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Synthesis of new fluorescently labeled glycosylphosphatidylinositol (GPI) anchors

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ABSTRACT

The borondipyrromethene (BODIPY) labeled new glycosylphosphatidylinositol (GPI) molecules were synthesized as cellular probes to study the chemical basis of microdomain organization of GPI-anchored proteins and cholesterol in plasma membrane. The synthesis enabled by a new stereo-selective glycosylation of *myo*-D-inositol acceptor led to the preparation of optically pure glucosaminyl-(1-6)-α-phosphatidyl-*myo*-D-inositol and its unnatural stereoisomer.

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Glycosylphosphatidylinositols (GPIs) are a unique class of natural glycophospholipids which provide an alternative mechanism for anchoring of a number of specialized proteins to the outer leaflet of the eukaryotic plasma membrane.[1–3] The GPI-anchored proteins along with cholesterol and sphingolipids create functional microdomains (lipid-rafts) as signaling platforms at the cell surface.[4] The lipid-raft model has provided a new view of the plasma membrane generating significant interest in cell and membrane biology as to how the GPIs interact with cholesterol leading to the formation of ordered domains in biological membranes.[5,6] The molecular understanding of the mechanism of the localized clustering of GPI-anchored proteins[7] and cholesterol in lipid rafts requires synthetic probes based on GPI structures. In view of the great interest in the biochemistry and cell biology of GPI anchors, a number of GPI analogs have been synthesized.[8–11] In our research on chemistry and biology of GPI molecules, we have designed new approaches for the synthesis of the full-length GPI anchors and their structural and functional mimics and used them to address specific questions of GPI biosynthesis, inhibitor design, and membrane lipid-raft organization.[7,12–17] First synthesis of a head-group labeled GPI intermediate was reported by Schmidt et al.[18] followed by our synthesis of lipid-tail labeled GPIs.[13] In continuation to our collaborative work[7] showing that nanoclusters of GPI-anchored proteins are formed by cortical-actin driven activity and to elucidate the stereo-chemical role of GPIs in clustering of cholesterol-rich ordered domains, we required a BODIPY labeled fluorescent GPI probe **15a** and its unnatural stereoisomer **15b**. The rationale for designing the GlcN-PI structure was based on: (a) the glucosaminylation of phosphatidylinositol by UDP-GlcNAc is the first committed step of GPI bio-synthesis and (b) this step imparts asymmetric transbilayer distribution of GPI biosynthetic intermediates across the plasma-membrane.[13] The unique feature of GPI anchor, compared to other glyco-conjugates in the living cells, is the presence of a 'free amine' containing glucosamine residue instead of the *N*-acetylglucosamine. Interestingly, during the biosynthesis of GPIs, the *N*-acetylglycosamine is first transferred to a phosphatidylinositol (PI) precursor from UDP-GlcNAc to generate GlcNAc-PI intermediate. The *N*-acetyl group is then removed by a unique enzyme *N*-de-acetylase to produce $GlcNH_2$-PI (with free amine). This N-de-acetylation is a critical and necessary requirement for further mannosylation steps, toward full length GPI anchor.[3] From our previous work,[7,13] we have built a hypothesis that the positive charge on glucosamine and the stereochemical orientation of the D-*myo*-inositol residue of GPI anchor are involved in cholesterol recognition. Therefore, it would be of great interest to have both isomers of the probe, GlcN-(1-6)-α-D-*myo*-inositol and GlcN-(1-6)-α-L-*myo*-inositol.

Here, we report the synthesis of fluorescent labeled glucosaminyl-(1-6)-α-phosphatidyl-*myo*-D-inositol **15a** and glucosaminyl-(1-6)-α-phosphatidyl-*myo*-L-inositol **15b** with fluorescent label at 6-OH position of glucosamine residue. For preliminary studies,

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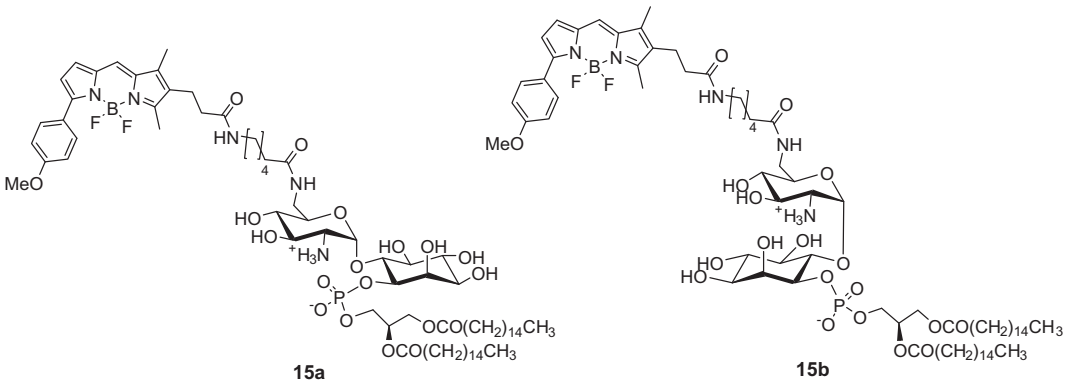
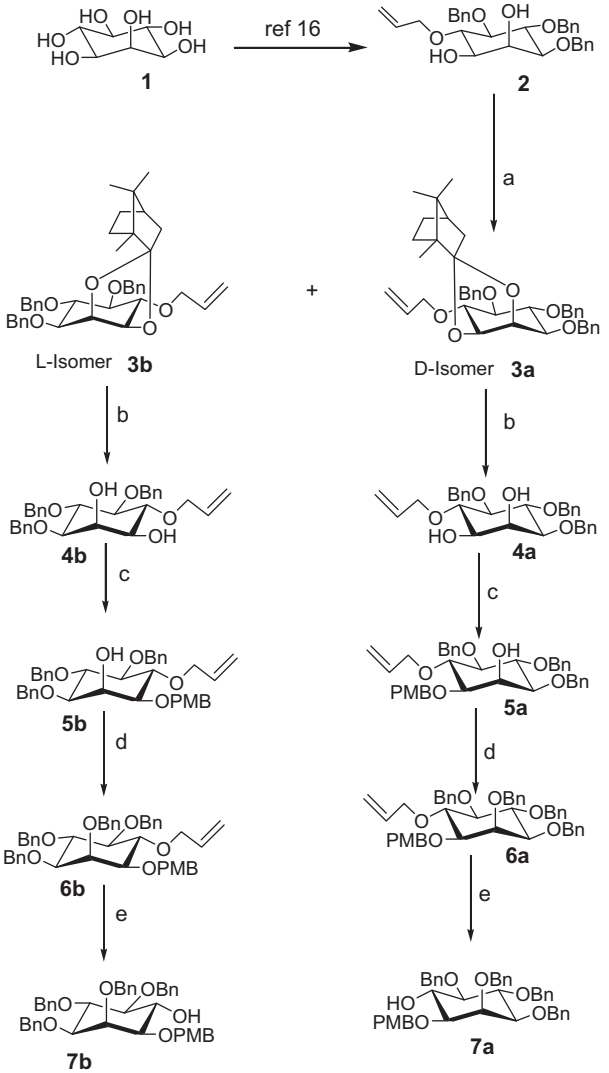


GlcN-PI with BODIPY at 6-OH position of glucosamine was designed as this position offers reasonable distance from the membrane surface and also gives optional site (4-OH of GlcN residue) for further biosynthetic elongation of GPI anchor. Furthermore, choice of 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*S*-indacene (BODIPY) as a fluorophore was based on two reasons: (a) the relatively small size of BODIPY probe should minimize the impact on binding affinity of the resulting GPI probe to the membrane and (b) BODIPY probe having good photo-physical properties (high photo-stability, quantum yield, extinction coefficient, and narrow emission bandwidth).[19]

In our earlier approach[15] for the synthesis of key glucosamine–inositol intermediate, the racemic 1-*O*-p-methoxybenzyl-2,3,4,5-tetra-*O*-benzyl-*myo*-inositol was glycosylated with a 2-azidoglycosyl donor to get a pseudo-disaccharide which was resolved by converting into 4,6-cyclic acetal protected disaccharide. Now, we have improved this method significantly by: (a) introducing a new simpler method for the resolution of *myo*-inositol intermediate **2**, (b) 6-*O*-acetyl group directed stereo-selective glycosylation[20] of *myo*-inositol intermediate **7a** with azidoglycosyl α/β trichloroacetimidate TCA **8** (without cumbersome isolation of minor β-TCA isomer), and (c) improved *H*-phosphonate coupling of the glycero-lipid donor.

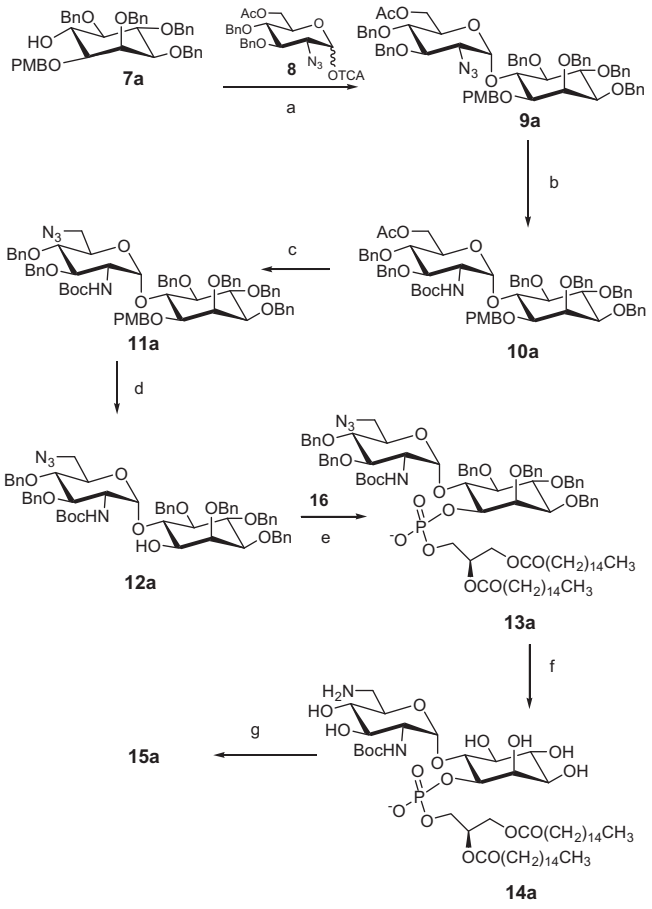
Accordingly, optically pure D and L inositol intermediates **7a** and **b** (Scheme 1) were obtained by reaction of **2**[21] with camphor dimethyl acetal.[22] Now the camphor protection of *myo*-inositol intermediate **3a** was removed and 1-*O*-position was selectively protected by a PMB group by dibutyltin chemistry followed by 2-O-benzylation to give **6a**. Removal of 6-*O*-allyl group from **6a** provided the acceptor **7a**. Similarly, the L-*myo*-inositol intermediate **7b** was prepared from **3b**. The absolute configuration of the *myo*-inositol intermediates **7a** and **b** was confirmed by removing PMB group and comparing the optical rotation with that of the reported compounds.[21]

The azidoglycosyl donor **8** was prepared by a known method.[20b] Initially we isolated β-isomer of trichloroacetamidate for glycosylation with inositol acceptor **7a**. However, later we found that the separation of α/β isomer of donor is not required and glycosylation using α/β mixture of **8** proceeds smoothly at low temperature to give α-pseudodisaccharide **9a** (Scheme 2). This is the first report of such stereochemically controlled synthesis of key glucosamine–inositol intermediate of the GPI anchor synthesis.[20] However, such a method for directed glycosylation has been reported for heparin synthesis.[20b] Therefore, the reaction of inositol acceptor **7a** with donor **8** in the presence of catalytic TMSOTf at −78 °C resulted in formation of the desired product **9a** in excellent yield (Scheme 2). Now the azido group of pseudo-disaccharide **9a** was transformed into the amino function by reduction with propanedithiol in pyridine/water using catalytic amounts of triethylamine followed by its protection with Boc group affording the desired



Scheme 1. Synthesis of enantio-pure D and L *myo*-inositol building block. Reagents and conditions: (a) camphor dimethyl ketal, dry CH_2Cl_2, PTSA, reflux, 2 h, 43% (**3a**) and 42% (**3b**); (b) PTSA, MeOH, 50 °C, 3 h, 86% (**4a**) and 85% (**4b**); (c) (i) $(Bu)_2SnO$, MeOH, reflux, 2 h; (ii) PMBCl, $(Bu)_4NBr$, dry toluene, 4 Å MS, reflux 6 h, 90% (**5a**) and 89% (**5b**); (d) BnBr, NaH, DMF, rt, 3 h, 93% (**6a** and **b**); (e) (i) *t*-BuOK, DMSO, 80 °C, 3 h; (ii) 1 M HCl:acetone (1:9), 80 °C, 30 min, 82% (**7a**) and 85% (**7b**).

N-Boc protected intermediate **10a**. The primary hydroxyl group of **10a** was converted to azido intermediate by 6-O-deacetylation, mesylation followed by azide substitution (**10a**–**11a**). The PMB



Scheme 2. Synthesis of Bodipy-GlcN-PI (**15a**). Reagents and conditions: (a) TMSOTf, CH_2Cl_2, −70 °C, 1 h, 75%; (b) 1,3-propanedithiol, pyridine, TEA, H_2O, 12–18 h and then $(Boc)_2O$, dry toluene, 4 h, 66%; (c) (i) NaOMe, MeOH, 1 h, 95%; (ii) mesyl chloride, pyridine, 80 °C, 3 h, 97%; (iii) NaN_3, DMF, 120 °C 4 h, 96%; (d) DDQ, CH_2Cl_2, H_2O, 2 h, 55%; (e) 2,3-di-*O*-palmitoyloxy-*sn*-glycero-*H*-phosphonate (16), pivaloyl chloride, dry pyridine, dry ACN, 1 h, and then I_2, pyridine, water, 2 h, 90%; (f) $Pd(OH)_2$, H_2, DCM:MeOH:H_2O, 18 h, 80%; (g) (i) 6-((4,4-difluoro-1,3-dimethyl-5-(4-methoxyphenyl)-4-bora-3a,4a-diaza-*S*-indacene-2-propionyl)amino)-hexanoic acid, succinimidyl ester, TEA, 6–8 h; (ii). 15% TFA, 1 h, 60%.

group was now removed (**11a**–**12a**) and the resulting free 1-OH was reacted with 2,3-di-*O*-palmitoyloxy-*sn*-glycero-*H*-phosphonate **16**.[12] The efficiency of this *H*-phosphonate coupling was significantly improved (from low yield to >90%) by the addition of acetonitrile during *H*-phosphonate coupling.

The final steps involved the global deprotection and 6-*O*-azide-reduction of **13a** by Pearman's hydrogenolysis method [$Pd(OH)_2$, CH_2Cl_2–MeOH–H_2O, H_2] followed by coupling of resulting **14a** with the required BODIPY ester, that is, 6-((4,4-difluoro-1,3-dimethyl-5-(4-methoxyphenyl)-4-bora-3a,4a-diaza-*S*-indacene-2-propionyl)amino)-hexanoic acid, succinimidyl ester (BODIPY TMR-X, SE) and deprotection of Boc group providing the target BODIPY-GlcN-PI **15a** viz. 6-*O*-(2-amino-2-deoxy-6-BODIPY-amino-6-deoxy-α-D-glucopyranosyl)-*myo*-D-inositol-1-[*sn*-2,3-bis(palmitoyloxy)propyl phosphate]. The final fluorescent product **15a** was purified by preparative TLC using EtOAc/$(Me)_2CO$/MeOH/H_2O (7:1:1:1) as eluent followed by reversed phase HPLC. The structure of final probe was supported by the presence of peaks at *m*/*z* 1464 (M) and 1444 (M−F) in negative mode of MALDI mass spectrum.

An identical synthetic approach was utilized to prepare the unnatural BODIPY-GlcN-PI **15b** viz. 6-*O*-(2-amino-2-deoxy-6-BODIPY-amino-6-deoxy-α-D-glucopyranosyl)-*myo*-L-inositol-1-[*sn*-2,3-bis(palmitoyloxy)propyl phosphate] from its respective intermediate **7b** (Scheme 3, Supplementary data).

In conclusion, BODIPY labeled GPI probes **15a** and **15b** were synthesized in an efficient manner. Our initial cell biology experiments have demonstrated the utility of the GPI probe **15a** in its insertion in plasma-membrane, the full biological study will be published in due course.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2011.06.005.

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