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Design and synthesis of biotinylated inositol 1,3,4,5-tetrakisphosphate targeting Grp1 pleckstrin homology domain

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ABSTRACT

A bifunctional molecule containing biotin and D-*myo*-inositol 1,3,4,5-tetrakisphosphate was synthesized. This molecule was designed on the basis of X-ray structure of the complex of D-*myo*-inositol 1,3,4,5-tetrakisphosphates, Ins(1,3,4,5)P_4, and Grp1 PH (general receptor of phosphoinositides pleckstrin homology) domain for the application to the widely employed biotin–avidin techniques. The building block of inositol moiety was synthesized starting with *myo*-inositol and assembled with the biotin-linker moiety through a phosphate linkage. The equilibrium dissociation constant K_D of biotinylated Ins(1,3,4,5)P_4 binding of original Grp1 PH domain was 0.14 μM in pull-down analysis, which was comparable to that of unmodified Ins(1,3,4,5)P_4. Furthermore, biotinylated Ins(1,3,4,5)P_4 had an ability to distinguish Grp1 PH domain from PLCδ_1 PH domain. Thus, biotinylated Ins(1,3,4,5)P_4 retained the binding affinity and selectivity of original Grp1 PH domain, and realized the intracellular Ins(1,3,4,5)P_4 despite a tethering at the 1-phosphate group of inositol.

1. Introduction

Inositol phosphates play important roles as second messengers in intracellular signal transduction.[1,2] In the family of inositol phosphates, D-*myo*-inositol 1,4,5-trisphosphate, Ins(1,4,5)P_3, is formed by the hydrolysis of the phosphatidylinositol 4,5-bisphosphate by the phospholipase C (PLC). While hydrophilic Ins(1,4,5)P_3 diffuses into cytosol and triggers Ca^{2+} release from internal store, D-*myo*-inositol 1,3,4,5-tetrakisphosphate, Ins(1,3,4,5)P_4, is formed by the direct phosphorylation of the Ins(1,4,5)P_3 by the Ins(1,4,5)P_3 3-kinase.[1,3] The synergistic involvement of Ins(1,3,4,5)P_4 in the Ins(1,4,5)P_3-mediated mobilization of Ca^{2+} and the subsequent regulation of the store-operated Ca^{2+}-influx[4–6] still remains as a subject of controversy. Previously, in order to study the detailed analysis of Ins(1,4,5)P_3-protein binding, we synthesized a bifunctional molecule, namely biotinylated Ins(1,4,5)P_3, which has both Ins(1,4,5)P_3 and biotin moiety.[7] This immobilizable inositol phosphate derivative based on the biotin–avidin techniques[8] would address the binding analysis of inositol phosphate and PH domain, exploring the inhibitors for PH domain, search for novel PH domain. Indeed, biotinylated Ins(1,4,5)P_3 exhibited the binding affinity for PLCδ_1 pleckstrin homology (PH) domain comparable to that of unmodified Ins(1,4,5)P_3. In this report, we designed and synthesized biotinylated Ins(1,3,4,5)P_4, and studied its binding to Grp1 (general receptor of phosphoinositides 1) PH domain[1] by pull-down analysis. Furthermore, we utilized both biotinylated Ins(1,3,4,5)P_4 and biotinylated Ins(1,4,5)P_3 for SPR (surface plasmon resonance) analyses to study the binding selectivity of Grp1 PH domain and PLCδ_1 PH domain.

2. Results and discussion

2.1. Design and synthetic strategy of biotinylated Ins(1,3,4,5)P_4

Biotinylated Ins(1,3,4,5)P_4 was designed based on the X-ray crystal structure of Ins(1,3,4,5)P_4-Grp1 PH domain complex.[9] This complex 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 Grp1 PH domain, while 1-phosphate lies out of the binding pocket. Thus, it is reasonable to assume that a biotin-linker moiety through the

Abbreviations: Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P_4, D-*myo*-inositol 1,3,4,5-tetrakisphosphate; Grp1, general receptor of phosphoinositides 1; PLC, phospholipase C; PH, pleckstrin homology; SPR, surface plasmon resonance.

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phosphate group at the 1-position of Ins(1,3,4,5)P_4 would not affect the complex formation with Grp1 PH domain. Our synthetic strategy of biotinylated Ins(1,3,4,5)P_4 (Fig. 1) is to differentiate the six hydroxyl groups of *myo*-inositol through the diacetal intermediate[10] to obtain a suitably protected intermediate that could be coupled with the biotin-linker moiety by a bifunctional phosphorylating agent.[11]

2.2. Syntheses of Ins(1,3,4,5)P_4 moiety

The syntheses of racemic Ins(1,3,4,5)P_4 moiety was carried out as shown in Scheme 1. The starting material DL-3-*O*-benzyl-1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol **1** was prepared by the method of Billington et al.[10] Allylation of the alcohol **1** provided **2**, which was further treated with *p*-toluenesulfonic acid and H_2O to give deacetalyzed **3** in 67% yield (for two steps). The *cis*-1,2-diol of **3** was regioselectively *p*-methoxybenzylated by means of the dibutyltin oxide procedure.[12,13] Thus the tin complex of the 1,2-diol was reacted with *p*-methoxybenzyl chloride in the presence of cesium fluoride to give regioselectively protected **4** in 88% yield. The introduction of isopropylidene acetal to the 4,5-vicinal alcohol under the usual condition gave **5** in 87% yield. Isomerization of the allyl group of **5** followed by the treatment with HgO and $HgCl_2$ gave **6** in 68% yield.[14] Acetylation of the resulting alcohol provided diacetate **7** in 91% yield. The selective deprotection of benzyl group of **7** by the method of Oikawa et al.[15] gave **8** in 64% yield, accompanying a small amount of rearranged 3-*O*-acetyl by-product. Treatment of **8** with *p*-toluenesulfonic acid and ethylene glycol gave the intermediate **9** in 89% yield. The 3,4,5-trihydroxy compound **9** was converted to the corresponding trisphosphonate **10** by treatment with bis(2-cyanoethyl) *N,N*-diisopropylphosphoramidite and 1*H*-tetrazole and subsequent oxidation with mCPBA in 51% yield. Oxidative cleavage of *p*-methoxybenzyl group with CAN[16] gave the Ins(1,3,4,5)P_4 fragment **11** in 86% yield.

2.3. Coupling reaction of Ins(1,3,4,5)P_4 moiety and biotin-linker moiety

A coupling reaction of the Ins(1,3,4,5)P_4 moiety and the biotin-linker moiety was carried out as shown in Scheme 2. The biotin-linker moiety, 6-{N^1-(4,4′-dimethoxytrityl)biotinyl}amino-1-hexanol, prepared by the method of Pon [17] was reacted with bifunctional phosphorylating agent 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphordiamidite[11] and 1*H*-tetrazole to give rather labile phosphoramidite. This compound was condensed with Ins(1,3,4,5)P_4 moiety **11** without further purification. Oxidation of the condensed product with *tert*-BuOOH gave the fully protected biotinylated inositol tetrakisphosphate **12** in 80% yield. The removal of the dimethoxytrityl group of biotin with acid treatment gave **13** in 96% yield. Finally, all protecting groups were removed in one step by reaction with NH_3 to give water-soluble biotinylated inositol phosphate. The biotinylated Ins(1,3,4,5)P_4 was efficiently purified by anion-exchange chromatography with gradient elution of ammonium formate to give DL-biotinylated Ins(1,3,4,5)P_4 in 27% yield. Biotinylated D-Ins(1,3,4,5)P_4 used for the protein binding study was synthesized by the same procedure starting with optically resolved (−)-**1**.[10]

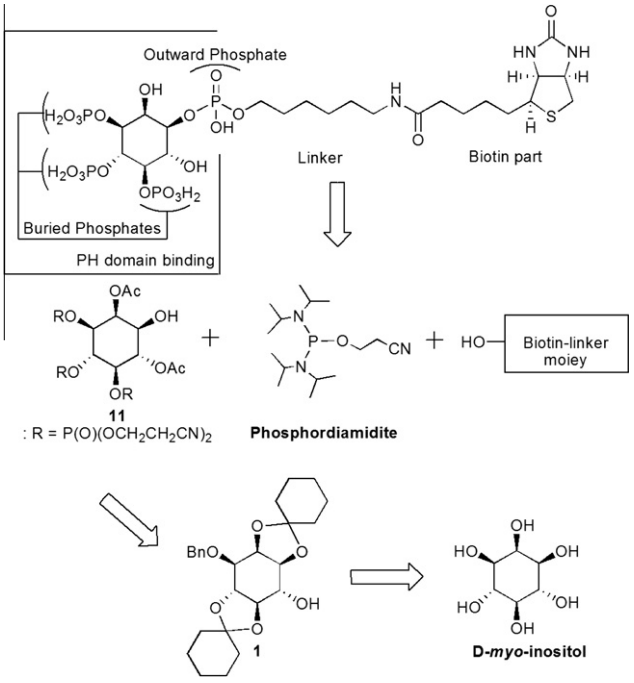


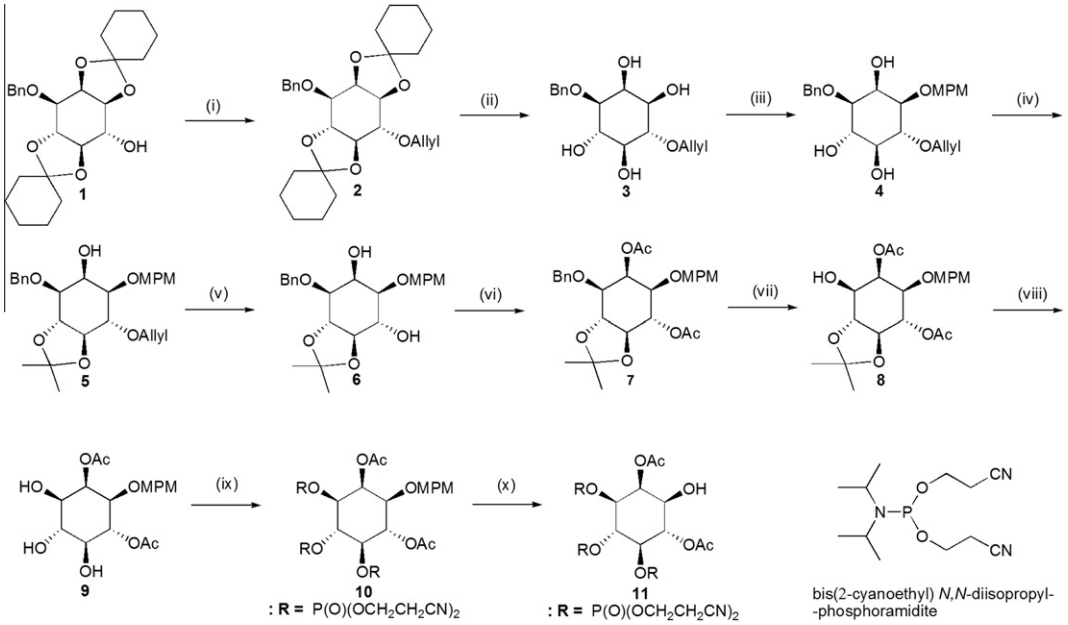
Figure 1. Design and synthetic strategy of biotinylated D-*myo*-inositol 1,3,4,5-tetrakisphosphate.

2.4. Verification of protein binding activity of biotinylated Ins(1,3,4,5)P_4

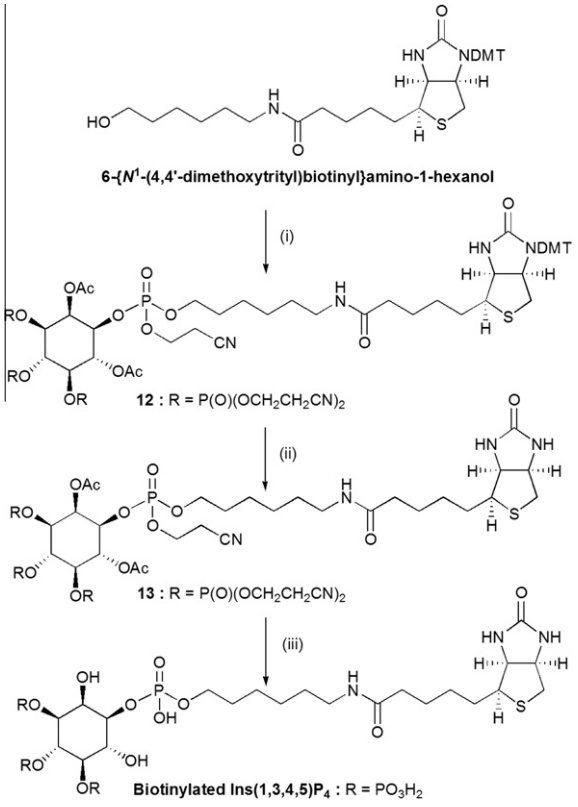
The biochemical study of the biotinylated D-*myo*-inositol 1,3,4,5-tetrakisphosphate obtained through the optical resolution of the *myo*-inositol diacetal intermediate[10] was carried out using streptavidin coated beads. Grp1 PH domain was incubated with various amounts of biotinylated Ins(1,3,4,5)P_4 immobilized beads. The binding ratio was calculated by dividing the bound fraction [**B**] by the sum of bound fraction [**B**] and unbound supernatant fraction [**S**] obtained from the SDS–polyacrylamide electrophoretic analyses. (Fig. 2a) An equilibrium dissociation constant K_D for the complex of biotinylated Ins(1,3,4,5)P_4 and Grp1 PH domain was obtained (0.14 ± 0.04 μM) from titration analyses of Grp1 PH domain by biotinylated Ins(1,3,4,5)P_4. (Fig. 2b) According to the previous report,[18] the K_D value for the complex of Grp1 PH domain and Ins(1,3,4,5)P_4 is approximately 0.10 μM. The K_D value in the present pull-down analysis (0.14 μM) is in good agreement with the reported value despite the fact that biotin is tethered at the 1-phosphate position of inositol. Thus, the result demonstrates that biotinylated Ins(1,3,4,5)P_4 successfully reproduces the protein binding characteristics of unmodified 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.

2.5. Application of biotinylated Ins(1,3,4,5)P_4 to SPR analysis

We demonstrated that optically active biotinylated Ins(1,3,4,5)P_4 was effectively immobilized on streptavidin beads, and that the resulting beads-bound Ins(1,3,4,5)P_4 was the active substrate for Grp1 PH domain. Biotinylated Ins(1,3,4,5)P_4 was next applied to SPR-based PH domain binding analysis using commercially available pre-immobilized streptavidin coated sensorchip. After biotinylated Ins(1,3,4,5)P_4 was immobilized partially on the sensorchip, the binding affinity was determined by injecting various concentration of Grp1 PH domain over the sensorchip. The equilibrium binding constant (K_D) was obtained by using the average of Response Unit (RU) at the equilibrium state at various concentrations of Grp1 PH domain. The K_D was deduced to be 0.3 μM (Fig. 3a, open squares). To confirm whether the biotinylated Ins(1,3,4,5)P_4 retained the binding activity, biotinylated Ins(1,4,5)P_3 and biotinylated Ins(3,5,6)P_3 [L-isomer of Ins(1,4,5)P_3][7] were also immobilized on the sensorchip. Grp1 PH domain barely bound biotinylated Ins(1,4,5)P_3 (K_D >>1.0 μM) (Fig. 3a, filled circles) and biotinylated Ins(3,5,6)P_3 (K_D >>1.0 μM) (Fig. 3a, open circles). (All sensorgrams were shown in Supplementary data Fig. S1) We also prepared PLCδ_1 PH domain, which is the well-characterized PH domain that binds specifically to Ins(1,4,5)P_3. PLCδ_1 PH domain bound biotinylated Ins(1,4,5)P_3 with high affinity (K_D = 2.2 ± 0.1 μM)



Scheme 1. Reagents and conditions: (i) Allyl bromide, NaH, DMF, rt, 24 h, 82%; (ii) *p*-TsOH·H_2O, THF–H_2O, reflux, 3 h, 82%; (iii) (a) Bu_2SnO, toluene, reflux, 3 h; (b) CsF, MPM-Cl, DMF, −78 °C then rt, 24 h, 88%; (iv) 2-methoxypropene, *p*-TsOH·H_2O, DMF, rt, 24 h, 87%; (v) (a) $(Ph_3P)_3RhCl$, DABCO, EtOH–benzene–H_2O, reflux, 5 h; (b) HgO, $HgCl_2$, acetone–H_2O, rt, 5 min, 68%; (vi) Ac_2O, DMAP, pyridine, rt, 12 h, 91%; (vii) H_2/W-2 Raney–Ni, MeOH, 50 °C, 3 h, 64%; (viii) TsOH, ethylene glycol, CH_2Cl_2, rt, 10 min, 89%; (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, 54%; (x) CAN, CH_3CN–H_2O, rt, 1 h, 87%.



Scheme 2. Reagents and conditions: (i) (a) 2-Cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite, 1*H*-tetrazole, CH_2Cl_2, rt, 15 min; (b) **11**, 1*H*-tetrazole, CH_2Cl_2, rt, 2 h; (c) *tert*-BuOOH, CH_2Cl_2, rt, 5 min, <80%; (ii) TCA, CH_2Cl_2, rt, 1 h, 96%; (iii) aq NH_3, MeOH, 55 °C, 10 h, 27%.

(Fig. 3b, filled circles), but it bound modestly to biotinylated Ins(1,3,4,5)P_4 (K_D >5.0 μM) (Fig. 3b, open squares) and biotinylated Ins(3,5,6)P_3 (K_D >5.0 μM) (Fig. 3b, open circles). (All sensorgrams were shown in Supplementary data Fig. S2) Thus, biotinylated Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 retain the inherent biochemical characteristics for PLCδ_1 domain and Grp1 PH domain, respectively.

2.6. Theoretical binding analysis of Grp1 PH domain and biotinylated Ins(1,3,4,5)P_4

To confirm theoretical binding mode of Grp1 PH domain and biotinylated Ins(1,3,4,5)P_4, molecular docking study was adapt to the Grp1 PH domain-Ins(1,3,4,5)P_4 and Grp1 PH domain-Ins(1,3,4,5)P_4 with hexyl linker (C_6) complexes. The structures are shown in Figure 4, wherein the lines in magenta indicate the interaction between amino acid and phosphate of Ins(1,3,4,5)P_4 (or Ins(1,3,4,5)P_4 with hexyl linker) shorter than 3.0 Å. The 3-phosphate group of Ins(1,3,4,5)P_4 interacts with Lys273 (2.0, 2.7 Å: NH_2), Arg284 (1.8 Å: NH, 1.9 Å: NH) and Arg305 (2.2 Å: NH_2). The 4-phosphate interacts with Lys273 (1.9 Å: NH_2), Lys343 (2.0, 2.6 Å: NH_2), His355 (1.6 Å: NH), and Tyr295 (1.6 Å: OH). The 5-phosphate interacts with Lys343 (1.8 Å: NH_2) and Asn354 (2.0 Å: NH_2), while the 1-phosphate group interacts with Thr280 (2.1, 2.2 Å: OH) (Fig. 4a). The 3-phosphate group of Ins(1,3,4,5)P_4 with hexyl linker interacts with Lys273 (1.2 Å: NH_2) and Arg284 (1.2 Å: NH, 2.4 Å: NH). The 4-phosphate interacts with Lys343 (1.3, 2.6 Å: NH_2) and His355 (1.3 Å: NH). The 5-phosphate interacts with Lys343 (1.2 Å: NH_2) and Asn354 (1.8 Å: NH_2). Although the 1-phosphate group does not interact with Thr280 unlike the case of unmodified Ins(1,3,4,5)P_4, the hexyl group attached through the phosphodiester linkage at the 1-position of Ins(1,3,4,5)P_4 lied out of the binding pocket of Grp1 PH domain (Fig. 4b, arrow). The average of distances between amino acid and Ins(1,3,4,5)P_4 with hexyl linker was shorter than that of unmodified Ins(1,3,4,5)P_4 and the numbers of interaction were comparable to that of unmodified Ins(1,3,4,5)P_4. In this context, Ins(1,3,4,5)P_4 with hexyl linker would retain a high affinity to Grp1 PH domain. Judging from the results of the docking score based on the electric interaction, van der Waals attraction and strain energy of ligand, Grp1 PH domain-Ins(1,3,4,5)P_4 complex was slightly more stable than Ins(1,3,4,5)P_4 with hexyl linker complex (−567 kcal and −565 kcal

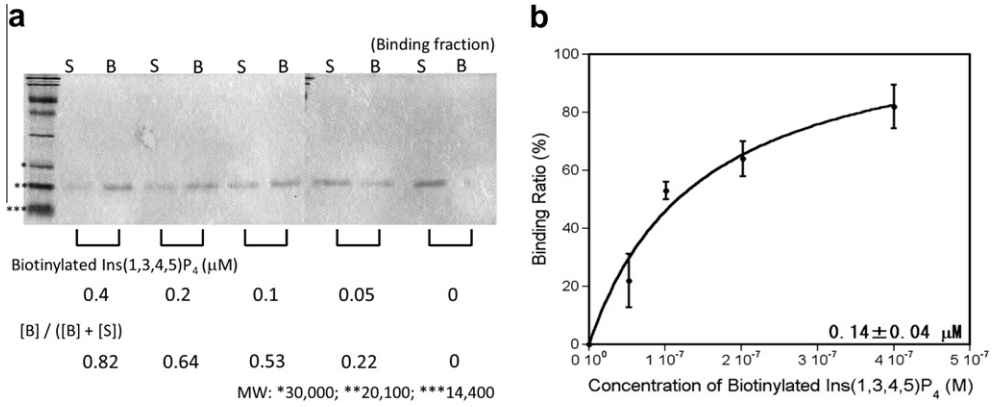


Figure 2. A SDS–polyacrylamide electrophoretic analysis for the Grp1 PH domain and biotinylated Ins(1,3,4,5)P_4 binding. (a) After biotinylated Ins(1,3,4,5)P_4 immobilized beads was incubated with 0.10 μM Grp1 PH domain, the unbound supernatant fraction [**S**] was obtained, then bound fraction [**B**] was obtained by eluting the beads using 50 μM unmodified Ins(1,3,4,5)P_4 solution. Each fraction was subjected to 15% SDS–polyacrylamide gel electrophoresis followed by quantitation with CBB staining. The binding ratio [**B**]/([**B**]+[**S**]) was calculated by the bound fraction [**B**] divided by the sum of bound fraction [**B**] and nonbinding fraction [**S**]. (b) The fraction of Grp1 PH domain bound to biotinylated Ins(1,3,4,5)P_4 immobilized beads [**B**]/([**B**]+[**S**]) was plotted against the biotinylated Ins(1,3,4,5)P_4 concentrations. A solid curve represents the best fit for the theoretical dissociation equation.

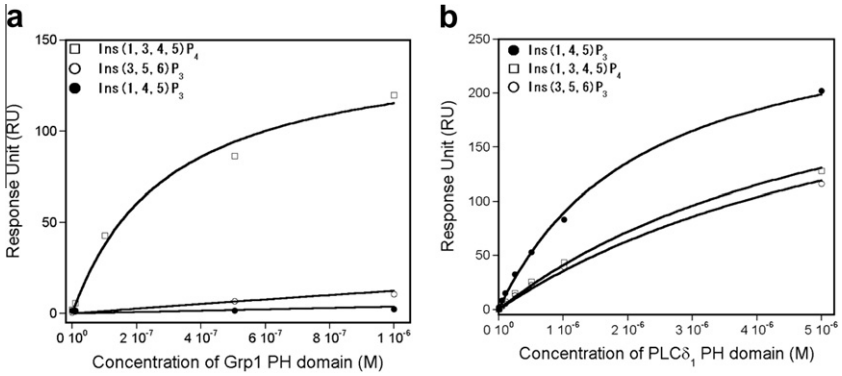


Figure 3. SPR analyses (BIACORE) for the binding of PH domains (Grp1 and PLCδ_1) to biotinylated Ins(1,3,4,5)P_4, Ins(1,4,5)P_3, and Ins(3,5,6)P_3. (a) The average of Response Unit (RU) at the equilibrium state (260–270 s, Supplementary data Fig. S1) of Grp1 PH domain bound to biotinylated Ins(1,3,4,5)P_4, Ins(3,5,6)P_3, and Ins(1,4,5)P_3 were plotted against concentrations of Grp1 PH domain. (b) The average of Response Unit (RU) at the equilibrium state (260–270 s, Supplementary data Fig. S2) of PLCδ_1 PH domain bound to biotinylated Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, and Ins(3,5,6)P_3 were plotted against concentrations of PLCδ_1 PH domain.

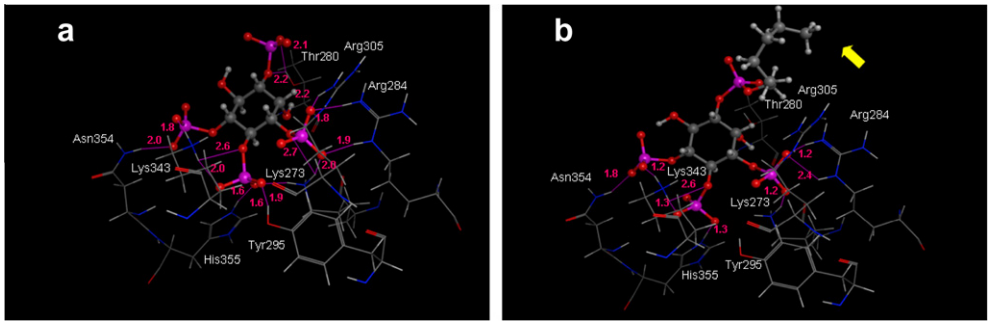


Figure 4. Docking studies of Grp1 PH domain-unmodified Ins(1,3,4,5)P_4 (a) and Grp1 PH domain- Ins(1,3,4,5)P_4 with hexyl linker (**b**) complexes. The lines show the interaction between amino acids and phosphates within 3.0 Å distance. The arrow indicates the position of hexyl linker (C_6).

as the U_dock values, respectively). These data suggested that both biotinylated Ins(1,3,4,5)P_4 and unmodified Ins(1,3,4,5)P_4 were reasonably accommodated in the same binding pocket of Grp1 PH domain. Furthermore, the biotin-linker moiety and avidin binding was also calculated by docking analysis, the linker of biotin lied out of the binding pocket of streptavidin (data not shown). Thus, the length of the linker (C_6) is suitable for the complex formation of biotinylated Ins(1,3,4,5)P_4 and Grp1 PH domain.

3. Conclusion

We have designed and synthesized biotinylated Ins(1,3,4,5)P_4 so that the modified Ins(1,3,4,5)P_4 exhibits essentially the same biochemical activity as that of unmodified Ins(1,3,4,5)P_4. Biotinylated Ins(1,3,4,5)P_4 has been designed based on the X-ray crystal structure of the complex of Ins(1,3,4,5)P_4 and Grp1 PH domain[9] and synthesized based on the strategy we have developed for

biotinylated Ins(1,3,4,5)P_4 by using the phosphoramidite method.[11] The building block of inositol moiety was synthesized starting with *myo*-inositol and assembled with the biotin-linker moiety through a phosphate linkage by a bifunctional phosphorylating agent. The synthesized biotinylated Ins(1,3,4,5)P_4 showed a high affinity for Grp1 PH domain (0.14 μM), which was comparable to that of unmodified Ins(1,3,4,5)P_4 in the pull-down analysis. This K_D value also reflected the fact that biotinylated Ins(1,3,4,5)P_4 mimics the unmodified Ins(1,3,4,5)P_4 despite of possessing a tether at the 1-phosphate group of the inositol ring. We also carried out the binding analysis of biotinylated Ins(1,4,5)P_3 as well as biotinylated Ins(1,3,4,5)P_4 for PH domains in the SPR analyses. Biotinylated Ins(1,3,4,5)P_4 was captured by the Ins(1,3,4,5)P_4 specific Grp1 PH domain with high affinity, but not by the Ins(1,4,5)P_3 specific PLCδ_1 PH domain. Whereas biotinylated Ins(1,4,5)P_3 was targeted by PLCδ_1 PH domain with relatively high affinity, but moderately by Grp1 PH domain. It was thus clearly shown that each biotinylated inositol phosphates was distinguished by its specific binding PH domain. Such immobilizable inositol phosphate derivatives with the inherent biochemical activity would be quite useful for exploring the inhibitors for Grp1 PH domain and PLCδ_1 PH domain.

4. Materials and methods

4.1. General Methods

Chemicals were purchased from Aldrich, Fluka, Kanto Chemical, Nacalai tesque, and Wako. Anhydrous CH_2Cl_2 was distilled from calcium hydride. Thin layer chromatography (TLC) was performed on precoated plates (Merck TLC sheets silica 60 F_{254}): products on the TLC were sprayed with phosphomolybdic acid in EtOH or basic potassium permanganate, and then visualized by heating at high temperature. Chromatography was carried out on Silica Gel 60 N (40–100 mesh). Anion exchange chromatography was performed using Dowex 1X8 (Cl^-, 50–100 mesh). Specific rotation of optically active compound was recorded by JASCO Dip-1000. NMR spectra (JEOL JNM-AL300) were referenced to $SiMe_4$ or (HDO). Infrared spectra were recorded on a JASCO FT/IR-410. The samples were prepared as KBr discs or thin films between sodium chloride discs. High resolution MS (HRMS) were recorded by a JEOL JMS-DX303HF by using positive and negative FAB with 3-nitrobenzyl alcohol (NBA) (containing HMPA or not) as the matrix.

4.2. DL-6-*O*-Allyl-3-*O*-benzyl-1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol (2)

To a solution of **1** (0.50 g, 1.2 mmol) in DMF (30 ml) was added 60% NaH (0.58 g, 14 mmol) followed by allyl bromide (1.0 ml, 12 mmol) and the resulting mixture was stirred at room temperature under argon for 24 h. The reaction was quenched with MeOH, concentrated under reduced pressure and the residue was diluted with AcOEt. The organic phase was washed with H_2O and saturated aqueous NaCl, dried over Na_2SO_4 and then concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (hexane/AcOEt = 5:1) to afford **2** (0.45 g, 82%) as a white solid.

^{1}H NMR ($CDCl_3$) δ: 1.42–1.75 (20H, m), 3.28 (1H, dd, J = 9.9, 9.5 Hz), 3.63 (1H, dd, J = 10.64, 6.6 Hz), 3.73 (1H, dd, J = 10.3, 4.2 Hz), 3.97–4.06 (2H, m), 4.29–4.35 (3H, m), 4.81 (1H, d, J = 12.5 Hz) , 4.89 (1H, d, J = 12.5 Hz), 5.18 (1H, d, J = 10.5 Hz), 5.33 (1H, d, J = 17.4 Hz), 5.89–6.00 (1H, m), 7.25–7.43 (5H, m). ^{13}C NMR ($CDCl_3$) δ: 23.5, 23.8, 23.9, 24.9, 25.0, 35.2, 36.3, 36.4, 37.6, 71.1, 71.5, 74.6, 76.2, 76.6, 78.4, 80.3, 80.7, 110.4, 112.5, 117.0, 127.6, 128.1, 128.3, 134.9, 138.1. IR (KBr) 2930, 2860, 1650, 1110, 740 cm^{-1}. MS (FAB) m/z 471 $(M+H)^+$. Anal. Calcd for $C_{28}H_{38}O_6$: C, 71.46; H, 8.14. Found: C, 71.52; H, 8.27.

4.3. DL-6-*O*-Allyl-3-*O*-benzyl-*myo*-inositol (3)

To a solution of **2** (0.45 g, 0.95 mmol) in THF–H_2O (5:1, 60 ml) was added *p*-toluenesulfonic acid monohydrate (*p*-TsOH·H_2O) (0.30 g, 1.6 mmol). The resulting mixture was refluxed for 3 h, and then neutralized with Et_3N and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 10:1) to afford **3** (0.24 g, 82%) as a white solid.

^{1}H NMR ($CDCl_3$) δ: 3.09–3.29 (3H, m), 3.39 (1H, dd, J = 9.5, 9.4 Hz), 3.67 (1H, dd, J = 9.5, 9.54 Hz), 4.00 (1H, dd, J = 2.8, 2.7 Hz), 4.23 (2H, d, J = 5.9 Hz), 4.54 (1H, d, J = 11.7 Hz), 4.62 (1H, d, J = 11.7 Hz), 4.99 (1H, dd, J = 10.5, 2.8 Hz), 5.16 (1H, dd, J = 17.2, 2.0 Hz), 5.87–5.96 (1H, m), 7.15–7.34 (5H, m). ^{13}C NMR ($CDCl_3$) δ: 71.0, 73.0, 73.1, 73.8, 75.1, 76.3, 80.9, 82.4, 116.7, 128.6, 129.1, 129.3, 137.1, 139.9. IR (KBr) 3410, 2870, 1640, 1120, 750 cm^{-1}. Anal. Calcd for $C_{16}H_{22}O_6$: C, 61.92; H, 7.15. Found: C, 61.72; H, 7.23.

4.4. DL-6-*O*-Allyl-3-*O*-benzyl-1-*O*-(*p*-methoxybenzyl)-*myo*-inositol (4)

A mixture of **3** (0.47 g, 1.5 mmol) and dibutyltin oxide (0.45 g, 1.8 mmol) in toluene (50 ml) was refluxed for 3 h in a Dean–Stark apparatus to remove water. The mixture was concentrated under reduced pressure. To the residue was added cesium fluoride (0.30 g, 2.0 mmol) and the mixture was suspended in heated DMF (30 ml) at 100 °C, and then cooled to −78 °C. To the resulting suspension was added *p*-methoxybenzyl chloride (0.25 ml, 1.8 mmol) at −78 °C and the mixture was stirred at room temperature under argon for 24 h. The reaction mixture was diluted with $CHCl_3$, filtered through a pad of celite and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 14:1) to afford **4** (0.58 g, 88%) as a colorless oil.

^{1}H NMR ($CDCl_3$) δ: 2.54 (1H, br s), 2.92 (2H, br s), 3.19 (1H, dd, J = 9.5, 2.8 Hz), 3.29 (1H, dd, J = 9.5, 2.9 Hz), 3.37 (1H, d, J = 9.4 Hz), 3.66 (1H, dd, J = 9.4, 9.4 Hz), 3.81 (3H, s), 3.94 (1H, dd, J = 9.9, 9.5 Hz), 4.16 (1H, s), 4.26 (1H, dd, J = 12.5, 5.9 Hz), 4.40 (1H, dd, J = 12.5, 5.5 Hz), 4.59 (2H, s), 4.64 (1H, d, J = 11.7 Hz), 4.71 (1H, d, J = 11.7 Hz), 5.16 (1H, dd, J = 10.5, 1.8 Hz), 5.28 (1H, dd, J = 17.2, 1.6 Hz), 5.92–6.01 (1H, m), 6.88 (2H, d, J = 8.8 Hz), 7.25–7.35 (7H, m). ^{13}C NMR ($CDCl_3$) δ: 55.2, 67.0, 71.8, 72.2 (3-*O*–*C*H_2–C_6H_4–OMe and 6-*O*–*C*H_2–CH=CH_2), 74.1, 74.2, 79.0, 79.3, 80.0, 113.8, 116.9, 127.9 (2,6-*C*H, methoxyphenyl and 2,6-*C*H, phenyl), 128.0, 128.5, 129.5, 129.9, 135.1, 137.7, 159.4. IR (KBr) 3400, 3030, 2920, 1610, 1030, 760 cm^{-1}. Anal. Calcd for $C_{24}H_{30}O_7$: C, 66.96; H, 7.02. Found: C, 66.86; H, 7.04.

4.5. DL-6-*O*-Allyl-3-*O*-benzyl-1-*O*-(*p*-methoxybenzyl)-4,5-*O*-isopropylidene-*myo*-inositol (5)

To a solution of **4** (0.75 g, 1.7 mmol) in DMF (30 ml) was added 2-methoxypropene (0.52 ml, 10 mmol) followed by dehydrated *p*-toluenesulfonic acid (0.25 g, 1.3 mmol). The resulting mixture was stirred at room temperature under argon for 24 h. The mixture was neutralized with triethylamine (Et_3N) and concentrated under reduced pressure, and then the residue was diluted with AcOEt. The organic phase was washed with H_2O and saturated aqueous NaCl, dried over Na_2SO_4, and then concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (hexane/AcOEt = 2:1) to afford **5** (0.72 g, 87%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ: 1.43 (3H, s), 1.45 (3H, s), 2.63 (1H, br s), 3.27–3.37 (2H, m), 3.50 (1H, dd, J = 9.9, 3.1 Hz), 3.80 (3H, s), 3.86 (1H, dd, J = 9.7, 9.2 Hz), 4.07 (1H, dd, J = 9.7, 9.7 Hz), 4.14 (1H, s), 4.25 (1H, dd, J = 12.8, 5.5 Hz), 4.36 (1H, dd, J = 12.8, 5.3 Hz), 4.50

(3H, m), 4.82 (1H, d, J = 12.1 Hz) 5.16 (1H, d, J = 11.8 Hz), 5.32 (1H, dd, J = 17.2, 1.5 Hz), 5.89–6.00 (1H, m), 6.86 (2H, d, J = 8.6 Hz), 7.26–7.38 (7H, m). ^{13}C NMR ($CDCl_3$) δ: 26.9, 27.0, 55.2, 69.8, 71.5, 72.4, 72.9, 75.9, 77.1, 78.0, 79.0, 80.6, 11.6, 113.8, 116.5, 127.7, 127.9, 128.3, 129.6, 129.9, 135.1, 138.0, 159.4. IR (KBr) 3450, 2930, 1610, 1070, 790 cm^{-1}. HRMS(FAB) m/z calcd for $C_{27}H_{34}O_7Na$ $(M+Na)^+$ 493.2202. Found: 493.2216.

4.6. DL-3-*O*-Benzyl-1-*O*-(*p*-methoxybenzyl)-4,5-*O*-isopropylidene-*myo*-inositol (6)

To a solution of **5** (0.10 g, 0.21 mmol) in EtOH–benzene–H_2O (7:3:1, 22 ml) was added diazabicyclo[2.2.2]octane (DABCO) (0.034 g, 0.30 mmol) followed by tris(triphenylphosphine)rhodium(I) chloride (0.028 g, 0.030 mmol) and the resulting mixture was refluxed for 5 h. The mixture was concentrated under reduced pressure and the residue was diluted with AcOEt. The organic phase was washed with H_2O and saturated aqueous NaCl, dried over Na_2SO_4, and then concentrated under reduced pressure. To a solution of the residue in acetone–H_2O (10:1, 5 ml) was added mercury(II) oxide (0.046 g, 0.21 mmol). To the resulting mixture was added dropwise a solution of mercury(II) chloride (0.057 g, 0.21 mmol) in acetone–H_2O (10:1, 5 ml). The resulting mixture was stirred at room temperature for 5 min. The mixture was neutralized with aqueous NaOH, filtered through a pad of celite and concentrated under reduced pressure. The residue was diluted with saturated aqueous NaCl. The aqueous phase was extracted with CH_2Cl_2 and the organic phase was dried over Na_2SO_4, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 15:1) to afford **6** (0.061 g, 68%) as a white solid.

^{1}H NMR ($CDCl_3$) δ: 1.45 (3H, s),1.47 (3H, s), 2.60 (1H, br s), 2.62 (1H, br s), 3.22–3.24 (2H, m), 3.56 (1H, dd, J = 10.1, 2.8 Hz), 3.80 (3H, s), 4.08 (2H, t, J = 9.7 Hz), 4.24 (1H, s), 4.50 (1H, d, J = 11.4 Hz), 4.66 (1H, d, J = 11.4 Hz), 4.69 (1H, d, J = 12.1 Hz), 4.85 (1H, d, J = 12.1 Hz), 6.89 (2H, d, J = 8.1 Hz), 7.25–7.38 (7H, m). ^{13}C NMR ($CDCl_3$) δ: 27.2, 27.3, 55.7, 69.0, 70.7, 72.1, 72.5, 76.5, 77.6, 78.6, 81.7, 112.5, 114.4, 128.3, 128.4, 128.8, 129.7, 130.1, 138.3, 160.0. IR (KBr) 3570, 2990, 2930, 1070, 790 cm^{-1}. HRMS(FAB) m/z calcd for $C_{24}H_{35}O_7$ $(M+H)^+$ 431.2070. Found: 431.2048.

4.7. DL-3-*O*-Benzyl-2,6-di-*O*-acetyl-1-*O*-(*p*-methoxybenzyl)-4,5-*O*-isopropylidene-*myo*-inositol (7)

To a solution of **6** (0.33 g, 0.76 mmol) in pyridine (10 ml) was added 4-dimethylaminopyridine (DMAP) (0.024 g, 0.20 mmol) and acetic anhydride (Ac_2O) (0.19 ml, 2.0 mmol), and the resulting mixture was stirred at room temperature for 12 h. After azeotropic removal of pyridine with toluene under reduced pressure, the residue was diluted with AcOEt. The organic phase was washed with H_2O and saturated aqueous NaCl, dried over Na_2SO_4, and then concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (hexane/AcOEt = 2:1) to afford **7** (0.36 g, 91%) as a white solid. ^{1}H NMR ($CDCl_3$) δ: 1.41 (3H, s),1.46 (3H, s), 2.05 (3H, s), 2.16 (3H, s), 3.34–3.40 (2H, m), 3.61 (1H, dd, J = 10.3, 3.1 Hz), 3.80 (3H, s), 4.03 (1H, t, J = 10.9, 9.8 Hz), 4.32 (1H, d, J = 11.7 Hz), 4.59–4.74 (3H, m), 5.38 (1H, dd, J = 10.3, 9.9 Hz), 5.83 (1H, dd, J = 3.3, 3.2 Hz), 6.87 (2H, d, J = 8.6 Hz), 7.16–7.19 (2H, d, J = 8.6 Hz), 7.26–7.38 (5H, m). ^{13}C NMR ($CDCl_3$) δ: 21.0, 21.1, 26.8, 26.8, 55.2, 67.5, 71.2, 71.2, 71.6, 74.6, 76.6, 76.9, 77.0, 112.2, 113.8, 127.7 (2,6-*C*H, methoxyphenyl and 2,6-*C*H, phenyl), 128.4, 129.2, 129.5, 137.5, 159.4, 169.8, 170.3. IR (KBr) 2990, 1740, 1100, 740 cm^{-1}. MS (FAB) m/z 515 $(M+H)^+$. Anal. Calcd for $C_{28}H_{34}O_9$: C, 65.36; H, 6.66. Found: C, 65.06; H, 6.72.

4.8. DL-2,6-Di-*O*-acetyl-1-*O*-(*p*-methoxybenzyl)-4,5-*O*-isopropylidene-*myo*-inositol (8)

To a solution of **7** (0.38 g, 0.74 mmol) in MeOH (25 ml) was added W-2 Raney Nickel (Ni) (0.20 g) and the resulting mixture was stirred at 50 °C under hydrogen for 3 h. The mixture was filtered through a pad of celite and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/AcOEt = 1:1) to afford **8** (0.20 g, 64%) as a colorless oil.

^{1}H NMR ($CDCl_3$) δ: 1.42 (3H, s), 1.46 (3H, s), 2.07 (3H, s), 2.18 (3H, s), 2.51 (1H, br s), 3.41–3.47 (2H, m), 3.80 (3H, s), 3.92 (2H, d, J = 7.3 Hz), 4.35 (1H, d, J = 11.7 Hz), 4.60 (1H, d, J = 11.7 Hz), 5.36 (1H, dd, J = 10.3, 9.6 Hz), 5.65 (1H, s), 6.87 (2H, d, J = 8.6 Hz), 7.19 (2H, d, J = 8.6 Hz). ^{13}C NMR ($CDCl_3$) δ: 20.9, 21.1, 26.7 (–CH_3 × 2, acetal), 55.2, 68.9, 70.1, 71.2, 71.7, 76.3, 76.8, 77.6, 112.4, 113.8, 129.1, 129.6, 159.4, 169.8, 170.8. HRMS(FAB) m/z calcd for $C_{21}H_{28}O_9Na$ $(M+Na)^+$ 447.1631. Found: 447.1624.

4.9. DL-2,6-Di-*O*-acetyl-1-*O*-(*p*-methoxybenzyl)-*myo*-inositol (9)

To a solution of **8** (0.20 g, 0.47 mmol) in CH_2Cl_2 (10 ml) was added ethylene glycol (28 μl, 0.50 mmol) followed by *p*-toluenesulfonic acid monohydrate (0.0095 mg, 0.05 mmol). The resulting mixture was stirred at room temperature for 10 min. The mixture was neutralized with Et_3N (0.10 ml, 0.72 mmol) and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 7:1) to afford **9** (0.16 g, 89%) as a white solid.

^{1}H NMR ($CDCl_3$) δ: 1.91 (3H, s), 2.02 (3H, s), 3.20–3.27 (3H, m), 3.41–3.57 (3H, m), 3.67 (3H, s), 4.21 (1H, d, J = 11.2 Hz), 4.47 (1H, d, J = 11.4 Hz), 5.00 (1H, t, J = 9.9 Hz), 5.55 (1H, dd, J = 6.0, 2.9 Hz), 6.77 (2H, d, J = 8.6 Hz, 2H), 7.19 (2H, d, J = 8.6 Hz). ^{13}C NMR ($CDCl_3$) δ: 21.0, 21.1, 55.7, 71.2, 71.8, 72.4, 74.2, 74.4, 75.3, 76.8, 114.7, 130.6, 130.8, 131.3, 172.4. HRMS(FAB) m/z calcd for $C_{18}H_{24}O_9Na$ $(M+Na)^+$ 407.1318. Found: 407.1319.

4.10. Preparation of bis(2-cyanoethyl) *N,N*-diisopropyl aminophosphoramidite

(Diisopropylamino)dichlorophosphine was prepared by the method of Uhlmann and Engels[11] by adding two equivalents of diisopropylamine to a solution of phosphorus trichloride in dry ethyl ether at −78 °C. The crude product was purified by distillation under reduced pressure and could be stored as a crystalline solid at −20 °C. Two equivalents of 2-cyanoethanol were reacted with the purified product in dehydrated CH_2Cl_2 in the presence of diisopropylethylamine to afford bis (2-cyanoethyl) *N,N*-diisopropylaminophosphoramidite.

4.11. D-2,6-Di-*O*-acetyl-1-*O*-(*p*-methoxybenzyl)-3,4,5-tri-*O*-[bis(2-cyanoethyl)phosphoryl]-*myo*-inositol (10)

To a solution of **9** (0.25 g, 0.66 mmol) in CH_2Cl_2 (10 ml) was added bis(2-cyanoethyl) *N,N*-diisopropylphosphoramidite (0.64 ml, 2.5 mmol) followed by 1*H*-tetrazole (0.21 g, 3.0 mmol), and the resulting mixture was stirred at room temperature for 1.5 h. To the mixture was added *m*-chloroperbenzoic acid (0.087 g, 0.51 mmol) in small portions and the resulting mixture was stirred for 5 min. The mixture was diluted with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$. The organic phase was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 12:1) to afford **10** (0.33 g, 54%) as a colorless oil.

^{1}H NMR ($CDCl_3$) δ: 2.07 (3H, s), 2.21 (3H, s), 2.77–2.86 (12H, m), 3.61 (1H, d, J = 10.1 Hz), 3.80 (3H, s), 4.27–4.53 (16H, m), 4.81 (1H,

dd, J = 18.9, 9.4 Hz), 5.33 (1H, dd, J = 9.9, 9.8 Hz), 5.90 (1H, s), 6.86 (2H, d, J = 8.6 Hz), 7.17 (2H, d, J = 8.6 Hz). ^{13}C NMR ($CDCl_3$) δ: 19.5, 19.6, 19.6, 20.8, 21.0, 55.2, 62.7, 62.8, 62.9, 63.0, 63.1, 67.1, 70.4, 71.8, 73.4, 73.9, 76.1, 76.7, 113.7, 116.7, 116.9, 117.0, 117.1, 117.2, 129.0, 129.6, 159.6, 169.9, 170.1. HRMS(FAB) m/z calcd for $C_{36}H_{45}N_6O_{18}P_3Na$ $(M+Na)^+$ 965.1901. Found: 965.1913.

4.12. DL-2,6-Di-*O*-acetyl-3,4,5-tri-*O*-[bis(2-cyanoethyl) phosphoryl]-*myo*-inositol (11)

To a solution of **10** (0.14 g, 0.15 mmol) in CH_3CN–H_2O (9:1, 10 ml) was added diammonium cerium(IV) nitrate (CAN) and the resulting mixture was stirred at room temperature for 1.5 h. The mixture was concentrated under reduced pressure and the residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 7:1) to afford **11** (0.11 g, 87%) as a colorless oil.

^{1}H NMR ($CDCl_3$) δ: 2.08 (3H, s), 2.12 (3H, s), 2.87 (12H, t, J = 6.1 Hz), 3.86 (1H, dd, J = 5.8, 2.9 Hz), 4.23–4.38 (12H, m), 4.58 (1H, dd, J = 17.8, 9.0 Hz), 4.70–4.75 (2H, m), 5.22 (1H, dd, J = 9.7, 9.7 Hz), 5.68 (1H, s). ^{13}C NMR ($CDCl_3$) δ: 20.1 (–*C*H_2–CH_2–CN × 2), 20.2 (–*C*H_2–CH_2–CN × 2), 20.3 (–*C*H_2–CH_2–CN × 2), 20.9, 21.4, 64.8 (–CH_2–*C*H_2–CN × 2), 64.9 (–CH_2–*C*H_2–CN × 2), 64.9 (–CH_2–*C*H_2–CN × 2), 65.0, 68.2, 72.5, 73.4, 75.4, 77.8, 78.4, 118.6 (–CH_2–CH_2–*C*N × 2), 118.8 (–CH_2–CH_2–*C*N × 2), 118.8 (–CH_2–CH_2–*C*N × 2), 171.8, 172.3. HRMS(FAB) m/z calcd for $C_{28}H_{37}N_6O_{17}P_3Na$ $(M+Na)^+$ 845.1326. Found: 845.1349.

4.13. Preparation of 6-{N^1-(4,4′-dimethoxytrityl)biotinyl} amino-1-hexanol

6-{N^1-(4,4′-dimethoxytrityl)biotinyl}amino-1-hexanol was prepared by the method of Pon [17] *N*-biotinyloxysuccinimide was reacted with 6-amino-1-hexanol to give 6-biotinylamino-1-hexanol, which was further converted into the dimethoxytrityl derivative by silylation of OH group, N^1-protection with dimethoxytrityl group and removal of *O*-silyl group.

4.14. DL-2, 6-Di-*O*-acetyl-3,4,5-tri-*O*-[bis(2-cyanoethyl) phosphoryl]-*myo*-inositol 1-{′[6-(N^1-(4,4′-dimethoxytrityl) biotinyl)amino-1-hexyl] (2-cyanoethyl)phosphate} (12)

To a solution of 6-{N^1-(4,4′-dimethoxytrityl)biotinyl}amino-1-hexanol (0.33 g, 0.51 mmol) in CH_2Cl_2 (5 ml) was added 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (162 μl, 0.51 mmol) followed by 1*H*-tetrazole (0.035 g, 0.50 mmol) and the resulting mixture was stirred at room temperature under argon for 15 min. The resulting mixture was diluted with dry AcOEt (30 ml). The organic phase was washed with saturated aqueous NaCl and dried over Na_2SO_4. The dried mixture was evaporated under reduced pressure and the resulting residue further dissolved with CH_2Cl_2 (10 ml). To the mixture was added completely dissolved compound **11** (0.054 g, 0.070 mmol) in CH_2Cl_2 (10 ml) with MS4A, followed by adding 1*H*-tetrazole (0.10 g, 1.4 mmol) and the resulting mixture was stirred at room temperature for further 2 h. To the mixture was added *tert*-butylhydroperoxide (0.10 ml, 0.78 mmol) and stirred at room temperature for further 5 min, and then concentrated under reduced pressure to a half volume. The resulting residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 12:1, 0.5%Et_3N) to afford crude compound **12** (0.084 g, <80%) as a colorless oil.

^{1}H NMR ($CDCl_3$) δ: 1.14–1.60 (14H, m), 1.92–2.23 (10H, m), 2.38 (1H, d, J = 12.8 Hz), 2.69–2.77 (14H, m), 3.04–3.18 (3H, m), 3.72 (6H, s), 3.94–4.32 (18H, m), 4.51–4.76 (4H, m), 5.34–5.47 (1H, m), 5.87 (2H, br s). 6.75 (4H, d, J = 8.8 Hz), 7.04–7.22 (9H, m). IR (KBr) 3450, 2950, 2250, 1750, 1650, 790 cm^{-1}. MS (FAB) m/z 1583 $(M+H)^+$.

4.15. DL-2, 6-Di-*O*-acetyl-3,4,5-tri-*O*-[bis(2-cyanoethyl) phosphoryl]-*myo*-inositol 1-[(6-biotinylamino-1-hexyl) (2-cyanoethyl)phosphate] (13)

To a solution of compound **12** (0.075 g, 0.050 mmol) in CH_2Cl_2 (10 ml) was added Trichloroacetic acid (TCA) (0.10 g, 0.61 mmol) and the resulting mixture was stirred at room temperature for 30 min. The mixture was concentrated under reduced pressure and the resulting residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 3:1) to afford **13** as a colorless oil. (0.058 g, 96%).

^{1}H NMR ($CDCl_3$) δ: 1.19–1.63 (14H, m), 2.06–2.15 (8H, m), 2.62 (1H, d, J = 12.6 Hz), 2.78–2.89 (15H, m), 3.08–3.20 (m, 3H), 3.97–4.42 (18H, m), 4.66–4.80 (2H, m), 5.41 (1H, dd, J = 9.6, 9.5 Hz), 5.87 (1H, s). ^{13}C NMR ($CDCl_3$) δ: 20.0, 20.1, 20.2, 20.2, 20.7, 21.6, 26.1, 26.9, 27.4, 29.5, 29.8, 30.2, 31.0, 31.1, 36.9, 40.2, 40.3, 41.1, 57.0, 61.6, 63.4, 64.3, 64.9, 65.0, 65.0, 65.2, 70.5, 70.6, 70.7, 71.4, 74.2, 77.5, 79.4, 118.6, 118.6, 118.7, 118.8, 118.9, 166.0, 171.4, 171.8, 171.9, 175.9, 176.0. IR (KBr) 3420, 2950, 2860, 2250, 1750, 1700, 1650 cm^{-1}. MS (FAB) m/z 1281 $(M+H)^+$.

4.16. DL-1-*O*-[(6-biotinylamino-1-hexyl) hydrogen phosphoryl]-*myo*-inositol 3,4,5-tris(hydrogenphosphate): Biotinylated DL-*myo*-inositol 1,3,4,5-tetrakisphosphate

To a solution of **13** (0.15 g, 0.11 mmol) in MeOH (5 ml) was added 28% aqueous NH_4OH solution (5 ml) and the resulting mixture was stirred at 55 °C for 10 h. The mixture was concentrated under reduced pressure and the residue was purified by the anion-exchange chromatography, eluting with 0.5–2.5 M ammonium formate containing 0.05–0.25 M formic acid. Column fractions containing biotinylated phosphate were assayed for phosphate by the Briggs test.[19] The fractions containing phosphate group were lyophilized to afford the ammonium salts of biotinylated DL-*myo*-inositol 1,3,4,5-tetrakisphosphate (0.028 g, 27%) as a white solid.

^{1}H NMR (D_2O) δ: 1.22–1.61 (14H, m), 2.10 (2H, t, J = 7.2 Hz), 2.64 (1H, d, J = 13.0 Hz), 2.86 (1H, dd, J = 13.2, 5.0 Hz), 3.03 (2H, t, J = 6.5 Hz), 3.16–3.23 (1H, m), 3.76–3.99 (5H, m), 4.28 (2H, dd, J = 8.1, 4.4 Hz), 4.45–4.60 (5H, m). HRMS(FAB) m/z calcd for $C_{22}H_{42}N_3O_{20}P_4S$ 824.1033. Found: 824.0990 $(M-H)^+$.

4.17. Preparation of the optically active D-3-*O*-benzyl-2,3:5,6-di-*O*-cyclohexylidene-*myo*-inositol (−)-1

Compound (−)-**1** was prepared by the method of Billington et al.[10] Treatment of the racemic **1** with (*R*)-camphanic acid chloride gave a mixture of the diastereomeric camphanate esters. Separation of the diastereomeric esters by recrystallization and chromatography and subsequent hydrolysis of each ester gave the corresponding enatiomeric forms of the alcohol **1** in quantitative yield. {$[\alpha]_D^{25}$ = +23.32 and −23.16 (c 0.1, $CHCl_3$)}. The latter compound was used as the optically active material.

4.18. D-1-*O*-[(6-Biotinylamino-1-hexyl) hydrogen phosphoryl]-*myo*-inositol 3,4,5-tris(hydrogenphosphate): Biotinylated D-*myo*-inositol 1,3,4,5-tetrakisphosphate

The optically resolved D-3-*O*-benzyl-1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol (−)-**1** gave biotinylated D-*myo*-inositol 1,3,4,5-tetrakisphosphate by the same procedure.

^{1}H NMR (D_2O) δ: 1.34–1.72 (14H, m), 2.23 (2H, t, J = 7.3 Hz), 2.77 (1H, d, J = 12.8 Hz), 2.99 (1H, dd, J = 13.4, 4.9 Hz), 3.15–3.19 (2H, m), 3.30–3.34 (1H, m), 3.90–4.20 (5H, m), 4.40–4.65 (5H, m). HRMS(FAB) m/z calcd for $C_{22}H_{42}N_3O_{20}P_4S$ 824.1033. Found: 824.2983 $(M-H)^+$. $[\alpha]_D^{25}$ = +19.6 (c 0.1, H_2O).

4.19. Binding assay of biotinylated Ins(1,3,4,5)P_4 against Grp1 PH domain

4.19.1. Plasmid constructs

cDNA encoding the PH domain of Grp1 (residues 1–120) and PLCδ_1 (residue 11–140) were amplified with PCR from rat brain cDNA library (CLONTECH Labs. Inc.) and subcloned into pBLZ vector as described by Sakaguchi et al.[18] and Sugimoto et al.[20]

4.19.2. Protein purification

The plasmids of Grp1 PH domain were transformed in *E. coli* BL21 (DE)-pLysS star cells, and proteins were extracted by gel filtration using a Hitrap Desalting column (Amersham Pharmacia), with a linear gradient of 0–1 M NaCl in 10 mM phosphate buffer (pH 7.0). The resulting proteins were purified by using Mono S cation exchange column (Amersham Pharmacia), with a linear gradient of 0–1 M NaCl in 10 mM phosphate buffer (pH 7.0), and fractions containing PH domain was collected. The PLCδ_1 PH domain was purified by the same procedure. The purified PH domains were identified by MALDI-TOF mass (Grp1; calcd 13829, found 13834, PLCδ_1; calcd 15723, found 15700) and 15% SDS/polyacrylamide gel visualized with CBB staining.

4.19.3. Pull-down analysis

The K_D of biotinylated Ins(1,3,4,5)P_4 binding of the original Grp1 PH domain was estimated based on SDS–polyacrylamide gel electrophoretic analysis. Aliquot of streptavidin beads (200 μl) which has binding capacity up to 85 nmol/ml free biotin was incubated with 5 nmol biotinylated Ins(1,3,4,5)P_4 solution for 6 h at 4 °C in 30 mM HEPES, 50 mM NaCl, 0.005% Tween 20, 3 mM EDTA (pH 7.4). The beads were washed with the same buffer and divided up into five different volumes (100, 50, 25, 10, 0 μl) in separate tubes, which volumes of beads were converted to biotinylated Ins(1,3,4,5)P_4 concentrations (0.40, 0.20, 0.10, 0.04, 0 μM), respectively. The divided beads were incubated with 500 μl of the Grp1 PH domain (0.10 μM) for 10 min at 4 °C in the same buffer. The beads were separated from the supernatant (**S**, nonbinding fraction) by centrifugation at 1000 g for 1 min at room temperature, the Grp1 PH domain was eluted from the beads with 50 μM unmodified Ins(1,3,4,5)P_4 to give the binding fraction (**B**). The nonbinding fraction (**S**) and (**B**) were precipitated by addition of 500 μL of 20% trichloroacetic acid and centrifugation, and were quantified by analysis of 15% SDS–polyacrylamide gel electrophoresis followed by CBB staining. The quantitation of each band was performed by analyzing the scaled gel data with NIH image (version 1.6) software to integrate the intensity of the dots of which each band was composed. The binding fraction Q = [**B**]/([**B**] + [**S**]) for the Grp1 PH domain to biotinylated Ins(1,3,4,5)P_4 was plotted against biotinylated Ins(1,3,4,5)P_4 concentration. The K_D was derived from the best fit for a theoretical dissociation equation; Qfit = [biotinylated Ins(1,3,4,5)P_4]/(K_D + [biotinylated Ins(1,3,4,5)P_4]), where [biotinylated Ins(1,3,4,5)P_4] is the concentration of biotinylated Ins(1,3,4,5)P_4.

4.19.4. SPR analysis

A BIACORE2000 (GE Healthcare, BIACORE AB., Uppsala, Sweden) was used as the surface plasmon resonance biosensor. To prepare the biotinylated InsP_n (biotinylated Ins(1,3,4,5)P_4, Ins(1,4,5)P_3, and Ins(3,5,6)P_3) immobilized sensorchip surface for the BIACORE, the streptavidin covalently immobilized upon the sensorchip surface (Sensor Chip SA, GE Healthcare, BIACORE AB., Uppsala, Sweden) was washed by injecting three 60-s pulses of 50 mM NaOH in 1 M NaCl at a flow rate of 5 μl/min at 25 °C, subsequently injected 5 μl of 25 nM biotinylated InsP_n in HEPES buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA and 0.005% Tween 20, pH 7.4) over the sensorchip surface at a flow rate of 5 μl/min at 25 °C. The flow buffer contained 10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20 (pH 7.4). Purified PH domains (Grp1 or PLCδ_1) were dialyzed against the flow buffer and injected over the immobilized-InsP_n sensorchip. Association was followed for 3 min and dissociation was measured at a flow rate of 20 μl/min at 25 °C. The surfaces were regenerated by injecting a 15-s pulse of 50 mM NaOH in 1 M NaCl. Analysis of the response was performed using evaluation software supplied with the instrument (BIAevaluation version 3.1). To eliminate small bulk refractive change differences at the beginning and end of each injection, binding responses were referenced by subtracting the response generated across a surface modified with biotin. The K_Ds of biotinylated InsP_n were calculated by the following procedure. The average of Response Unit (RU) at 260–270s of PH domains bound to biotinylated InsP_n were plotted against concentrations of PH domains. To calculate K_Ds, 1.0 μM for Grp1 PH domain and 5.0 μM for PLCδ_1 PH domain were taken over as the maximum concentration based on ten times K_D obtained by pull-down assay (K_D = 0.14 μM for Grp1 PH domain and 0.25 μM for PLCδ_1 PH domain,[7] respectively). The K_Ds (reciprocal of K_As) were obtained by fitting the data to a Steady state affinity model using BIAevaluation 3.1; Req = $K_A * \text{Conc} * \text{Rmax}/(K_A * \text{Conc} * n+1)$, where Req = average of RU at 260–270s of PH domains bound to biotinylated InsP_n, Conc = concentration of PH domains, Rmax = maximum PH domains binding capacity, and n = steric interference factor. The fitting model used [Fit Local] for the binding constant (K_A) and [Fit Global] for the Rmax, respectively, since the amount of immobilized biotinylated InsP_3s and Ins(1,3,4,5)P_4 were identical as described above.

4.19.5. Molecular docking methodology

Docking studies were performed using MOE 2009.10. Crystal structure of Grp1 PH domain and Ins(1,3,4,5)P_4 complex (PDB code: 1FGY) was obtained from the Protein Data Bank to prepare protein for docking studies. Docking procedure was followed using the standard protocol implemented in MOE 2009.10. Grp1 PH domain and Ins(1,3,4,5)P_4 complex was loaded. To the complex was added hydrogen atom and electric charge by Protonate 3D, and the resulting complex was optimized by MMFF94x, and then the dummy atoms were disposed in the docking site by Site finder (alpha site setting; probe radius 1: 1.4 Å, probe radius 2: 1.8 Å, isolated donor/acceptor: 3 Å, connection distance: 2.0 Å, minimum size: 3 Å, and radius: 2 Å). The docking simulation was carried out by ASEDock. The targeting ligands were assigned in ASEDock, and the conformations were integrated by LowModeMD based on the algorithm of conformation analysis (Step1; cutoff: 4.5 Å, RMS (root mean square) gradient: 10 kcal/mol/Å, energy threshold: 500 kcal/mol, Step2; optimize 5 lowest energy or 5 best score conformation, cutoff: 8 Å, RMS gradient: 0.1 kcal/mol/Å).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.09.035.

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