Molecular Recognition at the Phosphatidylinositol 3,4,5-Trisphosphate-Binding Site. Studies Using the Permuted Isomers of Phosphatidylinositol Trisphosphate

Da-Sheng Wang, Ao-Lin Hsu, Xueqin Song, Chi-Ming Chiou, and Ching-Shih Chen*

Division of Medicinal Chemistry and Pharmaceutics, College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536-0082

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Permuted isomers of L-α-phosphatidyl-D-*myo*-inositol trisphosphate (PtdInsP_3), including PtdIns-(3,4,5)P_3, PtdIns(3,4,6)P_3, PtdIns(3,5,6)P_3, and PtdIns(4,5,6)P_3, have been synthesized as part of our effort to understand the underlying principles governing ligand selection for PtdIns(3,4,5)P_3-specific binding proteins. These PtdInsP_3 isomers are examined by using two PtdIns(3,4,5)P_3-dependent functional assays: binding to the C-terminal SH2 domain of the p85 regulatory subunit of PI 3-kinase and platelet aggregation. Our data show that all these isomers bind to the SH2 domain with comparable affinity despite variation in the regioisomeric distribution of phosphate functions. Moreover, all these phospholipids are capable of triggering platelet aggregation with the relative potency of PtdIns(3,4,5)P_3 > PtdIns(3,5,6)P_3 > PtdIns(4,5,6)P_3 > PtdIns(3,4,6)P_3. Evidence suggests that these PtdInsP_3's facilitate cell aggregation by activating Ca^{2+} influx across the plasma membrane. In contrast, other inositol lipids examined including PtdIns(3,4)P_2, PtdIns-(4,5)P_2, PtdIns(3)P, and PtdIns(4)P are ineffective in eliciting the aggregation even at much higher concentrations. Taken together, the present data suggest that the charge density on the phosphorylated inositol ring represents a key factor in determining the phosphoinositide binding specificity of target proteins. It is conceivable that the interaction with the PtdIns(3,4,5)P_3-binding motif requires the participation of all three phosphates on the headgroup of PtdIns(3,4,5)P_3. Consequently, other membrane phosphoinositides (e.g., the bis- and monophosphates) become thermodynamically unfavorable for the binding to these PtdIns(3,4,5)P_3 targets.

Introduction

It is well documented that the lipid products of phosphoinositide 3-kinase (PI 3-kinase) exert their physiological functions by recruiting and/or activating target proteins at the plasma membrane.[1] Among these targets are several Src homology-2 (SH2) domain-containing proteins (such as the p85 regulatory subunit of PI 3-kinase[1a] and PLC-γ1[2]) that specifically bind phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3] and Akt/PKB that specifically binds phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P_2] via its Pleckstrin (PH) domain.[1c] Considering the diversity of membrane phospholipids, selective recognition of the D-3 phosphoinositides represents a crucial issue in addressing the functional role of these signaling enzymes. In the literature, several approaches have been undertaken to explore the underlying mechanism of this discriminative binding. Prestwich and co-workers successfully developed 4-benzoyldihydrocinnamoyl-containing photoaffinity probes to map the phosphoinositide-binding domains.[3] Previously, we identified a unique PtdIns(3,4,5)P_3-binding peptide,[4] WAAKIQASFRGHMARKK (single-letter amino acid code), to which many PtdIns(3,4,5)P_3-binding SH2 domains contain homologous internal sequences (Chen, C.-S., submitted). This model peptide possesses discrete apolar and polybasic segments, confirming that the interfacial binding to PtdIns(3,4,5)P_3 micelles entails both hydrophobic and electrostatic interactions.[4] It is worthy to note that all PtdIns(3,4,5)P_3-binding proteins examined so far exhibit a certain degree of tolerance with respect to the aliphatic chain length. Generally speaking, the C_8- and C_4-analogues of PtdIns(3,4,5)P_3 retain the activity of their C_{16}-counterpart. However, the deacylated product cannot interact with PtdIns(3,4,5)P_3 targets in a productive manner.[5] This observation suggests that while hydrophobic interactions with the fatty-acyl chains are important to the binding, electrostatic bonding plays a relatively more prominent role in the ligand selection. Thus, to gain insight into the mode of interaction between the highly charged headgroup and its binding domain, we have synthesized four permuted analogues of phosphatidylinositol trisphosphates (PtdInsP_3) to scrutinize the regioisomeric effect of phosphate functions on two PtdIns-(3,4,5)P_3-dependent processes: binding to the p85 C-terminal SH2 domain[6] and platelet activation through the activation of the putative Ins(1,3,4,5)P_4 receptor.[7] The present study indicates that the charge density on the

* To whom correspondence should be addressed. Tel.: (606) 257-2300 (ext. 261). Fax: (606) 257-2489. E-mail:cchen1@pop.uky.edu.

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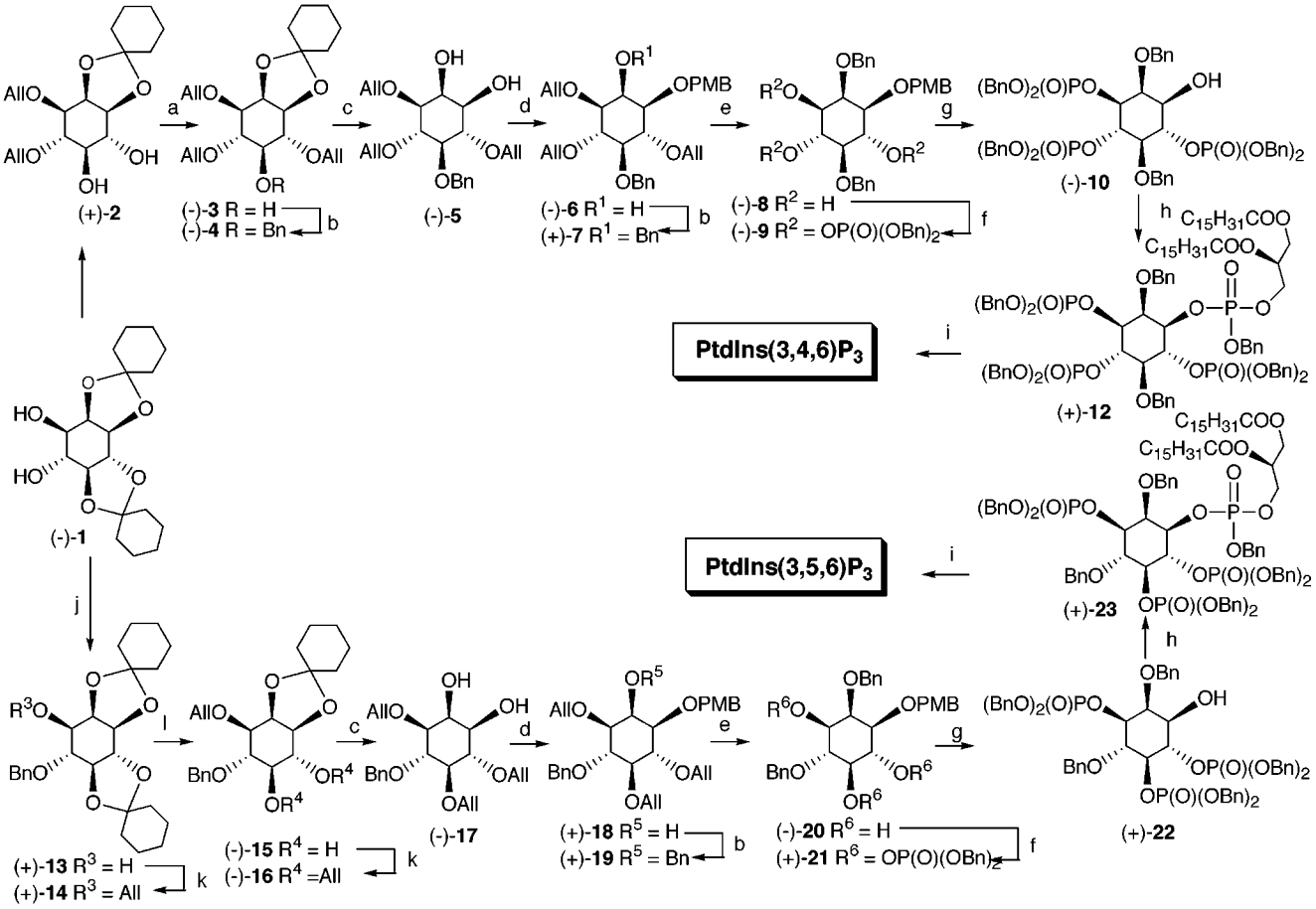
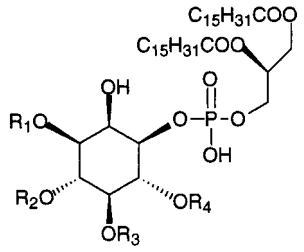
Scheme 1. Synthesis of PtdIns(3,4,6)P_3 and PtdIns(3,5,6)P_3[a]



[a] Key: (a) Bu_2SnO, AllBr, CsF; (b) NaH, BnBr; (c) AcCl/$CH_3OH-CH_2Cl_2$; (d) Bu_2SnO, PMBCl, CsF, NaI; (e) $RhCl(PPh_3)_3$, DABCO, 1 N HCl−acetone; (f) $(BnO)_2PN(i\text{-}Pr)_2$, 1*H*-tetrazole, *m*-CPBA; (g) TFA−CH_2Cl_2−CH_3OH; (h) **11**, 1*H*-tetrazole, *m*-CPBA; (i) palladium black/H_2 (55 psi); (j) Bu_2SnO, BnBr, CsF; (k) NaH, AllBr; (l) AcCl/$CH_3OH-CH_2Cl_2$.

phosphorylated inositol ring represents a key factor in dictating the binding specificity.



PtdIns(3,4,5)P_3: $R_1 = R_2 = R_3 = PO_3H_2$, $R_4 = H$
PtdIns(3,4,6)P_3: $R_1 = R_2 = R_4 = PO_3H_2$, $R_3 = H$
PtdIns(3,5,6)P_3: $R_1 = R_3 = R_4 = PO_3H_2$, $R_2 = H$
PtdIns(4,5,6)P_3: $R_2 = R_3 = R_4 = PO_3H_2$, $R_1 = H$

Results and Discussion

Synthesis of L-α-Phosphatidyl D-*myo*-Inositol Trisphosphates. The four regioisomers were synthesized using optically active **1** as precursors, i.e., PtdIns(3,4,5)P_3, PtdIns(3,4,6)P_3, and PtdIns(3,5,6)P_3 from (−)-**1** (Scheme 1) and PtdIns(4,5,6)P_3 from (+)-**1** (Scheme 2). Both enantiomers were prepared by a facile enzymatic resolution and have versatile applications in the preparation of inositol phosphates and inositol lipids.[8,9] The synthesis of PtdIns(3,4,5)P_3 from (−)-**1** has been described elsewhere[9] and will not be elaborated here.

PtdIns(3,4,6)P_3 and PtdIns(3,5,6)P_3. The efficient use of (−)-**1** to prepare PtdIns(3,4,6)P_3 and PtdIns(3,5,6)-P_3 was made of via key intermediates (−)-**5** and (−)-**17**, respectively.

For PtdIns(3,4,6)P_3 synthesis, (−)-**1** was first subjected to di-O-allylation. Selective removal of the *trans*-cyclohexylidene group to give (+)-**2** was achieved by brief exposure to CH_3COCl/CH_3OH at room temperature.[8a] Since an extended exposure to the acid resulted in the concomitant hydrolysis of the *cis*-1,2-ketal, the reaction was carefully monitored by TLC and was stopped when all the substrate disappeared. Stannylidene-activated alkylation by reacting (+)-**2** with *n*-Bu_2SnO, followed by allyl bromide and CsF, selectively introduced an allyl function at the 6-OH over 5-OH,[8a,9a,10] yielding (−)-**3**. Subsequent 5-O-benzylation and removal of the *cis*-ketal gave the diol (−)-**5**. Regioselective 1-O-*p*-methoxybenzylation of **5** via the aforementioned stannylidene activation followed by 2-O-benzylation afforded a fully protected

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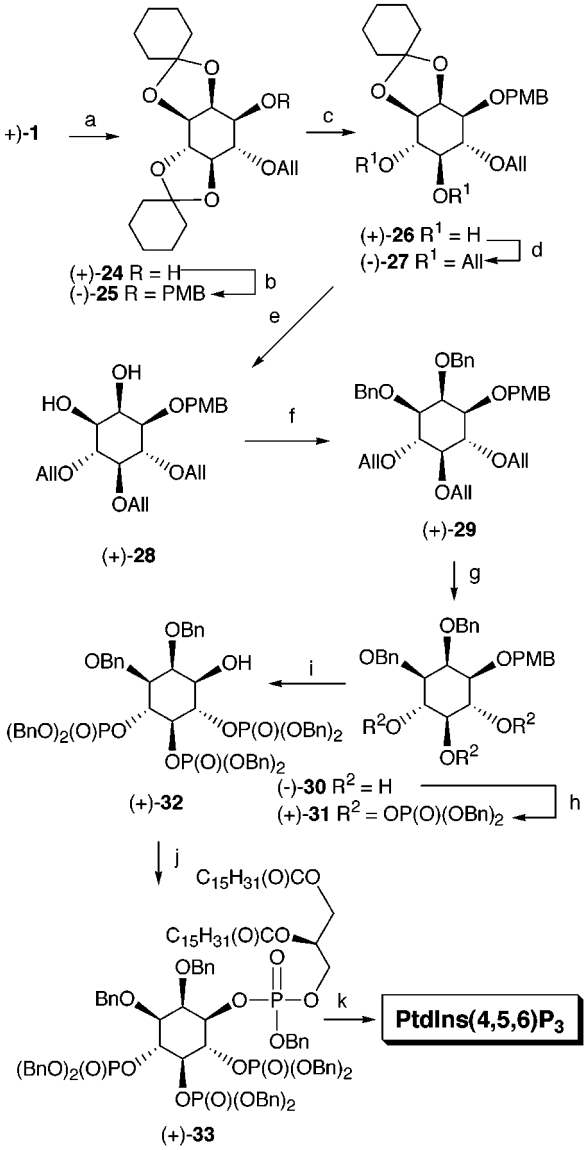
Scheme 2. Synthesis of PtdIns(4,5,6)P_3[a]



[a] Key: (a) Bu_2SnO, AllBr, CsF; (b) NaH, PMBBr; (c) AcCl/$CH_3OH-CH_2Cl_2$; (d) NaH, AllBr; (e) AcCl/$CH_3OH-CH_2Cl_2$; (f) NaH, BnBr; (g) $RhCl(PPh_3)_3$, DABCO, 1 N HCl−acetone; (h) $(BnO)_2PN(i\text{-}Pr)_2$, 1*H*-tetrazole, *m*-CPBA; (i) TFA−CH_2Cl_2−CH_3OH; (j) **11**, 1*H*-tetrazole, *m*-CPBA; (k) palladium black/H_2 (55 psi).

intermediate (+)-**7**. Deallylation of **7** with $RhCl(PPh_3)_3$ and diazabicyclo[2.2.2]octane (DABCO) under reflux, followed by 1 N HCl−acetone (1:9), gave the desired triol (−)-**8**. Phosphorylation of **8** by reacting with dibenzyl *N*,*N*-diisopropylphosphoramidite and 1*H*-tetrazole, followed by *m*-CPBA, afforded the corresponding 3,4,6-tris-(dibenzyl phosphate) (−)-**9**. The *p*-methoxybenzyl function was removed by trifluoroacetic acid (TFA)−CH_2Cl_2−CH_3OH (6:3:1; v/v) to furnish (−)-**10** in order for the subsequent formation of the phosphotriester linkage at the C-1 position. Reaction of **10** with 1,2-di-*O*-palmitoyl-*sn*-glycerol-3-(benzyl *N*,*N*-diisopropylphosphoramidite) (**11**) in the presence of 1*H*-tetrazole, followed by *m*-CPBA, gave the perbenzylated derivative (+)-**12** that underwent hydrogenolysis to afford PtdIns(3,4,6)P_3 with an overall yield of 30% from (−)-**1**.

Alternatively, treatment of (−)-**1** with *n*-Bu_2SnO followed by benzyl bromide and CsF resulted in regioselective benzylation at the 4-OH over 3-OH,[8b] affording (+)-**13**. Subsequent 3-O-allylation gave (+)-**14** that was subjected to the selective removal of the *trans*-ketal and di-O-allylation, in tandem, to furnish (−)-**16**. Hydrolysis of the *cis*-1,2-di-*O*-cyclohexylidene group afforded the key intermediate (−)-**17**. The synthetic route for PtdIns-(3,5,6)P_3 from **17** paralleled that converting (−)-**5** to PtdIns(3,4,6)P_3. The overall yield of converting (−)-**1** into PtdIns(3,5,6)P_3 was 22%.

PtdIns(4,5,6)P_3. It has been demonstrated in the literature that stannylidene-activated alkylation of **1** is highly selective for the 6-OH over 1-OH.[9b,11] Consequently, (+)-**24** was provided by reacting (+)-**1** with *n*-Bu_2SnO, followed by allyl bromide and CsF. Subsequent 1-*O*-*p*-methoxybenzylation gave (−)-**25**. Selective hydrolysis of the *trans*-ketal of **25** by a brief acid treatment yielded (+)-**26**. The diol was subjected to 4,5-di-*O*-allylation, followed by acid hydrolysis and bisbenzylation, to afford the fully protected intermediate (+)-**29**. Subsequent transformations leading to PtdIns(4,5,6)P_3 were similar to that described above for converting (+)-**7** to PtdIns(3,4,6)P_3. The overall yield was 27% from (+)-**1**.

Binding to the p85 C-Terminal SH2 Domain. SH2 domains are conserved sequences of approximately 100 amino acids that are able to bind phosphotyrosine in a sequence-specific manner.[12] Previously, Cantley and coworkers reported that PtdIns(3,4,5)P_3 bound directly and selectively to the SH2 domains of the 85 kDa subunit of PI 3-kinase.[6] It is noteworthy that this binding provides a negative feedback control of PI 3-kinase by disrupting the binding of p85 to phosphotyrosine via the SH2 domains.[6] In addition, such PtdIns(3,4,5)P_3 recognition is of implied significance to the membrane localization or activation of SH2-containing enzymes. For example, PtdIns(3,4,5)P_3 has been demonstrated to activate PLC-γ isozymes by interacting with their SH2 domains.[2]

The p85 subunit of PI 3-kinase contains two SH2 domains, designated as the N-terminal (NT) and C-terminal (CT) SH2 domains.[6] The CT-SH2 domain has been reported to bind PtdIns(3,4,5)P_3 with high affinity, while its affinity with phosphatidylinositol bisphosphates such as PtdIns(3,4)P_2 and PtdIns(4,5)P_2 was significantly lower.[6] Considering the stringent requirement of the three phosphate groups for binding, we examined the stereochemical effect on the protein−phospholipid interaction. The full-length CT-SH2 domain was prepared by expression as a glutathione S-transferase (GST) fusion protein in *Escherichia coli* according to a reported procedure,[6] followed by affinity purification and Factor Xa hydrolysis to liberate the SH2 domain from the immobilized GST. To assess the binding affinity with individual PtdInsP_3 isomers, equilibrium binding based on the quenching of SH2 intrinsic tryptophan fluorescence was performed. These phospholipids alone did not have appreciable emission (data not shown), but induced a dose-dependent and saturable reduction in the fluorescence intensity of the CT-SH2 domain in a similar manner. Figure 1 A−D displays the spectral change induced by PtdIns(3,4,5)P_3, PtdIns(3,5,6)P_3, PtdIns(3,4,6)-P_3, and PtdIns(4,5,6)P_3, respectively. The dissociation constants (K_d) were estimated from the linear relationship between $1/[1 - (\Delta F/\Delta F_{max})]$ and [phosphoinositide-

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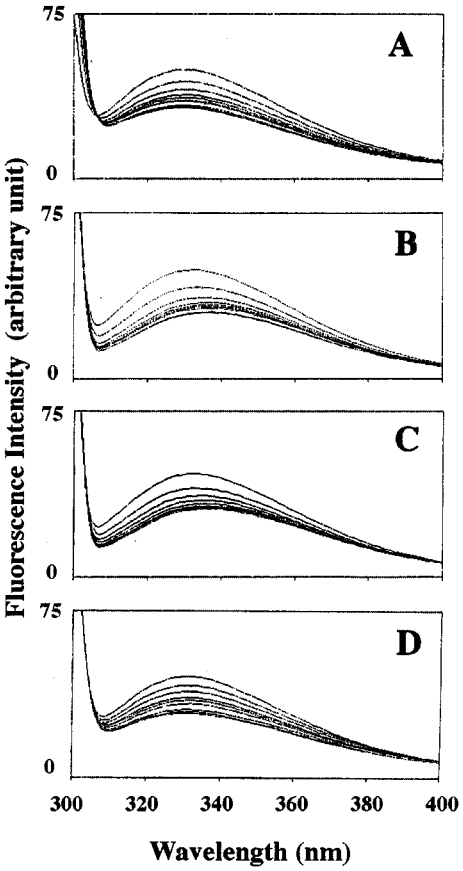

Figure 1. Tryptophan fluorescence emission spectra of the p85 CT-SH2 domain in the presence of various amounts of (A) PtdIns(3,4,5)P_3, (B) PtdIns(3,5,6)P_3, (C) PtdIns(3,4,6)P_3, and (D) PtdIns(4,5,6)P_3. Spectra were recorded with 12.5 μM p85 CT-SH2 domain according to the method described in the Experimental Section. Molar ratios of the phospholipid to the SH2 domain were (top to bottom) (A) for PtdIns(3,4,5)P_3: 0:1, 1:1, 1.5:1, 2.5:1, 3.5:1, 4.5:1, 5.5:1, 6.5:1, and 7:1 (saturation); (B) for PtdIns(3,5,6)P_3: 0:1, 2:1, 2.5:1, 3.5:1, 4.5:1, 5.5:1, 6.5:1, and 8:1 (saturation); (C) for PtdIns(3,4,6)P_3: 0:1, 1:1, 2:1, 3:1, 4.5:1, 6.5:1, and 8:1 (saturation); (D) for PtdIns(4,5,6)P_3: 0:1, 1:1, 1.5:1, 2.5:1, 3.5:1, 4.5:1, 6:1, 7:1, and 8:1 (saturation).

$]_{total}/(\Delta F/\Delta F_{max})$. The estimated K_d values were as follows: PtdIns(3,4,5)P_3, 15.3 $\pm$ 1.7 μM; PtdIns(3,5,6)P_3, 14.2 $\pm$ 2.5 μM; PtdIns(3,4,6)P_3, 15.1 $\pm$ 4.4 μM; PtdIns(4,5,6)P_3, 23.8 $\pm$ 3.0 μM. In contrast, the binding affinity for PtdIns(3,4)P_2 and PtdIns(4,5)P_2 was at least 10-fold lower (spectra not shown), indicating that the lipid binding effect on SH2 fluorescence was specific.

These binding data indicate that the regioisomeric arrangement of the phosphates did not have a significant impact on the binding to the CT-SH2 domain. Apparently, the charge density on the polyphosphorylated inositol ring represented a key factor in the phosphoinositide recognition. The effective binding requires the presence of three adjacent phosphates, regardless of their relative positions on the ring. This information is thus of importance to the rational design of inhibitors to intervene the binding of the p85 subunit to tyrosine-phosphorylated proteins via the SH2 domain.

Platelet Activation. Previously, we reported that exogenous PtdIns(3,4,5)P_3-induced platelet aggregation in a Ca^{2+} and dose-dependent manner.[7] This platelet activation was highly structurally specific and was not noted with other inositol lipids examined including

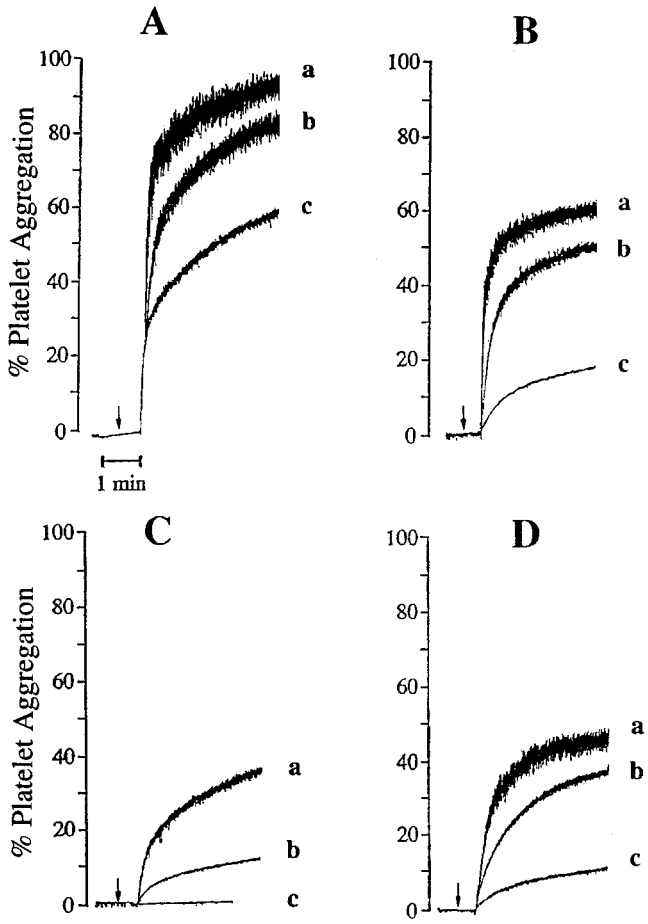

Figure 2. PtdInsP_3-induced platelet aggregation. Washed rabbit platelets were treated with (A) PtdIns(3,4,5)P_3, (B) PtdIns(3,5,6)P_3, (C) PtdIns(3,4,6)P_3, or (D) PtdIns(4,5,6)P_3 at 80 μM (trace a), 40 μM (trace b), or 20 μM (trace c). The arrow indicates the time point at which individual phosphoinositides were added.

PtdIns(3,4)P_2, PtdIns(4,5)P_2, PtdIns(3)P, and PtdIns(4)P. Evidence suggests that PtdIns(3,4,5)P_3 triggered platelet activation by facilitating transmembrane Ca^{2+} influx. It is documented that platelets contain an Ins(1,3,4,5)P_4-mediated Ca^{2+} transport system on plasma membranes.[13] Although the mechanism by which Ins(1,3,4,5)P_4 operates remains enigmatic, the putative Ins(1,3,4,5)P_4 receptor has been purified and characterized.[13] PtdIns(3,4,5)P_3 was able to compete with Ins(1,3,4,5)P_4 for the receptor binding with an identical potency.[7] This cross-reactivity may in part be attributable to the largely shared structural motifs between these two molecules. In fact, Ins(1,3,4,5)P_4-binding sites on platelet plasma membranes might represent PtdIns(3,4,5)P_3 receptors since PtdIns(3,4,5)P_3 contained Ins(1,3,4,5)P_4 as its headgroup.[13b]

In this study, we examined the potency of permuted PtdInsP_3 analogues vis-à-vis PtdIns(3,4,5)P_3 in inducing platelet activation. Washed rabbit platelets were exposed to various exogenous phospholipids in the presence of 1 mM Ca^{2+}. Figure 2 A−D displays the dose-dependent effect of PtdIns(3,4,5)P_3, PtdIns(3,5,6)P_3, PtdIns(3,4,6)-

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P_3, and PtdIns(4,5,6)P_3, respectively, on platelet aggregation (trace a, 80 μM; trace b, 40 μM, trace c, 20 μM).

Among these, the relative potency was in the order of PtdIns(3,4,5)P_3 > PtdIns(3,5,6)P_3 > PtdIns(4,5,6)P_3 > PtdIns(3,4,6)P_3. PtdIns(3,4,5)P_3 was severalfold more effective than other isomers in mediating the platelet aggregation. It is worth mentioning that PtdIns(4,5)P_2 and PtdIns(3,4)P_2 at comparable concentrations failed to elicit appreciable cell activation (data not shown). The fact that all PtdInsP_3's tested were active in triggering platelet activation while PtdIns(3,4)P_2 and PtdIns(4,5)-P_2 were not active underscores the notion that the charge density on the inositol ring was crucial to the receptor binding. Moreover, in this case, the ligand specificity also depended on the regioisomeric distribution of phosphate functions on the inositol ring. It appears that the phosphate function on the 5-OH played a major role in the activity.

Taken together, these observations suggest that the ligand specificity of PtdIns(3,4,5)P_3-binding proteins is achieved through the participation of all three phosphate functions of PtdIns(3,4,5)P_3 in the binding. In principle, a difference in the binding energy ($\Delta\Delta G_b$) of a few kcal/mol will lead to a difference of several orders of magnitudes in the dissociation constants (K_d) [$\Delta\Delta G_b = -RT \ln(K_d/K_d')$]. Consequently, PtdIns(3,4)P_2 and PtdIns(4,5)-P_2 become thermodynamically unfavorable for binding to PtdIns(3,4,5)P_3-specific targets. Further investigations on the mode of interaction by NMR are currently in progress in the laboratory.

Experimental Section

Material and Methods. Racemic 1,2:5,6-di-*O*-cyclohexylidene-*myo*-inositol ((±)-**1**) was prepared according to the procedure by Garegg et al.[14] Optically active **1** was prepared with good yield by an enzymatic method in which the 6-butyryl ester of **1** was subjected to enantioselective hydrolysis by porcine pancreatic lipase (sigma, Type II) in a biphasic system consisting of hexanes−ether/water.[8] The optical purity of (+)- and (−)-**1** thus prepared was greater than 98% enantiomeric excess after recrystallization. 1,2-Di-*O*-palmitoyl-*sn*-glycerol 3-(benzyl *N*,*N*-diisopropylphosphoramidite) (**11**) was prepared using a method described by Dreef et al.[15] (+)-3,4-Di-*O*-allyl-1,2-*O*-cyclohexylidene-*myo*-inositol (+)-**2** was synthesized from (−)-**1** as previously described.[8a]

(−)-3,4,6-Tri-*O*-allyl-1,2-*O*-cyclohexylidene-*myo*-inositol (3). A mixture of (+)-**2** (158 mg, 0.41 mmol), Bu_2SnO (128 mg, 0.51 mmol), and toluene (25 mL) was stirred under reflux, with azeotropic removal of water, for 2 h, and then concentrated to dryness. To the residue were added DMF (4 mL), CsF (177 mg, 1.16 mmol), and allyl bromide (60.5 μL, 0.52 mmol) at −15 °C. After being stirred at −15 °C for 1 h, the reaction mixture was allowed to warm to room temperature and stirred for an additional 16 h. The solution was diluted with CH_2Cl_2 (20 mL), washed with water, dried, and concentrated. Column chromatography (hexanes−ether, 20:1 → 10:1) of the residue gave (−)-**3** (syrup, 166 mg, 90%): $[\alpha]^{23}_D$ = −2.3° (*c* 1.3, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.26−1.79 (m, 10 H), 2.62 (s, 1 H), 3.33 (t, *J* = 9.6 Hz, 1 H), 3.48−3.60 (m, 3 H), 3.99 (t, *J* = 6.6 Hz, 1 H), 4.13−4.21 (m, 4H), 4.26−4.36 (m, 3 H), 5.14 (d, *J* = 9.9 Hz, 3 H), 5.26 (d, *J* = 17.4 Hz, 3 H), 5.81−5.97 (m, 3 H); MS (EI) *m*/*z* (rel inten) 380 (M^+, 10), 337.0 (33), 41 (100).

(−)-3,4,6-Tri-*O*-allyl-5-*O*-benzyl-1,2-*O*-cyclohexylidene-*myo*-inositol (4). A solution of (−)-**3** (160 mg, 0.42 mmol) in DMF (4 mL) was treated with NaH (20.2 mg, 0.82 mmol) at 0 °C under argon for 30 min, followed by benzyl bromide (106 μL, 0.84 mmol). The reaction mixture was allowed to attain 40 °C and stirred for an additional 2 h. Excess NaH was destroyed with CH_3OH, and the solution was diluted with ethyl acetate (25 mL), washed with water, dried, and concentrated. Column chromatography (hexanes−ether, 25:1) of the residue afforded (−)-**4** (syrup, 194 mg, 98%): $[\alpha]^{23}_D$ = −10.6° (*c* 3.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.40−1.80 (m, 10 H), 3.31 (t, *J* = 9.6 Hz, 1 H), 3.57 (dd, *J* = 3.6, 8.7 Hz, 1 H), 3.63 (dd, *J* = 7.2, 9.6 Hz, 1 H), 3.74 (t, *J* = 8.4 Hz, 1 H), 4.05 (dd, *J* = 5.4, 6.9 Hz, 1 H), 4.23−4.40 (m, 7 H), 4.79 (s, 2 H), 5.15−5.35 (m, 6 H), 5.91−6.02 (m, 3 H), 7.26−7.39 (m, 5 H); MS (EI) *m*/*z* (rel inten) 470 (M^+, 0.1), 427 (6), 379 (2), 91 (100).

(−)-3,4,6-Tri-*O*-allyl-5-*O*-benzyl-*myo*-inositol (5). A solution of (−)-**4** (157 mg, 0.33 mmol) in $CH_3OH-CH_2Cl_2$ (1:3, 12 mL) was stirred with acetyl chloride (25 μL) at 23 °C for 1 h. Triethylamine (100 μL) was added, and the solution was concentrated. Column chromatography (hexanes−ether, 10:1 → 1:3) of the residue yielded (−)-**5** (amorphous, 114 mg, 87%): $[\alpha]^{23}_D$ = −6.9° (*c* 1.3, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 2.44 (br s, 2 H), 3.35 (dd, *J* = 9, 18 Hz, 2 H), 3.47 (m, 1 H), 3.62−3.76 (m, 2 H), 4.19−4.45 (m, 7 H), 4.85 (dd, *J* = 10.5, 29.1 Hz, 2 H), 5.20 (dd, *J* = 9.6, 17.4 Hz, 3 H), 5.30 (t, *J* = 17.4 Hz, 3 H), 5.91−6.01 (m, 3 H), 7.26−7.36 (m, 5 H); MS (EI) *m*/*z* (rel inten) 390 (M^+, 1.5), 347 (12), 299 (6.5), 91 (100).

(−)-3,4,6-Tri-*O*-allyl-5-benzyl-1-*O*-(*p*-methoxybenzyl)-*myo*-inositol (6). A solution of (−)-**5** (110 mg, 0.28 mmol), Bu_2SnO (84 mg, 0.34 mmol), and toluene (25 mL) was stirred under reflux, with azeotropic removal of water, for 2 h, and then concentrated to dryness. To the residue were added DMF (4 mL), CsF (107 mg, 0.71 mmol), NaI (5 mg, 0.03 mmol), and 4-methoxybenzyl chloride (61 μL, 0.42 mmol) at −15 °C. After being stirred at −15 °C for 1 h, the reaction mixture was allowed to warm to room temperature and stirred for an additional 16 h. The solution was diluted with CH_2Cl_2 (20 mL), washed with water, dried, and concentrated. Column chromatography (hexanes−ether, 25:1 → 10:1) of the residue provided (−)-**6** (amorphous, 124 mg, 84%): $[\alpha]^{23}_D$ = −1.3° (*c* 1.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 2.38 (br s, 1 H), 3.17 (d, *J* = 9.9 Hz, 1 H), 3.27 (t, *J* = 9.6 Hz, 2 H), 3.68−3.73 (m, 2 H), 3.75 (s, 3 H), 4.10−4.12 (m, 4 H), 4.26−4.29 (m, 3 H), 4.59 (s, 2 H), 4.76 (d, *J* = 0.9 Hz, 2 H), 5.06−5.26 (m, 6 H), 5.85−5.95 (m, 3 H), 6.84 (d, *J* = 8.1 Hz, 2 H), 7.20−7.40 (m, 7 H); MS (EI) *m*/*z* (rel inten) 509 (M^+ − H, 2), 469 (0.5), 419 (0.7), 121 (100).

(+)-3,4,6-Tri-*O*-allyl-2,5-di-*O*-benzyl-1-*O*-(*p*-methoxybenzyl)-*myo*-inositol (7). Conventional benzylation of (−)-**6** (124.5 mg, 0.24 mmol), as described for (−)-**4**, gave (+)-**7** (syrup, 140 mg, 96%): $[\alpha]^{23}_D$ = +2.7° (*c* 2.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 3.13 (dd, *J* = 2.4, 9.9 Hz, 1 H), 3.25 (dd, *J* = 2.4, 9.9 Hz, 1 H), 3.29 (t, *J* = 9.0 Hz, 1 H), 3.73−3.84 (m, 5 H), 3.89 (t, *J* = 2.1 Hz, 1 H), 4.00−4.04 (m, 2 H), 4.22−4.34 (m, 4 H), 4.53 (q, *J* = 11.4, 21.3 Hz, 2 H), 4.77 (s, 4 H), 5.06−5.13 (m, 3 H), 5.18−5.27 (m, 3 H), 5.78−5.98 (m, 3 H), 6.84 (d, *J* = 8.7 Hz, 2 H), 7.20−7.35 (m, 12 H); MS (EI) *m*/*z* (rel inten) 559.2 (M^+ − 41, 0.1), 509.2 (0.2), 91 (100).

(−)-2,5-Di-*O*-benzyl-1-*O*-(*p*-methoxybenzyl)-*myo*-inositol (8). A mixture of (+)-**7** (80 mg, 0.13 mmol), $RhCl(PPh_3)_3$ (55.5 mg, 0.60 mmol), diazabicyclo[2.2.2]octane, and ethanol (2 mL) was refluxed for 4 h and cooled to room temperature. The solution was diluted with ether (20 mL), washed with water, dried, and concentrated. The residue was mixed with acetone−1 N HCl (9:1, 30 mL), refluxed for 30 min, and cooled to room temperature, and $NaHCO_3$ was added. The mixture was concentrated, diluted with water, and extracted with ether (3 × 10 mL). The organic phase was dried and concentrated. Column chromatography (hexanes−ether, 10:1 → 0:1) of the residue yielded (−)-**8** (amorphous, 56 mg, 88%): $[\alpha]^{23}_D$ = −2.9° (*c* 2.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 2.30 (br s, 1 H), 2.54 (br s, 2 H), 3.21−3.31 (m, 2 H), 3.40−3.44 (m, 2 H), 3.82 (s, 3 H), 4.05−4.13 (m, 2 H), 4.52−4.82 (m, 4 H), 4.94−4.99 (m, 2 H), 6.88 (d, *J* = 7.8 Hz, 2 H), 7.26−7.38 (m, 12 H); MS (EI) *m*/*z* (rel inten) 479.9 (M^+, 0.1), 388.9 (3), 268.9 (5), 91 (100).

(14) Garegg, P. J.; Iversen, T.; Johansson, R.; Lindberg, B. *Carbohydr. Res.* **1984**, *130*, 322.

(15) Dreef, C. E.; Elie, C. J. J.; Hoogerhout, P.; van der Marel, G. A.; van Boom, J. H. *Tetrahedron Lett.* **1988**, *29*, 6513.

(−)-2,5-Di-*O*-benzyl-1-*O*-(*p*-methoxybenzyl)-*myo*-inositol-3,4,6-tris(dibenzyl phosphate) (9). A solution of 1*H*-tetrazole (159 mg, 0.23 mmol) and dibenzyl *N*,*N*-diisopropylphosphoramidite (455 mg, 0.13 mmol) in dry dichloromethane (4 mL) was stirred at 23 °C under argon for 30 min, and (−)-**8** (50 mg, 0.10 mmol) was added in one portion. The mixture was kept under the same conditions for an additional 4 h, cooled to −40 °C, and then treated with *m*-CPBA (500 mg, 57% purity, 0.94 mmol). The solution was stirred at −40 °C for 30 min and then allowed to attain room temperature. After 1 h, the mixture was diluted with CH_2Cl_2 (20 mL), washed with, in tandem, 10% aqueous Na_2SO_3, 10% aqueous $NaHCO_3$, and brine, dried, and concentrated. Column chromatography (hexanes−ether, 25:1 → 1:1) of the residue provided (−)-**9** (syrup, 119 mg, 91%): $[\alpha]^{23}_D = -0.1°$ (*c* 3, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 3.32−3.40 (m, 1 H), 3.43−3.50 (m, 1 H), 3.80 (s, 3 H), 4.05−4.13 (m, 2 H), 4.53−4.99 (m, 20 H), 6.78 (d, J = 7.8 Hz, 2 H), 6.94−7.36 (m, 42 H); ^{31}P NMR ($CDCl_3$, 85% H_3PO_4 as external standard) δ −1.30, −1.64, −1.75; HRMS (MALDI) *m*/*z* (rel inten) 1261.405 (M + H, 100).

(−)-2,5-Di-*O*-benzyl-3,4,6-tris(dibenzyl phosphate)-*myo*-inositol (10). Removal of the *p*-methoxybenzyl function was achieved by exposing (−)-**9** (110 mg, 0.083 mmol) to trifluoroacetic acid−CH_2Cl_2−CH_3OH (6:3:1, 10 mL) at 0 °C for 1 h, and 10% aqueous $NaHCO_3$ was added. The organic layer was washed with 10% aqueous $NaHCO_3$ and water, dried, and concentrated. Column chromatography (hexanes−ether, 5:1 → 1:2) of the residue afforded (−)-**10** (syrup, 91 mg, 92%): $[\alpha]^{23}_D = -2.1°$ (*c* 2.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 3.32−3.40 (m, 1 H), 3.43−3.50 (m, 1 H), 4.05−4.13 (m, 2 H), 4.53−4.99 (m, 18 H), 6.94−7.36 (m, 40 H); ^{31}P NMR ($CDCl_3$, 85% H_3PO_4 as external standard) δ 1.08, −1.34, −1.62; HRMS (MALDI) *m*/*z* (rel inten) 1141.339 (M + H, 100).

(+)-1-*O*-(1,2-Di-*O*-palmitoyl-*sn*-glycerol-3-benzyloxyphosphoryl)-2,5-di-*O*-benzyl-*myo*-inositol-3,4,6-tris(dibenzyl phosphate) (12). A solution of 1,2-di-*O*-palmitoyl-*sn*-glycerol 3-(benzyl *N*,*N*-diisopropylphosphoramidite) (**11**) (56 mg, 0.069 mmol) and 1*H*-tetrazole (8.2 mg, 0.053 mmol) in dry CH_2Cl_2 (2 mL) was stirred at 23 °C under argon for 30 min, and (−)-**10** (20 mg, 0.018 mmol) was added in one portion. The mixture was kept under the same conditions for an additional 4 h, cooled to −40 °C, and treated with *m*-CPBA (30 mg, 57% purity, 0.056 mmol). The mixture was stirred at −40 °C for 30 min and then allowed to attain room temperature for 1 h, diluted with CH_2Cl_2 (20 mL), washed with, in tandem, 10% aqueous Na_2SO_3, 10% aqueous $NaHCO_3$, and brine, dried, and concentrated. Column chromatography (hexanes−ether, 25:1 → 1:1) of the residue provided (+)-**12** (syrup, 28 mg, 86%): $[\alpha]^{23}_D = +0.2°$ (*c* 2.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 6.6 Hz, 6 H), 1.21−1.28 (m, 48 H), 1.40−1.58 (m, 4 H), 2.15−2.24 (m, 4 H), 3.47−3.60 (m, 2 H), 3.96−4.44 (m, 6 H), 4.68−5.08 (m, 21 H), 6.85−7.40 (m, 45 H); ^{31}P NMR ($CDCl_3$, 85% H_3PO_4 as external standard) δ −0.351 (2P), −0.89 (br, 2 P); HRMS (MALDI) *m*/*z* (rel inten) 1862.858 (M + 2 H, 100).

(+)-L-α-Phosphatidyl-D-*myo*-inositol 3,4,6-Triphosphate, Dipalmitoyl [PtdIns(3,4,6)P_3]. A solution of (+)-**12** (28 mg, 0.015 mmol) and palladium black (20 mg) in 80% aqueous ethanol (2 mL) was shaken under H_2 (55psi) for 16 h, filtered, and concentrated. The filtrate was lyophilized to furnish PtdIns(3,4,6)P_3 (lyophilized powder, 15.5 mg, 98%): $[\alpha]^{23}_D = +3.6°$ (*c* 6, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.96 (t, J = 7.2 Hz, 6 H), 1.31 (s, 48 H), 1.52−1.68 (m, 4 H), 2.30−2.37 (m, 4 H), 3.45 (t, J = 9.3 Hz, 1 H), 3.86 (t, J = 9.6 Hz, 1 H), 4.02−4.25 (m, 5 H), 4.41−4.57 (m, 3 H), 5.22−5.30 (m, 1 H); ^{31}P NMR ($CDCl_3$, 85% H_3PO_4 as external standard) δ −0.715 (2P), −1.063 (1P), −2.93 (1P); HRMS MALDI) *m*/*z* (rel inten) 1049.395 (M − H, 100).

(+)-4-*O*-Benzyl-1,2:5,6-di-*O*-cyclohexylidene-*myo*-inositol (13). A mixture of (−)-**1** (180 mg, 0.53 mmol), Bu_2SnO (144.7 mg, 0.59 mmol), and toluene (25 mL) was stirred under reflux, with azeotropic removal of water, for 2 h and then concentrated to dryness. To the residue were added DMF (4 mL), CsF (201 mg, 1.34 mmol), and benzyl bromide (94 μL, 1.13 mmol) at −15 °C. After being stirred at −15 °C for 1 h, the reaction mixture was allowed to warm to room temperature and stirred for 16 h. The solution was then diluted with CH_2Cl_2 (25 mL), washed with water, dried, and concentrated. Column chromatography (hexanes−ether, 20:1 → 10:1) of the residue afforded (+)-**13** (syrup, 193 mg, 85%): $[\alpha]^{23}_D = +0.8°$ (*c* 2.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.41−1.75 (20 H), 2.65 (d, J = 1.5 Hz, 1 H), 3.58 (dd, J = 7.6, 10.5 Hz, 1 H), 3.93 (dd, J = 2, 7.8 Hz, 1 H), 4.06−4.07 (m, 1 H), 4.23 (dd, J = 7.6, 10.5 Hz, 1 H), 4.38 (t, J = 7.5 Hz, 1 H), 4.46 (dd, J = 3.6, 7.5 Hz, 1 H), 4.75 (q, J = 11.8, 35 Hz, 2 H), 7.28−7.43 (m, 5 H); MS (EI) *m*/*z* (rel inten) 430 (M^+, 6), 91 (100).

(+)-3-*O*-Allyl-4-*O*-benzyl-1,2:5,6-di-*O*-cyclohexylidene-*myo*-inositol (14). A solution of (+)-**13** (180 mg, 0.42 mmol) in DMF (3 mL) was treated with NaH (24 mg, 85% purity, 0.84 mmol) at 0 °C under argon for 30 min, followed by allyl bromide (73 μL, 0.84 mmol), and then the reaction mixture was allowed to attain 40 °C for 1 h. Excess NaH was destroyed with CH_3OH. The solution was diluted with ethyl acetate (25 mL), washed with water, dried, and concentrated. Column chromatography (hexanes−ether, 50:1 → 20:1) of the residue gave (+)-**14** (syrup, 190 mg, 97%): $[\alpha]^{23}_D = +6.3°$ (*c* 4, $CHCl_3$); ^{1}H NMR δ 1.34−1.73 (m, 20 H), 3.50 (dd, J = 7.7, 10.7 Hz, 1 H), 3.69 (t, J = 3.1 Hz, 1 H), 3.82 (dd, J = 2.7, 7.7 Hz, 1 H), 4.01−4.12 (m, 3 H), 4.31 (t, J = 7.0 Hz, 1 H), 4.39 (dd, J = 3.8, 6.9 Hz, 1 H), 4.75 (q, J = 11.9, 43.3 Hz, 2 H), 5.13−5.31 (m, 2 H), 5.80−5.93 (m, 1 H), 7.25−7.39 (m, 5 H); MS (EI) *m*/*z* (rel inten) 470.2 (M^+, 0.5), 427.1 (6), 390.1 (10), 379.1 (4), 91 (100).

(−)-3-*O*-Allyl-4-*O*-benzyl-1,2-*O*-cyclohexylidene-*myo*-inositol (15). A solution of (+)-**14** (176 mg, 0.37 mmol) in CH_3OH−CH_2Cl_2 (1:3, 6 mL) was stirred with acetyl chloride (8 μL) at 23 °C for 10 min. Triethylamine (30 μL) was then added, and the solution was concentrated. Column chromatography (hexanes−ether, 10:1 → 1:3) of the residue yielded (−)-**15** (amorphous, 117 mg, 80%): $[\alpha]^{23}_D = -3.0°$ (*c* 2.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.30−1.80 (m, 10 H), 2.74 (br s, 2 H), 3.32 (t, J = 8.6 Hz, 1 H), 3.63−3.76 (m, 3 H), 3.98 (dd, J = 2.2, 7.5 Hz, 1 H), 4.21−4.25 (m, 2 H), 4.44 (dd, J = 3.8, 5.2 Hz, 1 H), 4.71 (d, J = 11.1 Hz, 1 H), 5.01 (d, J = 11.1 Hz, 1 H), 5.19−5.35 (m, 2 H), 5.90−6.03 (m, 1 H), 7.26−7.37 (m, 5 H); MS (EI) *m*/*z* (rel inten) 389.1 (M^+ − H, 2), 299.1 (12), 91 (100).

(−)-3,5,6-Tri-*O*-allyl-4-*O*-benzyl-1,2-*O*-cyclohexylidene-*myo*-inositol (16). A solution of (−)-**15** (100 mg, 0.26 mmol) in DMF (3 mL) was treated with NaH (30 mg, 85% purity, 1.04 mmol) at 0 °C under argon for 30 min, followed by allyl bromide (90 μL, 1.04 mmol). The reaction mixture was allowed to warm to 40 °C and stirred for 1 h. Excess NaH was destroyed with CH_3OH. The mixture was diluted with ethyl acetate (25 mL), washed with water, dried, and concentrated. Column chromatography (hexanes−ether, 50:1 → 20:1) of the residue provided (−)-**16** (syrup, 114 mg, 95%): $[\alpha]^{23}_D = -4.2°$ (*c* 2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.37−1.78 (m, 10 H), 3.21 (dd, J = 8.8, 9.7 Hz, 1 H), 3.55−3.63 (m, 2 H), 3.81 (t, J = 8.7 Hz, 1 H), 4.02 (dd, J = 5.4, 7.0 Hz, 1 H), 4.21−4.28 (m, 5 H), 4.31−4.38 (m, 2 H), 4.80 (q, J = 10.7, 15.8 Hz, 2 H), 5.13−5.32 (m, 6 H), 5.89−6.01 (m, 3 H), 7.28−7.40 (m, 5 H); MS (EI) *m*/*z* (rel inten) 470 (M^+, 2), 380 (12), 91 (100).

(−)-3,5,6-Tri-*O*-allyl-4-*O*-benzyl-*myo*-inositol (17). Removal of the *cis*-cyclohexylidene group of (−)-**16** (100 mg, 0.21 mmol) with acetyl chloride, as described for (−)-**5**, yielded (−)-**17** (amorphous, 75 mg, 90%): $[\alpha]^{23}_D = -6.0°$ (*c* 2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 2.61 (d, J = 6.7 Hz, 2 H), 3.25 (t, J = 9.4 Hz, 1 H), 3.32 (dd, J = 2.9, 9.5 Hz, 1 H), 3.38−3.46 (m, 1 H), 3.64 (t, J = 9.5 Hz, 1 H), 3.81 (t, J = 9.5 Hz, 1 H),4.17−4.46 (m, 7 H), 4.81 (q, J = 10.6, 16.7 Hz, 2 H), 5.13−5.33 (m, 6 H), 5.86−6.03 (m, 3 H), 7.28−7.39 (m, 5 H); MS (EI) *m*/*z* (rel inten) 389.1 (M^+, 1), 299.1 (2), 91 (100).

(+)-3,5,6-Tri-*O*-allyl-4-*O*-benzyl-1-*O*-(*p*-methoxybenzyl)-*myo*-inositol (18). Regioselective introduction of a *p*-methoxybenzyl group to the C-1 of (−)-**17** (69 mg, 0.018 mmol), as described for (−)-**6**, afforded (+)-**18** (syrup, 83 mg, 92%): $[\alpha]^{23}_D = +0.5°$ (*c* 2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 2.43 (s, 1 H), 3.14 (dd, J = 2.5, 10 Hz, 1 H), 3.22−3.34 (m, 2 H), 3.79−3.82 (m, 4 H), 3.90−3.97 (m, 2 H), 4.04−4.06 (m, 2 H), 4.23−4.34 (m, 4 H), 4.50−4.57 (m, 2 H), 4.79−4.88 (m, 4 H), 5.14−5.19 (m, 2 H), 5.21−5.29 (m, 2 H), 5.83−6.03 (m, 3 H), 6.81−6.85 (m, 2

H), 7.24−7.40 (m, 7 H); MS (EI) *m*/*z* (rel inten) 509.2 (M^+ − H, 0.2), 91 (100).

(+)-3,5,6-Tri-*O*-allyl-2,4-di-*O*-benzyl-1-*O*-(*p*-methoxybenzyl)-*myo*-inositol (19). Conventional benzylation of (+)-**18** (72 mg, 0.014 mmol), as described for (−)-**4**, gave (+)-**19** (syrup, 81 mg, 96%): $[\alpha]^{23}_D$ = +4.4° (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 3.16 (dd, *J* = 2.3, 9.6 Hz, 1 H), 3.23−3.31 (m, 2 H) 3.76−3.86 (m, 4 H), 3.90−3.97 (m, 2 H), 4.04−4.07 (m, 2 H), 4.25−4.34 (m, 4 H), 4.50−4.57 (m, 2 H), 4.77−4.17 (m, 4 H), 5.12−5.15 (m, 3 H), 5.23−5.29 (m, 3 H), 5.83−6.02 (m, 3 H), 6.81−6.86 (m, 2 H), 7.24−7.40 (m, 12 H); MS (EI) *m*/*z* (rel inten) 600 (M^+, 0.1), 559 (0.4), 91 (100).

(−)-2,4-Di-*O*-benzyl-1-*O*-(p-methoxybenzyl)-*myo*-inositol (20). Deallylation of (+)-**19** (76 mg, 0.013 mmol) with $RhCl(PPh_3)_3$, as described for (−)-**8**, yielded (−)-**20** (amorphous, 44 mg, 72%): $[\alpha]^{23}_D$ = −0.4° (*c* 2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.85 (br s, 3 H), 3.46 (t, *J* = 9.2 Hz, 1 H), 3.55 (m, 1 H), 3.73−3.82 (m, 4 H), 3.88 (t, *J* = 9.2 Hz, 3 H), 4.06 (t, *J* = 2.7 Hz, 1 H), 4.65−4.87 (m, 5 H), 6.81−6.85 (m, 2 H), 7.25−7.40 (m, 12 H); MS (EI) *m*/*z* (rel inten) 479.0 (M^+ − H, 0.5), 389.0 (3), 359.0 (2), 91 (100).

(+)-2,4-Di-*O*-benzyl-1-*O*-(*p*-methoxybenzyl)-*myo*-inositol 3,5,6-Tris(dibenzyl phosphate) (21). Phosphorylation of (−)-**20** (40 mg, 0.008 mmol) via the phosphoramidite method, as described for (−)-**9**, yielded (+)-**21** (syrup, 90 mg, 86%): $[\alpha]^{23}_D$ = +0.5° (*c* 2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 3.43−3.50 (m, 1 H), 3.75 (s, 3 H), 4.06−4.13 (m, 1 H), 4.24−5.03 (m, 22 H), 6.74−6.78 (m, 2 H), 6.90−7.40 (m, 42 H); ^{31}P NMR ($CDCl_3$, 85% H_3PO_4 as external standard) δ −1.45, −1.61, −1.86; HRMS (MALDI) *m*/*z* (rel intel) 1261.392 (M + H, 100).

(+)-2,4-Di-*O*-benzyl-*myo*-inositol 3,5,6-Tris(dibenzyl phosphate) (22). Removal of the *p*-methoxybenzyl function of (+)-**21** (80 mg, 0.006 mmol) with TFA, as described for (−)-**10**, afforded (+)-**22** (syrup, 63 mg, 87%): $[\alpha]^{23}_D$ = +3.6° (*c* 2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 3.67−3.70 (m, 1 H), 4.01−4.07 (t, *J* = 9.9 Hz, 1 H), 4.25−4.33 (m, 2 H), 4.52 (q, *J* = 9.6, 18.9 Hz, 1 H), 4.59−5.08 (m, 17 H), 6.94−7.33 (m, 40 H); ^{31}P NMR ($CDCl_3$, 85% H_3PO_4 as external standard) δ 1.16, −1.05, −1.63; HRMS (MALDI) *m*/*z* (rel inten) 1141.337 (M + H, 100).

(+)-1-*O*-(1,2-Di-*O*-palmitoyl-*sn*-glycerol-3-benzyloxyphosphoryl)-2,4-di-*O*-benzyl-*myo*-inositol 3,5,6-Tris(dibenzyl phosphate) (23). Coupling of (+)-**22** (55 mg, 0.005 mmol) with the 1,2-di-*O*-palmitoyl-*sn*-glycerophosphoryl moiety, as described for (+)-**12**, provided (+)-**23** (syrup, 75 mg, 84%): $[\alpha]^{23}_D$ = +0.9° (c 2.6, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, *J* = 6.9 Hz, 6 H), 1.25 (s, 48 H), 1.43−1.62 (m, 4 H), 2.26−2.40 (m, 4 H), 3.97−4.26 (m, 6 H), 4.28−4.58 (m, 3 H), 4.60−5.07 (m, 20 H), 6.94−7.40 (m, 45 H); ^{31}P NMR ($CDCl_3$, 85% H_3PO_4 as external standard) δ −0.20, −0.50, −0.73 (d), −1.05 (d); HRMS (MALDI) *m*/*z* (rel inten) 1862.830 (M + 2 H, 100).

L-α-Phosphatidyl-D-*myo*-inositol 3,5,6-Trisphosphate, Dipalmitoyl [PtdIns(3,5,6)P_3]. The perbenzylated derivative (+)-**23** (65 mg, 0.003 mmol) was subjected to hydrogenolysis, as described for PtdIns(3,4,6)P_3, to provide PtdIns(3,5,6)P_3 (lyophilized powder, 36 mg, 98%): $[\alpha]^{23}_D$ = −10.9° (*c* 5, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.89 (t, *J* = 7.2 Hz, 6 H), 1.28 (s, 48 H), 1.52−1.66 (m, 4 H), 2.24−2.35 (m, 4 H), 3.96−4.28 (m, 7 H), 4.40−4.45 (m, 2 H), 4.63−4.75 (m, 1 H), 5.21−5.30 (m, 1 H); ^{31}P NMR ($CDCl_3$, 85% H_3PO_4 as external standard) δ −0.92 (2P), −1.31 (1P), −2.74 (1P); HRMS (MALDI) *m*/*z* (rel inten) 1049.399 (M − H, 100).

(+)-6-*O*-Allyl-2,3:4,5-di-*O*-cyclohexylidene-*myo*-inositol (24). Regioselective allylation of (+)-**1** (200 mg, 0.59 mmol), as described for (−)-**3**, afforded (+)-**24** (196.3 mg, 87%): $[\alpha]^{23}_D$ = +4.3° (*c* 2.8, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.31−1.77 (m, 20 H), 2.58 (d, *J* = 2.4 Hz, 1 H), 3.48 (dd, *J* = 12, 15.9 Hz, 1 H), 3.80 (dd, *J* = 2.7, 12 Hz, 1 H), 3.90−4.02 (m, 1 H), 4.12−4.23 (m, 3 H), 4.30−4.45 (m, 2 H), 5.16−5.36 (m, 2 H), 5.69−6.09 (m, 1 H); MS (EI) *m*/*z* (rel inten) 380.1 (M^+, 30), 337.1 (60), 55 (100).

(−)-6-*O*-Allyl-1-*O*-(*p*-methoxybenzyl)-2,3:4,5-di-*O*-cyclohexylidene-*myo*-inositol (25). A solution of (+)-**24** (180 mg, 0.47 mmol) in DMF (4 mL) was treated with NaH (21.2 mg, 85% purity, 0.71 mmol) at 0 °C under argon for 30 min, followed by *p*-methoxybenzyl chloride (71.8 μL, 0.71 mmol). The reaction mixture was warmed to 40 °C and stirred for 2 h. The excess NaH was destroyed with CH_3OH. The solution was diluted with ethyl acetate (50 mL), washed with water, dried, and concentrated. Column chromatography (hexanes−ether, 50:1 → 20:1) of the residue gave (−)-**25** (225 mg, 95%): $[\alpha]^{23}_D$ = −5.7° (*c* 1.4, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.30−1.77 (m, 20 H), 3.21−3.36 (m, 1 H), 3.55−3.80 (m, 5 H), 3.84−4.11 (m, 2 H), 4.20−4.35 (m, 2 H), 4.62−4.66 (m, 2 H), 5.12−5.20 (m, 1 H), 5.28 (d, *J* = 3 Hz, 2 H), 5.76−6.02 (m, 1 H), 6.87 (d, *J* = 13.2 Hz, 2 H), 7.30 (d, *J* = 13.2 Hz, 2 H); MS (EI) *m*/*z* (rel inten) 500.2 (M^+, 0.1), 457.2 (1), 379.2 (0.1), 121 (100).

(+)-6-*O*-Allyl-1-*O*-(*p*-methoxybenzyl)-2,3-*O*-cyclohexylidene-*myo*-inositol (26). Removal of the *trans*-cyclohexylidene group of (−)-**25** (195 mg, 0.39 mmol) with acetyl chloride, as described for (−)-**15**, afforded (+)-**26** (syrup, 126 mg, 77%): $[\alpha]^{23}_D$ = +6.5° (*c* 3, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.30−1.73 (m, 10 H), 2.01 (br s, 2 H), 3.21−3.30 (m, 1 H), 3.40−3.90 (m, 7 H), 4.10−4.30 (m, 2 H), 4.41−4.50 (m, 1 H), 4.67 (s, 2 H), 5.18−5.34 (m, 2 H), 5.85−6.10 (m, 1 H), 6.90 (d, *J* = 12.1 Hz, 2 H), 7.29 (d, *J* = 12.2 Hz, 2 H); MS (EI) *m*/*z* (rel inten) 420.1 (M^+, 0.2), 377.1 (1), 299.1 (0.5), 121 (100).

(−)-4,5,6-Tri-*O*-allyl-1-*O*-(*p*-methoxybenzyl)-2,3-cyclohexylidene-*myo*-inositol (27). Allylation of (+)-**26** (126 mg, 0.30 mmol) with NaH and allyl bromide, as described for (+)-**14**, yielded (−)-**27** (syrup, 142.5 mg, 95%): $[\alpha]^{23}_D$ = −9.0° (*c* 1, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.30−1.78 (m, 10 H), 3.12 (t, *J* = 10.2 Hz, 1 H), 3.49−3.57 (m, 2 H), 3.67 (t, *J* = 10.2 Hz, 1 H), 3.79 (s, 3 H), 3.90 (dd, *J* = 8.1, 10.5 Hz, 1 H), 4.12−4.66 (m, 7 H), 4.70 (q, *J* = 19.8, 33 Hz, 2 H), 5.10−5.32 (m, 6 H), 5.85−6.09 (m, 3 H), 6.83−6.88 (m, 2 H), 7.25−7.32 (m, 2 H); MS (EI) *m*/*z* (rel inten) 500.2 (M^+, 0.2), 457.2 (0.2), 444.2 (0.4), 417.2 (0.2), 121 (100).

(+)-4,5,6-Tri-*O*-allyl-1-*O*-(*p*-methoxybenzyl)-*myo*-inositol (28). Removal of the *cis*-cyclohexylidene group of (−)-**27** (172 mg, 0.34 mmol) with acetyl chloride, as described for (−)-**5**, gave (+)-**28** (amorphous, 130 mg, 90%): $[\alpha]^{23}_D$ = +10.3° (*c* 3.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 2.43 (s, 2 H), 3.19 (t, *J* = 14.2 Hz, 1 H), 3.28−3.40 (m, 2 H), 3.55−3.73 (m, 2 H), 3.81 (s, 3 H), 4.13−4.39 (m, 7 H), 4.63 (q, *J* = 5.5, 19 Hz, 2 H), 5.15−5.32 (m, 6 H), 5.89−6.03 (m, 3 H), 6.90 (d, *J* = 12.7 Hz, 2 H), 7,26 (d, *J* = 12.2 Hz, 2 H); MS (EI) *m*/*z* (rel inten) 420.1 (M^+, 0.1), 379.1 (0.5), 299.1 (0.2), 121 (100).

(+)-4,5,6-Tri-*O*-allyl-2,3-di-*O*-benzyl-1-*O*-(*p*-methoxybenzyl)-*myo*-inositol (29). Benzylation of (+)-**28** (100 mg, 0.24 mmol) with NaH and benzyl bromide, as described for (−)-**4**, gave (+)-**29** (syrup, 140 mg, 95%): $[\alpha]^{23}_D$ = +5.4° (*c* 3.7, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 3.16−3.21 (m, 3 H), 3.74−3.69 (m, 5 H), 4.17−4.42 (m, 7 H), 4.43−4.70 (m, 4 H), 4.78 (s, 2 H), 5.08−5.28 (m, 6 H), 5,75−6.09 (m, 3 H), 6.81−6.85 (m, 2 H), 7.19−7.42 (m, 12 H); MS (EI) *m*/*z* (rel inten) 600.1 (M^+, 0.), 559.1 (0.1), 509.1 (1), 479.1 (0.1), 91 (100).

(−)-2,3-Di-*O*-benzyl-1-*O*-(*p*-methoxybenzyl)-*myo*-inositol (30). Deallylation of (+)-**29** (134 mg, 0.22 mmol) with $RhCl(PPh_3)_3$, as described for (−)-**8**, afforded (−)-**30** (amorphous, 87,1 mg, 81%): $[\alpha]^{23}_D$ = −21.8° (*c* 2.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 2.57 (br s, 3 H), 3.13−3.24 (m, 2 H), 3.32−3.47 (m, 2 H), 3.78 (s, 3 H), 3.93−4.03 (m, 2 H), 4.39−4.75 (m, 6 H), 6.88 (d, *J* = 12.7, 2 H), 7.19−7.45 (m, 12 H); MS (EI) *m*/*z* (rel inten) 479.9 (M^+, 0.1), 388.9 (3), 268.9 (5), 91 (100).

(+)-2,3-Di-*O*-benzyl-1-*O*-(*p*-methoxybenzyl)-*myo*-inositol 4,5,6-Tris(dibenzyl phosphate) (31). Phosphorylation of (−)-**30** (82.1 mg, 0.17 mmol) via the phosphoramidite method as described for (−)-**9** yielded (+)-**31** (syrup, 215 mg, 99%): $[\alpha]^{23}_D$ = +2.0° (*c* 3.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 3.38−3.45 (m, 3 H), 3.78 (s, 3 H), 4.34−4.71 (m, 5 H), 4.86−5.07 (m, 16 H), 6.80 (d, *J* = 8.7 Hz, 2 H), 7.11−7.35 (m, 42 H); ^{31}P NMR ($CDCl_3$, 85% H_3PO_4 as external standard) δ 1.49, −0.86, −1.54; HRMS (MALDI) *m*/*z* (rel inten) 1261.401 (M + H, 100).

(+)-2,3-Di-*O*-benzyl-*myo*-inositol 4,5,6-Tris(dibenzyl phosphate) (32). Removal of the *p*-methoxybenzyl function of (+)-**31** (204 mg, 0.16 mmol) with TFA, as described for (−)-**10**, gave (+)-**32** (148 mg, 80%): $[\alpha]^{23}_D$ = +3.2° (*c* 2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 2.05 (br s, 1 H), 3.44−3.52 (m, 1 H), 3.67−3.74 (m, 1 H), 4.02 (br s, 1 H), 4.14−4.28 (m, 1 H), 4.40−5.08 (m, 18 H), 7.06−7.41 (m, 40 H); ^{31}P NMR ($CDCl_3$, 85% H_3PO_4

as external standard) δ 1.39, −0.80, −1.34; HRMS (MALDI) *m*/*z* (rel inten) 1141.335 (M + H, 100).

(+)-1-*O*-(1,2-Di-*O*-palmitoyl-*sn*-glycerol-3-benzyloxyphosphoryl)-2,3-di-*O*-benzyl-*myo*-inositol 4,5,6-Tris(dibenzyl phosphate) (33). Coupling of (+)-**32** (40 mg, 0.035 mmol) with the 1,2-di-*O*-palmitoyl-*sn*-glycerophosphoryl moiety, as described for (+)-**12**, yielded (+)-**33** (54.5 mg, 84%): $[\alpha]^{23}_D$ = +6.4° (*c* 1.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 6.0 Hz, 6 H), 1.14−1.36 (m, 48 H), 1.46−1.62 (m, 4 H), 2.16−2.28 (m, 4 H), 3.50−3.59 (m, 1 H), 3.91−4.24 (m, 4 H), 4.42−4.74 (m, 5 H), 4.77−5.08 (m, 19 H), 7.06−7.38 (m, 45 H); ^{31}P NMR ($CDCl_3$, 85% H_3PO_4 as external standard) δ −0.63 (d), −0.73 (d), −1.48 (d), −1.51 (d); HRMS (MALDI) *m*/*z* (rel inten) 1862.847 (M + 2 H, 100).

L-α-Phosphatidyl-D-*myo*-inositol 4,5,6-Trisphosphate, Dipalmitoyl [PtdIns(4,5,6)P_3]. The perbenzylated derivative (+)-**33** (34 mg, 0.018 mmol) was subjected to hydrogenolysis, as described for PtdIns(3,4,6)P_3, to provide PtdIns(4,5,6)P_3 (lyophilized powder, 18.8 mg, 98%): $[\alpha]^{23}_D$ = +4° (*c* 2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.89 (t, J = 7.2 Hz, 6 H), 1.28 (s, 48 H), 1.52−1.66 (m, 4 H), 2.24−2.35 (m, 4 H), 3.53−3.56 (m, 1 H), 3.64−3.68 (m, 2 H), 4.03−4.43 (m, 5 H), 4.46−4.60 (m, 1 H), 4.62−4.78 (m, 1 H), 5.22−5.30 (m, 1 H); ^{31}P NMR ($CDCl_3$, 85% H_3PO_4 as external standard) δ −0.22 (2P), −0.95 (1P), −1.51 (1 P); HRMS (MALDI) *m*/*z* (rel inten) 1049.382 (M − H, 100).

Preparation of the C-Terminal SH2 Domains of the p85 Subunit of PI 3-Kinase. A pGEX vector containing the cDNA sequence encoding the p85 CT-SH2 domain was expressed in *E. coli* as a GST fusion protein by IPTG induction.[6] The GST fusion protein and free SH2 domain were prepared as follows. The bacterial lysates were incubated with glutathione-coupled Sepharose 4B beads (Sigma) for 2 h at 37 °C and washed three times with 30 mM Hepes, pH 7.0, containing 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40, followed by the same buffer without NP-40 twice. Subsequently, the immobilized GST fusion protein was subjected to Factor Xa hydrolysis to isolate the free SH2 domain. After incubation with the protease at 4 °C overnight, the mixture was subjected to centrifugation at 200*g* for 5 min to separate the free SH2 domain from the GST bead. The SH2 domain was concentrated by ultrafiltration. The homogeneity of the isolated SH2 domain was indicated by a single band on 15% SDS−PAGE with molecular masses of 20 kDa for the CT-SH2 domain.

Fluorescence Spectroscopy. Fluorescence spectra were recorded at 30 °C with a fluorescence spectrophotometer according to a method previously described.[16] Interactions between the SH2 domain and individual PtdInsP_3 isomers were assessed by monitoring the tryptophan fluorescence with excitation wavelength at 292 nm. The buffer used for the fluorescence experiments consisted of 25 mM Tris/HCl, pH 7.5, 100 mM KCl, and 1 mM DTT. Varying amounts of the phospholipid, in 15 μL of the same buffer, were gradually introduced into 785 μL of the buffer containing the CT-SH2 domain (12.5 μM).

Changes in the fluorescence intensity were used as a measure of the binding affinity of the protein−lipid complex according to the following equation[17]

$$1/[[1 - (\Delta F/\Delta F_{max})]K_a] = [[\text{phosphoinositide}]\text{total}/(\Delta F/\Delta F_{max})] - [\text{CT-SH2}]_{total}$$

where K_a denotes the association constant.

Preparation of Washed Rabbit Platelets. Platelets were obtained from adult New Zealand White rabbits and washed using a modified method of Baenziger and Majerus.[18] In brief, whole blood was anticoagulated by treating 9 volumes of blood with 1 volume of anticoagulant that consisted of 3.8% (w/v) trisodium citrate and 140 mM dextrose. Platelet-rich plasma was obtained by centrifugation of whole blood at 200*g* for 15 min. The platelets were pelleted by centrifugation at 1500*g* for 10 min. The platelet pellets were resuspended in a platelet-washing buffer, consisting of 4.3 mM Na_2HPO_4, 24.3 mM NaH_2PO_4, 4.3 mM K_2HPO_4, pH 6.5, 113 mM NaCl, 5.5 mM glucose, and 0.5% bovine serum albumin, washed twice, and resuspended in buffer A consisting of 20 mM Tris/HCl, pH 7.0, containing 150 mM NaCl and 5 mM glucose. Platelet concentrations were determined by using a Coulter cell counter (Coulter Electronics, Inc., Hialeah, FL) and were adjusted to 2−3 × 10^8 cells/mL for all experiments. These washed platelets stood at room temperature for 1−2 h before use.

Platelet Aggregation. Washed platelets were added to an aggregation cuvette, and the reaction was initiated by adding indicated amounts of the phosphoinositide in the presence of 1 mM Ca^{2+} with stirring at 1000 rpm. Platelet aggregation was monitored on a two-channel Chrono-Log aggregometer (Chrono-Log Corp., Havertown, PA) at 37 °C.

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Supporting Information Available: NMR spectra for obtained compounds (33 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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