectrometer fitted with pulse-field gradient probe, and trimethylsilane (TMS) or residual resonance of deuterated solvent were used as internal reference. For ^{31}P NMR spectra, phosphoric acid was used as external reference. ^{13}C NMR spectra were broadband ^{1}H decoupled or inverse HMQC experiments. Chemical shifts are expressed in ppm and coupling constants *J* in Hz. ^{1}H and ^{13}C assignments were made by ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR analysis. Electrospray ionisation mass-spectra (ESMS) and High-resolution mass-spectra (HRMS) were obtained on quadrupole and LCT-TOF (time of flight) spectrometers respectively using acetonitrile-water (1:1) mobile phase. Optical rotations were measured on a digital polarimeter. TLC was performed on Merck Kieselgel 60 F_{254} plates, and compounds visualized by ammonium-molybdate/ceric-sulfate developing reagent. Preparative TLC was conducted on Analtech Uniplate silica-gel plates (20 x 20 cm). Silica column chromatography was carried out with silica gel 60 (60-120 mesh). Analytical and semi-preparative HPLC purification were carried out on a Shimadzu system using RP-18 column and a photodiode array detector.

Synthesis of NBD-labeled GPI probes:

1-*O*-Allyl-2,3,4,5-tetra-*O*-benzyl-D-*myo*-inositol (**3**). This chiral intermediate was prepared in following steps.

(a)1-O-[(1S)-(-)-camphanoyl]-2,3:4,5-di-O-cyclohexylidene-6-O-(p-methoxybenzyl)-D-myo-inositol. A solution of racemic 2,3:4,5-di-O-cyclohexylidene-*myo*-inositol (31.4 g, 92 mmol), prepared by a reported method[1] and dibutyltinoxide (24 g, 93 mmol) in anhydrous MeOH (200 mL) was heated to reflux for 4 h. Excess of solvent was removed and the residue was dried by repeated evaporation through toluene. The residue was dissolved in anhyd DMF and CsF (20 g, 120 mmol), KI (20 g, 120 mmol) and PMBCl (25 mL, 184 mmol) were added. The suspension was vigorously stirred for 24 h at room temperature. The solvent was removed under reduced pressure and the residue was purified by silica column chromatography to provide racemic 2,3:4,5-di-O-cyclohexyledene-6-O-(p-methoxybenzyl)-*myo*-inositol (16 g, 38%, Rf = 0.4 in 20% EtOAc-hexane). The above compound (8 g, 17.2 mmol) was dissolved in anyd CH_2Cl_2 (160 mL) and (1S)-(-)-camphanic chloride (4.6 g, 21.2 mmol), triethylamine (5 mL) and DMAP (catalytic) were added and mixture stirred for 1 h at rt. After completion of the reaction, the solvent was evaporated under reduced pressure and the residue was purified on a flash silica column (2% CH_2Cl_2-ether to 3% CH_2Cl_2-ether) to provide desired optically pure D-diastereoisomer 1-*O*-[(1S)-(-)-camphanoyl]-2,3:4,5-di-*O*-cyclohexylidene-6-*O*-(p-methoxybenzyl)-D-*myo*-inositol (4.0 g, 36%, Rf = 0.35 2% CH_2Cl_2-ether, $[a]^{20}_D$ = -35.8, c 1.4 $CHCl_3$ (lit.[2] $[a]^{20}_D$ = -36.5, c 1.4 $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): 7.28 (m, 2H), 6.87 (m, 2H), 5.21 (t, J = 5 Hz, 1H), 4.75 (d, J = 11 Hz, 1H), 4.67 (d, J = 11 Hz, 1H), 4.58 (dd, J = 4.7 and 6.3 Hz, 1H), 4.31 (dd, J = 6.2 and 8.4 Hz, 1H), 3.88-3.83 (m, 2H), 3.79 (s, OMe), 3.48 (dd, J = 8.8 and 10.6 Hz, 1H), 2.37 (m, 1H), 2.00-1.85 (m, 2H), 1.80-1.30 (m, 21H), 1.09 (s, Me), 1.01 (s, Me), 0.91 (s, Me); ^{13}C NMR (75 MHz, $CDCl_3$): 178.0, 166.51, 159.22, 129.70, 129.42, 113.67, 113.01, 111.45, 90.64, 77.82, 75.85, 74.12, 73.10, 71.73, 55.19, 54.80, 54.50, 36.45, 36.35, 30.56, 28.88, 24.87, 23.72, 16.7,

16.6, 9.6; HRMS (positive ion ESMS, $M+Na^+$) calcd for $C_{36}H_{48}O_{10}Na$ 663.3145, found 663.3150.

(b) *2,3:4,5-di-O-cyclohexylidene-6-O-(p-methoxybenzyl)-D-myo-inositol*. The above compound (1.4 g, 2.18 mmol) was treated with 2% NaOH solution in MeOH (100 mL) at 60-65 ^oC for 30 min. The solvent was removed and the residue dissolved in CH_2Cl_2 (100 mL) was washed with water, organic layer washed with H_2O, dried with Na_2SO_4 and concentrated to give optically pure 2,3:4,5-di-O-cyclohexyledene-6-*O*-(*p*-methoxy benzyl)*-D-myo*-inositol (0.72 g, 99% yield). ^1H NMR (300 MHz, $CDCl_3$): 7.29 (m, 2H), 6.88 (m, 2H), 4.7 (dd, J = 12 Hz, 1H), 4.60 (d, J = 12 Hz, 1H), 4.41 (m, 1H), 4.35 (t, J = 7.5 Hz, 1H), 4.16 (dd, J = 7.5 and 10.5 Hz, 1H), 4.00 (m, 1H), 3.88 (dd, J = 1.8 and 7.8 Hz), 3.79 (s, OMe), 3.52 (dd, J = 7.8 and 10.8 Hz, 1H), 2.58 (m, 1H), 1.70-1.55 (m, 21H); ^{13}C NMR (75 MHz, $CDCl_3$): 129.45, 129.42, 113.73, 112.83, 111.16, 90.64, 79.43, 78.85, 75.18, 72.22, 71.29, 55.18, 36.55, 36.50, 36.22, 33.36, 24.98, 23.72, 23.59, 23.28; HRMS (positive ion ESMS, $M+Na^+$) calcd for $C_{26}H_{36}O_7Na$ 483.2359, found 483.2370.

(c)*1-O-Allyl-2,3:4,5-di-O-cyclohexylidene-6-O-(p-methoxybenzyl)-D-myo-inositol.* The above compound (1.32 g, 2.86 mmol) was dissolved in anhyd DMF (25 mL) and solution was brought to 0 ^oC and then NaH (0.43 g) and allylbromide (1 mL) was added. The reaction mixture was stirred for 3 h at room temperature and brine (50 mL) was added, and extracted with ether (25 mL x 4). The organic extract was dried over Na_2SO_4, concentrated., and the residue was purified by a column to give desired compound (1.4 g, 99% yield). ^1H NMR (300 MHz, $CDCl_3$): 7.27 (m, 2H), 6.87 (m, 2H), 5.86 (m, 1H), 5.28-5.15 (m, 2H), 4.7 (dd, J = 12 Hz, 1H), 4.60 (d, J = 12 Hz, 1H), 4.38 (dd, J = 3.8 and 6.8 Hz, 1H), 4.30 (t, J = 7.3 Hz, 1H), 4.10-4.06 (m, 3H), 3.81 (m, 1H),

3.79 (s, OMe), 3.67 (t, J = 3.2 Hz, 1H), 3.49 (dd, J = 7.8 and 10 Hz, 1H), 1.90-1.32 (m, 20H); HRMS (positive ion ESMS, $M+Na^+$) calcd for $C_{29}H_{40}O_7Na$ 523.2672, found 523.2750.

(d)*1-O-Allyl-2,3,4,5-tetra-O-benzyl-D-myo-inositol* (**3**). The above bis-ketal (1.5 g, 3 mmol) was dissolved in a mixture of MeOH and CH_2Cl_2 (1:1) followed by the addition of pTSA (100 mg). The reaction was stirred for 24 h at room temperature, neutralized with triethylamine, concentrated. The residue was dissolved in anhyd DMF (40 mL) and cooled to 0 ^oC, followed by addition of benzyl bromide (5.5 mL) and NaH (1 g). The mixture was stirred for 4 h at room temp, cooled, quenched with MeOH (1 mL), diluted with brine, extracted into EtOAc. The organic layer was concentrated and the residue was cleaned by a quick silica column filtration to obtain pure benzylated product which was dissolved in CH_3CN (24 mL) and H_2O (6 mL) at 0 ^oC and treated with cerric ammonium nitrate (CAN, 2 g) for 1 h. The work-up involved washing with 5% sodium bicarbonate solution, concentration and silica column, which provided pure desired compound **3** (875 mg, 50 % yield over three steps). Rf = 0.41 in 20% EtOAc-hexane; $[a]^{20}_D$ = -8.5, c 1.2 $CHCl_3$. ^1H NMR (300 MHz, $CDCl_3$): 7.32-7.29 (m, 25H), 5.86 (m, 1H), 5.28-5.15 (m, 2H), 4.83 (m, 6H), 4.67 (m, 2H), 4.03 (m, 3H), 3.47 (m, 1H), 3.10 (dd, 1H); HRMS (positive ion ESMS, $M+Na^+$) calcd for $C_{37}H_{40}O_6Na$ 603.2723, found 603.2750.

(3,4,6-Tri-*O*-acetyl-2-azido-2-deoxy-α**-D-glucopyranosyl)-(1–6)-1-*O*-allyl-2,3,4,5-tetra-*O*-benzyl-D-*myo*-inositol** (**5**). A mixture of 1-*O*-allyl-2,3,4,5-tetra-*O*-benzyl-D-*myo*-inositol acceptor **3** (1.56 g, 2.7 mmol) and 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-β-D-glucopyranosyl-trichloroacetimidate donor **4**[3] (2.1 g, 4.41 mmol, 1.63 equiv), and freshly activated powdered 4A molecular sieves were dried by azeotropic removal of residual moisture through toluene. The

mixture was dissolved in anhyd CH_2Cl_2 (14 mL), stirred under argon at room temperature for 30 min, and then cooled to 0 ^oC. To the above was added a solution of TMSOTf (0.6 mL, 0.2M in CH_2Cl_2) drop-wise and the mixture was stirred further for 40 min at 0 ^oC. After completion, the reaction mixture was neutralized with triethyamine, filtered through celite and concentrated. The silica column chromatography (hexane/ethyl acetate) provided the product **3** (2.4 g, 90%) as colorless solid. ^1H NMR (300 MHz, $CDCl_3$): 7.42-7.27 (m, 20H), 5.90 (m, 1H), 5.78 (d, J = 3.8 Hz, 1H), 5.42 (dd, J = 10.5 Hz, 1H), 5.28-5.15 (m, 2H), 5.15-4.40 (m, 8H), 4.92 (dd,, J = 9.5 Hz), 4.30-4.21 (m, 2H), 4.13 (dd, J = 9.3 Hz, 1H), 4.05 (bs, 1H), 3.60 (m, 2H), 3.50 (dd, J = 1.8 and 9.5 Hz, 1H), 3.45 (dd, J = 9.5 Hz, 1H), 3.40 (dd, J = 1.8 and 9.5 Hz, 1H), 3.12 (dd, J = 3.6 and 10.6 Hz, 1H), 2.07 (s, 3H), 1.96 (s, 3H), 1.83 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): 170.43, 169.97, 169.47, 138.60, 138.35, 138.10, 138.30, 134.01, 128.45-126.74 (20 carbons), 117.14, 97.27, 81.83, 81.51, 81.09, 80.78, 75.69, 75.24, 75.18, 74.92, 74.08, 72.82, 70.71, 69.85, 66.72, 66.66, 60.95, 20.53, 20.44, 20.37; HRMS (positive ion ESMS, $M+Na^+$) calcd for $C_{49}H_{55}O_{13}N_3Na$ 916.3633, found 916.3644.

(2-Azido-3,4,6-tri-*O*-benzyl-2-deoxy-a**-D-glucopyranosyl)-(1–6)-1-*O*-allyl-2,3,4,5-tetra-*O*-benzyl-D-*myo*-inositol (6)**. The preceding pseudodisaccharide **3** (1.3 g, 1.4 mmol) dissolved in a solvent mixture of anhyd CH_2Cl_2 (3 mL) and MeOH (12 mL) was treated with saturated NaOMe solution (0.2 mL). The reaction was stirred for 24 h at room temperature. After completion, the reaction was neutralized with cation-exchange resin, filtered and concentrated. The residue was dissolved in anhyd DMF (32 mL) and cooled to 0 ^oC. This was followed by addition of benzyl bromide (2 mL) and NaH (0.6 g). The reaction was stirred at room temperature for 12 h, after which the excess of benzyl bromide was destroyed by addition of MeOH (1 mL). The reaction

was diluted with ethyl acetate (100 mL), organic layer washed with brine and water, dried over anhyd Na_2SO_4, and concentrated. The residue was purified on a silica column providing desired compound **6** (1.2 g, 80%). ^{1}H NMR (300 MHz, $CDCl_3$): 7.40-7.20 (m, 35H), 5.90 (m, 1H), 5.70 (d, J = 3.8 Hz, 1H), 5.28-5.15 (m, 2H), 5.04-4.23 (m, 14H), 4.25 (m, 1H), 4.11 (dd,, J = 9.4 Hz), 4.01 (dd, J = 2.2 Hz, 1H), 3.95 (m, 1H), 3.76 (m, 1H), 3.68 (m, 2H), 3.47 (dd, J = 2.2 and 9.7 Hz, 1H), 3.44 (m, 1H), 3.38 (dd, J = 2.2 and 9.7 Hz, 1H), 3.28 (dd, J = 34 and 9.8 Hz, 1H), 3.22 (m, 1H), 3.17 (dd, J = 3.8 and 9.8 Hz, 1H); HRMS (positive ion ESMS, $M+Na^+$) calcd for $C_{64}H_{67}O_{10}N_3Na$ 1060.4724, found 1060.4743.

(2-Azido-3,4,6-tri-*O*-benzyl-2-deoxy-a**-D-glucopyranosyl)-(1–6)-2,3,4,5-tetra-*O*-benzyl-D-*myo*-inositol (7)**. A mixture of the allylated pseudodisaccharide **6** (450 mg, 0.433 mmol), $PdCl_2$ (300 mg, 1.69 mmol) and NaOAc (310 mg, 3.83 mg) in AcOH-H_2O (20 mL, 19:1) was stirred under argon for 48 h. the reaction was diluted with ethyl acetate, filtered through celite, washed with 5% aqueous $NaHCO_3$, dried (Na_2SO_4), concentrated, and residue purified by column (hexane-ethylacetate, 2:1 to 3:2) to give de-allylated product **7** (280 mg, 64%) and remaining starting material. ^{1}H NMR (300 MHz, $CDCl_3$): 7.40-7.01 (m, 35H), 5.44 (d, J = 3.5 Hz, 1H), 5.10-4.11 (m, 14H), 4.10 (m, 1H), 4.00 (m, 1H), 3.98 (t, J = 9.5 Hz), 3.96 (m, 1H), 3.90 (m, 1H), 3.72 (t, J = 9.6 Hz, 1H), 3.61 (m, 1H), 3.50 (dd, J = 3.5 and 10 Hz, 1H), 3.44 (dd, J = 2.5 and 4.4 Hz, 1H), 3.37 (t, J = 9.5 Hz, 1H), 3.25 (m, 1H), 3.22 (dd, J = 2.5 Hz, 1H), 3.03 (dd, J = 2 and 11 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): 138.50, 138.42, 138.35, 138.15, 138.01, 137.79, 137.74, 128.35-127.35 (a cluster of peaks), 98.25, 81.87, 81.08, 80.85, 80.61, 80.29, 78.02, 77.11, 75.70, 75.31, 75.13, 74.69, 74.64, 73.45, 73.25, 72.81, 70.74, 67.40, 64.04; HRMS (positive ion ESMS, $M+Na^+$) calcd for $C_{61}H_{63}O_{10}N_3Na$ 1020.4411, found 1020.4312.

(3,4,6-Tri-*O*-benzyl-2-*N*-(*tert*-butyloxycarbonyl)-amino-2-deoxy-a-D-glucopyranosyl)-(1–6)-2,3,4,5-tetra-*O*-benzyl-D-*myo*-inositol (8): The azido compound **7** (280 mg, 0.28 mmol) was dissolved in a mixture of pyridine (14 mL), water (2 mL) and propane-dithiol (0.42 mL). The solution was made alkaline by addition of triethylamine (0.7 mL) and stirred at room temperature for 24 h. Now a mixture of toluene-ethanol (30 mL, 5:1) was added and reaction mixture concentrated to 25% volume keeping the bath temperature below 30 ^{o}C, and above procedure was repeated three times. This was followed by addition of di-tert-butyldicarbonate (Boc_2O, 0.56 mL, 2.5 mmol) and overnight stirring at room temperature. The reaction mixture was concentrated by co-evaporation with toluene and the residue was purified by column chromatography (20% ethyl-acetate in hexane) to give compound **8** (200 mg, 66% yield). ^{1}H NMR (300 MHz, $CDCl_3$): 7.35-7.03 (m, 35H), 5.41 (d, J = 3.6 Hz, 1H), 5.08-4.95 (m, 1H), 4.72-4.41 (m, 14H), 4.32-4.14 (m, 3H), 4.00-3.80 (m, 2H), 3.75-3.57 (m,, 2H), 3.52-3.30 (m, 3H), 3.25-3.18 (m, 1H), 1.40 (s, 9H); HRMS (positive-ion ESMS, M+Na^+) calcd $C_{66}H_{73}O_{12}NNa$ 1094.5030, found 1094.5130.

2-*O*-octadecanoyl-1-*O*-[6-(N-carbobenzyloxyamino)-hexanoyl]-sn-glyceryl-H-phosphonate (9). This lipid intermediate was prepared in 4 steps as described below

(a) *3-(p-methoxybenzyl)-sn-glycerol*: The commercially available chiral 1,2-isopropylidene-sn-glycerol (1S-2,2-dimethyl-1,3-dioxolane-4-methanol, 2 g, 15,2 mmol) dissolved in anhyd DMF (50 mL) was treated with NaH (910 mg, 22 mmol) and PMBCl (2.36 g, 18 mmol) and the mixture was stirred for 1h. The reaction was quenched with MeOH (2 mL) and diluted with CH_2Cl_2 (100 mL). The organic layer was washed with brine, dried (Na_2SO_4) and concentrated.

The residue was taken in MeOH (40 mL) and treated with pTSA (160 mg) and stirred for 2 h at room tempearure. The reaction was neutralized by the addition of solid $NaHCO_3$ (200 mg), solvent evaporated and residue purified by a silica column (50% to 80% EtOAc-hexane) to give desired compound (2.4 g). The ^1H and ^{13}C NMR spectra were identical to the reported[4] data. ^1H NMR (300 MHz, $CDCl_3$): 7.23 (d, 2H), 6.88 (d, 2H), 4.48 (s, 2H), 3.85 (brs, 1H), 3.80 (s, 3H), 3.64-3.45 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): 129.5, 113.8, 73.18, 71.45, 70.45, 64.03, 55.18. ESMS: 212.30 (M^+) for $C_{11}H_{16}O_4$.

(b) *1-O-[6-(N-carbobenzyloxyamino)-hexanoyl]-2-O-octadecanoyl-3-O-(p-methoxybenz yl)-sn-glycerol*: To a solution of 3-(p-methoxybenzyl)-*sn*-glycerol (1.54 g, 7.28 mmol) and 6-(*N*-carbobenzyloxyamino)-hexanoic acid (1.8 g, 7 mmol) in anhyd CH_2Cl_2 (100 mL) at 0 ^oC was added DCC (1.8 g, 8.6 mmol) and DMAP (1 g). The reaction mixture was stirred at 0 ^oC for 24 h. Now the temperature was raised to 30 ^oC and additional DCC (400 mg) was added followed by addition of stearic acid (octadecanoic acid, 1.2 g, 4.4 mmol). The reaction was stirred further overnight at 30 ^oC, concentrated and redissolved in anhyd EtOAc. The dicyclohexylurea (DCU) byproduct was removed by filtration and solution concentrated. The residue was purified on a silica column (25% EtOAc-CH_2Cl_2) to desired product (1.5 g, 55% yield). ^1H NMR (300 MHz, $CDCl_3$): 7.29 (m, 5H), 7.19, 7.15, 6.82, 6.79 (4s, 4H), 5.15 (m, 1H), 5.02 (s, 2H), 4.83 (brs, 1H), 4.40 (dd, J = 11.8, 2H), 4.26 (dd, J = 3.7 and 11.8 Hz, 1H), 4.12 (m, 1H), 3.73 (s, 3H), 3.49 (d, J = 5 Hz, 2H), 3.12 (m, 2H), 2.28 (m, 4H), 1.70-1.10 (m, 36H), 0.84 (t, 6.2 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): 173.1, 173.1, 159.3, 156.4, 136.6, 129.7, 129.3, 128.5, 128.0, 113.8, 72.9, 70.0, 67.8, 66.5, 62.8, 55.2, 40.8, 33.94, 29.5, 29.3, 29.0, 26.1, 24.9, 24.4, 22.7, 14.1; MS (positive-ion ESMS, $M+Na^+$) calcd $C_{43}H_{67}O_8NNa$ (M+Na) 748.4764, found 748.4734.

(c) *1-O-[6-(N-carbobenzyloxyamino)-hexanoyl]-2-O-octadecanoyl-sn-glycerol*: The above PMB protected glycerol intermediate (1.15 g, 1.59 mmol) was dissolved in CH_2Cl_2-H_2O (50 mL, 99:1) and treated with DDQ (750 mg) and the mixture stirred at room temperature overnight, diluted with CH_2Cl_2 (100 mL), washed three times with 10% $NaHCO_3$ solution. The organic layer was concentrated and the residue purified on a silica column to provide desired compound (900 mg, 93%). ^{1}H NMR (300 MHz, $CDCl_3$): 7.30 (m, 5H), 5.23-5.10 (m, 3H), 4.86 (brs, 1H), 4.25 (dd, J = 3.7 and 11.8, 1H), 4.17 (dd, J = 3.7 and 11.8 Hz, 1H), 3.65 (dd, J = 5.6 and 5.7 Hz, 2H), 3.14 (q, J = 6.5 Hz, 2H), 2.30-2.25 (m, 4H), 1.70-1.10 (m, 36H), 0.83 (t, 6.2 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): 173.4, 156.4, 156.4, 136.5, 128.5, 128.5, 128.1, 72.0, 66.6, 62.1, 61.4, 40.8, 33.9, 29.5, 29.3, 29.0, 26.1, 24.9, 24.4, 22.7, 14.1; MS (ESMS, $M+H^+$) calcd for $C_{35}H_{60}NO_7$ (M+H) 606.4370, found 606.4340.

(d) *2-O-octadecanoyl-1-O-[6-(N-carbobenzyloxyamino)-hexanoyl]-sn-glyceryl-H-phosph onate* (**9**). To a strirred solution of imidazole (42.5 mg, 0.61 mmol, dried through toluene evaporation) in anhyd toluene (0.5 mL) at 0 ^{o}C was added PCl_3 (12 µL, 0.135 mmol dissolved in 125 µL toluene) followed by anhyd triethyamine (48 µL, 0.35 mmol dissolved in 125 µL toluene). The stirring was continued for 10 min at 0 ^{o}C and the tempearature was lowered to –5 ^{o}C and then a solution of 1-*O*-[6-*(N*-carbobenzyloxy amino)-hexanoyl]-2-*O*-octadecanoyl-sn-glycerol (27 mg, 44 µmol dissolved in 0.5 mL toluene) was added dropwise. The reaction was stirred at –5 ^{o}C for 2 h and then quenched with pyridine-water (2.5 mL, 4:1) and stirred further for 30 min. This was followed by addition of $CHCl_3$ (7.5 mL), washing with H_2O (2.5 mL x 3). The $CHCl_3$ layer was dried (Na_2SO_4), concentrated and the residue purified through a silica column (1% MeOH-

CH_2Cl_2 to 10% MeOH-CH_2Cl_2 with 1% TEA) to provide the desired lipid H-phosphonate donor **9** (25 mg, 92%). ^{1}H NMR (300 MHz, $CDCl_3$): 7.25-7.01 (m, 5H), 6.80 (d, J_{PH} = 628 Hz, 1H), 5.15-4.98 (m, 3H), 4.26 (dd, J = 3.6 and 11.7 Hz, 1H), 4.00 (m, 1H), 3.88 (dd, J = 5.1 and 7.8 Hz, 2H), 3.49 (q, J = 67.2 Hz, 2H), 2.18 (t, J = 7.2 Hz 4H), 1.50-1.10 (m, 36H), 0.76 (t, 6.2 Hz, 3H); HRMS (negative ion ESMS, M-H$^-$) calcd for $C_{35}H_{59}O_9NP$ (M-H) 668.3927, found 668.3412.

Triethylammonium (3,4,6-Tri-*O*-benzyl-2-*N*-(*tert*-butyloxycarbonyl)-amino-2-deoxy-a**-D-glucopyranosyl)-(1–6)-2,3,4,5-tetra-*O*-benzyl-1-*O*-(2-octadecanoyl-1-*O*-[6-(N-carbobenzyloxyamino)-hexanoyl]-*sn*-glyceryl-phosphonato)-D-*myo*-inositol (10)**. Freshly prepared 2-*O*-octadecanoyl-1-*O*-[6-(*N*-carbobenzyloxyamino)-hexanoyl]-*sn*-glyceryl-H-phosphonate **9** (27 mg, 0.04 mmol) and the glucosamine-inositol intermediate **8** (64 mg, 0.06 mmol, 1.3 equiv) were dried by evaporation with anhyd pyridine three times, and dissolved in anhyd pyridine (0.4 mL). This was followed by addition of pivaloyl chloride (2 equiv, 9.6 μL, 0.08 mmol). After stirring at room temperature for 30 min, the reaction was treated with a iodine solution (2 equiv, 20 mg iodine in pyridine-water, 2.45:0.05) and the mixture was further stirred for 25 min. The reaction was diluted with $CHCl_3$ (12 mL) and organic layer washed with 5% sodium bisulfite solution (10 mL). The organic layer was dried over Na_2SO_4, concentrated and purified on a silica column using 5% MeOH-CH_2Cl_2 (with 1% triethylamine) solvent system, providing the desired phospho-coupled compound **10** (40 mg, 57%). ^{1}H NMR (300 MHz, $CDCl_3$): 7.45-6.98 (m, 40H), 5.40 (m, 2H), 5.26 (m, 1H), 4.77 (bt, 1H), 4.72-4.41 (m, 16H), 4,13 (m, 4H), 3.76 (m, 1H), 3.60 (m, 1H), 3.40 (m, 2H), 3.14 (m, 2H), 3.16 (m, 2H), 2.36-2.16 (m, 4H), 1.70-1.20 (m, 45H), 0.87

(t, J = 6.2, 3H); HRMS (negative-ion ESMS, M-H$^-$) calcd for $C_{101}H_{130}O_{21}N_2P$ 1737.8904, found 1737.8914.

(2-*N*-(*tert*-butyloxycarbonyl)-amino-2-deoxy-a**-D-glucopyranosyl)-(1–6)-1-*O*-(2-*O*-octadeca noyl-1-*O*-[6-aminohexanoyl]-*sn*-glyceryl-phosphonato)-D-*myo*-inositol (11)**. The protected GPI intermediate **10** (25 mg, 0.014 mmol) and the catalyst 20% $Pd(OH)_2$ (50 mg) were dissolved in a solvent mixture of MeOH (2 mL), CH_2Cl_2 (2 mL) and H_2O (0.05 mL). The residual and dissolved air from the flask was removed by repeated evacuations and reaction mixture was stirred under hydrogen atmosphere overnight. After completion of reaction, the mixture was filtered through a small celite pad, and concentrated under reduced pressure. The product was purified by a quick filtration through a silica column using MeOH-CH_2Cl_2 (1:1) to provide compound **11** (12 mg, 85%). ^{1}H NMR (300 MHz, CD_3OD-$CDCl_3$): 5,40 (d, J = 3.4 Hz, 1H), 5.10 (m, 1H), 4,17 (dd, 1H), 4.00 (m, 3H), 3.83 (m, 2H), 3.72-3.70 (m, 3H), 3.63 (m, 3H), 3.58 (m, 1H), 3.51 (m, 2H), 3.22 (t, J = 9.7 Hz, 1H), 3.10 (m, 2H), 2.90 (m, 2H), 2.82 (dd, 1H), 2.25-2.27 (m, 4H), 1.70-1.20 (m, 45H), 0.87 (t, 3H); HRMS (negative-ion ESMS, M-H$^-$) calcd for $C_{44}H_{82}O_{19}N_2P$ 973.5249, found 973.5253.

2-amino-2-deoxy-a**-D-glucopyranosyl-(1–6)-1-*O*-(2-octadecanoyl-1-*O*-[1-6'-[[6-[(7-nitro-2-oxa-1,3-diazolobenz-4-yl)-amino]hexanoyl]-*sn*-glyceryl-phosphonato)-D-*myo*-inositol (1)**: The amino compound **11** (12 mg, 0.0123 mmol) was dissolved in anhyd DMF (2 mL) containing triethylamine (0.05 mL). This was treated with N-hydroxy succinimidyl-6-[7-nitrobenz-2-oxa-1,3-diazolobenz-4-yl)amino]-hexanoate (NBD-aminocaproic NHS ester, 10 mg, 25.6 μmol) and the reaction was stirred at room temperature for 4 h, when negative-ion ESMS showed

completion of the reaction. The solvents were removed under reduced pressure and residual DMF was removed by repetitive evaporation with toluene. The residue was washed with hexane-ethylacetate (1:1) until the washed solvent showed no fluorescent active (NBD-aminocaproic acid) or UV-active (N-hydroxysuccinimide) byproducts. To remove NHBoc group, the NBD conjugated compound was treated with a 2 mL reagent mixture of CH_2Cl_2-CH_3CN-CF_3COOH (2:2:1) and stirred for 2h at room temperature. The reaction was cooled to 10 ^oC and excess of TFA neutralized with triethylamine (1.6 mL). The solvents were removed under reduced pressure and the product was purified by preparative TLC using, n-butanol-ethanol-ammonia-water (4:4:1:1) system to give the desired NBD-GlcN-PI compound **1** (7.7, 55%). The purity of the compound was further established by HPLC on a RP-18 column using CH_3CN-H_2O gradient system and photodiode array detector. TLC (n-BuOH-EtOH-NH_3-H_2O, 4:4:2:2): Rf = 0.45. ^1H NMR (300 MHz, $CDCl_3$): 8.42 (d, J = 8.4 Hz, 1H), 6.21 (d, J = 8.4 Hz), 5,30 (d, J = 3.8 Hz, 1H), 5.28 (m, 1H), 4,42 (dd, 1H), 4.29 (m, 1H), 4.21 (m, 2H), 4.19 (m, 1H), 4.10 (bt, 1H), 4.03 (m, 1H), 3.99 (m, 3H), 3.66 (m, 2H), 3.48 (m, 2H), 3.36 (m, 2H), 3.20 (m, 1H), 3.10 (m, 2H), 2.74 (t, J = 6.8 Hz, 2H), 2.30-2.37 (m, 6H), 1.40-1.20 (m, 42H), 0.87 (t, 3H); ^{31}P NMR (100 MHz, CD_3OD-$CDCl_3$): 0.90 ppm; HRMS (negative-ion ESMS, M-H^-) calcd for $C_{51}H_{86}O_{21}N_6P$ 1149.5584, found 1149.5590.

2-*N*-Acetylamino-2-deoxy-a**-D-glucopyranosyl-(1–6)-1-*O*-(2-octadecanoyl-1-*O*-[1-6'-[[6-[(7-nitro-2-oxa-1,3-diazolobenz-4-yl)-amino]hexanoyl]--*sn*-glyceryl-phosphonato)-D-*myo*-inositol (2)**: The NBD-labeled GlcN-PI (**1**, 0.2 mg, 0.17 micromol) was dissolved in a mixture of methanol (0.2 mL) and saturated $NAHCO_3$ solution (0.2 mL). This was treated with Ac_2O (10 µL) and stirred for 10 min at room temperature followed by addition of more Ac_2O (10 µL) and

10 min stirring. The solvents were evaporated and product dissolved in MeOH was filtered through a small column of silica gel. The reaction was essentially complete in 20 min without any side reaction to provide NBD labeled GlcNAc-PI (**2**) in quantitative yield. ^{1}H NMR (300 MHz, $CDCl_3$): 8.43 (d, J = 8.4 Hz, 1H), 6.22 (d, J = 8.4 Hz), 5,31 (d, J = 3.8 Hz, 1H), 5.30 (m, 1H), 4,42 (dd, 1H), 4.29 (m, 1H), 4.21 (m, 2H), 4.19 (m, 1H), 4.10 (bt, 1H), 4.03 (m, 1H), 4.00 (m, 3H), 3.66 (m, 2H), 3.48 (m, 2H), 3.36 (m, 2H), 3.20 (m, 1H), 3.10 (m, 2H), 2.74 (t, J = 6.8 Hz, 2H), 2.30-2.37 (m, 6H), 1.91 (s, 3H), 1.42-1.21 (m, 42H), 0.86 (t, 3H); HRMS (negative-ion ESMS, M-H$^-$) calcd for $C_{53}H_{88}O_{22}N_6P$ 1191.5689, found 1191.5621.

References for Section [B] of Supporting Information

(Synthesis of Fluorescent Glycosylphosphatidylinositol probes)

1. Massy, D. J. R.; Wyss, P. *Helv. Chim. Acta.* 1990, **73**, 1037-1057
2. Madsen, R.; Udogong, U. E.; Roberts, C.; Mootoo, D. R.; Konradsson, P.; Fraser Reid, B. *J. Am. Chem. Soc*. 1995, **117**, 1554-1565
3. Grundler, G.; Schmidt, R. R. *Liebigs Ann. Chem*. 1984, 1826-1847
4. Chen, J.; Profit, A. A.; Prestwich, G. D. *J. Org. Chem*. 1996, **61**, 6305-6312