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# Chemical biology of Glycosylphosphatidylinositol (GPI) anchors



Ram Vishwakarma

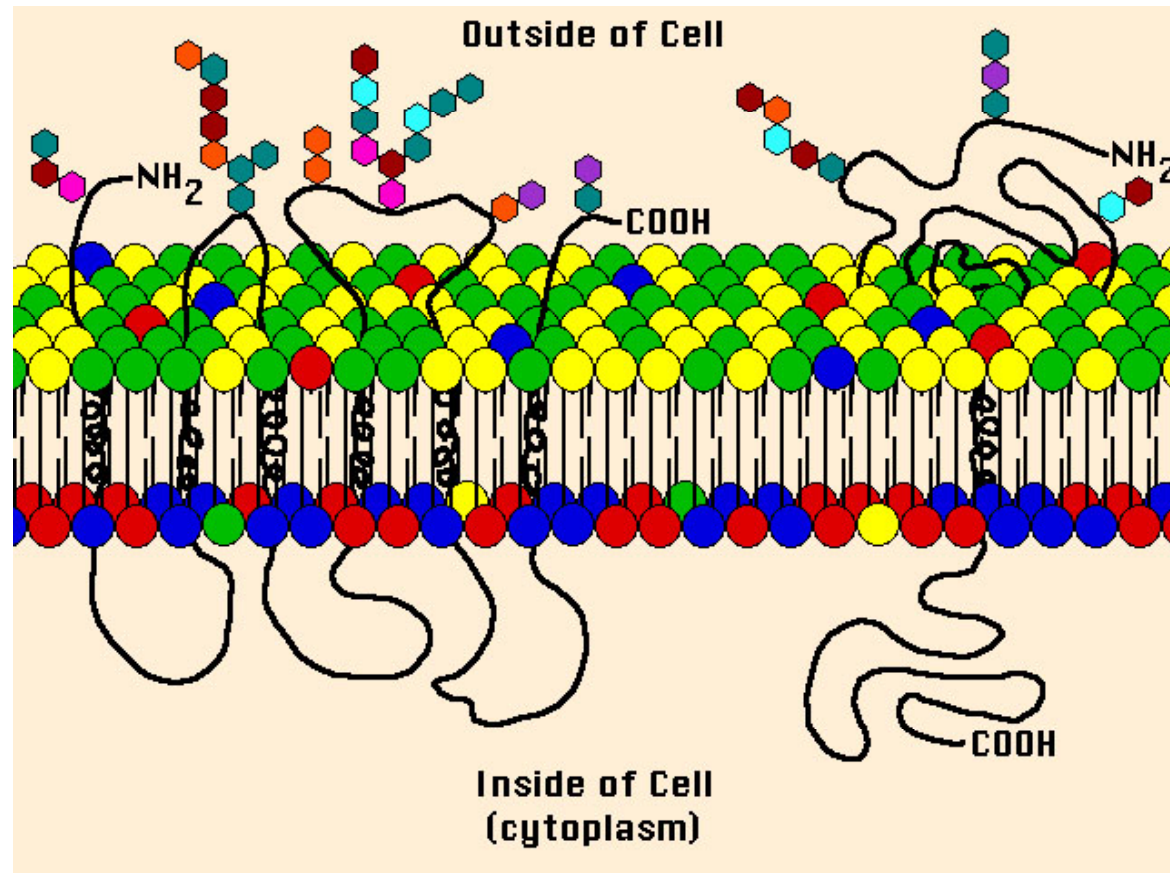
CSIR-Indian Institute of Integrative Medicine, Jammu

National Institute of Immunology, New Delhi

Piramal Life Sciences Ltd, Mumbai



# Topology of biological membrane

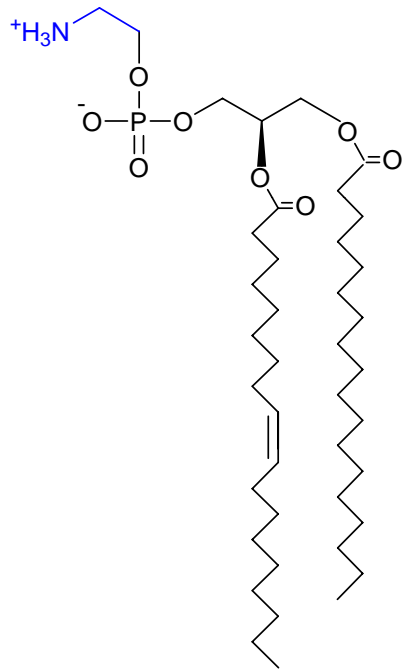


PC, PE, PS (major) and PI (minor)

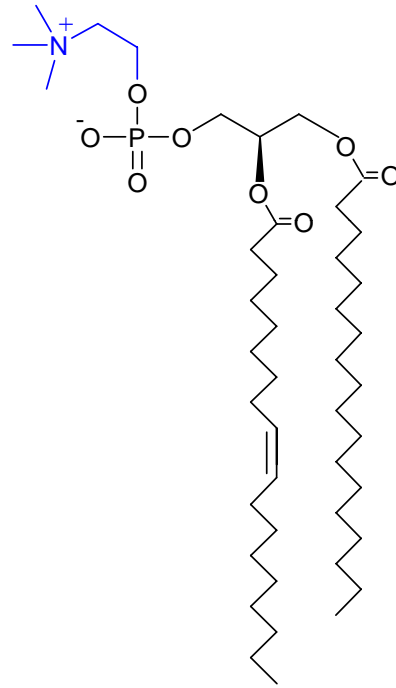


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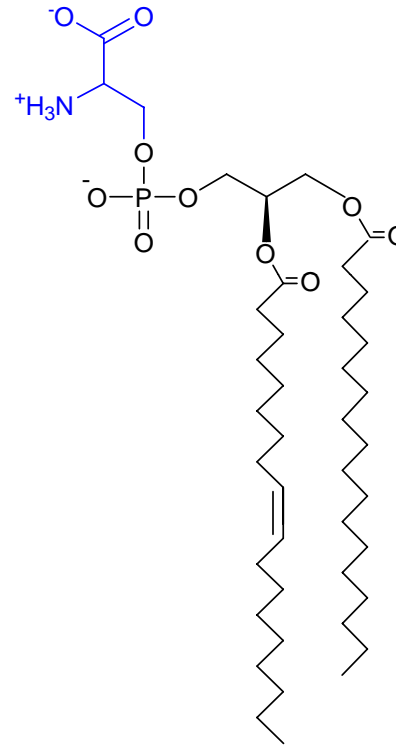
# Key building blocks of plasma membranes (Glycerophospholipids)



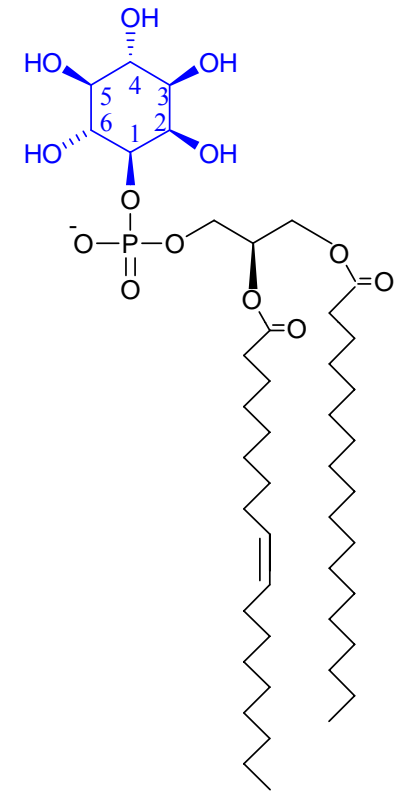
Phosphatidyl-ethanolamine



Phosphatidyl-choline



Phosphatidyl-serine

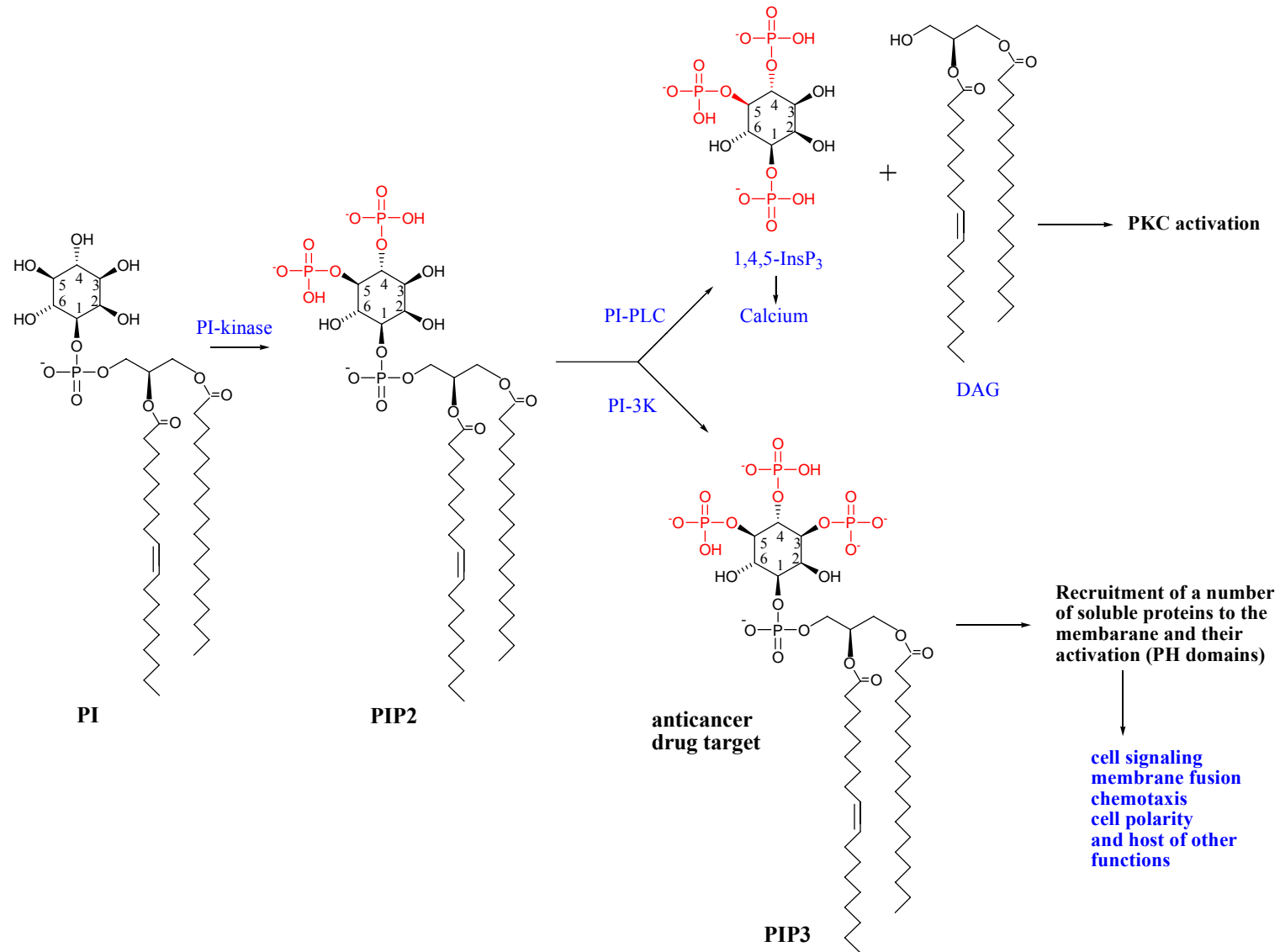


Phosphatidyl-Inositol



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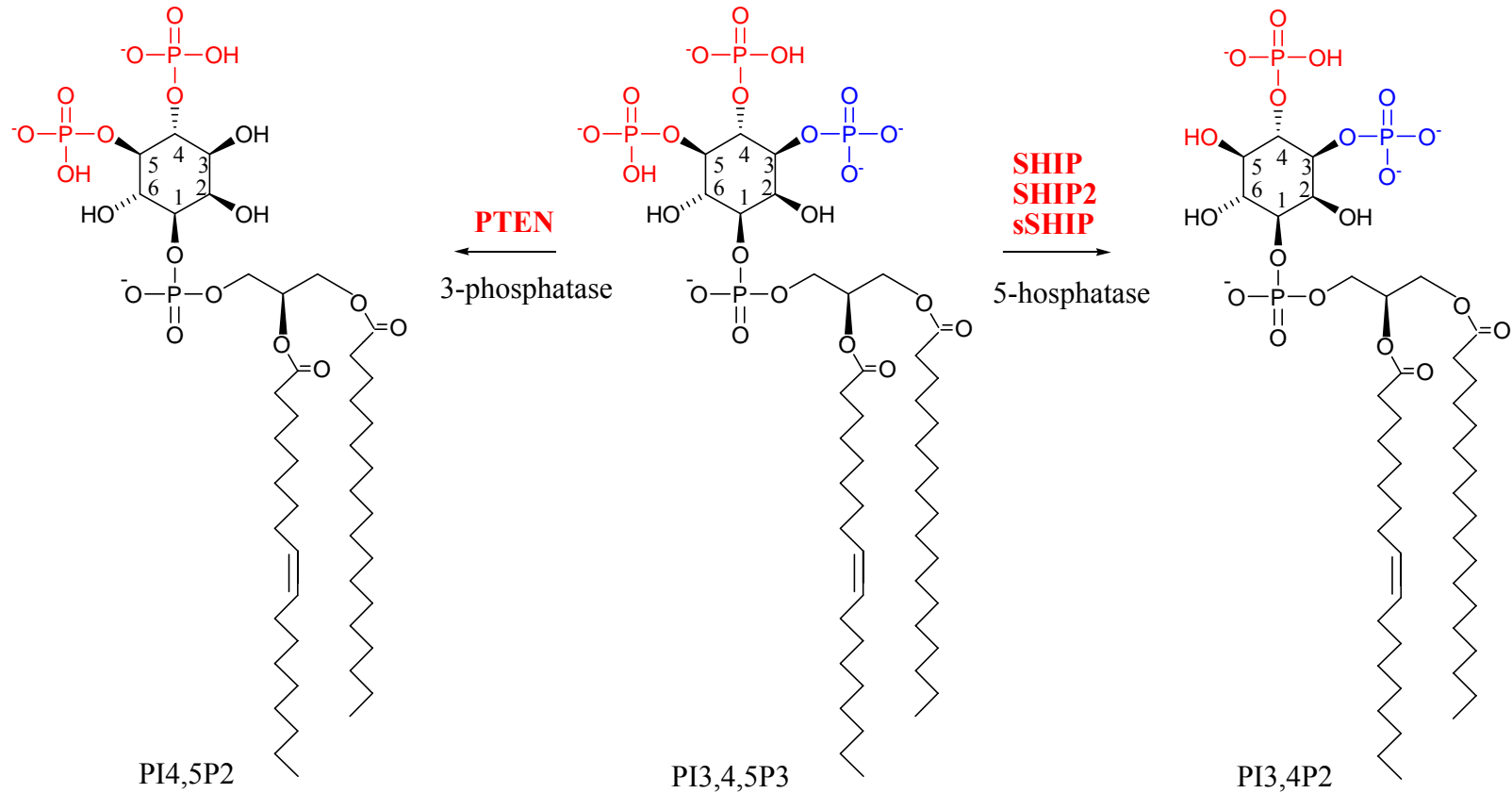
# Two major cell signaling pathways are mediated by Phosphatidylinositols and Inositol phosphates





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## Regulation of PI3K activity by phosphatases (PTEN and SHIP)

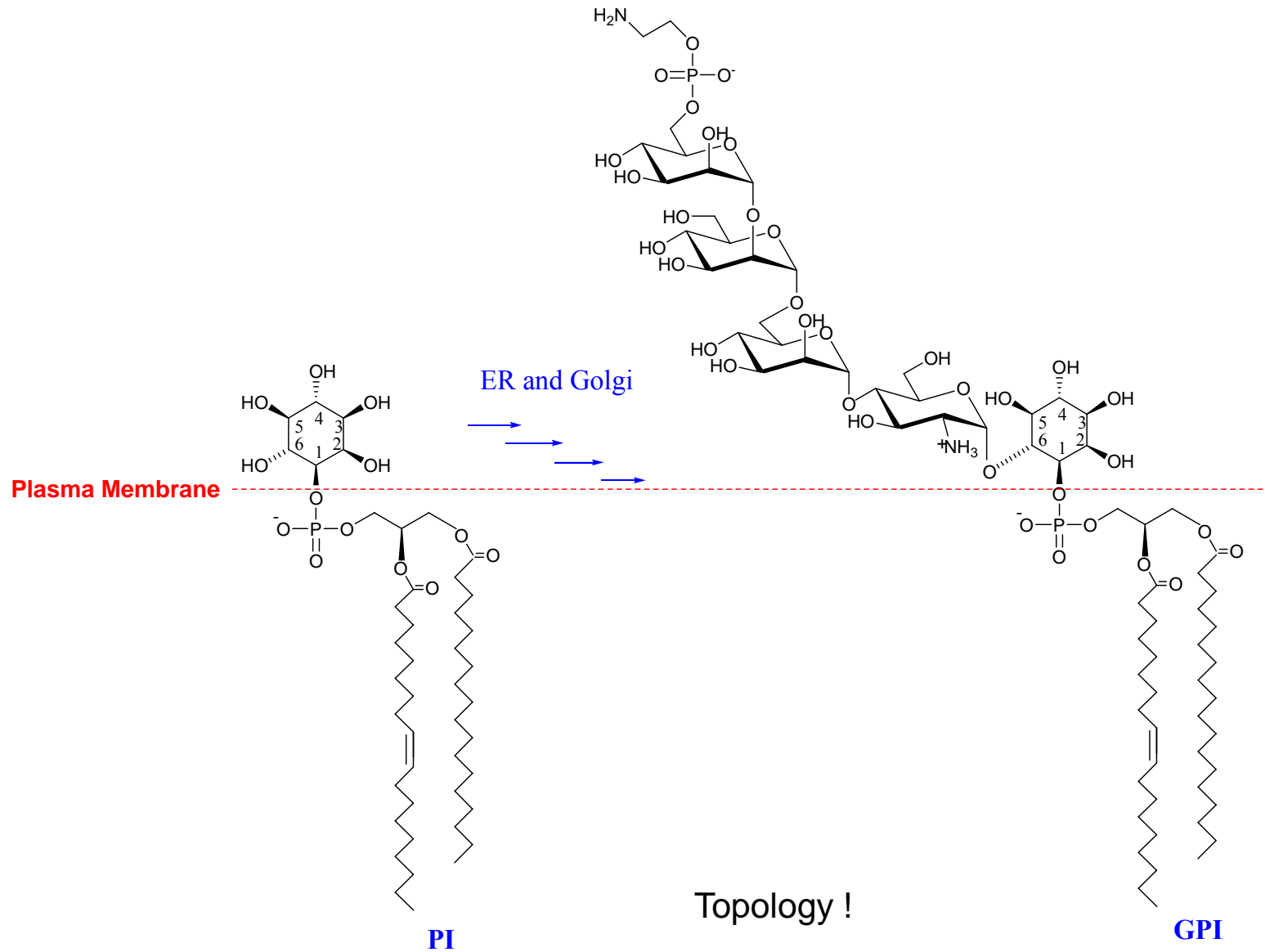


**PTEN and SHIP2 expressed in wide variety of cells**  
**SHIP is expressed only in blood cells**  
**sSHIP is expressed in stem cells**

**Approx 50% of human cancers contain PTEN mutations**



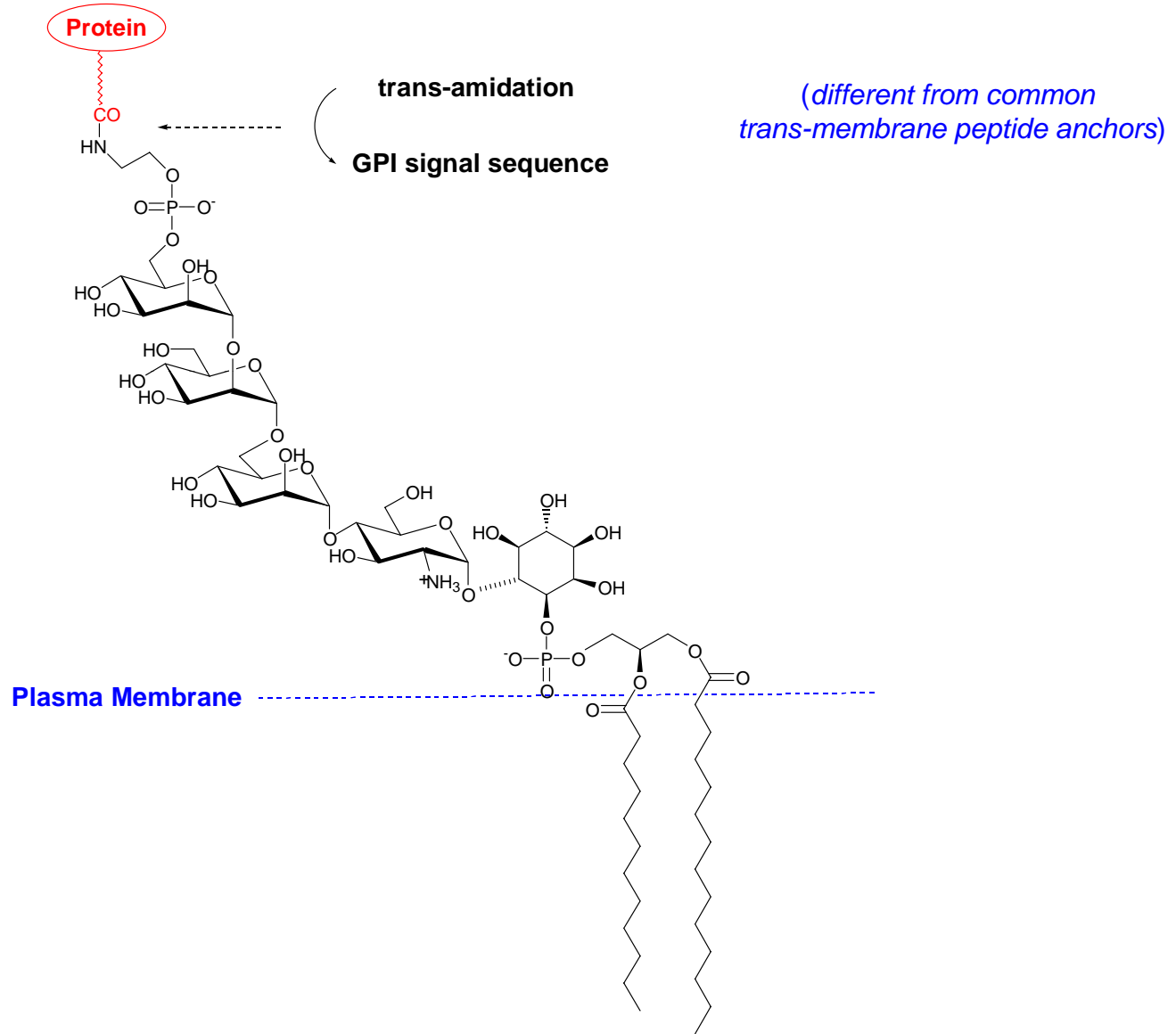
# Relationship of PI and GPI anchors (biosynthesis and topology)





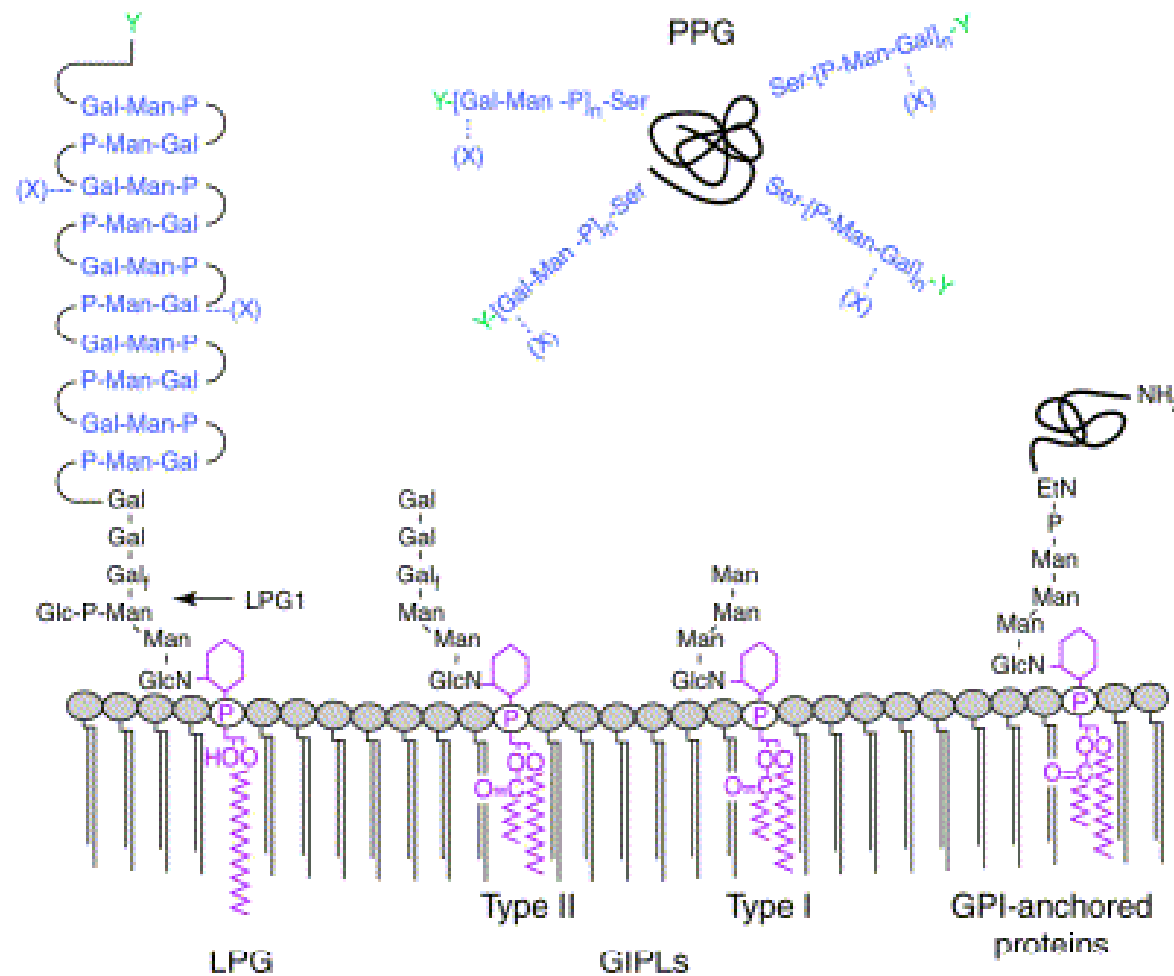
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# Novel mode of attachment of cell surface proteins and carbohydrate antigens





# GPI molecules are abundant in protozoan parasites (Leishmania, Trypanosoma and Malaria)



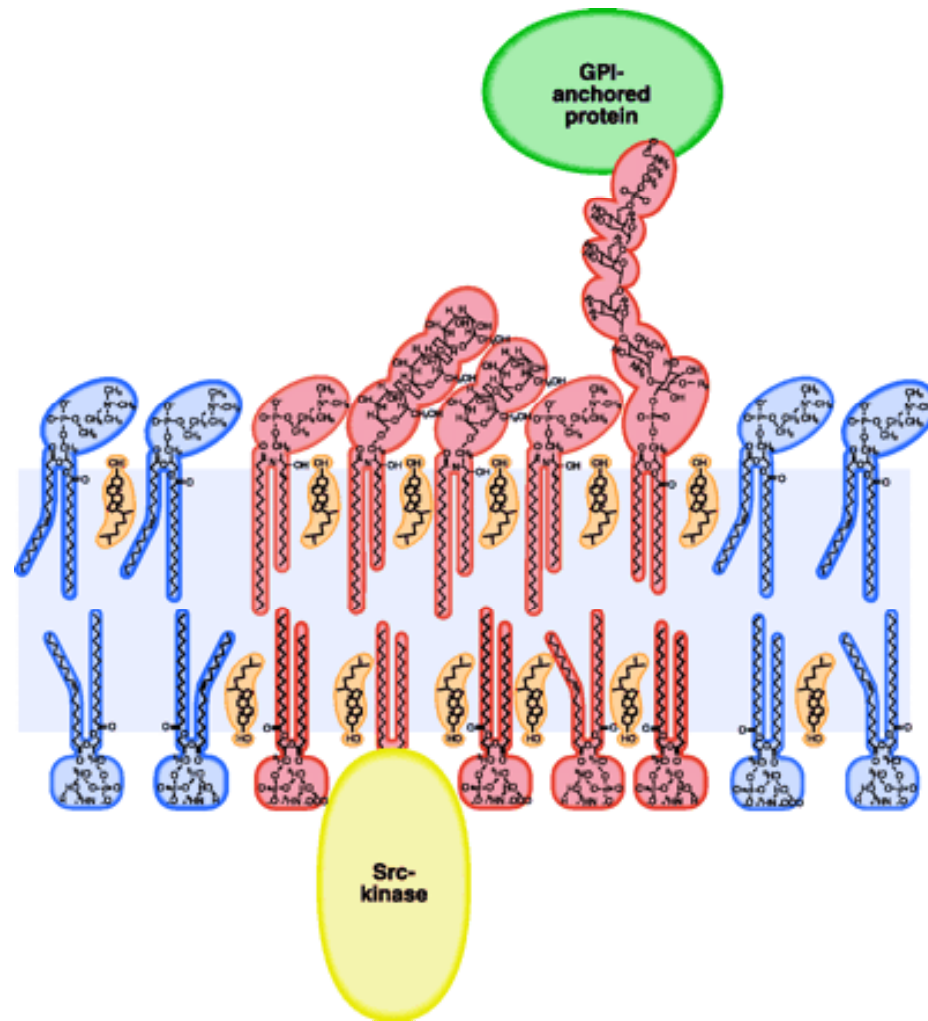


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## GPI anchors and cholesterol organize functional micro-domains (lipid rafts) in plasma membrane

A GPI-anchored protein is attached to the exoplasmic leaflet

A doubly acylated Src-kinase is attached to the cytoplasmic leaflet



**A model of a lipid raft with two intercalated proteins**



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## Chemical biology of GPI anchors

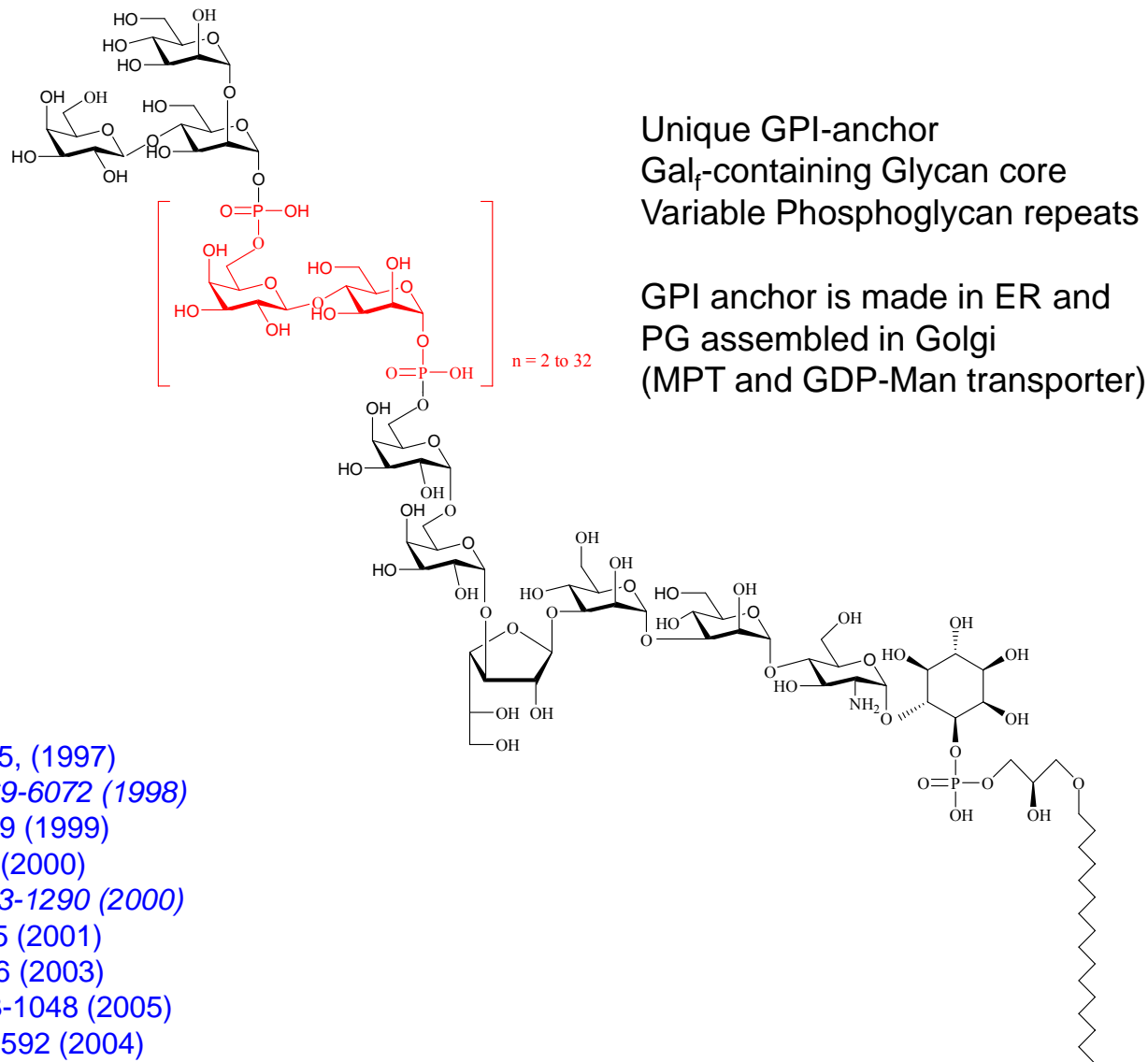
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- **Organic synthesis, biosynthesis and cell biology of PI/GPI molecules**
  - **Design and synthesis of structural and functional mimics of PI/GPIs to probe biological questions**
  - **Targeting PI3K/AKT/mTOR pathway for anticancer drug discovery**
  - **Role of PI3K isoforms ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ) in stem cell differentiation**
-



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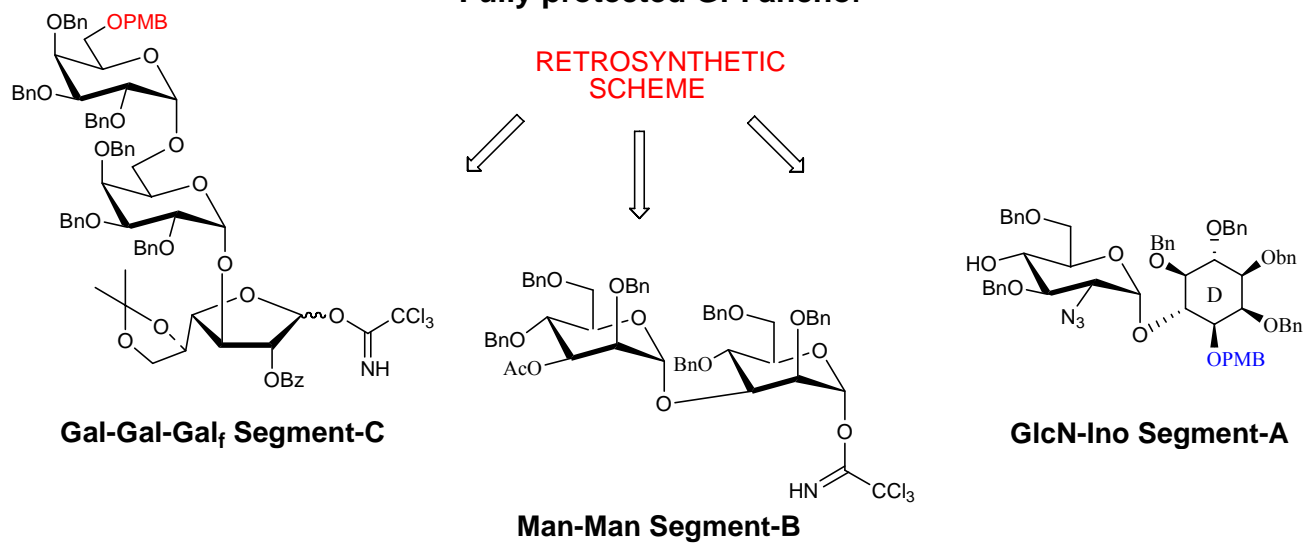
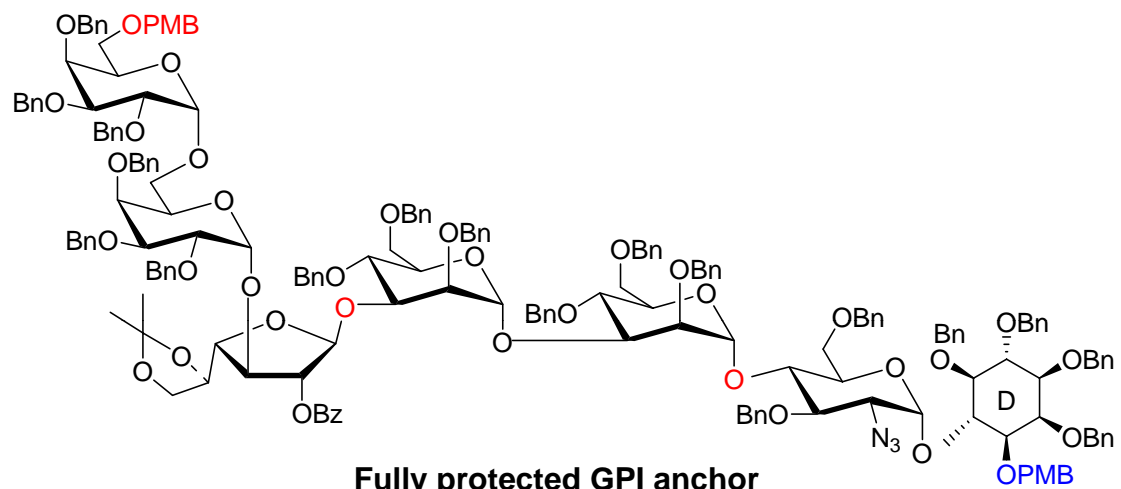
# Synthesis and biosynthesis of GPI-anchored Lipophosphoglycan (LPG) of Leishmania parasite



*J. Chem. Soc. Perkin-1*, 1845, (1997)  
*Tetrahedron Letters*, 39, 6069-6072 (1998)  
*Tetrahedron Letters*, **40**, 2619 (1999)  
*Tetrahedron*, **56**, 6577-6585 (2000)  
*J. Chem. Soc. Perkin 1*, 1283-1290 (2000)  
*Chem. Commun.*, 2024-2025 (2001)  
*J. Org. Chem.* **68**, 4446-4456 (2003)  
*Org. Biomol. Chem.*, **3**, 1043-1048 (2005)  
*Tetrahedron Lett.* **45**, 2589-2592 (2004)



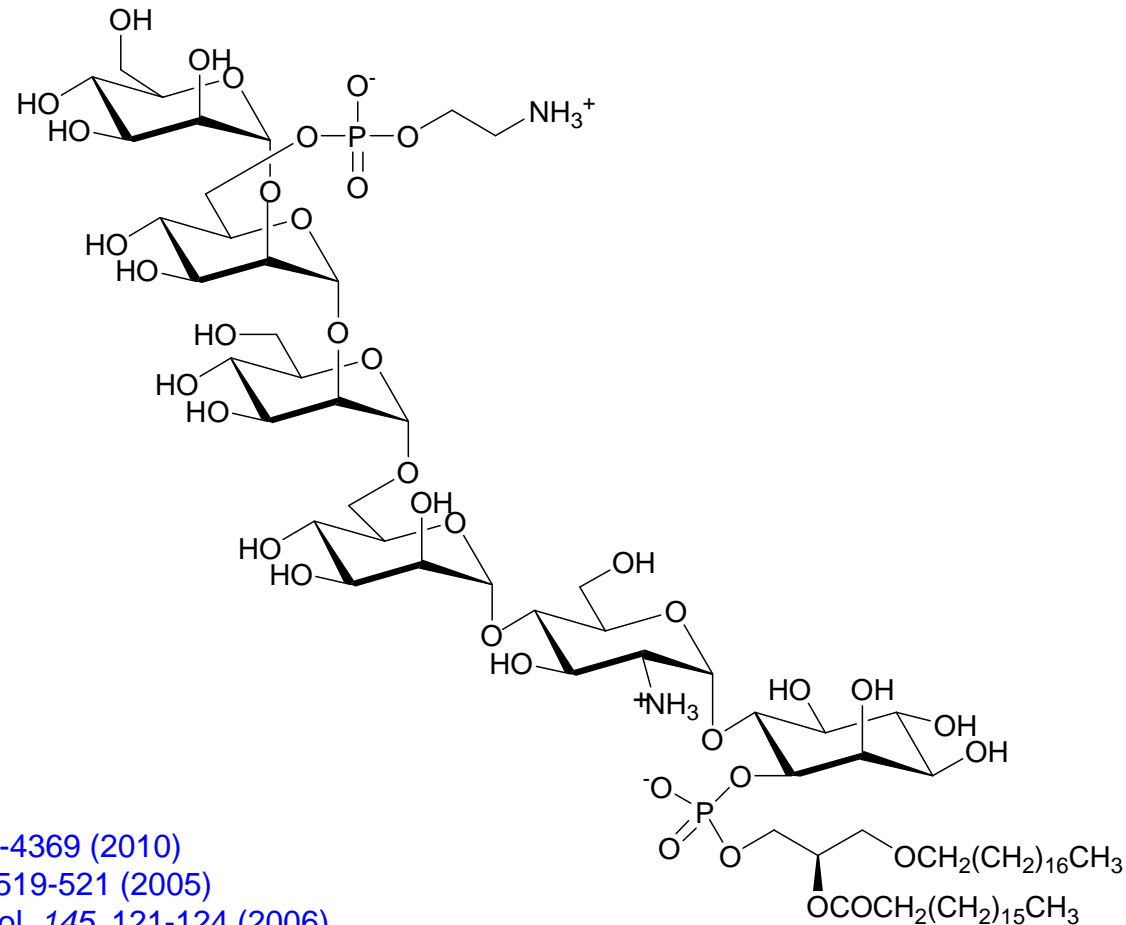
# Synthesis of GPI-anchor of LPG





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# Synthesis and biosynthesis of GPI-anchors of *L. donovani*, *T. cruzi* and *E. histolytica*

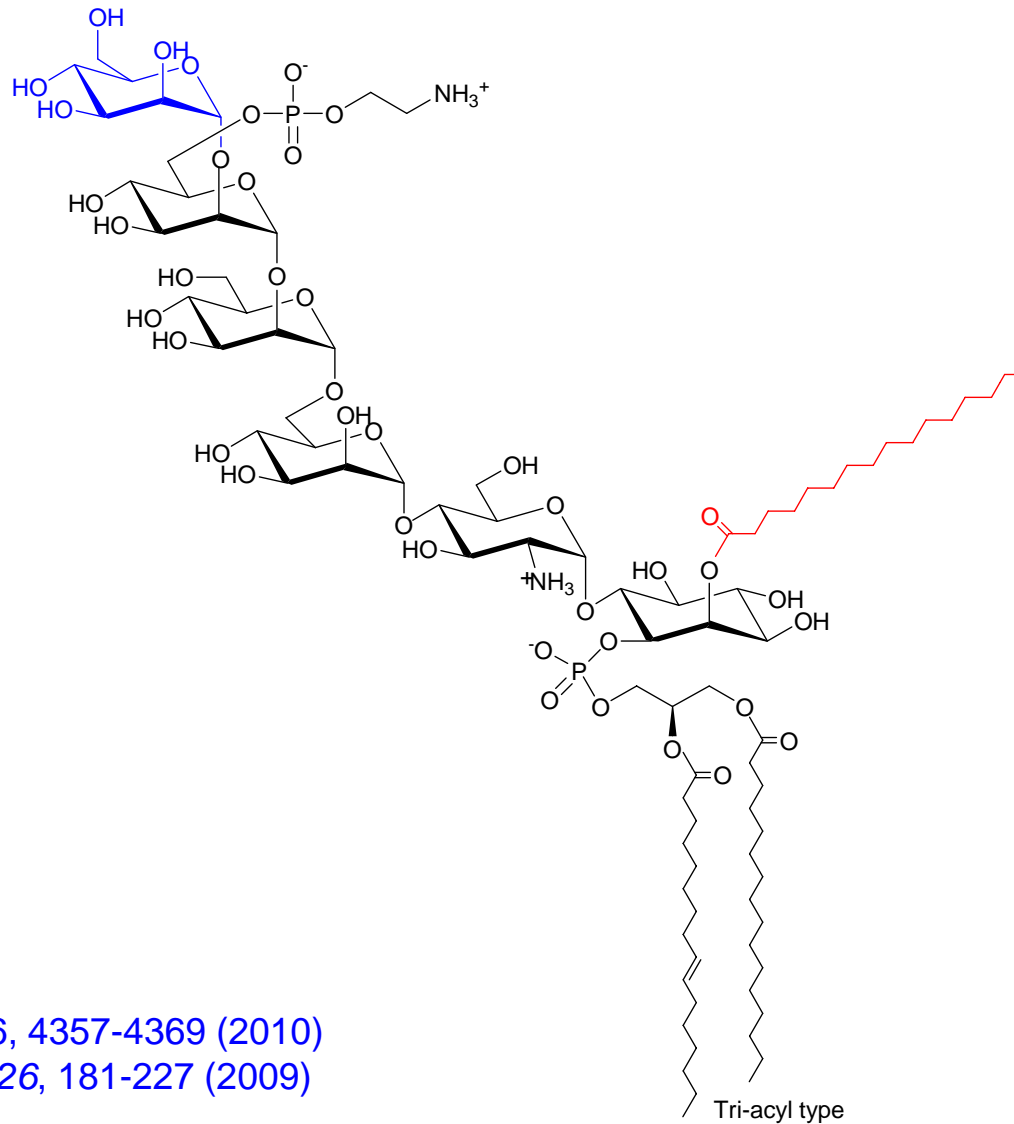


Tetrahedron, 66, 4357-4369 (2010)  
Chem. Commun. (4), 519-521 (2005)  
Mol. Biochem. Parasitol. 145, 121-124 (2006)  
Journal of Bacteriology, 187, 4173-4186 (2005)  
Infection and Immunity, 73, 8381-8392 (2005)  
J. Lipid. Res., 44, 594-600 (2003)



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# Synthesis of a full-length GPI anchor of malaria parasite (*Plasmodium falciparum*)



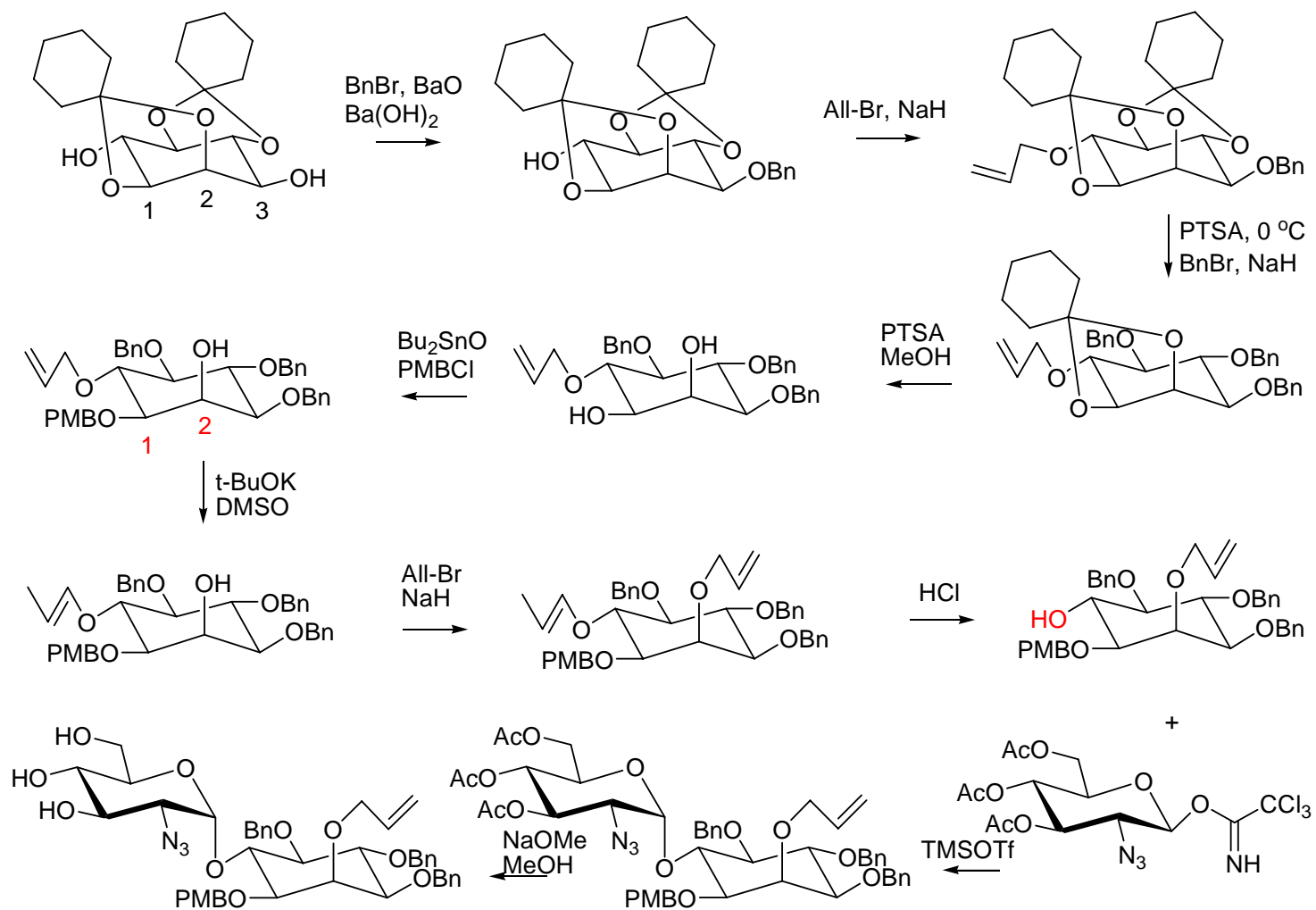
Tetrahedron, 66, 4357-4369 (2010)  
The Enzymes, 26, 181-227 (2009)





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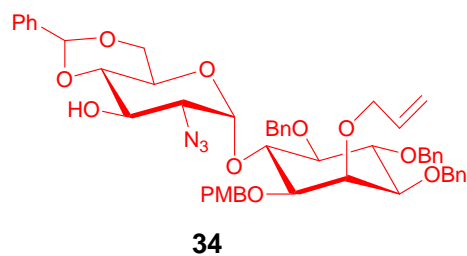
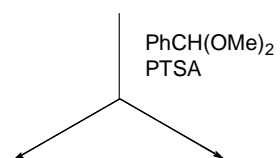
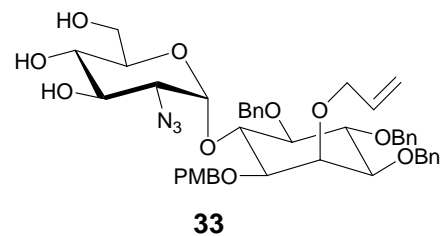
# Glucosamine-Inositol intermediate



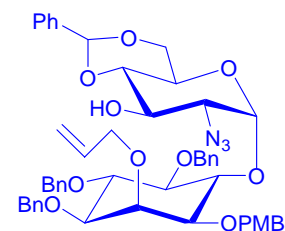


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# Resolution of GlcN-Inositol

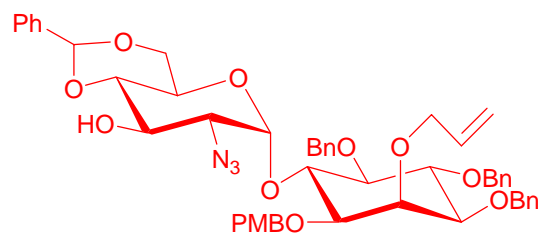


D-Inositol Isomer

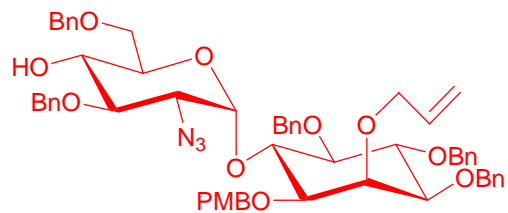
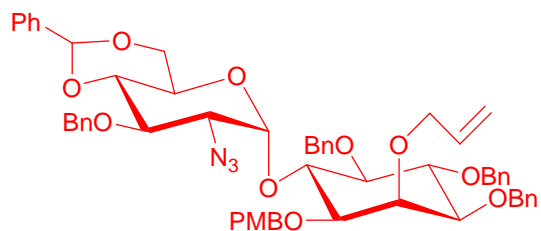
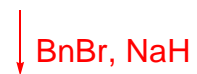


L-Inositol Isomer

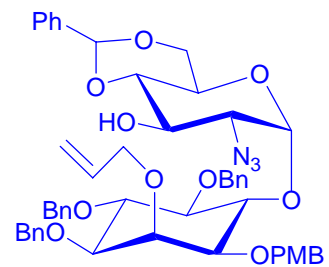
*Chem. Commun.*, 519-521 (2005)



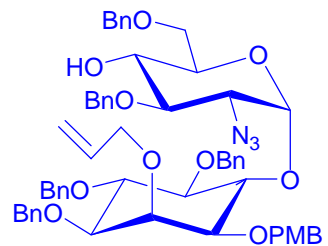
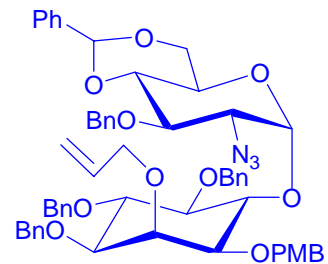
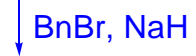
**34**



**36**



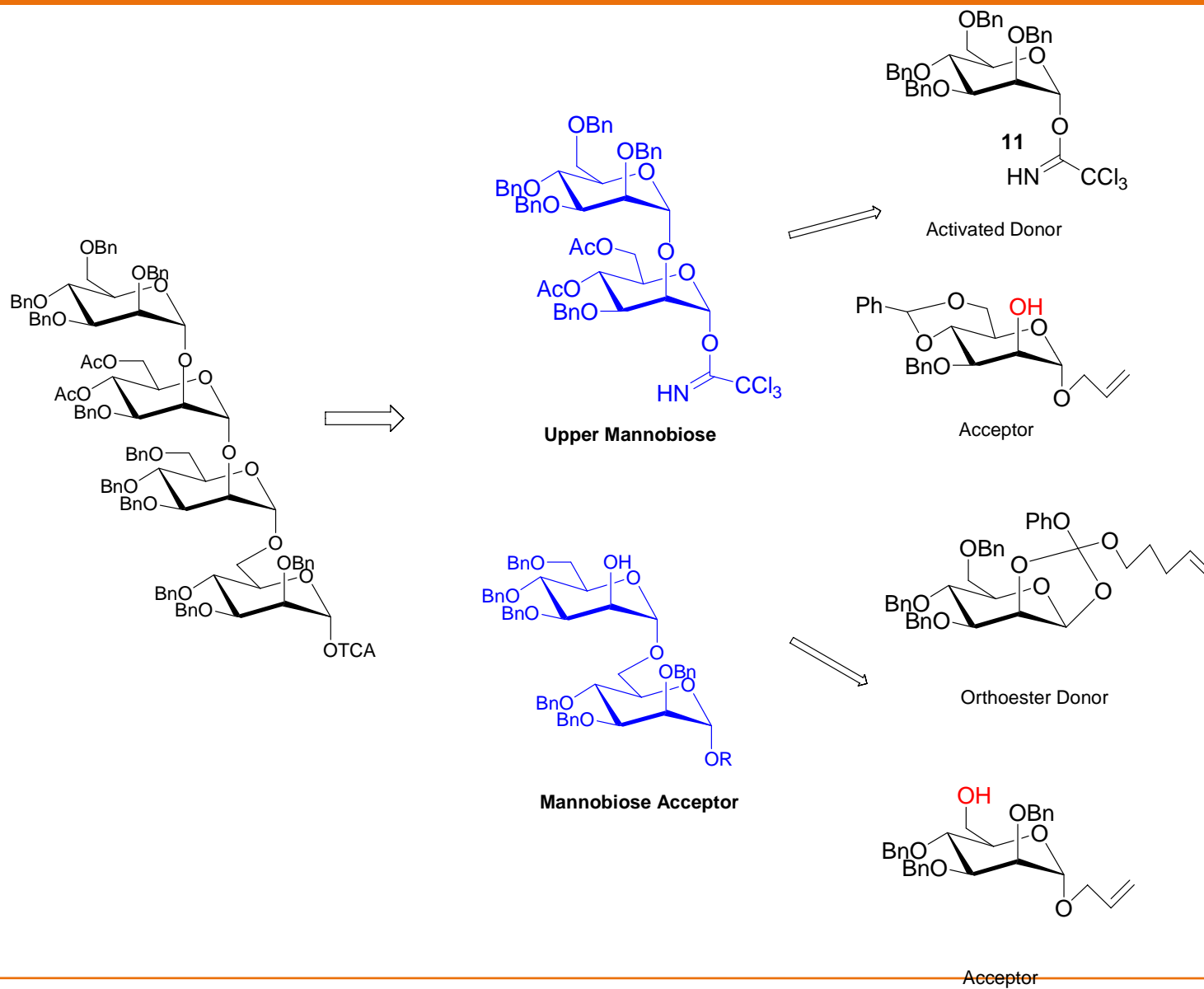
**35**



**37**



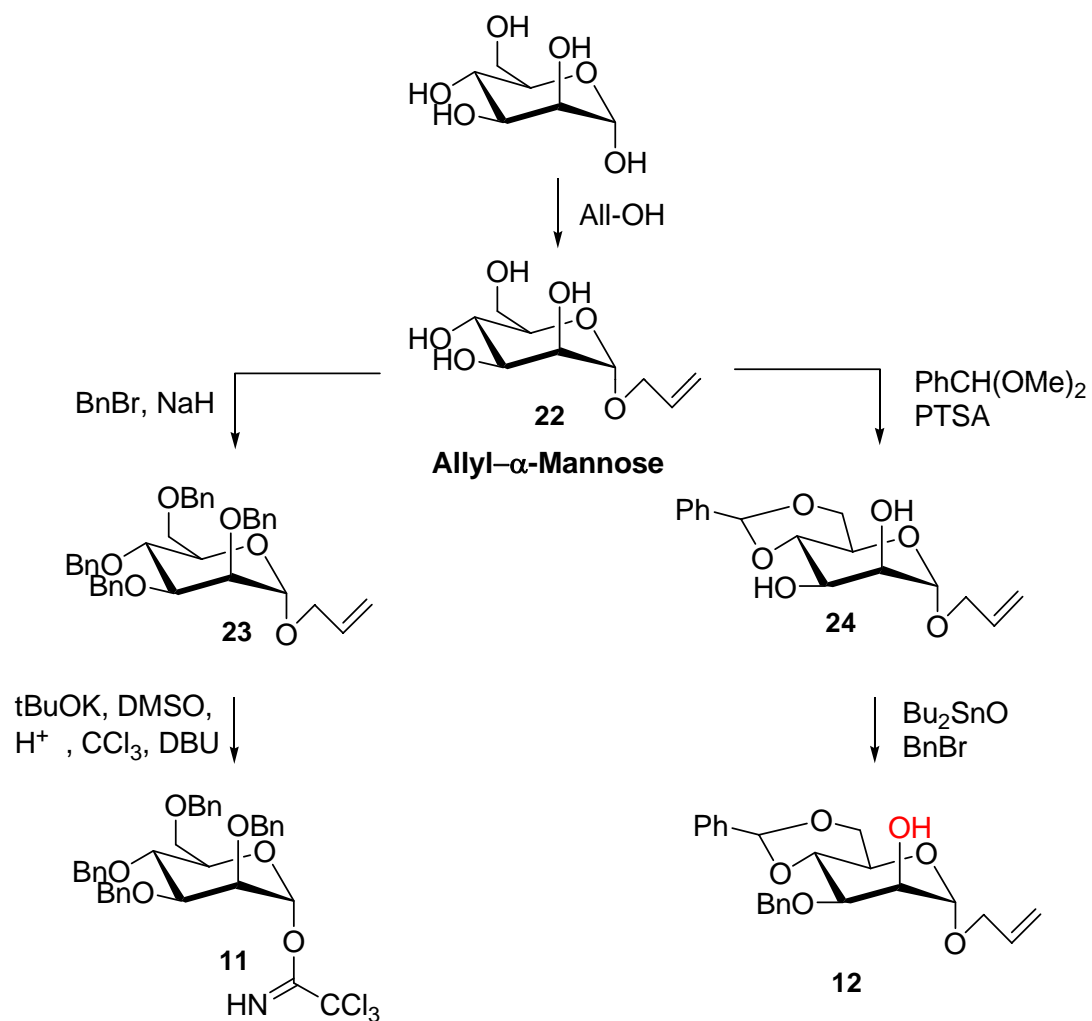
# Tetra-mannose Block



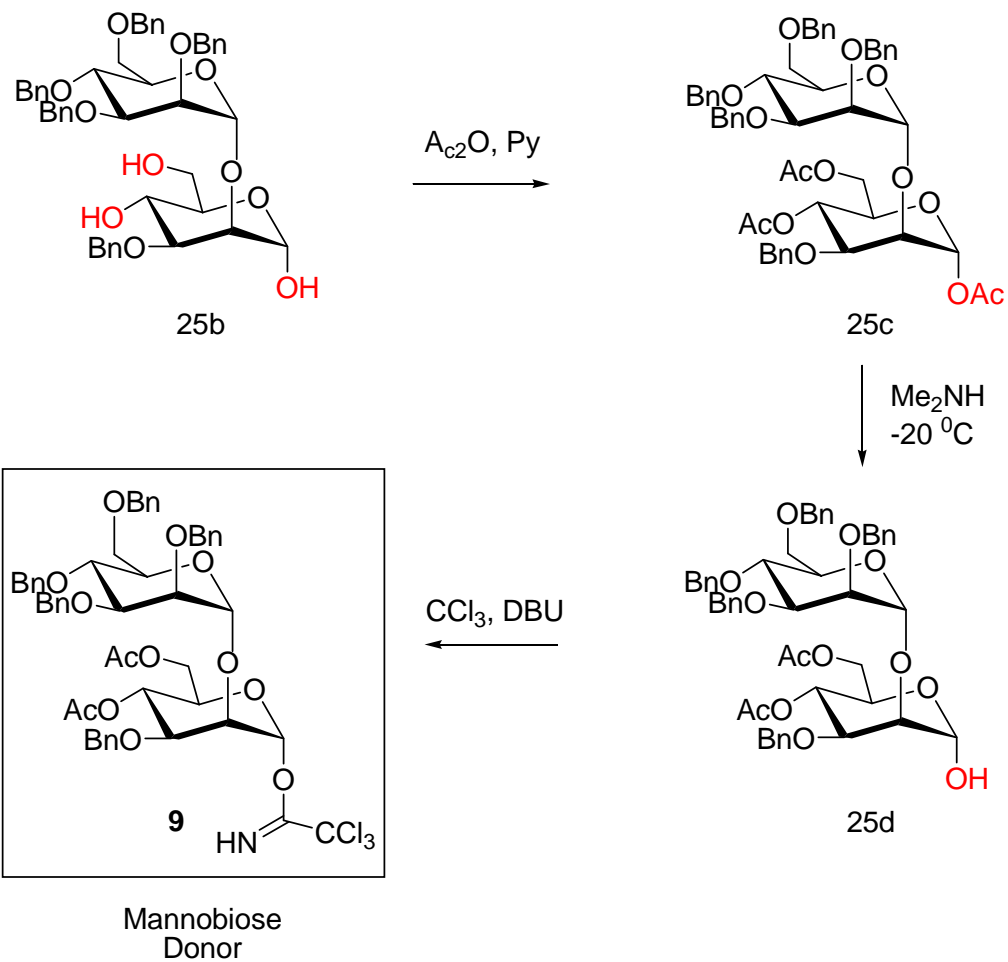


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## Synthesis of Upper Mannobiose Segment



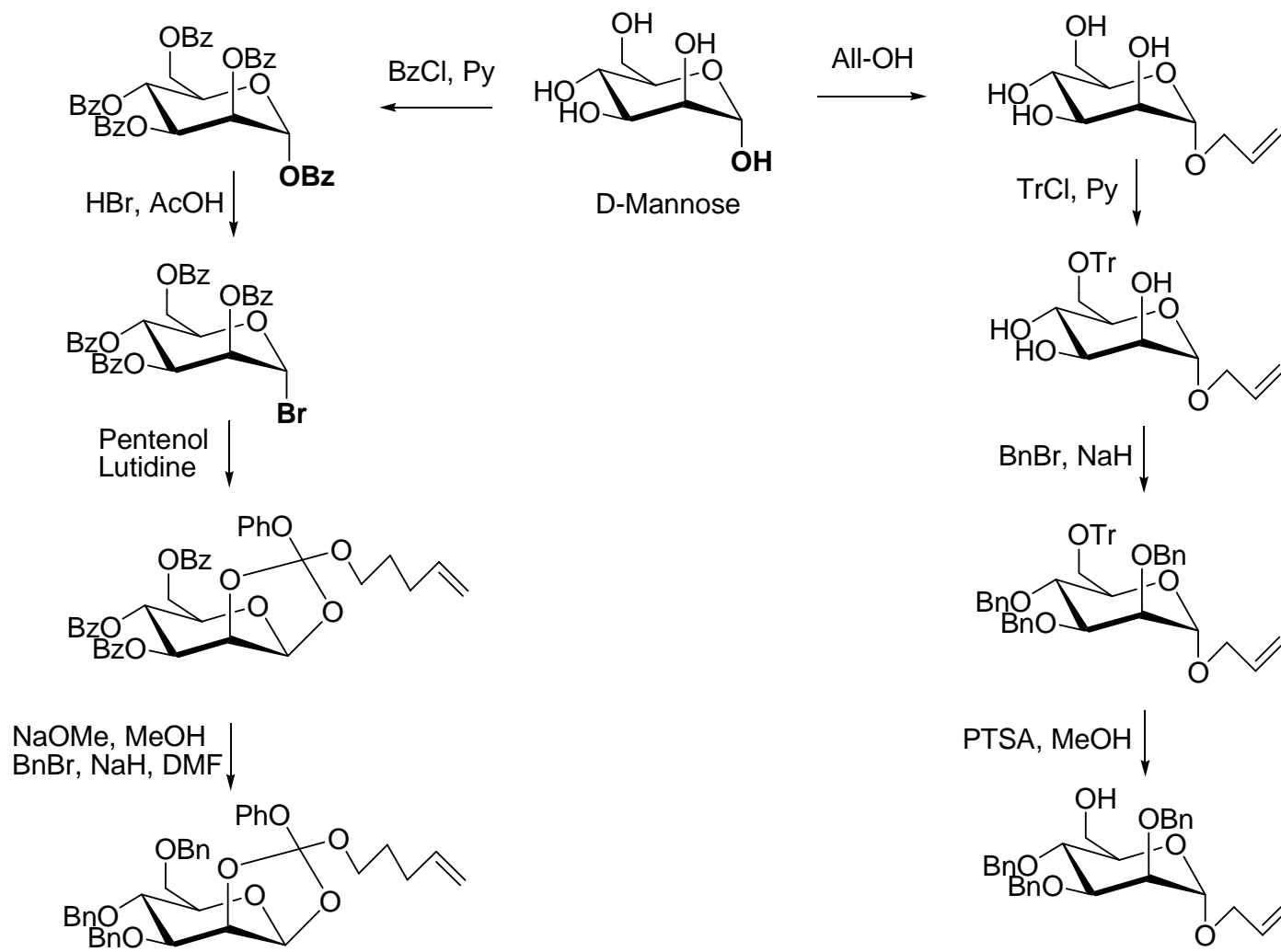






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## Synthesis of Lower Mannobiose Segment

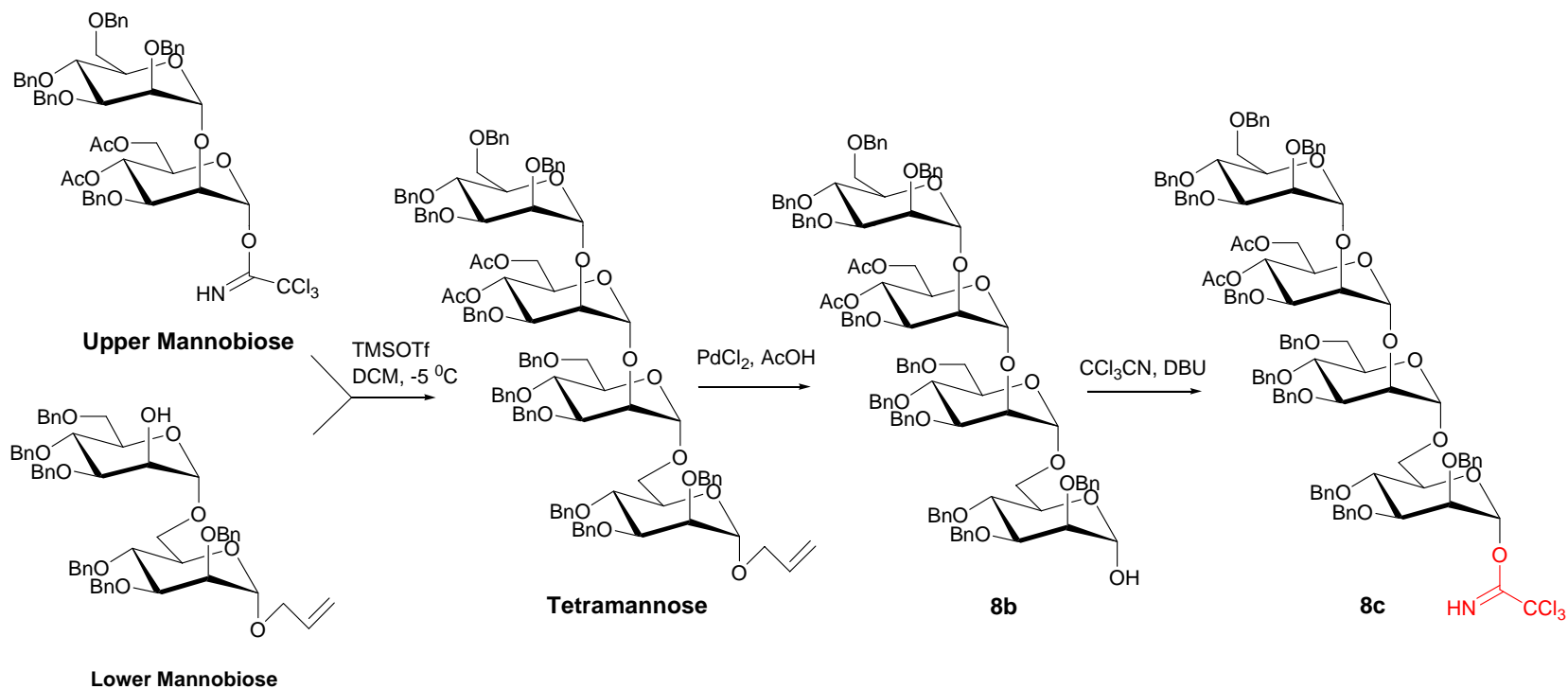






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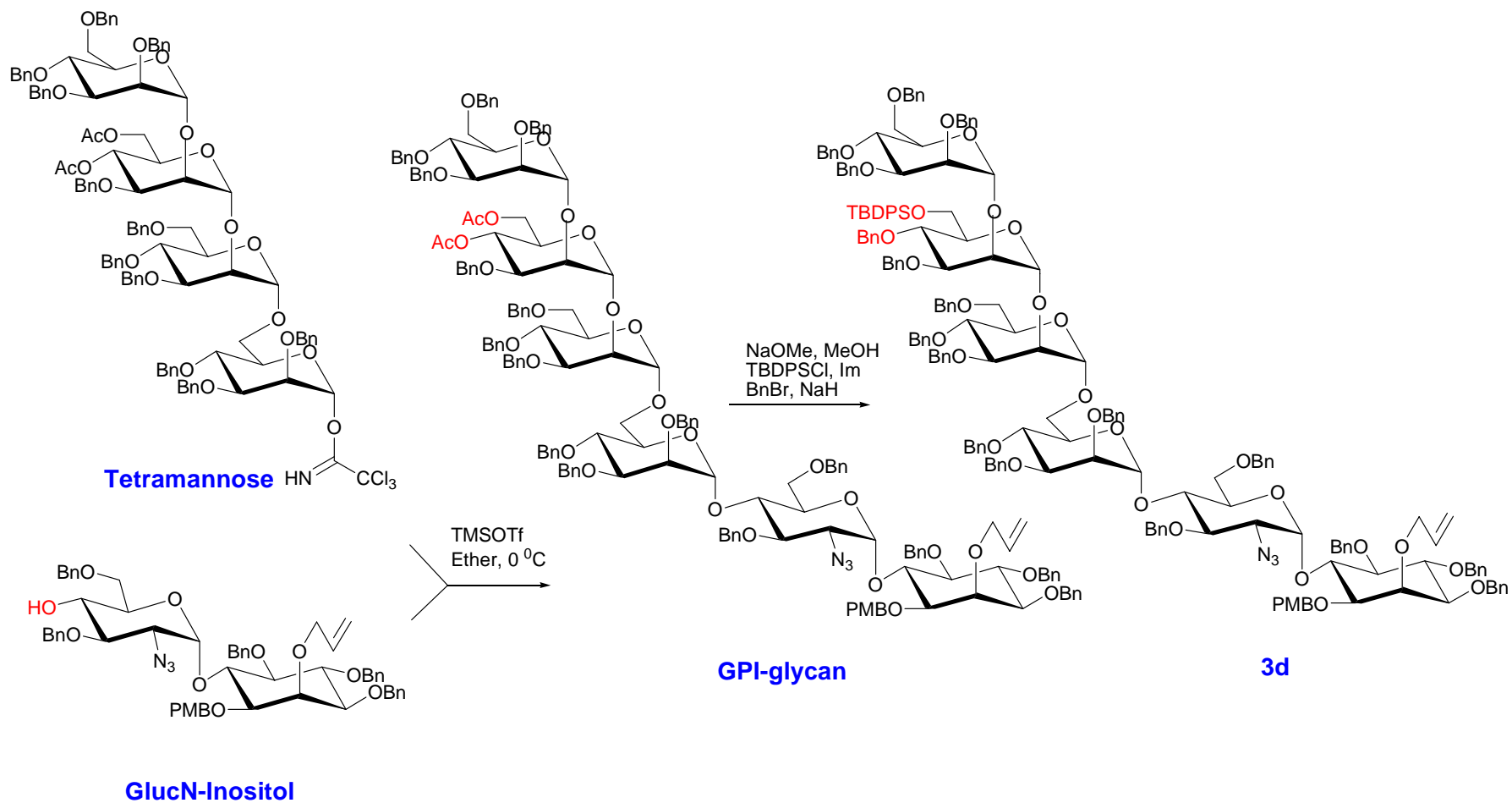
# Construction of Tetra-mannose Block

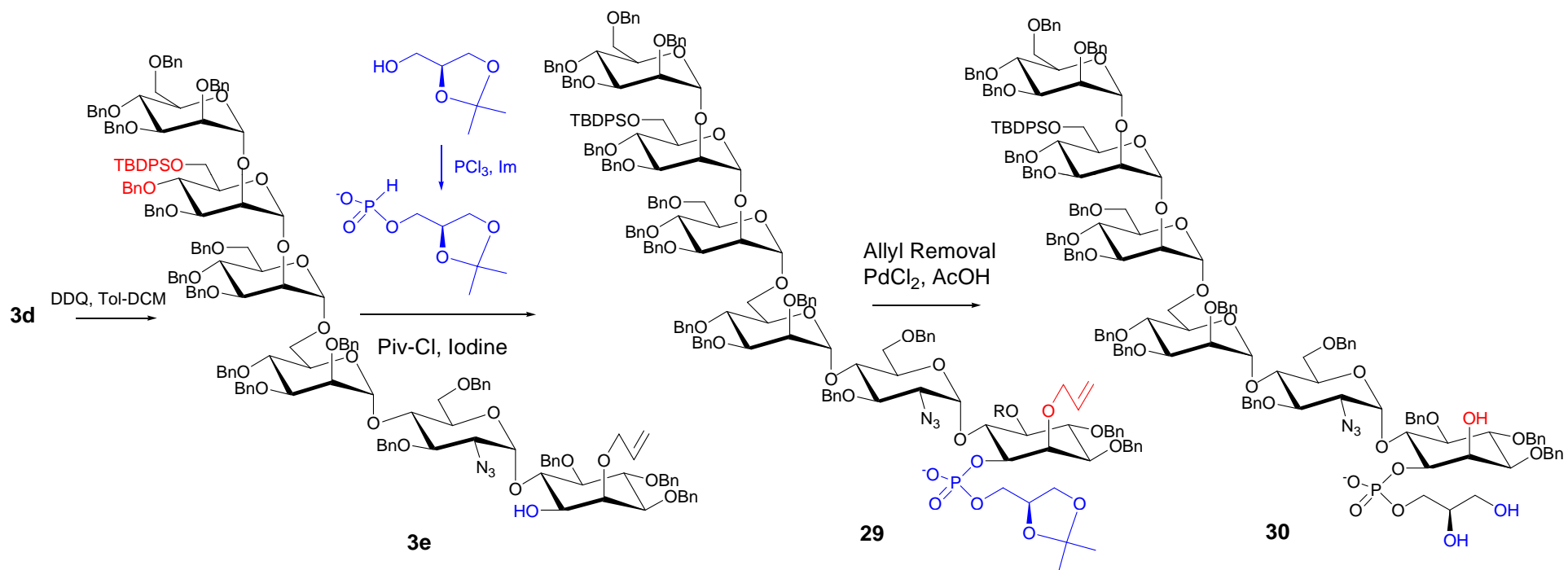




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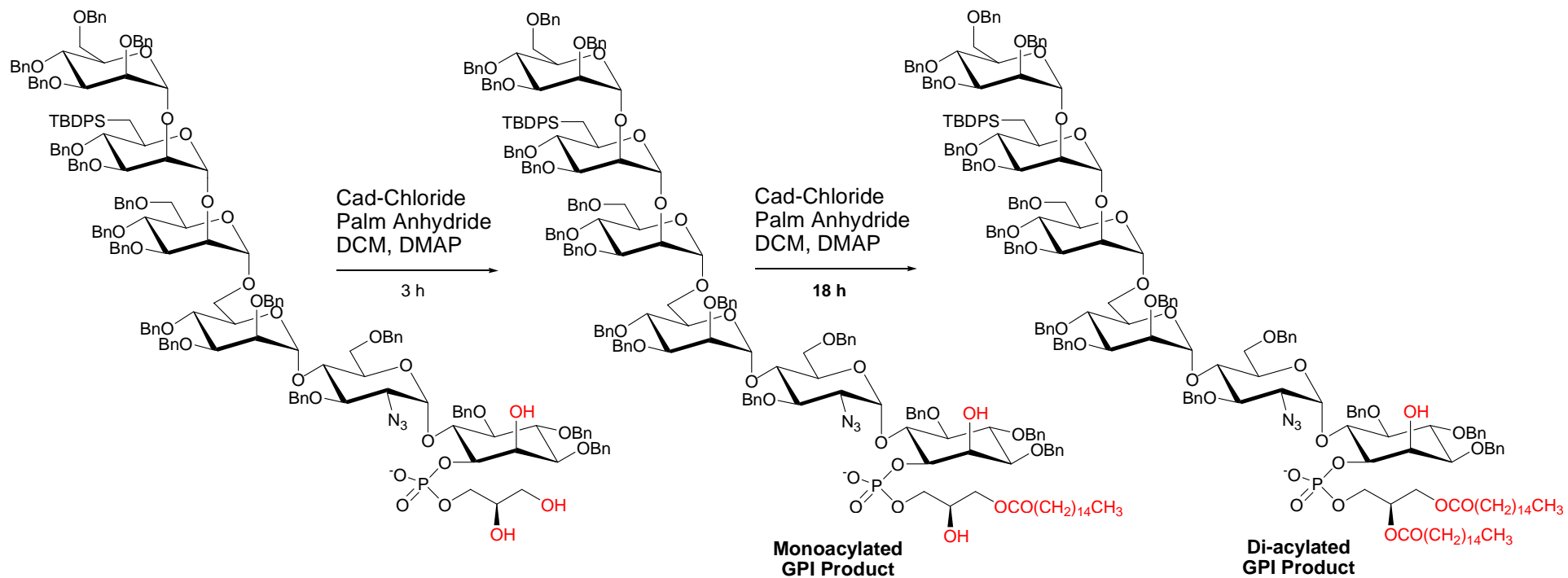
# Synthesis of GPI-Glycan





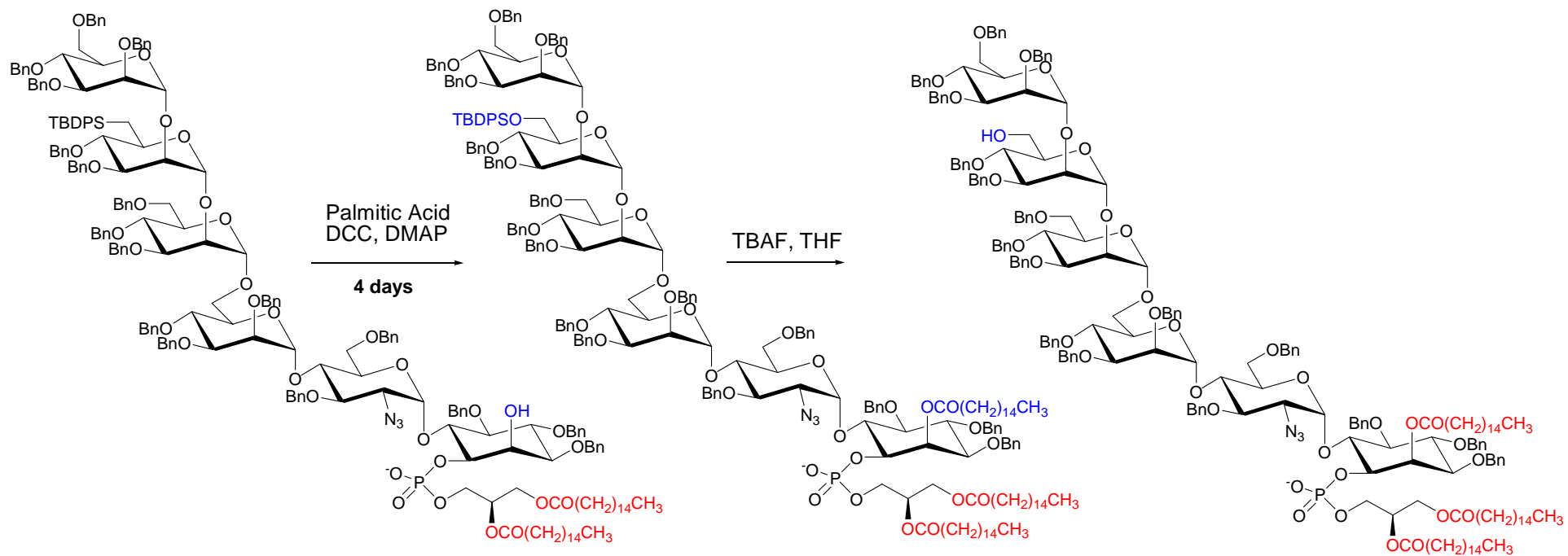


# Selective acylations





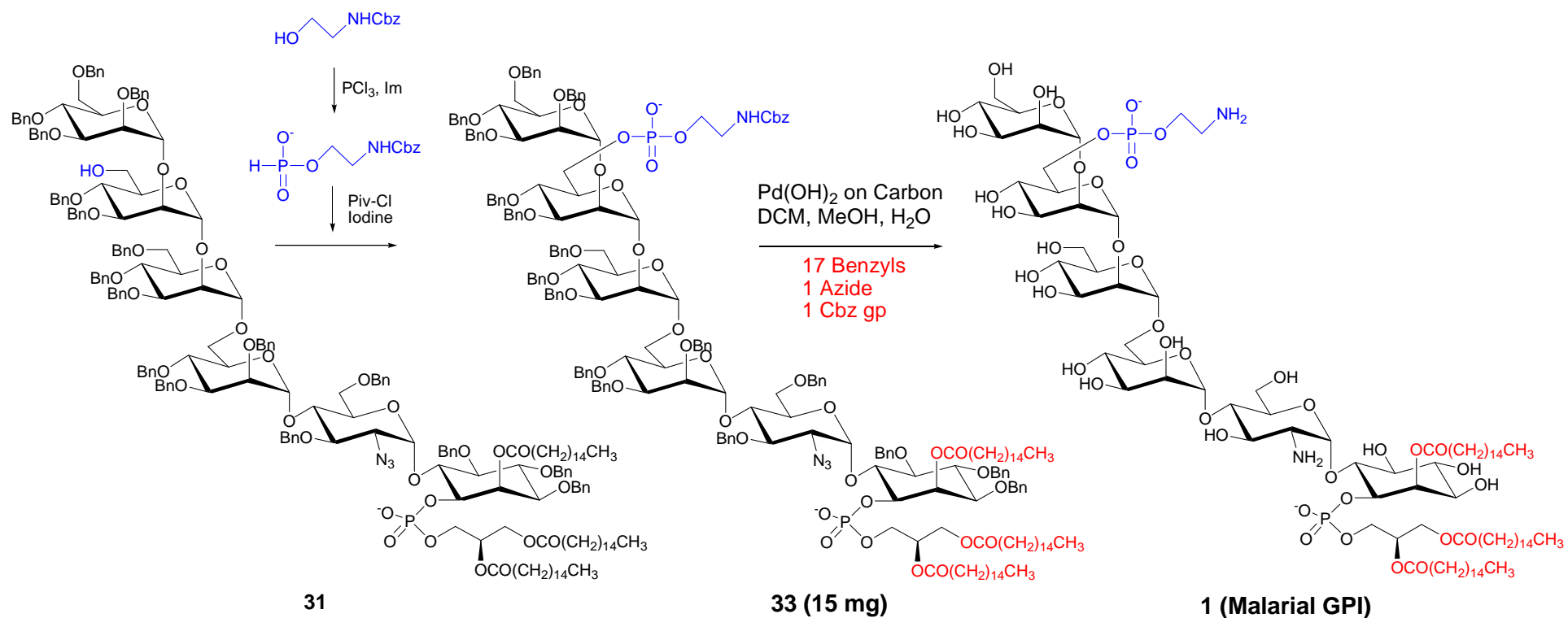
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# Final Assembly



*Chem. Commun.*, 519-521 (2005)

*Tetrahedron*, 66, 4357-4369 (2010)



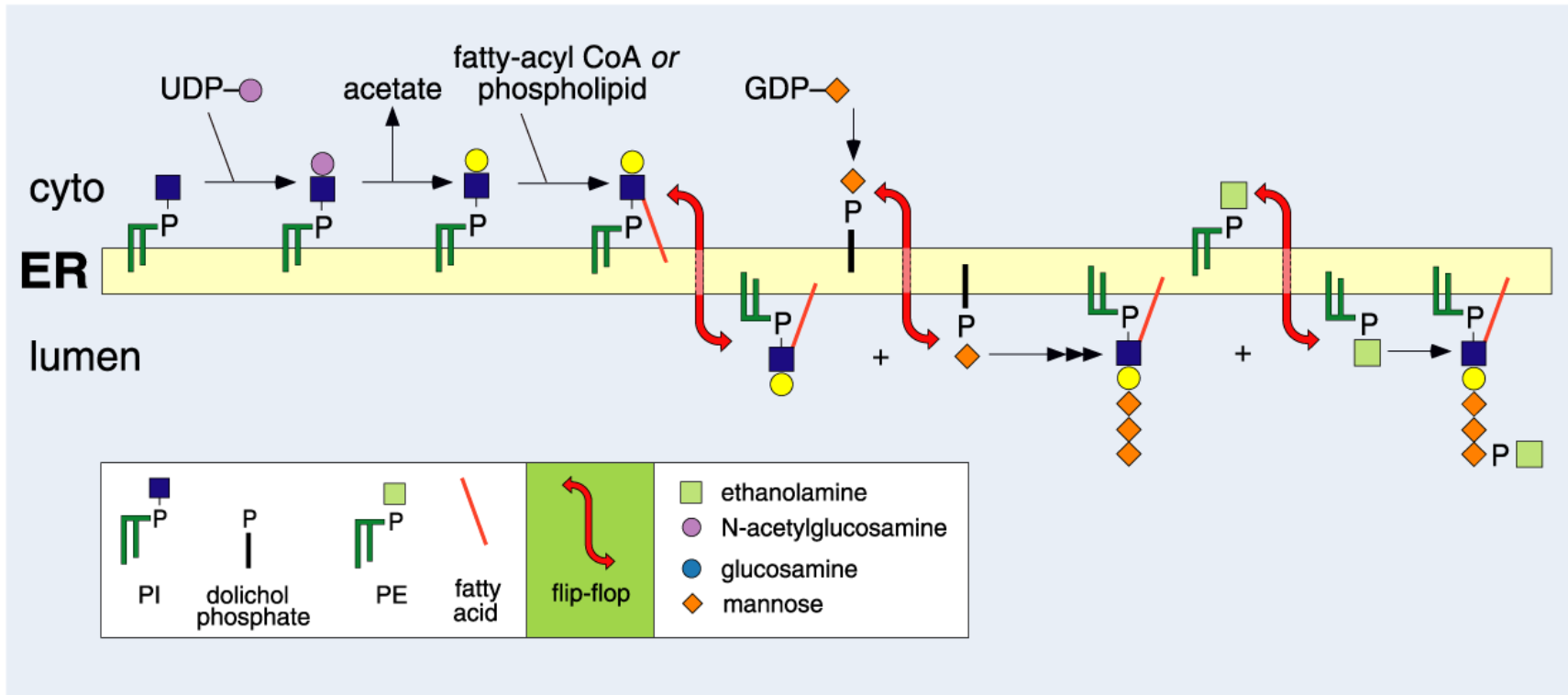
# Key questions of PI/GPI biology

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- **Topology of GPI biosynthetic pathway in Endoplasmic reticulum (ER) membrane**
  - **Stereo-chemical recognition PI in biogenic ER membrane**
  - **Role of GPIs in the organization of membrane micro-domains (lipid rafts) at the Plasma membrane**
  - **Golgi specific GDP-Man transporter in *Leishmania* species**
  - **Phosphatidylinositol pathway as key anticancer drug target**
-



# Topology of GPI biosynthesis

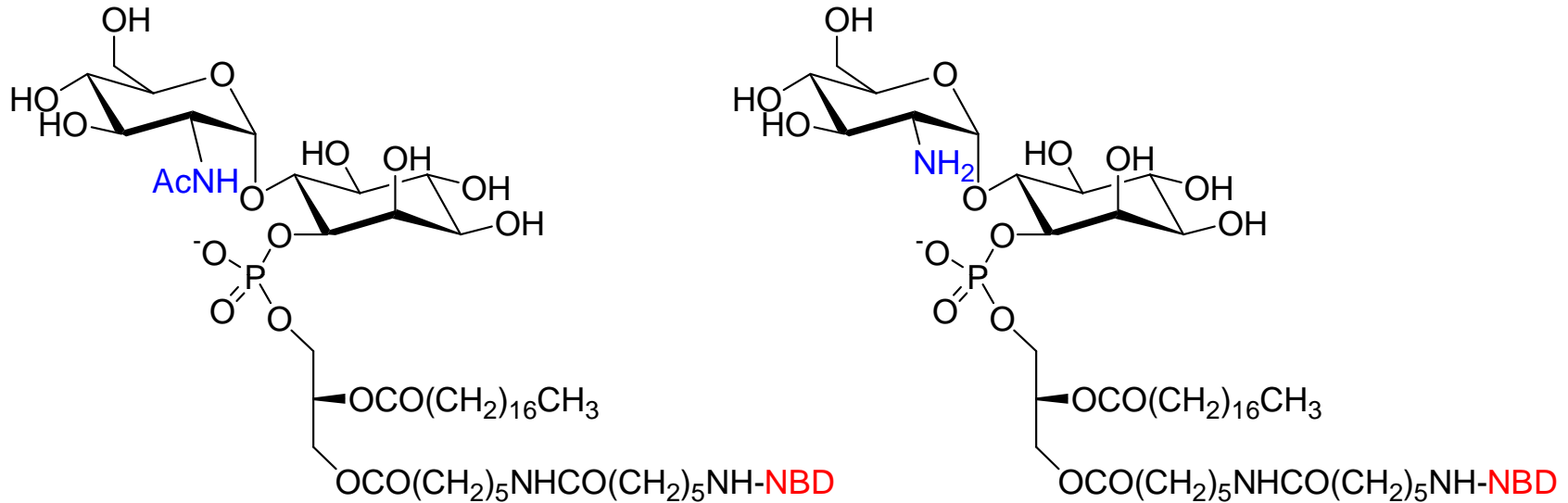


**Evidence for protein-mediated, ATP-independent  
Trans-bilayer movement of GPI intermediates**



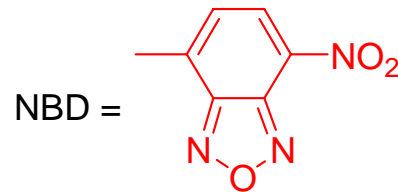
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# Fluorescent GPI probes with label on lipid domain



Fluorescent GlcNAc-PI

Fluorescent GlcN-PI



*J. Lipid Res.*, **44**, 594-600 (2003) and *Chem. Commun.*, (4) 453-455 (2005)



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# Biochemical Reconstitution of ER Flippase activity

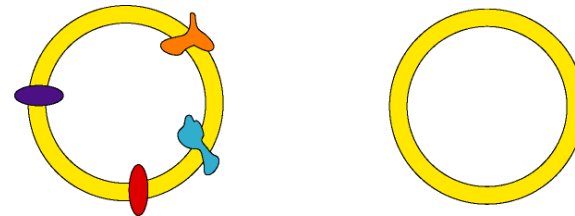
SWER  
↓  
TE  
  
**0.3 mole % of  
the NBD-PC probe**

Triton X-100 extract of ER or  
other biogenic membrane  
OR  
lipid extract of biogenic membrane  
dissolved in Triton X-100

+ egg phosphatidylcholine

remove detergent by adsorption  
using SM2 Bio-Beads

centrifuge to collect vesicles

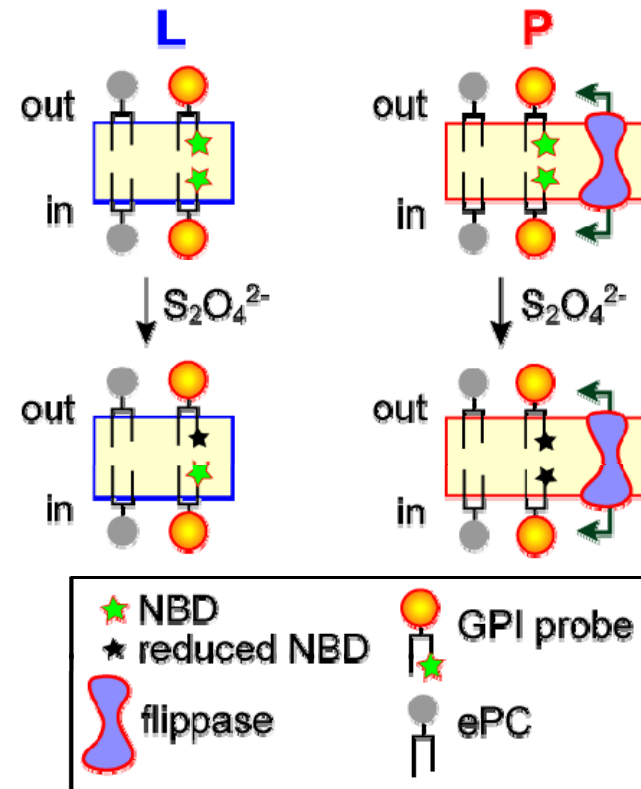
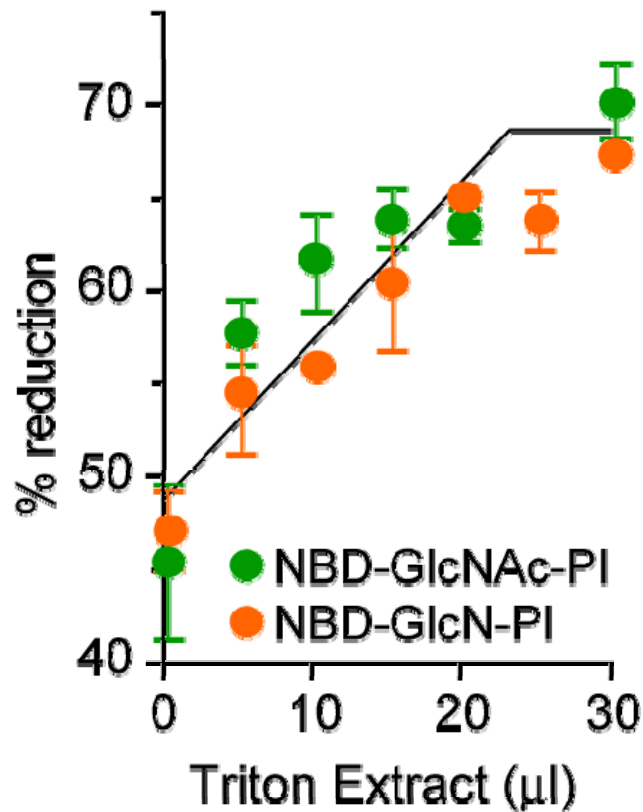


*Chem. Commun.*, (4) 453-455 (2005)



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## GlcNAc-PI and GlcN-PI both flip in proteoliposomes reconstituted from ER

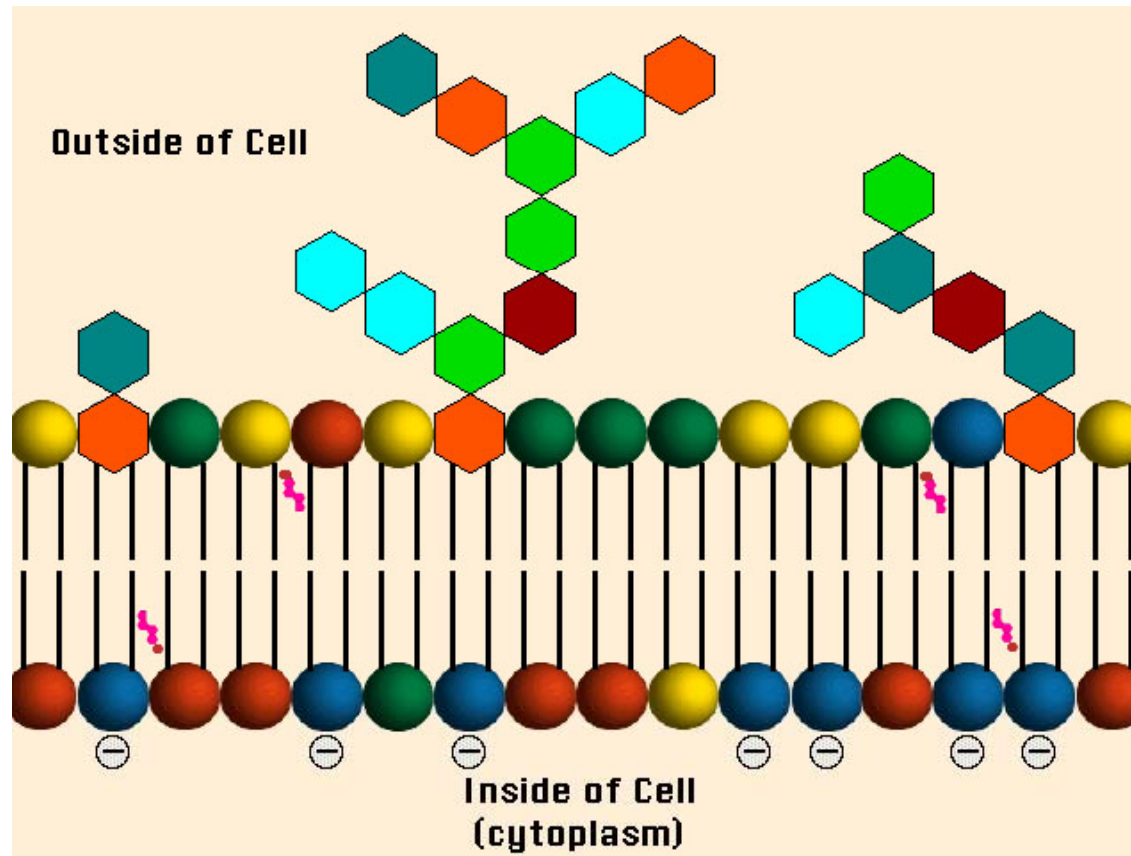


Evidence for protein-mediated, ATP-independent Trans-bilayer movement of GlcN-PI intermediates



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# Trans-bilayer movement of PI Across the ER Is stereochemistry important?





**Bishop and Bell (Cell, 1985)**

**BIOGENIC MEMBRANE**

*“PC flipping appears to be stereo-specific in ER”*

**Pagano (JBC, 1987)**

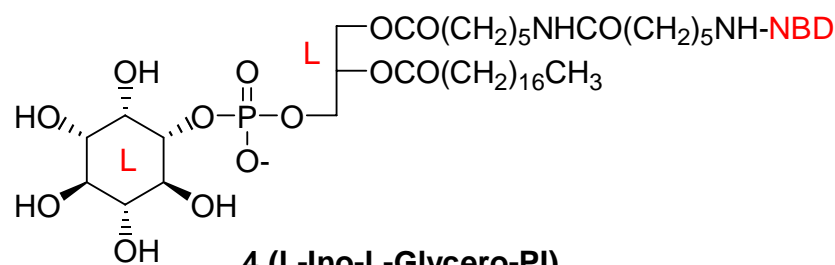
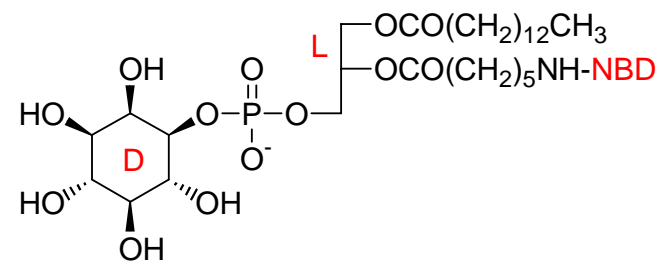
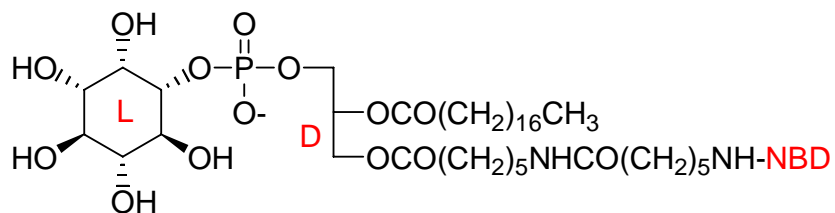
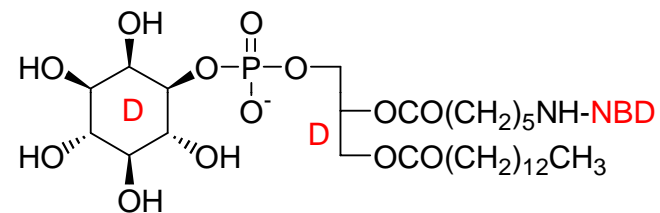
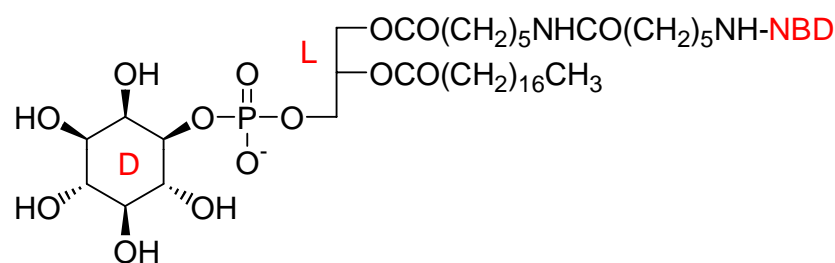
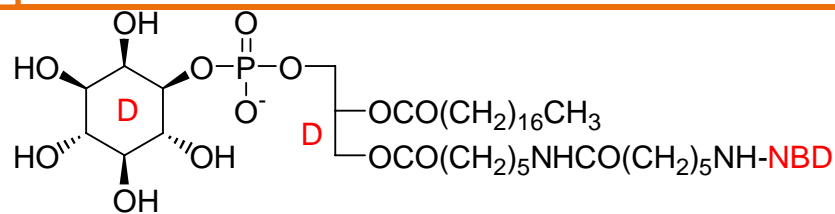
**PLASMA MEMBRANE (ABC transporters)**

*“Trans-membrane movement of PE and PS was Inhibited by their analogues, suggesting that the Process is stereo-specific”*



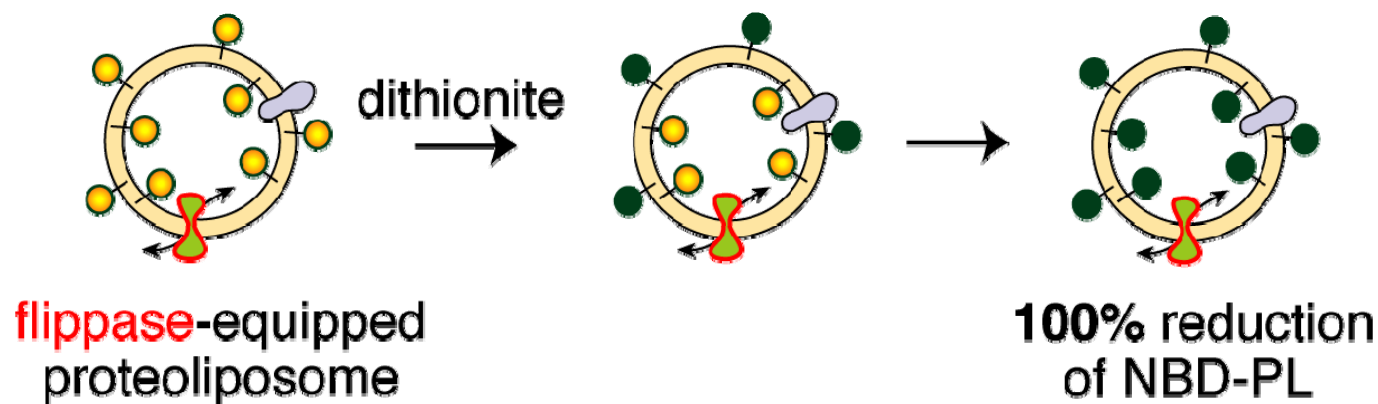
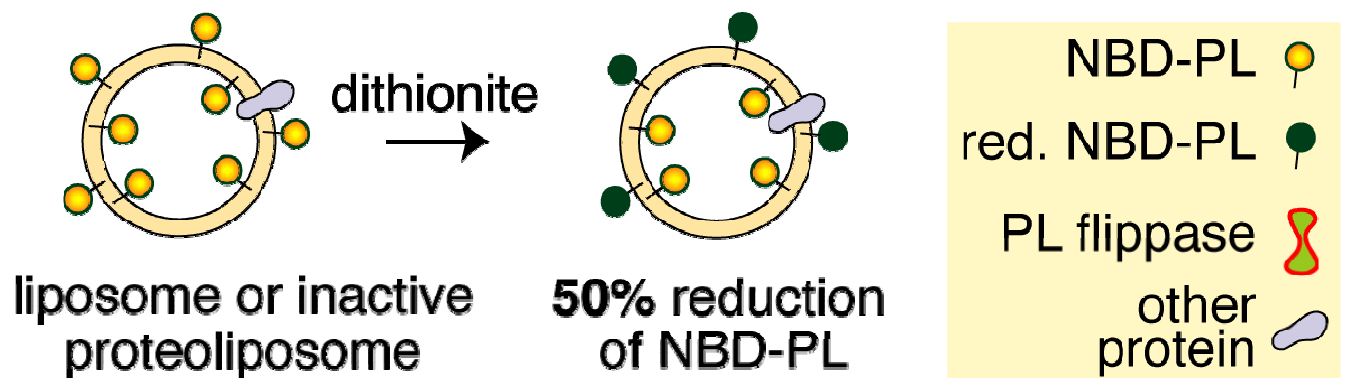
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# All four PI diastereo-isomers synthesized in fluorescent forms





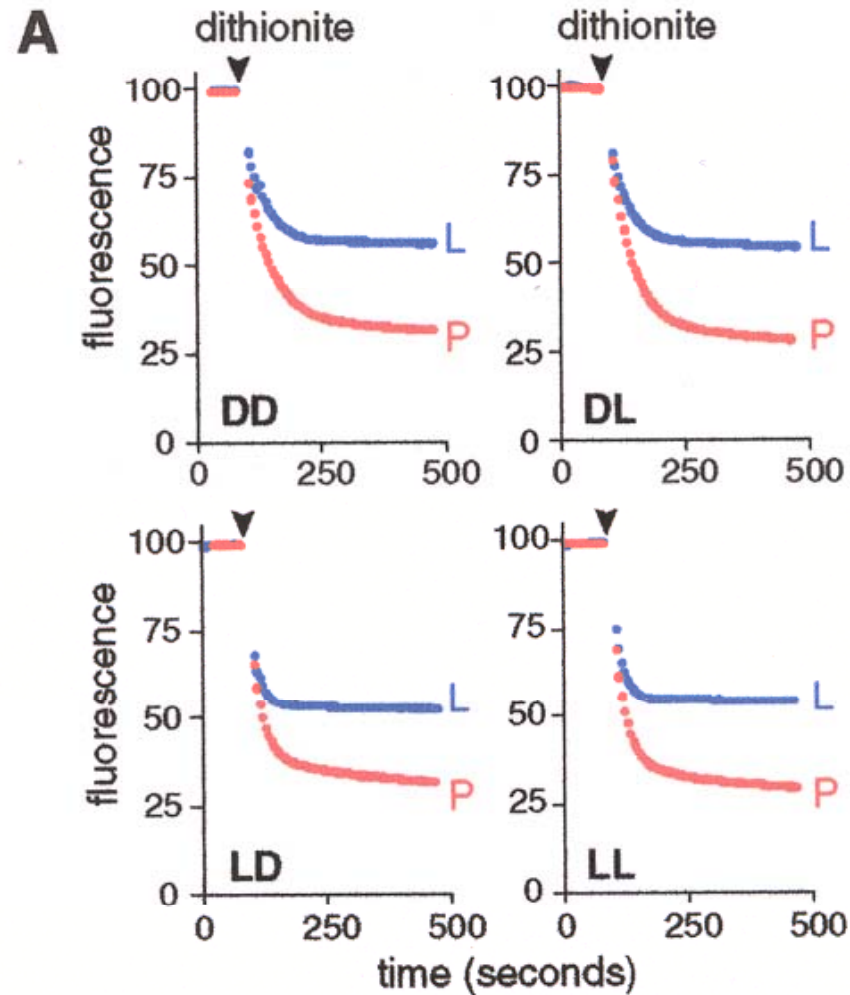
# Dithionite based Flippase assay





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## New fluorescent probes revealed that flip-flop of PI across the ER does *not* depend on the stereochemistry of the lipid





## Alternative Assays

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1. **BSA Back-extraction Assay after Reconstitution in Proteoliposomes**
2. **BSA Back-extraction Assay in Intact ER**
3. **Kinetics Measurements**

The rate constants were identical for these two *myr*-PI's, and similar to those for a *myr*-PC sample measured alongside

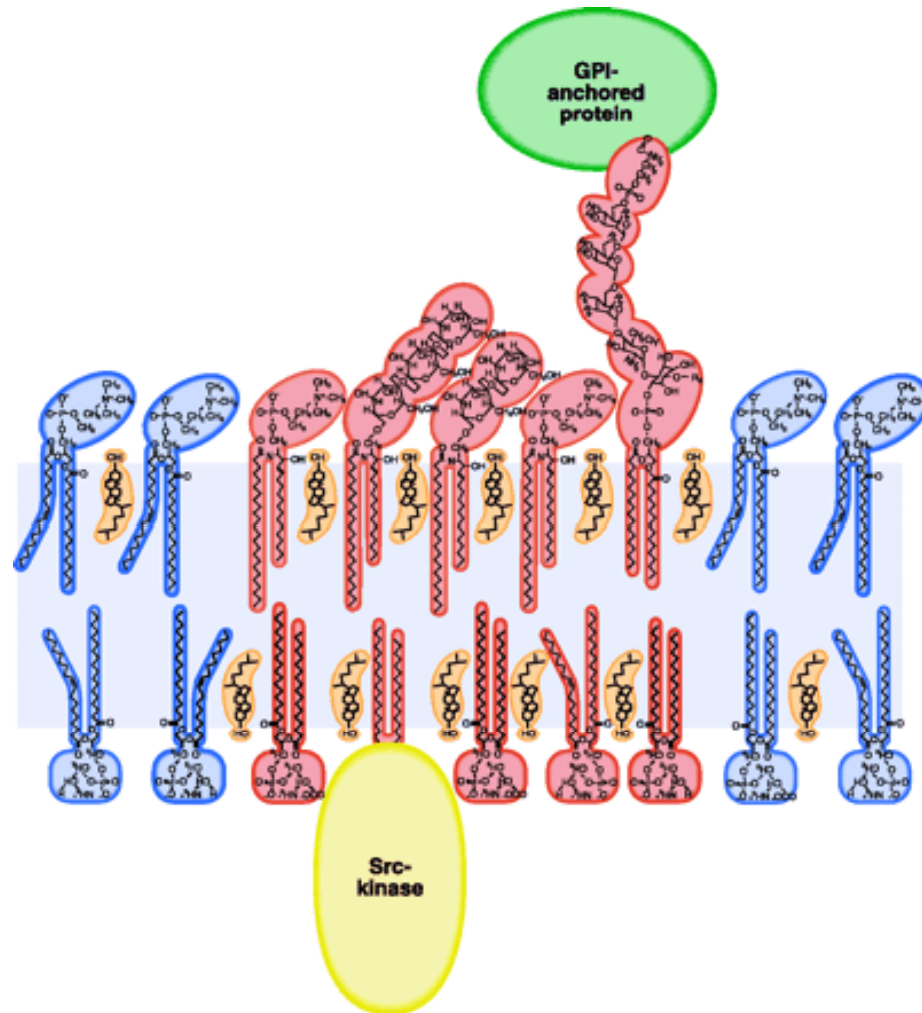


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# Role of GPI anchored proteins in Plasma Membrane Microdomain Organization

A GPI-anchored protein is attached to the exoplasmic leaflet

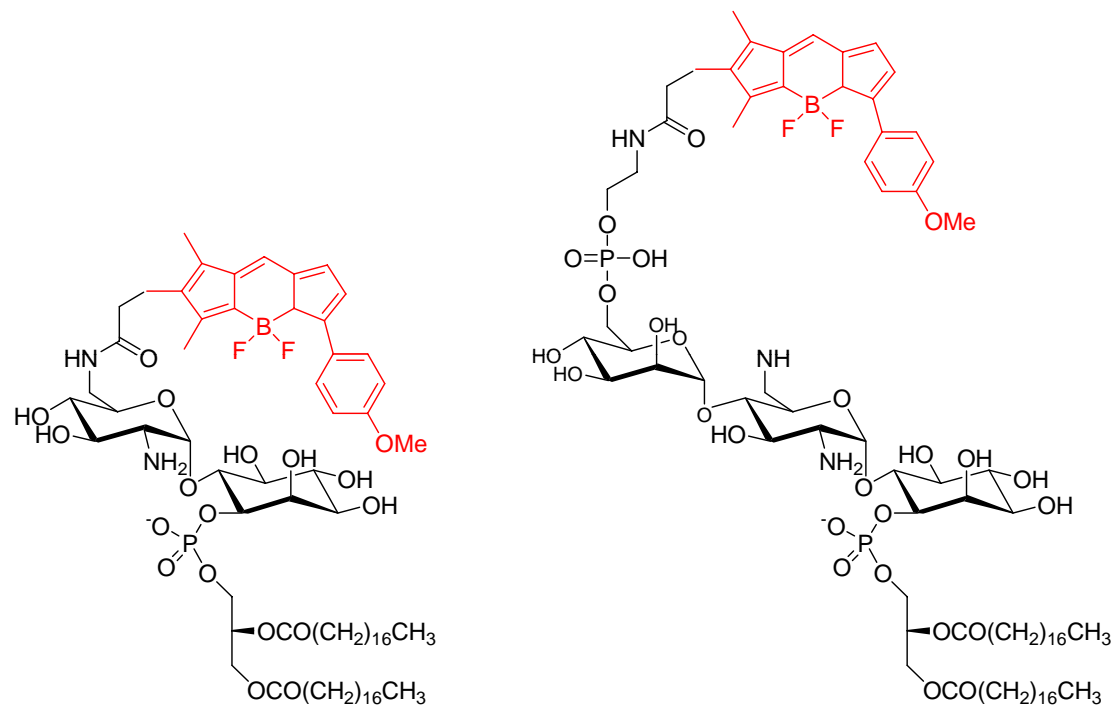
A doubly acylated Src-kinase to the cytoplasmic leaflet





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## Chemical basis of GPI structure recognition



Fluorescent GlcN-PI probe

Fluorescent Man-GlcN-PI probe

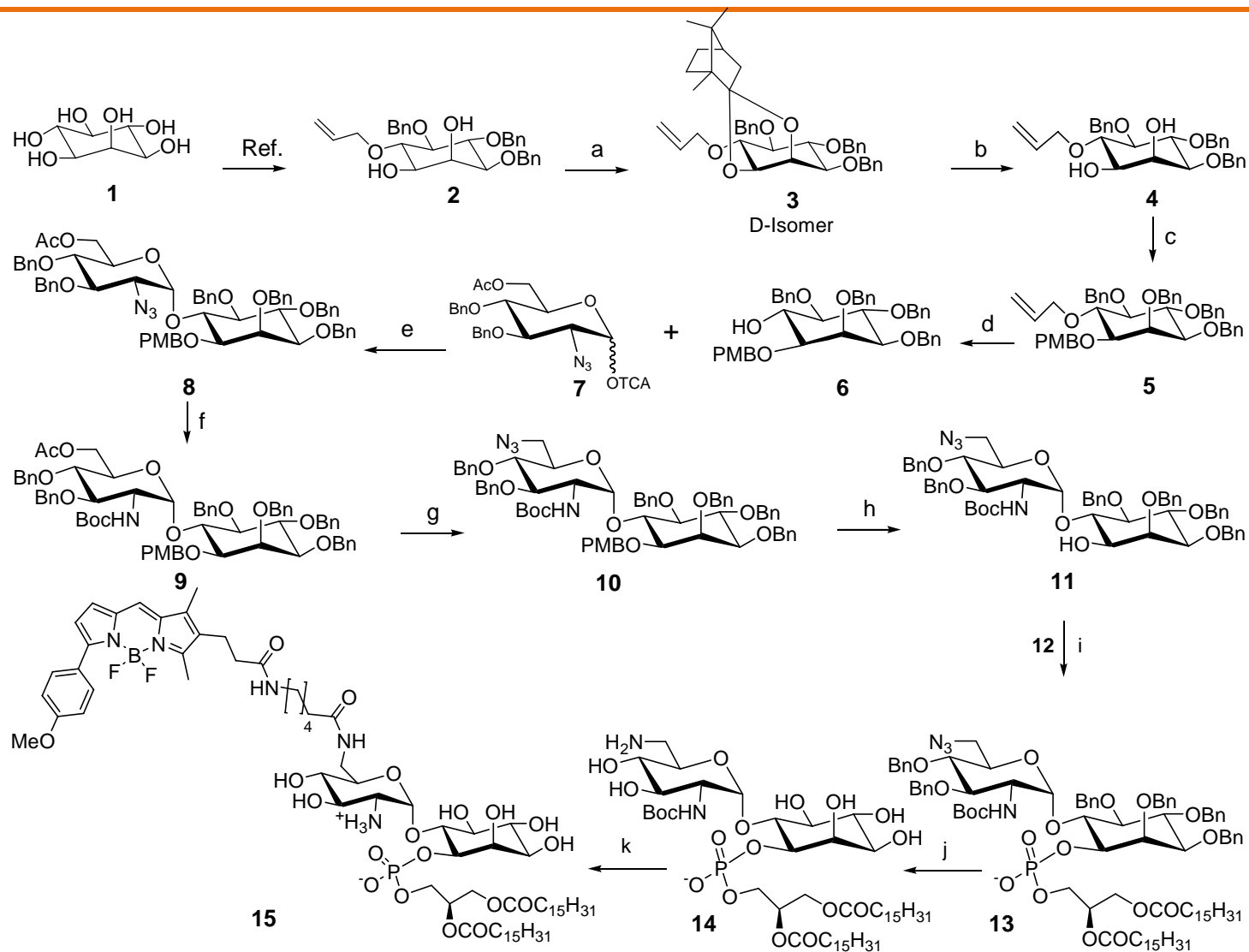
*Chem. Commun.*, 519 - 521 (2005)

*Chem. Commun.*, 5852-5854 (2011)



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# Synthesis of head group labeled GPI probes



Tetrahedron Lett., 52, 4277-4279 (2011)



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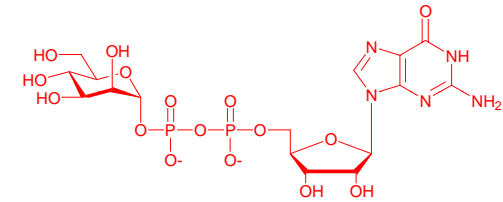
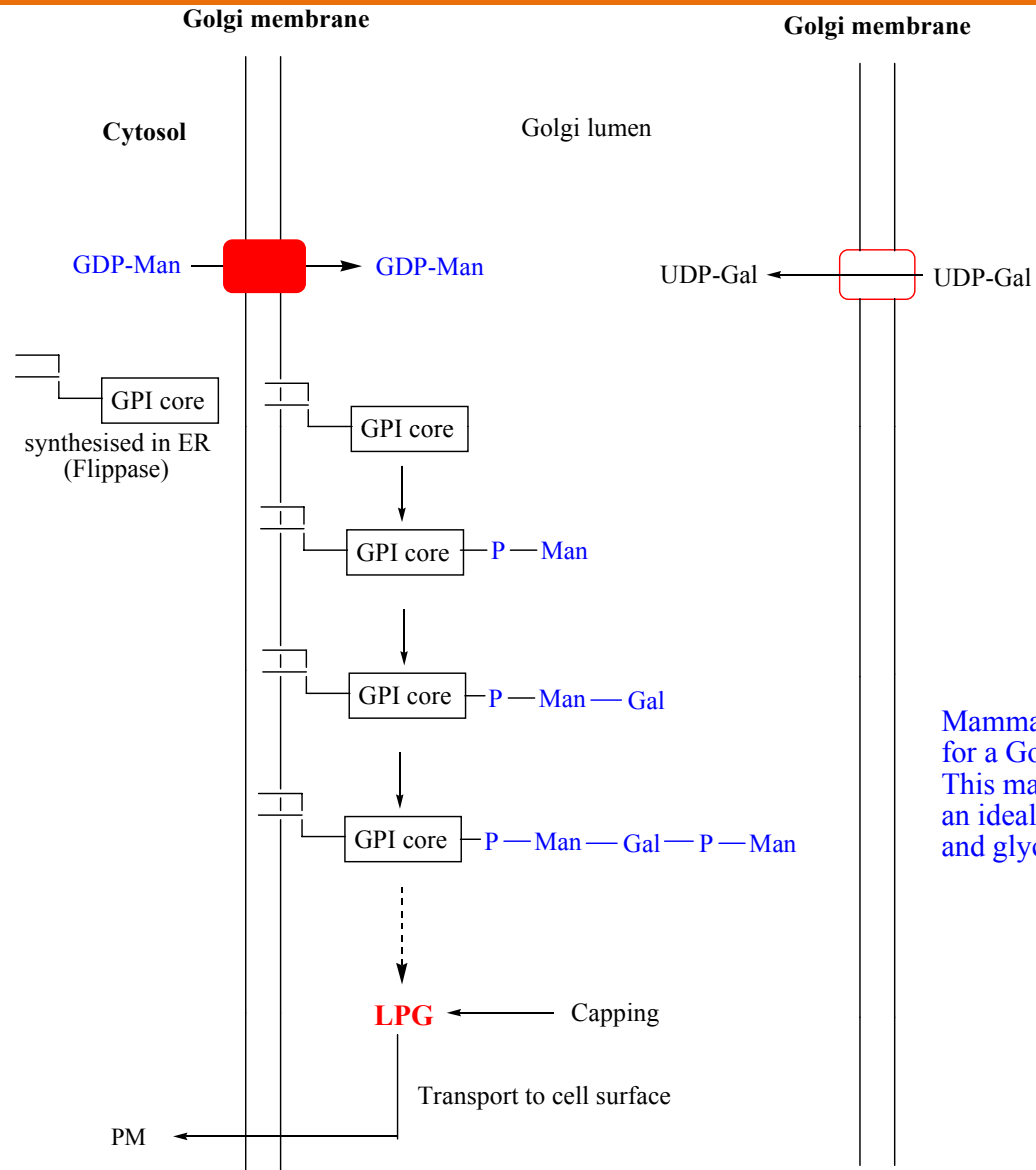
Nanoclusters of GPI-anchored proteins are formed  
by cortical actin-driven activity  
**Cell**, 135, 1085-1097 (2008)

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# GDP-Man transport in Leishmania during GPI biosynthesis

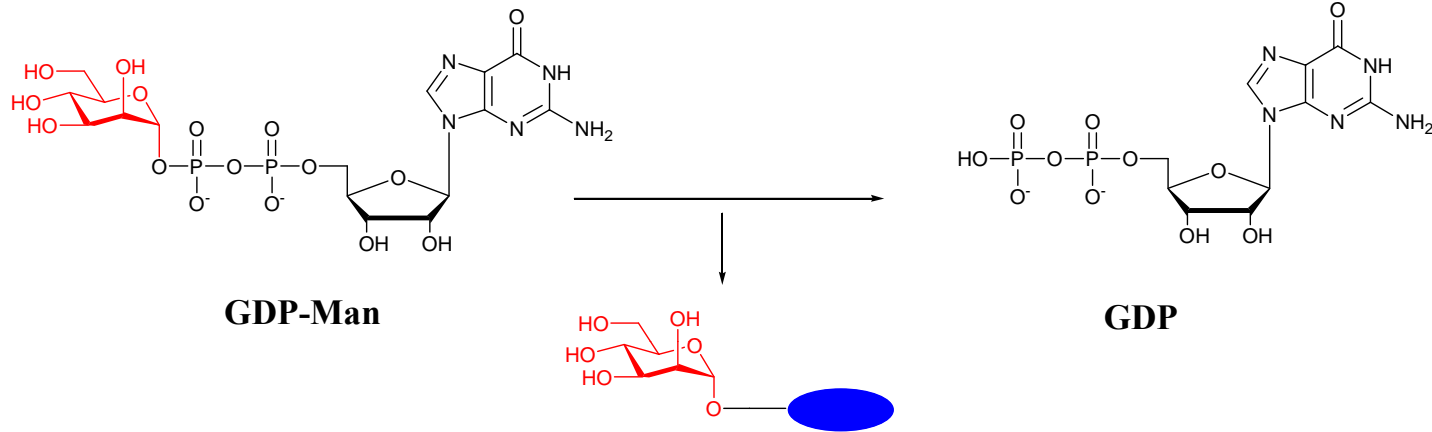


GDP-Man

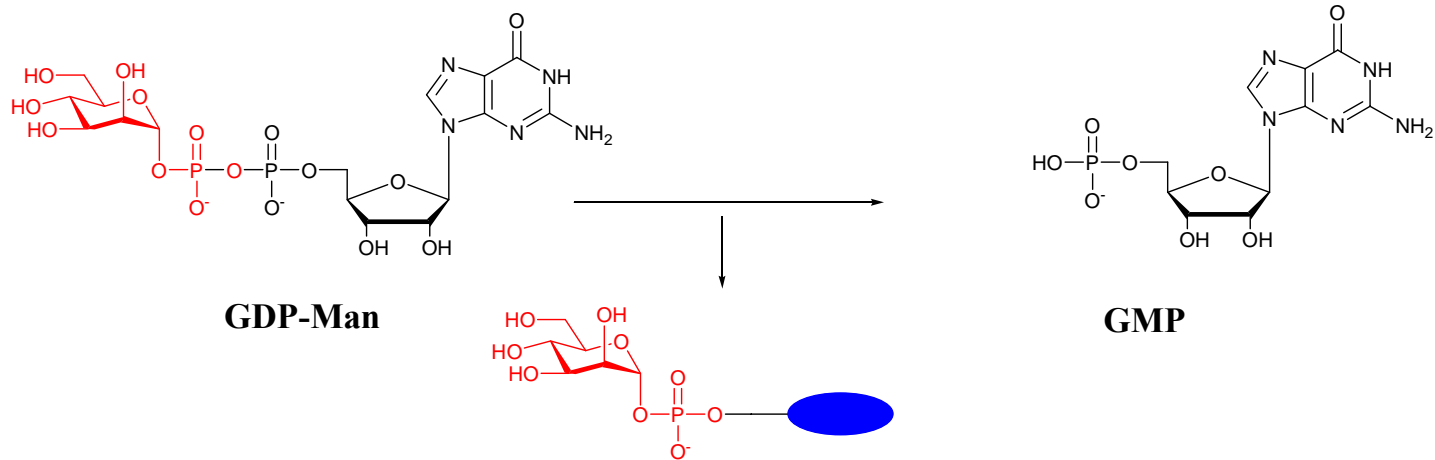
Mammalian cells have no requirement for a Golgi localised GDP-Man transporter. This makes *Leishmania* transporter an ideal target for drug-design and glycobiology



## Higher Organism



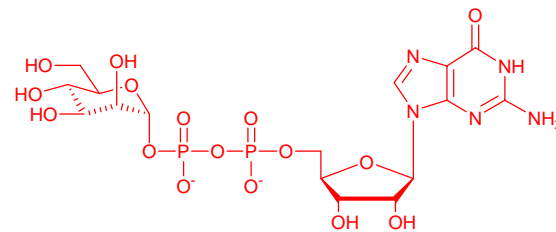
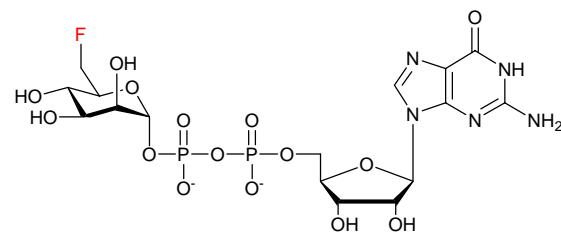
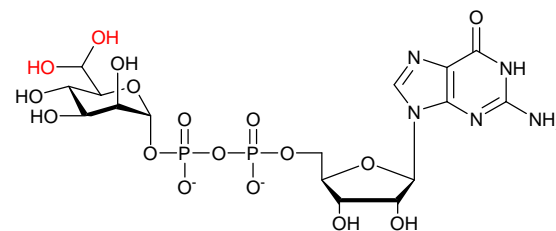
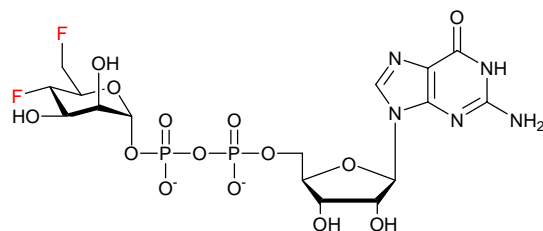
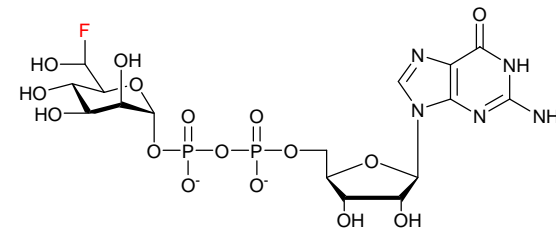
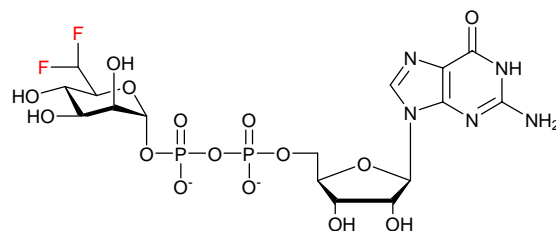
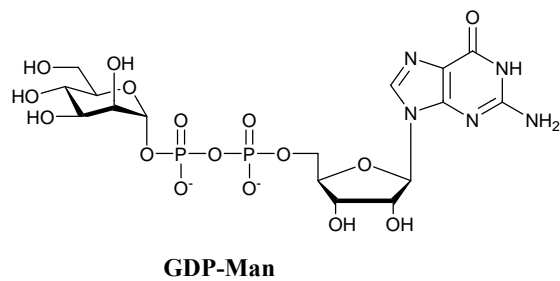
## Leishmania





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# Synthesis of novel GDP-Man probes





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## GDP-Man transporter assay (using Golgi vesicles)

Leishmania lysate (Membrane proteins)

Sucrose gradient fractionation  
(0.25 to 2.5 M gradient, 80000 rpm)

Golgi Vesicles  
(evaluated by GalT assay)

1M-1.5M fractions had  
maximum GalT activity

$[^3\text{H}]$ -GDP-Man  
(0.16  $\mu\text{Ci}$ )

Reaction mix contained 300  $\mu\text{g}$   
protein, 10 mM Tris-HCl, sucrose,  
TLCK, leupeptin, PMSF and DTT

Incubation at 28  $^{\circ}\text{C}$  for 6 min  
and snap-frozen

Filtered through HA filter, washed  
and bound radioactivity counted

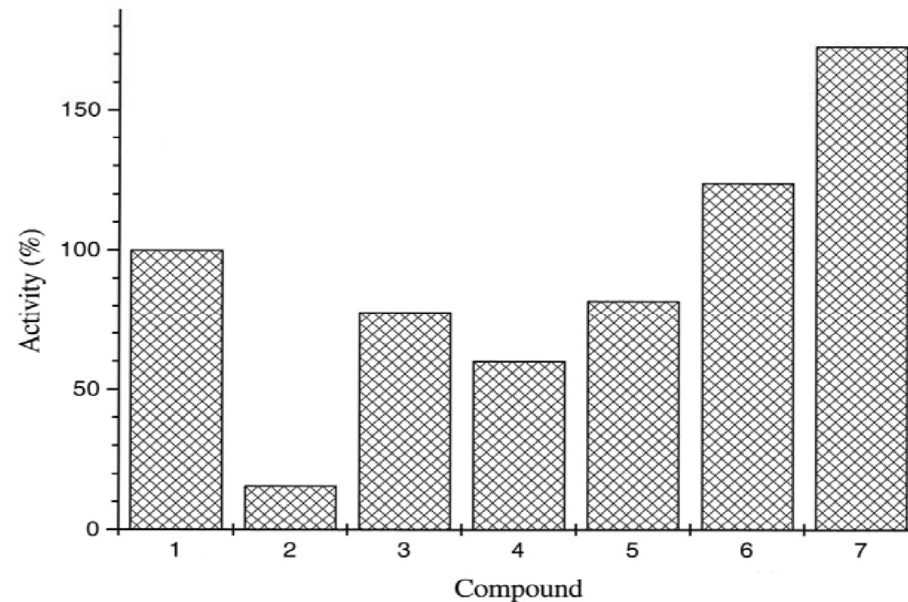
transport rate = 16 pmole  
GDP-Man/mg protein

Vesicles broken with CM treatment and applied to TLC  
along with standard GDP-Man and Bioscan



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# Transport of GDP-Man analogues



## GDP-Man Transport Assay Using Leishmania Golgi Vesicles

Transport rate 16 pmole/mg

1. Control (8  $\mu$ M GDP-Man)
2. DIDS (8  $\mu$ M)
3. [6-deoxy-6-fluoro]-GDP-Man (8  $\mu$ M)
4. [6-deoxy-6-fluoro]-GDP-Man (32  $\mu$ M)
5. [6-deoxy-6,6-difluoro]-GDP-Man (8  $\mu$ M)
6. [L-Man]-GDP-Man (8  $\mu$ M)
7. [L-Man]-GDP-Man (32  $\mu$ M)

Unpublished Results



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## Phosphatidylinositol 3-kinase (PI3K) pathway as clinically validated anticancer target

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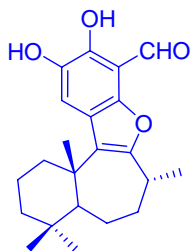
- Inhibition of PI-3K kinase (isoform specific inhibitors)
  - PI3K $\alpha$  for cancer and PI3K $\gamma$  for inflammation
  - Up-regulation of PTEN phosphatase
  - Inhibition of AKT activity
  - Inhibition of mTOR
-



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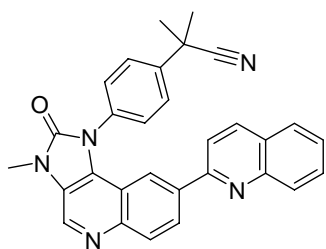
# Medicinal chemistry of natural products and clinically validated candidates for cancer drug discovery

## PI3K Inhibitors: Scaffolds selected for Med Chem Prog



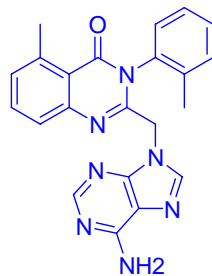
**Liphagal**  
Wyeth (Preclinical)

p110 $\alpha$  IC<sub>50</sub> - 100 nM  
p110 $\beta$  IC<sub>50</sub> - ND  
p110 $\gamma$  IC<sub>50</sub> - 1  
p110 $\delta$  IC<sub>50</sub> - ND



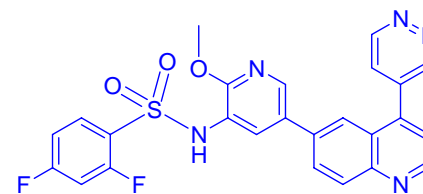
**BEZ235 as PI3K/mTOR inhibitors** (Novartis, Phase II)

p110 $\alpha$  IC<sub>50</sub> - 4nM  
p110 $\beta$  IC<sub>50</sub> - 76nM  
p110 $\gamma$  IC<sub>50</sub> - 7nM  
p110 $\delta$  IC<sub>50</sub> - 5nM



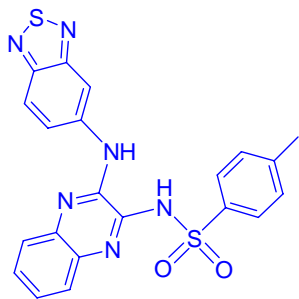
**CAL-101, PI3K inhibitor** (Calistoga, Phase I)

IC<sub>50</sub> - 2.5 nM in PI3K $\delta$



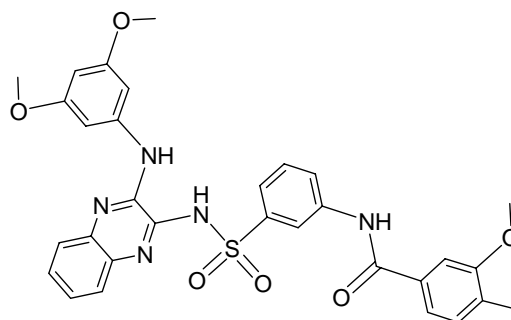
**GSK2126458**  
(Developed by GSK)

(PI3K $\alpha$  IC<sub>50</sub> - 0.04 nM)



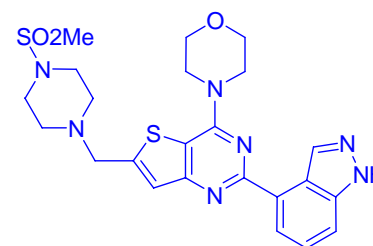
**XL147, PI3K inhibitor** (Exelixis, Phase I)

p110 $\alpha$  IC<sub>50</sub> - 39nM  
p110 $\beta$  IC<sub>50</sub> - 383nM  
p110 $\gamma$  IC<sub>50</sub> - 23nM  
p110 $\delta$  IC<sub>50</sub> - 36nM



**XL765 as PI3K inhibitor**, (Exelixis, Phase I)

p110 $\alpha$  IC<sub>50</sub> - 13nM  
p110 $\beta$  IC<sub>50</sub> - 113nM  
p110 $\gamma$  IC<sub>50</sub> - ND  
p110 $\delta$  IC<sub>50</sub> - 43nM



**GDC0941 as PI3K inhibitor**, (GenetechPhase I)

PI3K $\alpha$  IC<sub>50</sub> - 3nM  
1 log unit sensitive against  $\beta/\gamma$

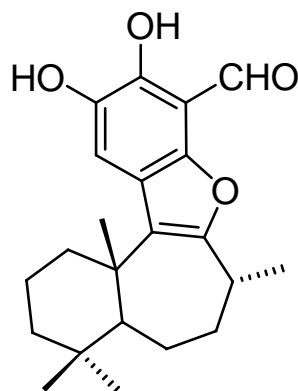
**Scaffolds in blue color: Synthesis completed at IIM-Jammu**  
**Patent search have been completed and analogs have been synthesized**



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# Medicinal chemistry of Liphagane scaffold

(First isoform selective PI3K inhibitors)



**p110 $\alpha$  IC<sub>50</sub> - 100 nM**  
**p110 $\beta$  IC<sub>50</sub> - ND**  
**p110 $\gamma$  IC<sub>50</sub> - 1**  
**p110 $\delta$  IC<sub>50</sub> - ND**

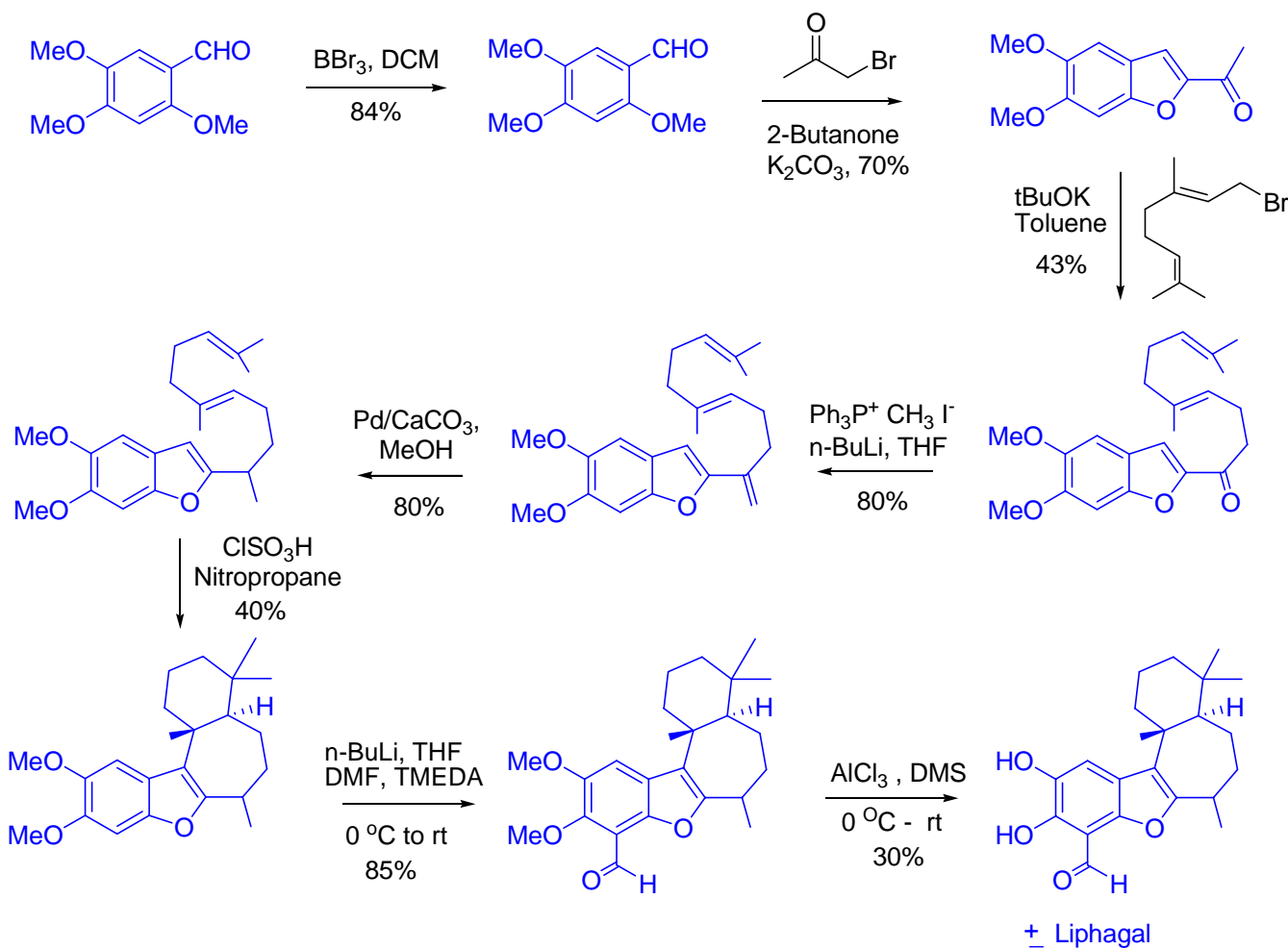
- A meroterpenoid isolated from marine sponge *Aka coralliphaga*  
Inhibitory activity against PI3K $\alpha$  (10 fold selectivity)
- More selective than the synthetic LY294002 and Wortmanin.
- IC<sub>50</sub>: 100 nM

**Org. Lett.** 2006 , 8, 321 (Isolation, synthesis)  
**Tetrahedron Lett.** 2009, 50, 5260-5262  
**Org. Lett.**, 2010, 12, 2394–2397  
**Org. Lett.**, 2010, 12, 4450-4453  
**J. Med. Chem.**, 2010, 53, 8523-8533  
(60 nM IC<sub>50</sub> and 27 fold selectivity)  
**Synth. Commun.**, 2011, 41, 177-183  
**Wyeth WO 2006/081659 A1**



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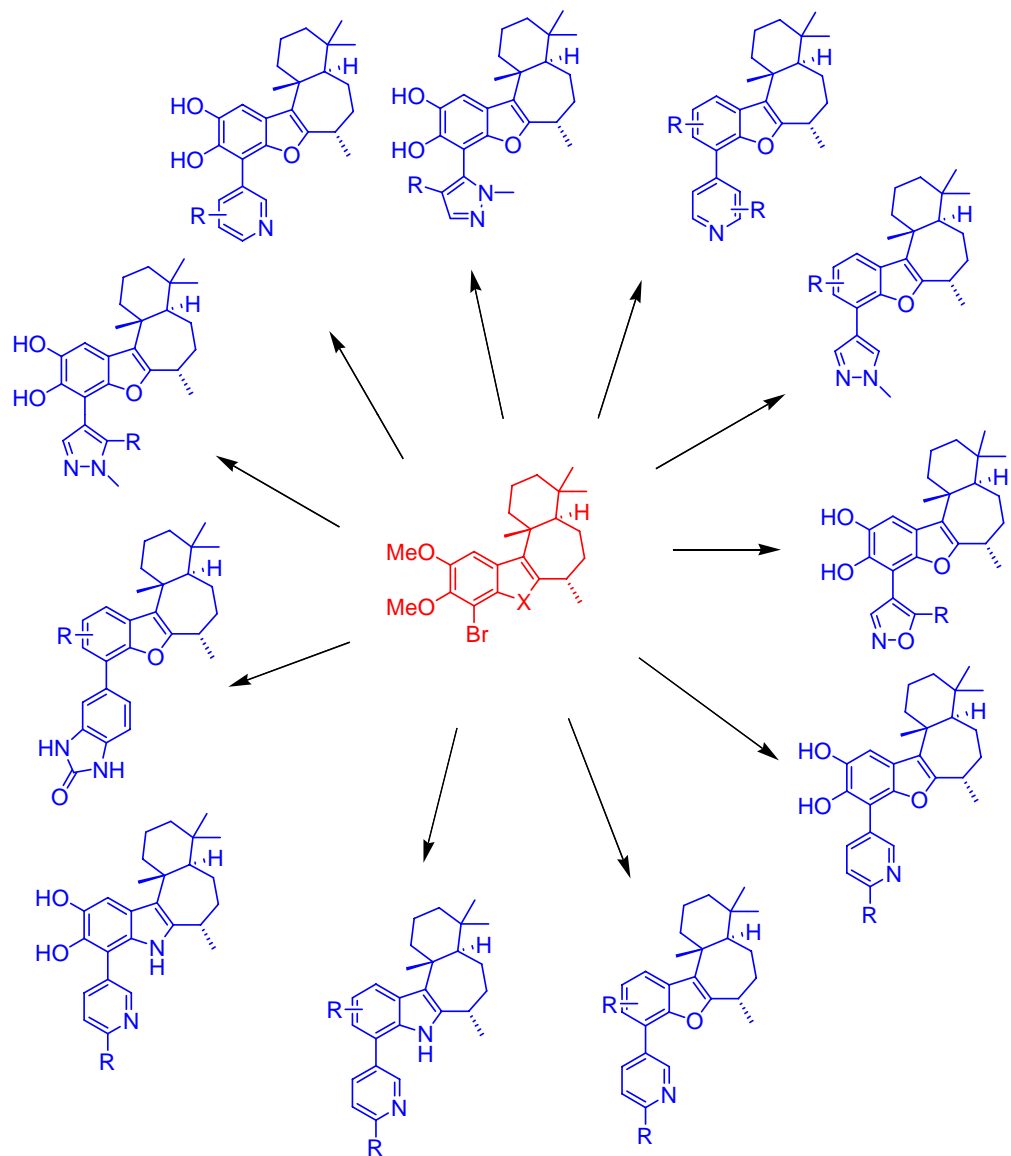
## Our synthesis of Liphagal





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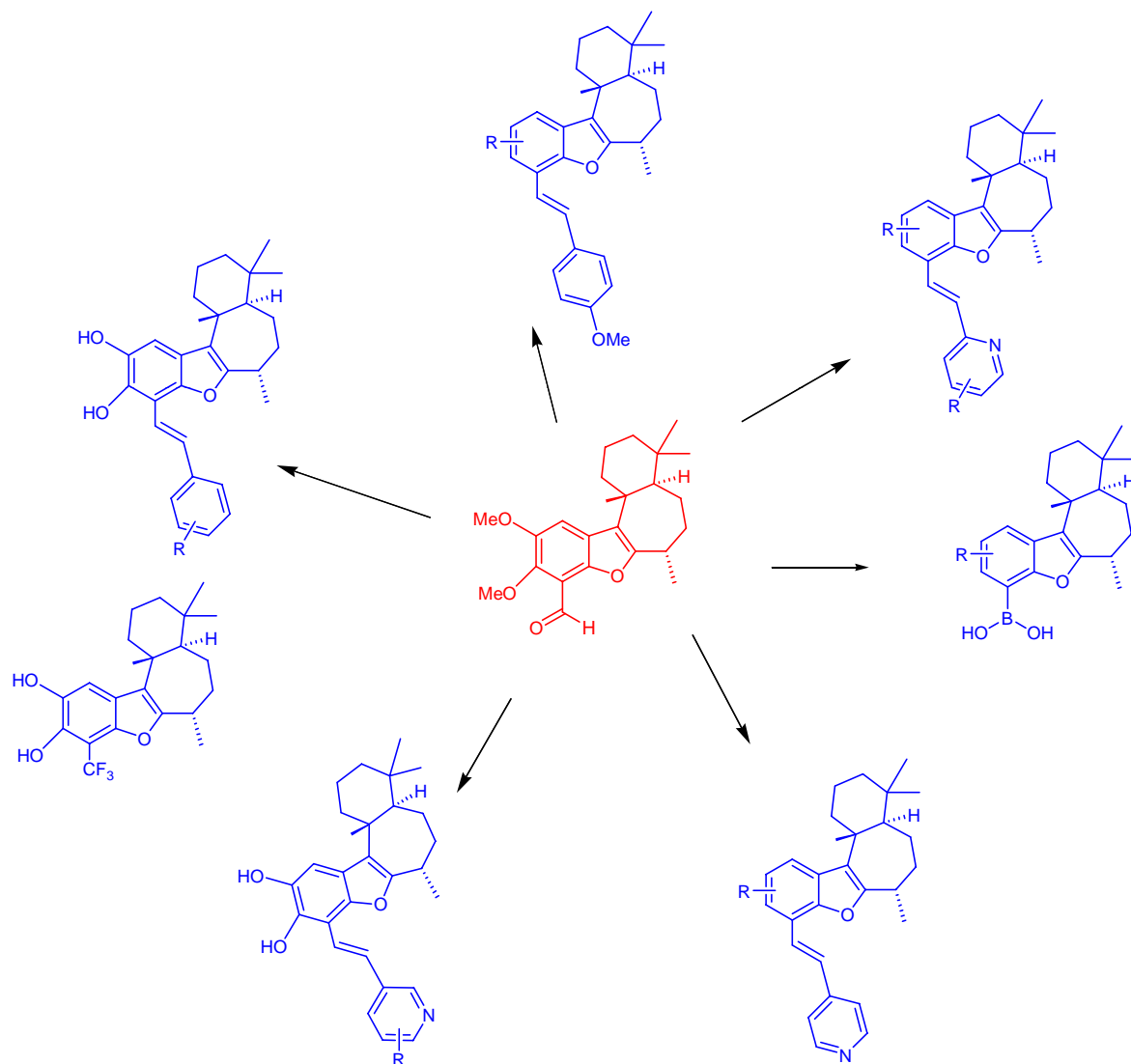
## NCEs based on Liphagal scaffold (first series)





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## NCEs Liphagal scaffold (second series)



IC<sub>50</sub> of two best compounds from Liphagal series

Control IC<sub>50</sub> 100 nM

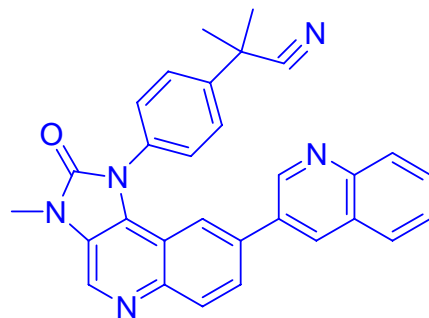
Events or Targets	Sample code	
	SIP-1003	SIP1004
<i>In vitro</i> enzyme assay IC <sub>50</sub>	PI3K $\alpha$ - 140 nM $\beta$ - 100 nM $\gamma$ - ND $\delta$ - ND	PI3K $\alpha$ - 102 nM $\beta$ - 30 $\mu$ M $\gamma$ - 25 $\mu$ M $\delta$ - 45 $\mu$ M
Cell based assay	2.6 $\mu$ M (MCF-7)	3.1 $\mu$ M (MCF-7)
Annexin-V	35-57% (1-9 $\mu$ M)	50-58% (1-9 $\mu$ M)
Cell Cycle	50-60% GI (1-9 $\mu$ M)	64-70% GI (1-9 $\mu$ M)
Wound Healing	Effective	Highly effective
Phospho-Akt	50-55% Down-regulation	60-68% Downregulation

IP Filed



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## Medicinal chemistry of Imidazo-quinoline scaffold



p110 $\alpha$	4 nM
p110 $\beta$	76 nM
p110 $\delta$	5 nM
p110 $\gamma$	7 nM

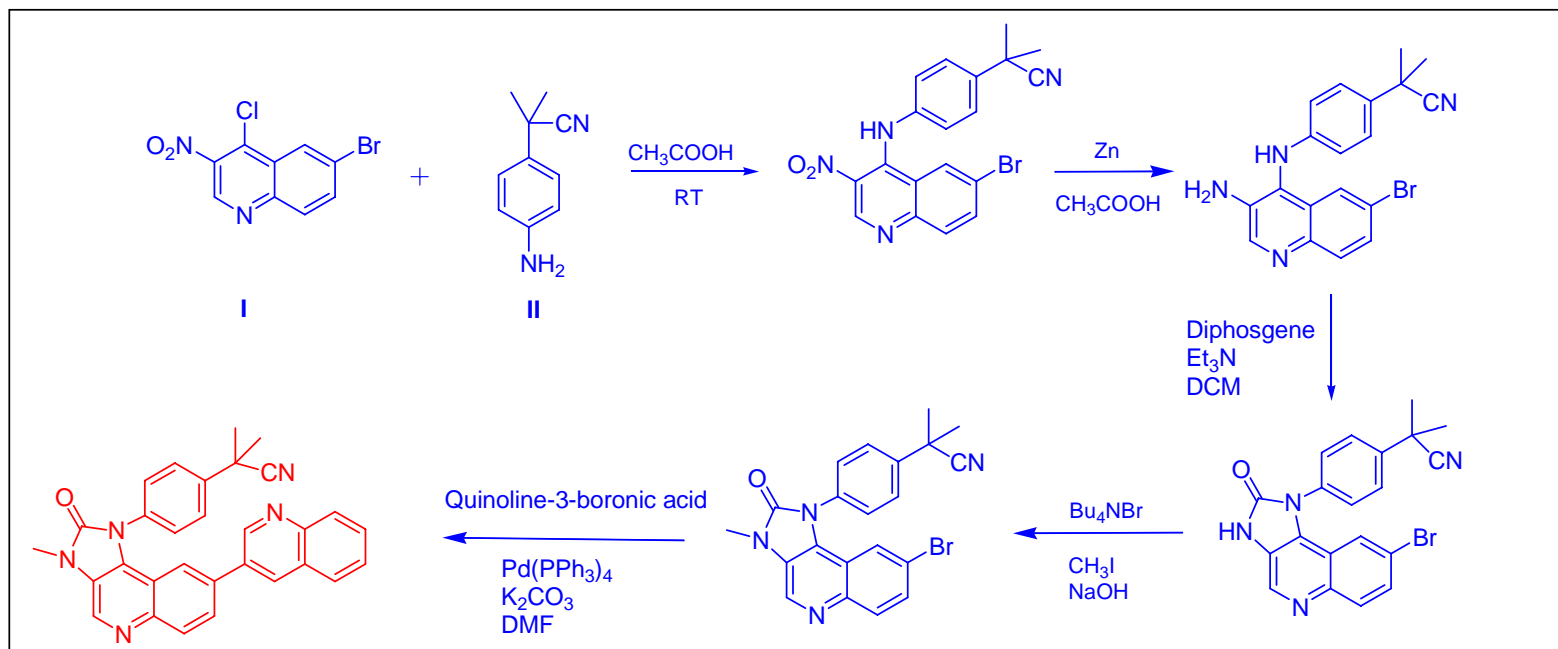
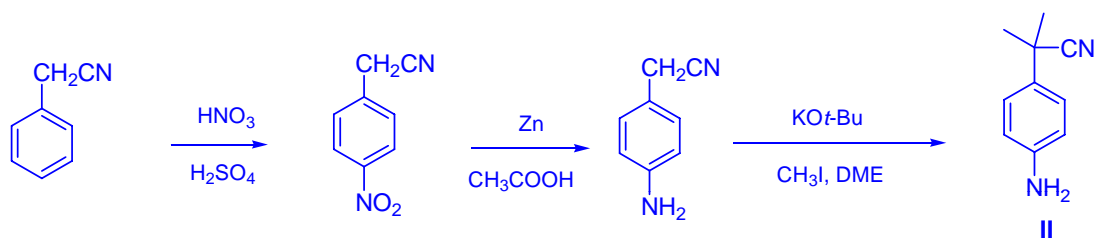
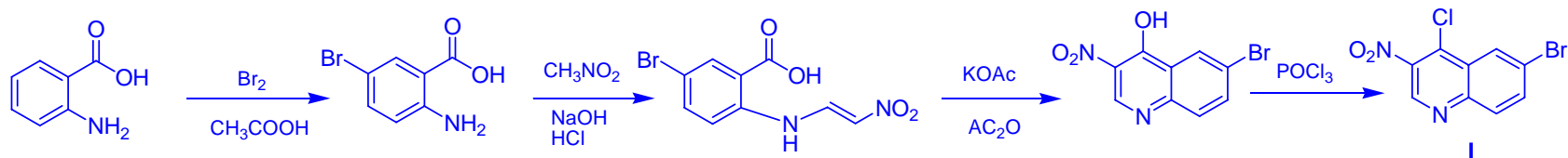
### BEZ-235 as PI3K/mTOR inhibitor

- BEZ235 is effective against advanced solid tumors, advanced breast cancer.
- Potent, orally active dual PI3K/mTOR inhibitor under phase II clinical trial.
- Inhibits PI3K and mTOR Kinase activity by binding to the ATP binding cleft of these enzymes



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# Synthesis of reference candidate BEZ-235

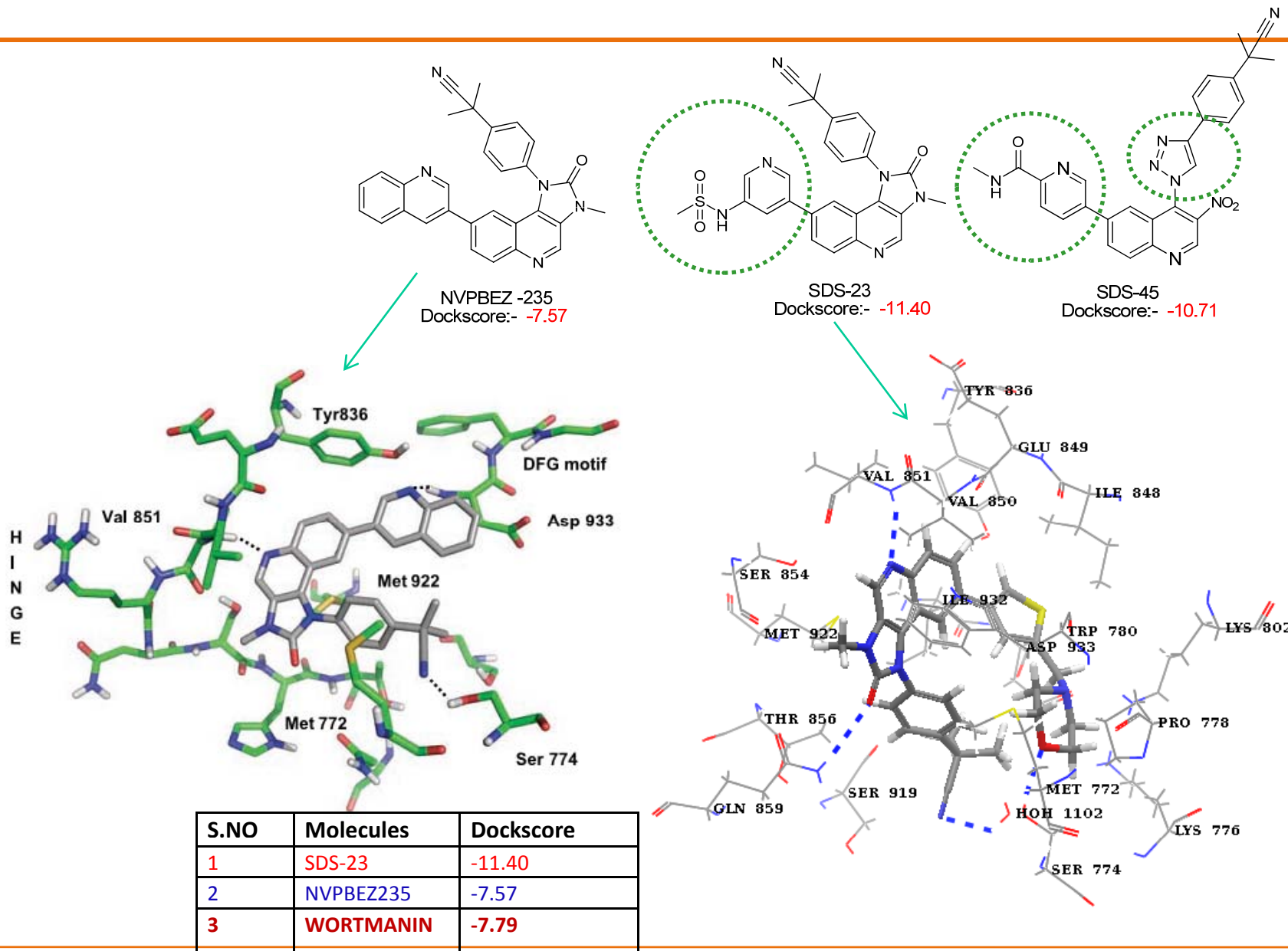


In house  
 $\text{IC}_{50}$  23 nM



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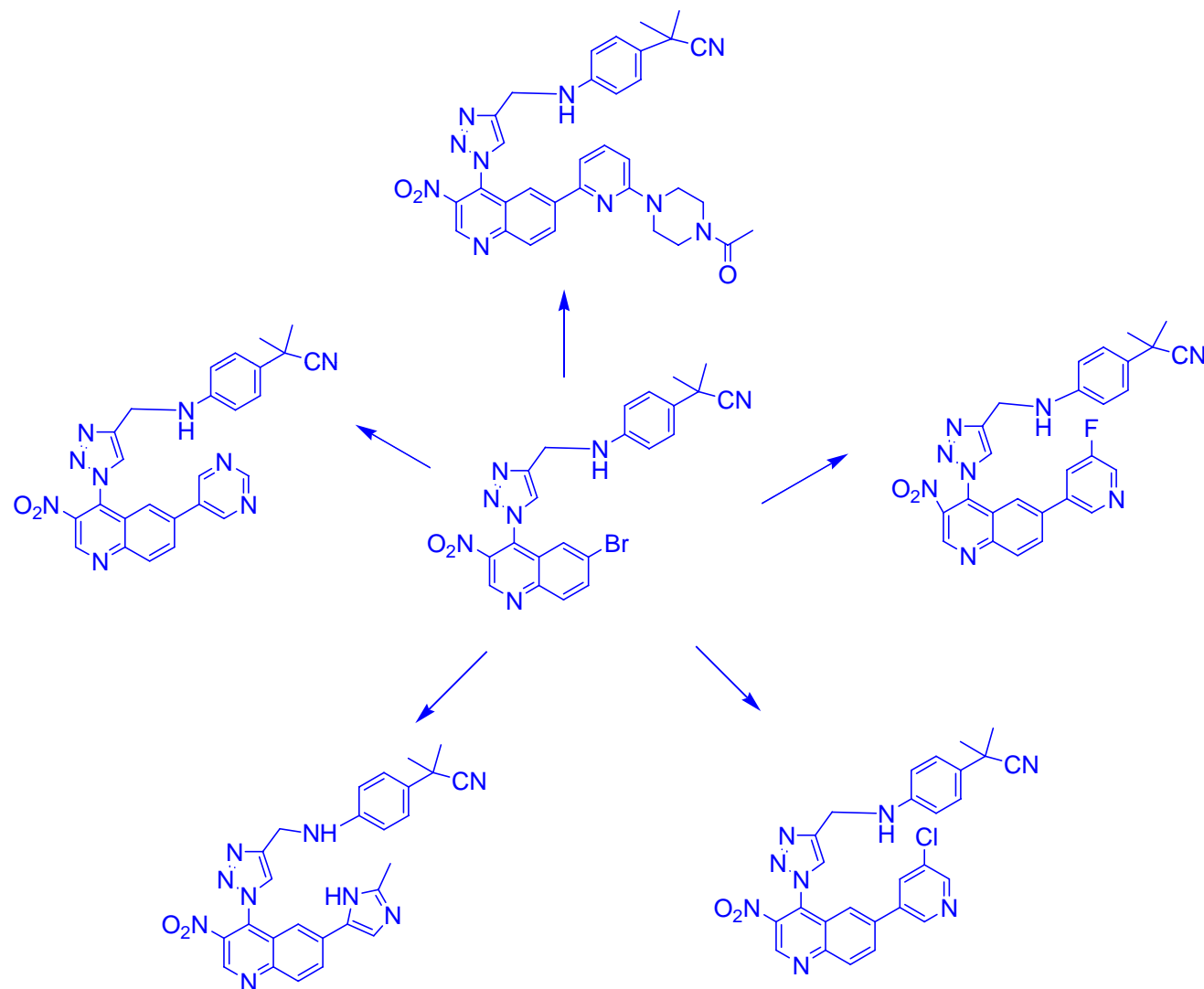
# Modeling of BEZ-235 with PI3K structure





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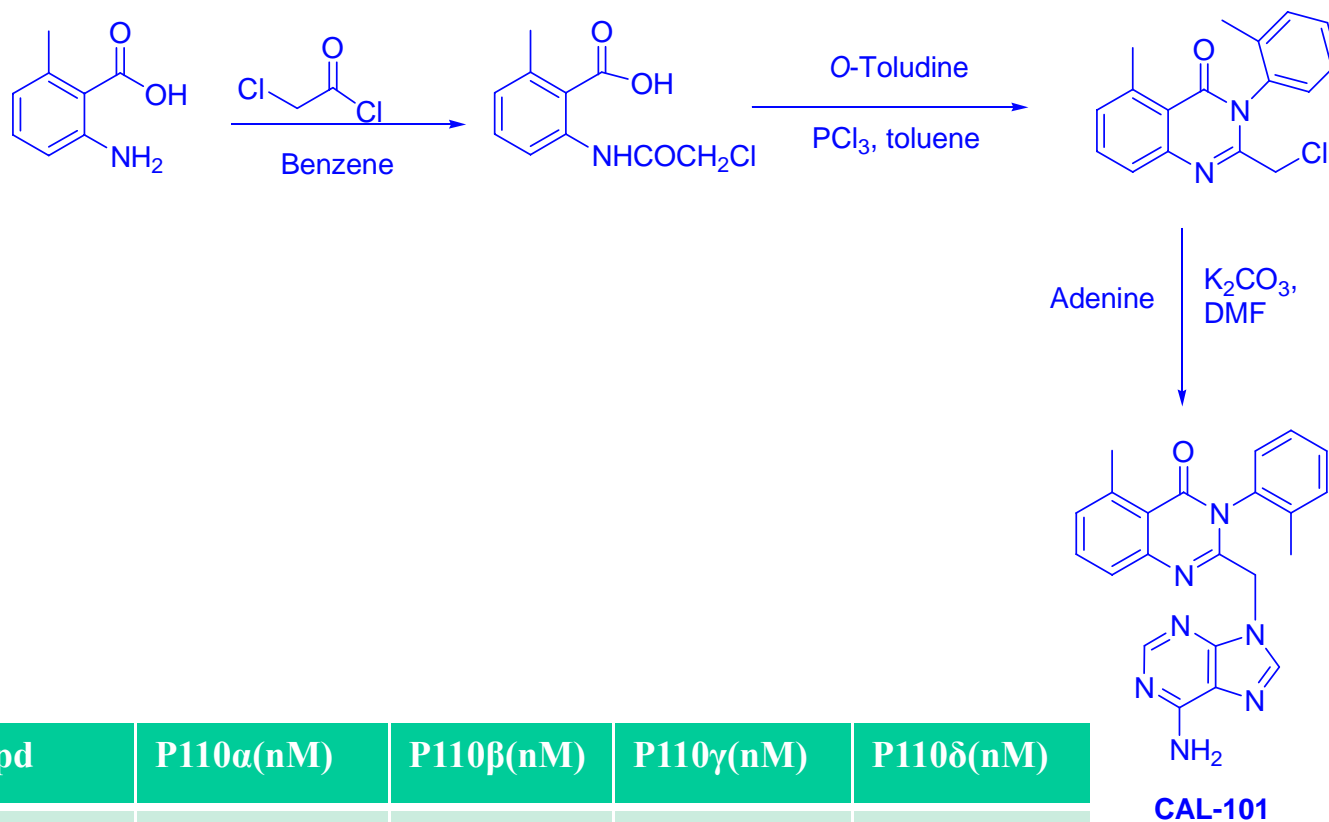
## Key NCEs from this scaffold





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## Medicinal chemistry of purinyl-quinazolinone scaffold



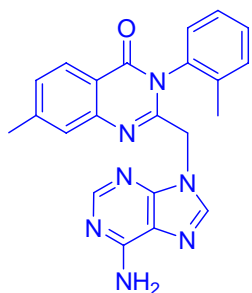
Compd	P110 $\alpha$ (nM)	P110 $\beta$ (nM)	P110 $\gamma$ (nM)	P110 $\delta$ (nM)
CAL101	200,000	16,000	61,000	2.5

•Patent space identified and NCEs have been prepared

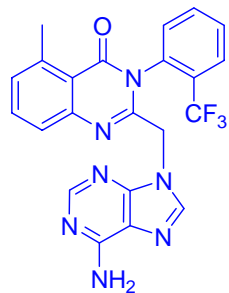


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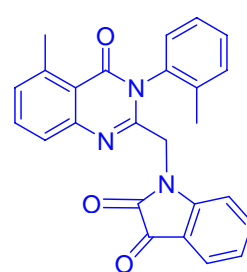
## NCEs synthesized



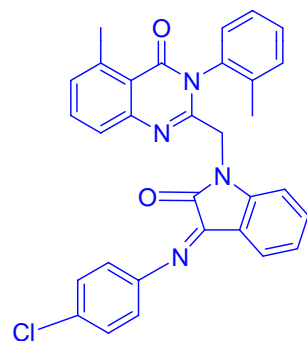
IIIM-Cal-101-1



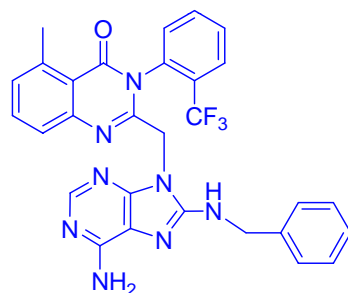
IIIM-Cal-101-2



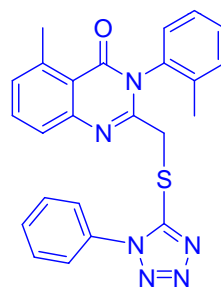
IIIM-Cal-101-3



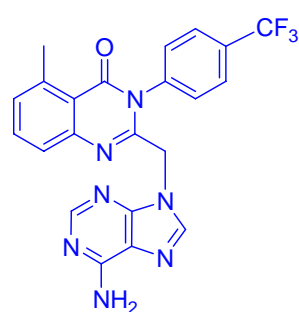
IIIM-Cal-101-4



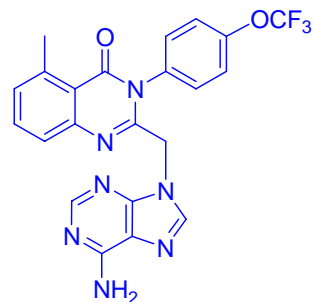
IIIM-Cal-101-5



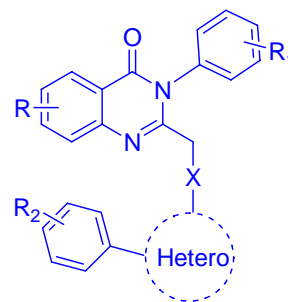
IIIM-Cal-101-6



IIIM-Cal-101-7



IIIM-Cal-101-8



IIIM-Cal-101-14

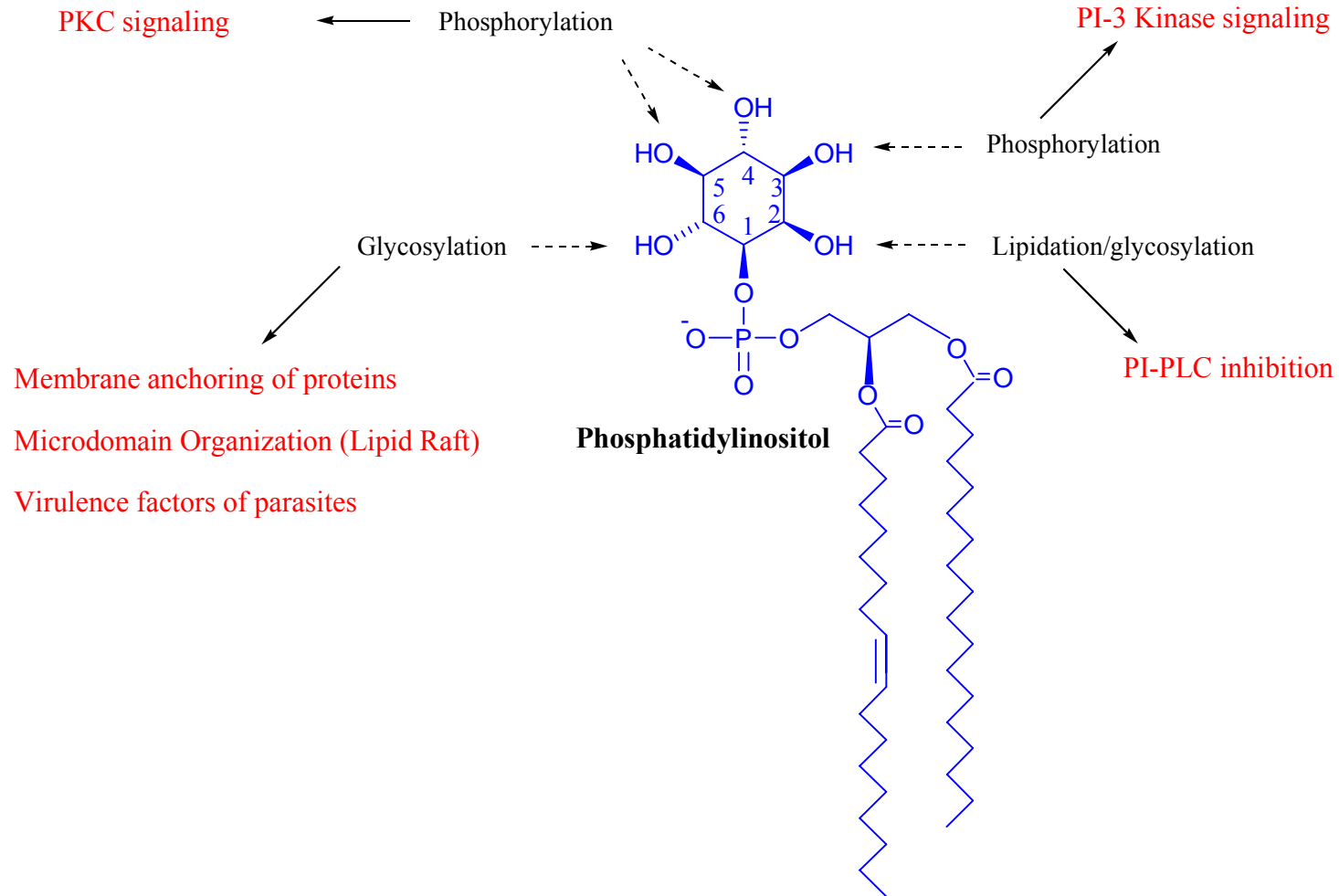
Two compounds shown potent  $IC_{50}$  value in *in vitro* and cell based assay

Events or Targets	Sample code	
	SIP-1107	SIP1114
<i>In vitro</i> $IC_{50}$	PI3K $\alpha$ - 57 nM $\beta$ - ND $\gamma$ - ND $\delta$ - 98 nM	PI3K $\alpha$ - 35 nM $\beta$ - ND $\gamma$ - ND $\delta$ - 200 nM
Cell based assay $IC_{50}$	Colo-205=200nM HL-60=250nM HCT-115=10 $\mu$ M Cac0-2=9.8 $\mu$ M THP-1=1.8 $\mu$ M	Colo-205=5 $\mu$ M A549=7.3 $\mu$ M HCT-115=7.2 $\mu$ M



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# Summary: Functional Diversity *within* PI class





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# Acknowledgements

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## Chemical biology team (NII)

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NIH (USA)

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