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Chapter 7

Design and Synthesis of Biotinylated Inositol Phosphates: Application to the Inositol Phosphate-Protein Binding Analysis

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Abstract

Inositol phosphates play important roles as second messengers in intracellular signal transduction. In order to study the relative affinity and specificity in binding inositol phosphates and diverse inositol phosphate-binding proteins by Surface Plasmon Resonance (SPR) analysis and pull-down analysis, biotinylated inositol phosphates, to be immobilized on avidin-based sensor chip or resin, were designed. The synthesis of the biotinylated inositol phosphates was accomplished by assembling the inositol phosphate building block, synthesized starting with optically resolved *myo*-inositol derivatives, and the biotin building block through a phosphate linkage. The inositol phosphate moiety of each biotinylated inositol phosphate showed specific binding to Pleckstrin Homology (PH) domain of PLCδ (phospholipase Cδ), PH domain of Grp1 (general receptor of phosphoinositides 1), and precursor of Gag protein ($Pr55^{Gag}$) of human immunodeficiency virus type 1 (HIV-1), as revealed by SPR analysis and pull-down analysis.

Introduction

D-*myo*-Inositol phosphates (InsP) and phosphatidylinositol phosphates (PtdInsP) are involved in diverse biological phenomena mediated by InsP binding proteins including, e.g., the pleckstrin homology (PH) domain-containing proteins often involved in various intracellular signaling and the $Pr55^{Gag}$ protein that initiates HIV replication. The elucidation of these InsP-binding proteins would not only be a clue to the unknown mechanism of the biological signaling but also lead to drug discovery. These InsP-binding proteins could be best studied by immobilizing InsP on appropriate carriers such as beads or sensor chips. This review deals with synthesis and application of biotinylated inositol phosphates that could be immobilized on various carriers via the biotin-streptavidin interaction.

PH Domain and Calcium Signaling

PH domain, found in over 100 proteins, is a structural module of about 120 amino acids homologous to the two regions in pleckstrin, the major protein kinase C substrate in platelets (Tyers et al. 1988, Haslam et al. 1993, Mayer 1993). PH domain plays significant roles in membrane recruiting of

proteins involved in intracellular signaling and the cytoskeleton organization (Kavran et al. 1998, Shaw 1996, Musacchio et al. 1993, Gibson et al. 1994), by binding specific phosphoinositides and their head groups (Harlan et al. 1994, Hyvönen et al. 1995, Lemmon et al. 1995).

Phospholipase C δ (PLCδ) PH domain is a representative D-Ins(1,4,5)P_3-binding motif which recognizes and binds to Ptd-D-Ins(4,5)P_2 with high affinity (Lemmon et al. 1995). D-Ins(1,4,5)P_3 plays a key role in the signaling cascade that links extracellular messengers to intracellular Ca^{2+} mobilization (Berrige 1993). Upon stimulation of a certain receptor, the associated G protein or tyrosine kinase activates a membrane-bound PLC, which hydrolyzes Ptd-D-Ins(4,5)P_2 into D-Ins(1,4,5)P_3 and diacylglycerol (DAG), bifurcating the signaling pathway. The hydrophilic Ins(1,4,5)P_3 diffused in the cytosol activates the receptor of a Ca^{2+} channel on the endoplasmic reticulum, resulting in the release of Ca^{2+} from an internal store.

On the other hand, D-Ins(1,3,4,5)P_4 is formed by the direct phosphorylation of the D-Ins(1,4,5)P_3 by the D-Ins(1,4,5)P_3 3-kinase (Batty et al. 1985, Irvine and Schell 2001). The synergistic involvement of D-Ins(1,3,4,5)P_4 in the D-Ins(1,4,5)P_3-mediated mobilization of Ca^{2+} and the subsequent regulation of the store-operated Ca^{2+}-influx (Kavran et al. 1998, Shaw 1996, Musacchio et al. 1993) still remains as a subject of controversy.

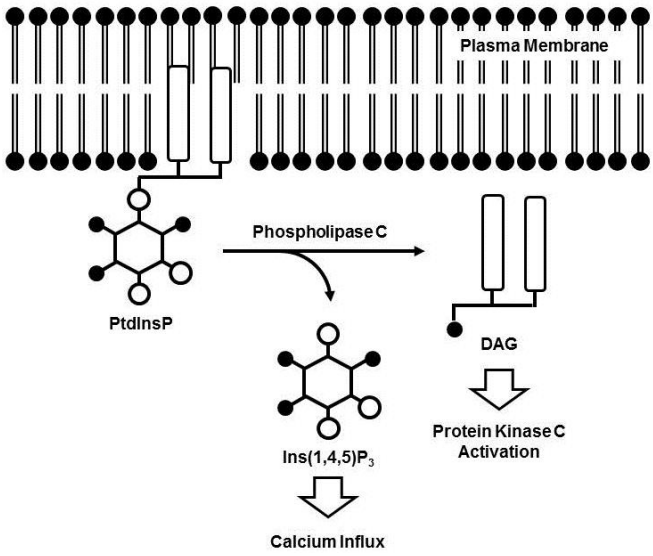


Figure 1. Calcium signaling mediated by D-Ins(1,4,5)P_3 produced by enzyme hydrolysis of membrane Ptd-D-Ins(4,5)P_2.

Gag and HIV-1 Replication

The HIV-1 genome-encoded $Pr55^{Gag}$ protein, the principal structural component required for virus assembly, is known to bind Ptd-D-Ins(4,5)P_2 (Wills and Craven 1991, Freed 1998). Following the ribosomal synthesis, $Pr55^{Gag}$ is directed to the plasma membrane of the host cell, where it is assembled with other components to form premature budding virions. $Pr55^{Gag}$ is composed of four major domains: matrix (MA), capsid (CA), nucleocapsid (NC), and p6, in addition to two spacer peptides: p2 and p1. The N-terminal MA domain plays a critical role in the binding of $Pr55^{Gag}$ to the plasma membrane (Conte and Matthews 1998).

During the membrane targeting, several thousand copies of $Pr55^{Gag}$ colocalize at lipid rafts on the plasma membrane (Nguyen and Hildreth 2000), mediated by the $Pr55^{Gag}$ MA domain N-terminal myristoyl moiety and the basic patch. The myristoyl group attached to the MA N-terminal glycine is flipped and exposed through the multimerization process of $Pr55^{Gag}$ to bind to the plasma membrane; a phenomenon known as the myristoyl switch (Resh 2004, Tang et al. 2004).

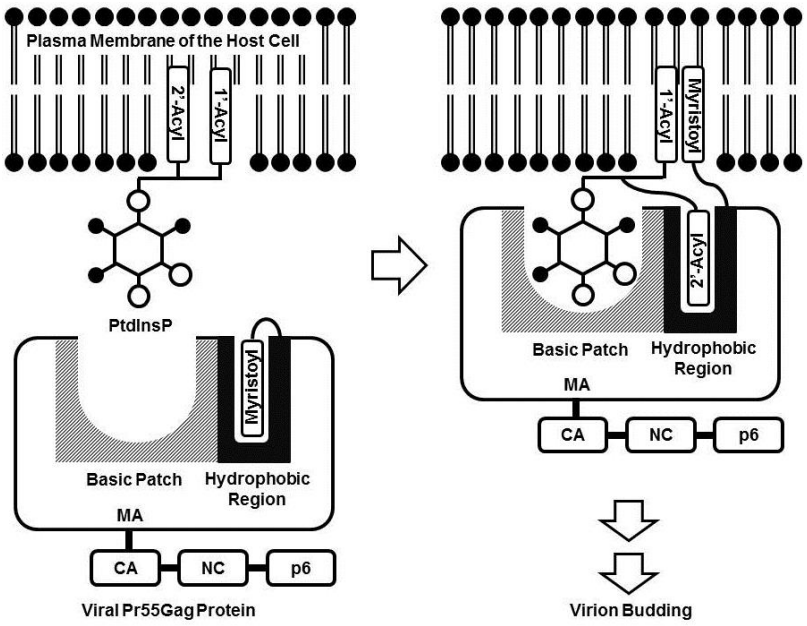


Figure 2. Interaction between the membrane PtdInsP and the MA domain of $Pr55^{Gag}$, leading to the budding of virion.

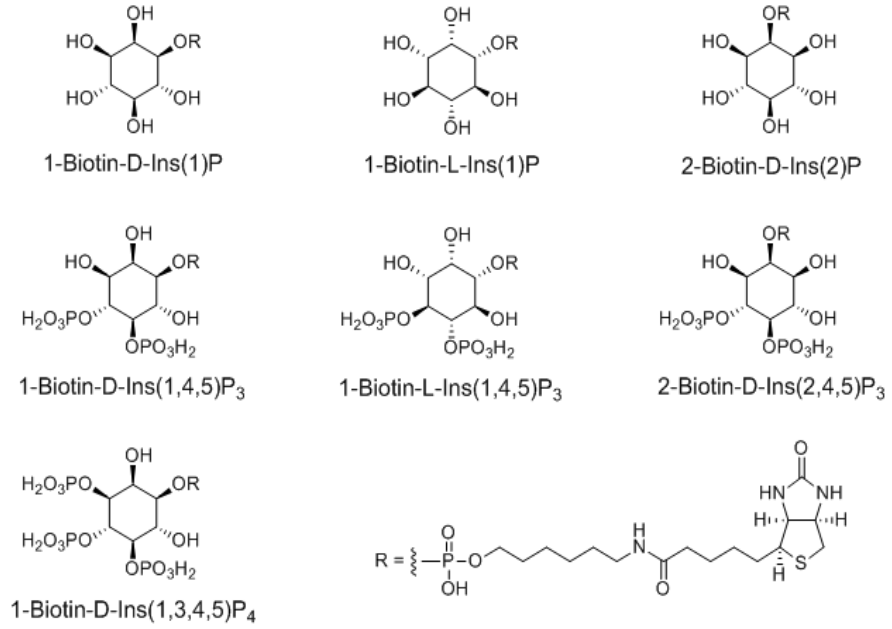


Figure 3. Structures of biotinylated inositol phosphates.

The MA hydrophobic region, in turn, interacts with the 2'-acyl chain of the membrane PtdInsP and the basic patch, on the other hand, accommodates the InsP moiety (Zhou et al. 1994). This mechanism of the MA domain-PtdInsP was supported by the following pioneering studies. Ono et al. identified Ptd-D-Ins(4,5)P_2 as a cellular factor regulating the targeting of $Pr55^{Gag}$ to the plasma membrane (Ono et al. 2004). These findings were supported by NMR analyses by Saad et al. that demonstrated direct interactions between $Pr55^{Gag}$ and Ptd-D-Ins(4,5)P_2, observing a conformational change in $Pr55^{Gag}$ induced by Ptd-D-Ins(4,5)P_2-MA binding that triggers myristate exposure (Saad et al. 2006).

Design of Biotinylated Inositol Phosphates

In order to study the relative affinity and specificity in the binding of InsPs and PH domain or $Pr55^{Gag}$ MA domain, we designed biotinylated

inositol phosphates that could be immobilized on avidin-modified beads or sensor chips (Wilchek and Bayer 1990) for the binding analysis.

These include biotin derivatives of inositol mono- and tris- and tetrakisphosphates, *i.e.*, 1-Biotin-D-Ins(1)P, 1-Biotin-L-Ins(1)P, 2-Biotin-D-Ins(2)P, 1-Biotin-D-Ins(1,4,5)P_3, 1-Biotin-L-Ins(1,4,5)P_3, 2-Biotin-D-Ins(2,4,5)P_3, and 1-Biotin-D-Ins(1,3,4,5)P_4 (Anraku et al., 2008, Anraku et al. 2010, Anraku et al. 2011).

Since the X-ray crystal structure of the Ins(1,4,5)P_3-PLCδ PH domain complex (Ferguson et al. 1995) revealed that the inositol and 4,5-phosphate groups of Ins(1,4,5)P_3 are accommodated in the binding pocket, we thought that a biotin-linker could be introduced at the 1-phosphate or 2-phosphate (the latter is an artificially created derivative) of inositol without affecting the PH domain binding.

The X-ray crystal structure of the Ins(1,3,4,5)P_4-Grp1 PH domain complex (Lietzke et al. 2000) revealed that the phosphate groups at the 3-, 4-, and 5-positions of Ins(1,3,4,5)P_4 are accommodated in the binding pocket of the Grp1 PH domain, while 1-phosphate lies out of the binding pocket. Thus, introduction of a biotin-linker at the 1-phosphate of Ins(1,3,4,5)P_4 would not affect the complex formation with Grp1PH domain.

Synthesis of Biotinylated Inositol Phosphates

D-Ins(1)P and L-Ins(1)P Fragments

For the synthesis of 2,3,4,5,6-pentaacetyl-D-inositol **4a**, we selected the optically resolved *myo*-inositol derivative (+)-**1** (Billington et al. 1987) as the starting material. Since (+)-**1** has a benzyl group only at the 1-hydroxyl group, the synthesis was straightforward.

Hydrolysis of the cyclohexylidene groups of (+)-**1** with acid provided pentaol **2a**, which was acetylated to give fully protected **3a**. Treatment of **3a** with H_2/Pd-C gave the debenzylated D-Ins(1)P fragment **4a**. The enantiomeric **4b** was prepared starting with (−)-**1** by the same procedure.

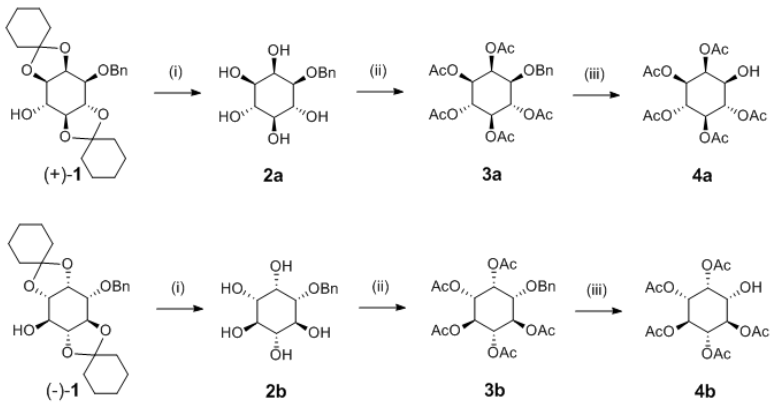


Figure 4. Synthesis of D- and L-Ins(1)P fragments. (i) TFA, MeOH, rt; (ii) Ac_2O, DMAP, Py, rt; iii) H_2/10% Pd-C, CH_3COOH, rt.

D-Ins(1,4,5)P_3 and L-Ins(1,4,5)P_3 Fragments

The synthesis of $InsP_3$ and $InsP_4$ was more complicated, necessitating the differentiation of 1- and 2-hydroxyl groups of the inositol. The Nagashima procedure (Nagashima and Ohno 1987) was successfully applied to the case. The stannylene acetal of the 1,2-diol formed was regioselectively alkylated with R^3-X in the presence of cesium fluoride to give 1-*O*-alkylated product.

Thus, the starting material **5a** was prepared by removing the benzyl group of (−)-**1**. Allylation of the diol **5a** provided **6a**, which was further treated with *p*-toluenesulfonic acid and H_2O to give deprotected **7a** in 92% yield (for 2 steps). The *cis*-1,2-diol of **7a** was regioselectively protected by a MPM group by means of the above mentioned Nagashima's dibutyltin oxide procedure (Nagashima and Ohno 1987, Liu and Potter 1997) to give **8a** exclusively in 97% yield.

The introduction of isopropylidene acetal to the 4,5-vicinal alcohol gave **9a** in 85% yield. The subsequent removal of the allyl groups (Corey and Suggs 1973) of **9a** gave **10a** in 67% yield. Acetylation of the 3,6-diol **10a** provided **11a**, which was treated with *p*-toluenesulfonic acid and ethylene glycol to give the 4,5-diol intermediate **12a** in 88% yield (for 2 steps). The subsequent phosphorylation of the 4,5-diol **12a** (Uhlmann and Engels 1986) afforded 4,5-bisphosphate **13a** in 93% yield. Oxidative cleavage of the *p*-methoxybenzyl group with CAN (Johansson and Samuelsson 1984) gave the D-Ins(1,4,5)P_3

fragment **14a** in 71% yield. The enantiomeric **14b** was prepared from (+)-**1** by the same procedure.

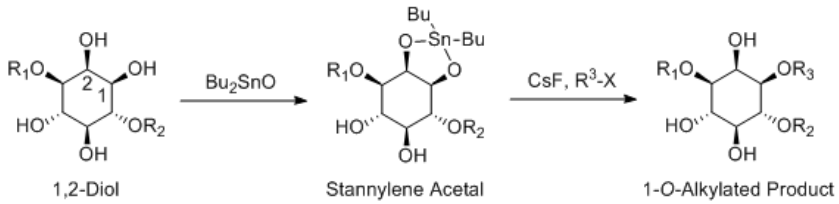


Figure 5. Regioselective alkylation of inositol 1,2-diol via the stannylene acetal formation.

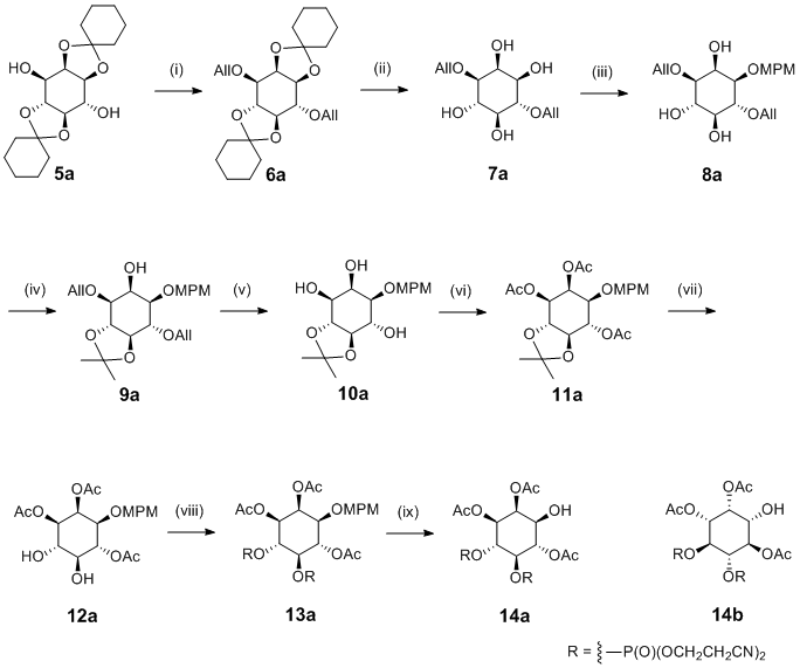


Figure 6. Synthesis of D- and L-Ins(1,4,5)P_3 fragments. (i) Allyl-Br, NaH, DMF, rt; (ii) TsOH, THF–MeOH, reflux; (iii) (a) Bu_2SnO, toluene, reflux, 3 h; (b) CsF, MPM-Cl, DMF, −40°C then rt; (iv) 2-methoxypropene, TsOH, DMF, rt; (v) (a) $(Ph_3P)_3RhCl$, DABCO, EtOH–benzene–H_2O, reflux, 5 h; (b) HgO, $HgCl_2$, acetone–H_2O, rt, 5 min; (vi) Ac_2O, DMAP, Py, rt; (vii) TsOH, ethylene glycol, CH_2Cl_2, rt, 10 min; (viii) (a) bis(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite, 1*H*-tetrazole, CH_2Cl_2, rt, 1.5 h; (b) MCPBA, CH_2Cl_2, rt, 5 min; (ix) CAN, CH_3CN–H_2O, rt, 1 h.

D-Ins(2,4,5)P_3 and D-Ins(2)P Fragments

The regioselective allylation (Nagashima and Ohno 1987, Liu and Potter 1997) at the 1-hydroxyl group of **7a** by the Nagashima procedure gave triallyl derivative **15a** in 59% yield. Introduction of isopropylidene acetal to the 4,5-diol **15a** provided **16a**, which was further protected with a MPM group to give the 2-MPM derivative **17a** in 66% yield (for 2 steps). Deprotection of the three allyl groups (Corey and Suggs 1973) of **17a** followed by acetylation gave **19a**. The isopropylidene acetal was removed to afford 4,5-diol **20a** in 42% yield (for 3 steps). Phosphorylation (Uhlmann and Engels 1986) of **20a** gave 4,5-bisphosphate **21a**, which was treated with CAN (Johansson and Samuelsson 1984) to give the D-Ins(2,4,5)P_3 fragment **22a** in 59% yield (for 2 steps).

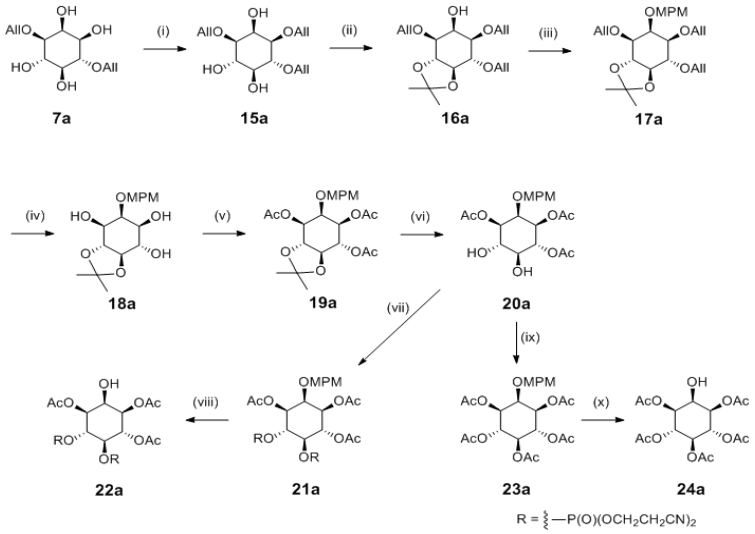


Figure 7. Synthesis of D-Ins(2,4,5)P_3 and D-Ins(2)P fragments. (i) (a) Bu_2SnO, toluene, reflux, 3 h; (b) CsF, Allyl-Br, DMF, −40 °C then rt; (ii) 2-methoxypropene, TsOH, DMF, rt; (iii) MPM-Cl, NaH, DMF, rt; (iv) (a) $(Ph_3P)_3RhCl$, DABCO, EtOH–benzene–H_2O, reflux, 5 h; (b) HgO, $HgCl_2$, acetone–H_2O, rt, 5 min; (v) Ac_2O, DMAP, Py, rt; (vi) TsOH, ethylene glycol, CH_2Cl_2, rt, 10 min; (vii) (a) bis(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite, 1*H*-tetrazole, CH_2Cl_2, rt, 1.5 h; (b) MCPBA, CH_2Cl_2, rt, 5 min; (viii) CAN, CH_3CN–H_2O, rt, 1 h; (ix) Ac_2O, DMAP, Py, rt; (x) H_2/10% Pd-C, CH_3COOH, rt.

On the other hand, exhaustive acetylation of **20a** and successive removal of the MPM group of **23a** gave D-Ins(2)P fragment **24a** in 64% yield (for 2 steps).

D-Ins(1,3,4,5)P_4 Fragment

Aiming at the synthesis of 2,5-*O*-diacetyl-1-ol **34**, we started with alcohol (−)-**1** (Billington et al. 1987**)** Compound **25**, obtained by allylation of the alcohol (−)-**1**, which was treated with *p*-toluenesulfonic acid and H_2O to give deacetalyzed **26** in 67% yield (for two steps). The cis-1,2-diol **26** was regioselectively protected by the Nagashima procedure (Nagashima and Ohno 1987, Liu and Potter 1997) to give regioselectively protected **27** in 88% yield. The introduction of isopropylidene acetal to the 4,5-diol gave **28** in 87% yield. Deprotection of the allyl group of **28** gave **29** in 68% yield (Corey and Suggs 1973).

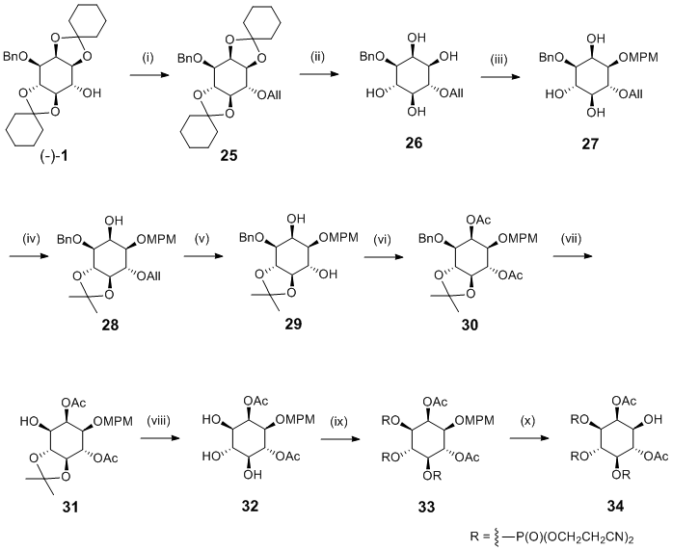


Figure 8. Synthesis of D-Ins(1,3,4,5)P_4 fragment. (i) Allyl-Br, NaH, DMF, rt, 24 h; (ii) TsOH, THF–H_2O, reflux, 3 h; (iii) (a) Bu_2SnO, toluene, reflux, 3 h; (b) CsF, MPM-Cl, DMF, −78 °C then rt, 24 h; (iv) 2-methoxypropene, TsOH, DMF, rt, 24 h; (v) (a) $(Ph_3P)_3RhCl$, DABCO, EtOH–benzene–H_2O, reflux, 5 h; (b) HgO, $HgCl_2$, acetone–H_2O, rt, 5 min; (vi) Ac_2O, DMAP, pyridine, rt, 12 h; (vii) H_2/W-2 Raney-Ni, MeOH, 50 °C, 3 h; (viii) TsOH, ethylene glycol, CH_2Cl_2, rt, 10 min; (ix) (a) bis(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite, 1*H*-tetrazole, CH_2Cl_2, rt, 1.5 h; (b) MCPBA, CH_2Cl_2, rt, 5 min; (x) CAN, CH_3CN–H_2O, rt, 1 h.

Acetylation of **29** provided 2,6-diacetate **30** in 91% yield. The selective deprotection of benzyl group of **30** (Oikawa et al. 1984) gave **31** (64% yield), which was treated with *p*-toluenesulfonic acid to give the 3,4,5-triol

intermediate **32** in 89% yield. The 3,4,5-triol **32** was converted to the corresponding 3,4,5-trisphosphate **33** by the usual procedure in 51% yield. Oxidative cleavage of MPM group with CAN (Johansson and Samuelsson 1984) gave the D-Ins(1,3,4,5)P_4 fragment **34** in 86% yield.

Synthesis of Biotinylated Inositol Phosphates

The biotin-linker fragment (Pon 1991) was reacted with bifunctional phosphorylating agent (2-cyanoethyl)-*N*,*N*,*N*,*N*-tetraisopropylphosphoramidite (Bannwarth and Trezeciak 1987) and 1*H*-tetrazole to give a rather labile phosphoramidite which was immediately condensed with inositol moiety **14a**, **4a**, **14b**, **4b**, **22a**, **24a** or **34**. Subsequent oxidation of the condensation products with *tert*-BuOOH gave the fully protected biotinylated inositol phosphates **35a** (93% yield), **36a** (78% yield), **35b** (83% yield), **36b** (85% yield), **37** (99% yield), **38** (52% yield), and **39** (<80% yield). The ^{1}H NMR and FABMS data of these compounds showed no oxidation of the biotin sulfide.

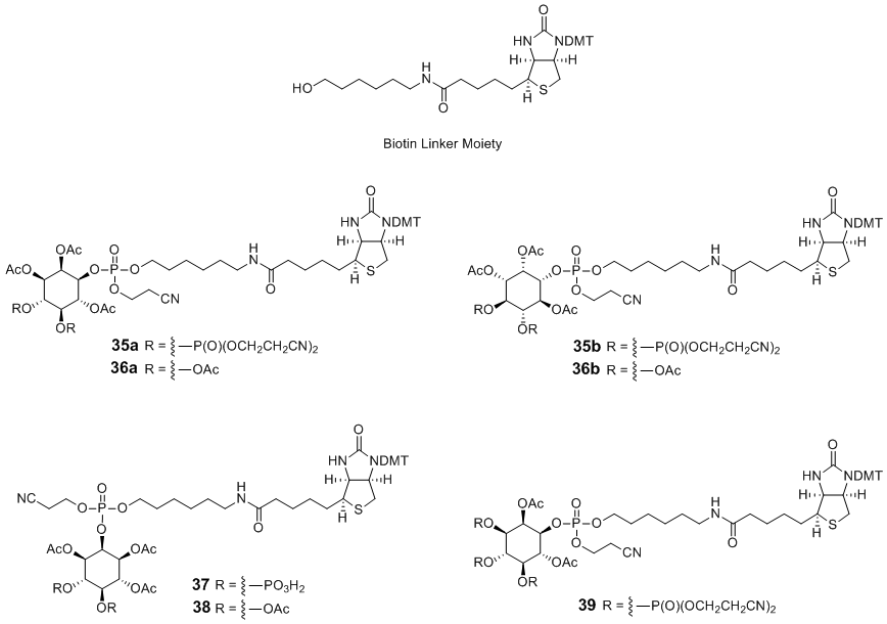


Figure 9. The penultimate precursors for the biotinylated inositol phosphates.

The final stage of the synthesis was carried out as follows. After removal of the trityl group of biotin with acid treatment, all protecting groups were removed in one step by reaction with NH_3 to give water-soluble biotinylated inositol phosphates. The biotinylated products were efficiently purified by anion-exchange chromatography with gradients of ammonium formate as eluent to give 1-Biotin-D-Ins(1,4,5)P_3 (32% yield), 1-Biotin-D-Ins(1)P (30% yield), 1-Biotin-L-Ins(1,4,5)P_3 (35% yield), 1-Biotin-L-Ins(1)P (46% yield), 2-Biotin-D-Ins(2,4,5)P_3 (32% yield), 2-Biotin-D-Ins(2)P (66% yield), and 1-Biotin-D-Ins(1,3,4,5)P_4 (26% yield) (for 2 steps).

Biological Study

PLCδ PH Domain: Surface Plasmon Resonance Analysis

Biological study of the synthetic biotinylated inositol phosphates using streptavidin beads was carried out. The original PLCδ PH domain bound efficiently to the prebound beads of 1-Biotin-D-Ins(1,4,5)P_3. The dissociation constant *K*d of 1-Biotin-D-Ins(1,4,5)P_3 binding of the PLCδ PH domain was 250±20 nM, which was comparable to that of non-tethered D-Ins(1,4,5)P_3 (Lemmon et al. 1995). Further, the Surface Plasmon Resonance (SPR) study of the synthetic biotinylated inositol phosphates against the PLCδ PH domains was carried out using a streptavidin-modified sensor chip. The results are summarized in the Table 1. 1-Biotin-D-Ins(1,4,5)P_3 and the PLCδ PH domain was bound the most tightly (*K*d = 126±23 nM) compared with 1-Biotin-L-Ins(1,4,5)P_3 (*K*d = 262±72 nM) and 2-Biotin-D-Ins(1,4,5)P_3 (*K*d = 303±93). Biotinylated inositol monophosphates did not show discernible binding. These results are consistent with those previously reported by Lemmon et al. (Lemmon et al. 1995).

Grp1 PH Domain: Pull-down Analysis

The biological study of the 1-Biotin-D-Ins(1,3,4,5)P_4 was carried out using streptavidin coated beads. Grp1 PH domain was incubated with various amounts of 1-Biotin-D-Ins(1,3,4,5)P_4 immobilized beads.

Table 1. Dissociation Constant (*K*d) of biotinylated inositol phosphates binding of the PLCδ PH domain (*Lemmon et al. 1995)

	*K*d (nM)	*K*d (nM)*
1-Biotin-D-Ins(1,4,5)P_3	126±23	210±120
1-Biotin-D-Ins(1)P	Not detected	−
1-Biotin-L-Ins(1,4,5)P_3	262±72	8,800
1-Biotin-L-Ins(1)P	Not detected	−
2-Biotin-D-Ins(2,4,5)P_3	303±93	9,600
2-Biotin-D-Ins(2)P	Not detected	>64,000

The binding ratio was calculated by dividing the bound fraction by the sum of bound fraction and unbound supernatant fraction obtained from the SDS–polyacrylamide electrophoretic analyses. An equilibrium dissociation constant *K*d for the complex of 1-Biotin-D-Ins(1,3,4,5)P_4 and Grp1 PH domain was obtained (0.14 ± 0.04 μM) by plotting the binding ratio against 1-Biotin-D-Ins(1,3,4,5)P_4 concentration. According to the previous report (Sakaguchi et al. 2010), the *K*d value for the complex of Grp1 PH domain and Ins(1,3,4,5)P_4 is approximately 0.10 μM, which is in good agreement with the *K*d value in the present pull-down analysis (0.14 μM) despite the presence of the biotin tethered at the 1-phosphate position of inositol. Thus, the result demonstrates that 1-Biotin-D-Ins(1,3,4,5)P_4 successfully reproduces the protein binding characteristics of unmodified D-Ins(1,3,4,5)P_4, and furthermore, the biotin-linker moiety does not perturb the Ins(1,3,4,5)P_4-binding of Grp1 PH domain.

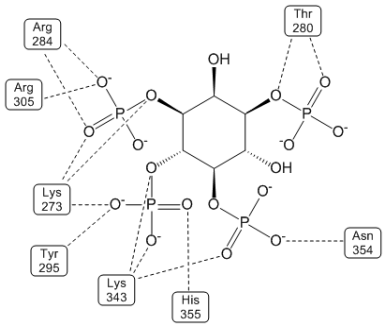
Grp1 PH Domain: Surface Plasmon Resonance Analysis

1-Biotin-D-Ins(1,3,4,5)P_4 was next applied to SPR-based PH domain binding analysis using a streptavidin coated sensor chip. After 1-Biotin-D-Ins(1,3,4,5)P_4 was partially immobilized on the sensor chip, the binding affinity was determined by injecting various concentrations of Grp1 PH domain over the sensor chip. The *K*d was deduced to be 0.3 μM. The reasons for the difference in *K*d values, between SPR-based analysis and Pull-down analysis, could be due to differences in the principle of measurement. To confirm whether the 1-Biotin-D-Ins(1,3,4,5)P_4 retained the binding activity, 1-Biotin-Ins(1,4,5)P_3 and 1-Biotin-L-Ins(1,4,5)P_3 were also immobilized on the sensor chip. Grp1 PH domain barely bound 1-Biotin-D-Ins(1,4,5)P_3 (*K*d >>1.0

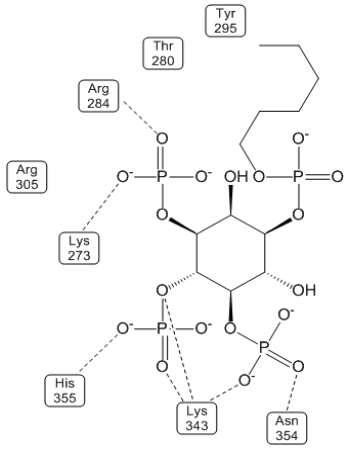
μM) and 1-Biotin-L-Ins(1,4,5)P_3 (*K*d >>1.0 μM). Thus, 1-Biotin-D-Ins(1,4,5)P_3 and 1-Biotin-D-Ins(1,3,4,5)P_4 retain the inherent biochemical characteristics for PLCδ domain and Grp1 PH domain, respectively.

Grp1 PH Domain: Docking Study

The mode of interaction of the Grp1 PH domain-D-Ins(1,3,4,5)P_4 and Grp1 PH domain-1-Hexyl-D-Ins(1,3,4,5)P_4 (D-Ins(1,3,4,5)P_4 with hexyl linker at C1 position through a phosphodiester linkage) complexes was examined by a molecular docking study.



(a)



(b)

Figure 10. Docking studies of Grp1 PH domain-unmodified D-Ins(1,3,4,5)P_4 (a) and Grp1 PH domain-1-Hexyl-D-Ins(1,3,4,5)P_4 (b) complexes. The lines show the interaction between amino acids and phosphates within 3.0 Å distance.

Table 2. Interactions between the phosphate/hexyl groups and the Grp1 PH domain

	D-Ins(1,3,4,5)P_4	1-Hexyl-D-Ins(1,3,4,5)P_4
1-Phosphate	Thr280 (2.1, 2.21 Å: OH)	No interaction
3-Phosphate	Lys273 (2.04, 2.66 Å: NH_2) Arg284 (1.84 Å: NH, 1.93 Å: NH) Arg305 (2.19 Å: NH_2)	Lys273 (1.21 Å: NH_2) Arg284 (1.24 Å: NH, 2.39 Å: NH)
4-Phosphate	Lys273 (1.94 Å: NH_2), Tyr295 (1.58 Å: OH) Lys343 (2.01, 2.65, 1.81 Å: NH_2) His355 (1.61 Å: NH)	Lys343 (1.26, 2.63 Å: NH_2) His355 (1.33 Å: NH)
5-Phosphate	Lys343 (1.81 Å: NH_2) Asn354 (1.99 Å: NH_2)	Lys343 (1.2 Å: NH_2) Asn354 (1.81 Å: NH_2)
Hexyl linker		Out of the binding pocket

The structures are shown in Figure 10, wherein the dotted lines indicate the interaction between amino acids and phosphate of D-Ins(1,3,4,5)P_4 (or 1-Hexyl-D-Ins(1,3,4,5)P_4) are shorter than 3.0 Å. Interactions between the phosphate and the hexyl groups and the Grp1 PH domain are summarized in Table 2.

The average of distances between the amino acids and the 1-Hexyl-D-Ins(1,3,4,5)P_4 was shorter than that of unmodified D-Ins(1,3,4,5)P_4 and the numbers of interaction were comparable to that of unmodified D-Ins(1,3,4,5)P_4. In this context, both 1-Biotin-D-Ins(1,3,4,5)P_4 and unmodified D-Ins(1,3,4,5)P_4 were reasonably accommodated in the same binding pocket of the Grp1 PH domain. The 1-phosphate group of 1-Hexyl-D-Ins(1,3,4,5)P_4 did not interact with Thr280 and the hexyl group located outside the binding pocket of Grp1 PH domain. Thus, the length of the linker (C_6) is suitable for the complex formation of 1-Biotin-D-Ins(1,3,4,5)P_4 and the Grp1 PH domain.

$Pr55^{Gag}$/MA: Surface Plasmon Resonance Analysis

1-Biotin-D-Ins(1,3,4,5)P_4 was immobilized to the streptavidin sensor chip in the SPR system through the biotin-streptavidin interaction (Anraku et al. 2011). MA and full length $Pr55^{Gag}$ were expressed by 293T cells transfected

with pEF/Myc-His A-based vector and pSG5-based vector, respectively (Anraku et al. 2010). The MA protein thus produced was myristoylated as confirmed by MALDI-TOF-MS. The *K*d of $Pr55^{Gag}$/MA-phosphoinositide complexes were calculated using a competition assay. The competitors used in this study are shown in Figure 11.

The *K*d values of $Pr55^{Gag}$ and MA, complexed with various phosphoinositides, are shown in Table 3. To more fully define these interactions, we examined the binding affinity between $Pr55^{Gag}$ and D-Ins(1,4,5)P_3, which has divalent phosphate groups at the 1,4,5-position of the inositol head group and lacks an acyl tail. We found that D-Ins(1,4,5)P_3 binds $Pr55^{Gag}$ very weakly (*K*d = 2170 μM). We then determined the binding affinity between $Pr55^{Gag}$ and di-C_8-Ptd-D-Ins, which is composed of a glycerol tail containing saturated C_6 acyl chains and inositol devoid of divalent phosphate groups. Unexpectedly, di-C_8-Ptd-D-Ins bound $Pr55^{Gag}$ with an affinity 12 times greater (*K*d = 186 μM) than that of D-Ins(1,4,5)P_3 (*K*d = 2170 μM). The *K*d for the interaction of $Pr55^{Gag}$ and di-C_8-Ptd-D-Ins(4,5)P_2, which has divalent phosphate groups at the 4,5-position of the inositol head group and saturated C_6 acyl chains, was 47.4 μM. McLaughlin and Aderem suggested that the myristoyl group of $Pr55^{Gag}$ binds to lipid bilayers with a *K*d of approximately 100 μM (McLaughlin and Aderem 1995); thus, the binding affinity we determined for di-C_8-Ptd-D-Ins(4,5)P_2 (*K*d = 47.4 μM) is sufficient for membrane binding of $Pr55^{Gag}$.

In addition, the binding affinity of di-C_8-Ptd-D-Ins(4,5)P_2 was 46 and 4 times greater than that of D-Ins(1,4,5)P_3 and di-C_8-Ptd-D-Ins, respectively. The same tendency was observed in the case of complexes of other phosphoinositide derivatives and MA.

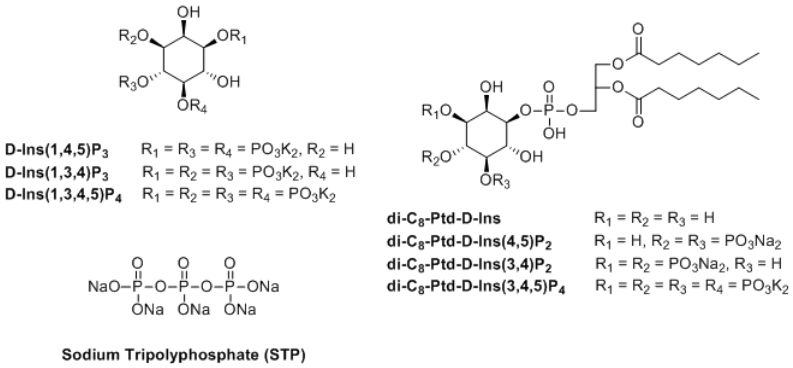


Figure 11. Structures of competitors used in the competition assay.

Table 3. K*d* Values for $Pr55^{Gag}$ or MA Complexed with Phosphoinositide-Related Compounds

Proteins	Competitors	*K*d (μM)
$Pr55^{Gag}$	InsPs	
	D-Ins(1,4,5)P_3	2,170±260
	D-Ins(1,3,4)P_3	579±48
	D-Ins(1,3,4,5)P_4	332±58
	Phosphate	
	STP	1,810±240
	PtdInsPs	
	di-C_8-Ptd-D-Ins	186±22
	di-C_8-Ptd-D-Ins(4,5)P_2	47.4±6.5
	di-C_8-Ptd-D-Ins(3,4)P_2	54.6±4.8
	di-C_8-Ptd-D-Ins(3,4,5)P_3	19.3±2.9
MA	InsPs	
	D-Ins(1,4,5)P_3	568±52
	D-Ins(1,3,4,5)P_4	526±57
	PtdInsPs	
	di-C_8-Ptd-D-Ins	178±29
	di-C_8-Ptd-D-Ins(4,5)P_2	5.64±1.43
	di-C_8-Ptd-D-Ins(3,4)P_2	2.51±1.56
	di-C_8-Ptd-D-Ins(3,4,5)P_3	6.02±0.97
	di-C_4-Ptd-D-Ins(4,5)P_2	86.6±2.2

These data suggest that both phosphate groups and acyl chains are essential for tight binding between $Pr55^{Gag}$ and phosphoinositides and that the presence of the acyl chains is more important than the inositol divalent phosphate groups.

Conclusion

Biotinylated inositol phosphates were designed to be immobilized on beads or SPR sensor chips. These were efficiently synthesized via routes utilizing the regioselective alkylation of 1,2-diol. Each biotinylated inositol phosphate synthesized showed specific binding to the PLCδ PH domain, Grp1 PH domain, and $Pr55^{Gag}$ protein as revealed by SPR analysis and pull-down

analysis. Biotinylated inositol phosphates would be applicable to function analysis of diverse inositol phosphate-binding proteins.

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