CHEMBIOCHEM

DOI: 10.1002/cbic.200900545

5-Stabilized Phosphatidylinositol 3,4,5-Trisphosphate Analogues Bind Grp1 PH, Inhibit Phosphoinositide Phosphatases, and Block Neutrophil Migration

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Metabolically stabilized analogues of PtdIns(3,4,5)P_3 have shown long-lived agonist activity for cellular events and selective inhibition of lipid phosphatase activity. We describe an efficient asymmetric synthesis of two 5-phosphatase-resistant analogues of PtdIns(3,4,5)P_3, the 5-methylene phosphonate (MP) and 5-phosphorothioate (PT). Furthermore, we illustrate the biochemical and biological activities of five stabilized PtdIns-(3,4,5)P_3 analogues in four contexts. First, the relative binding affinities of the 3-MP, 3-PT, 5-MP, 5-PT, and 3,4,5-PT$_3$ analogues to the Grp1 PH domain are shown, as determined by NMR spectroscopy. Second, the enzymology of the five analogues is explored, showing the relative efficiency of inhibition of SHIP1, SHIP2, and phosphatase and tensin homologue deleted on chromosome 10 (PTEN), as well as the greatly reduced ability of these phosphatases to process these analogues as substrates as compared to PtdIns(3,4,5)P_3. Third, exogenously delivered analogues severely impair complement factor C5a-mediated polarization and migration of murine neutrophils. Finally, the new analogues show long-lived agonist activity in mimicking insulin action in sodium transport in A6 cells.

Introduction

The phosphoinositide 3-kinase (PI 3-K) signaling pathway contains important therapeutic targets in human pathophysiology.[1,2] Phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P_3) is a ubiquitous signaling lipid found in higher eukaryotic cells[3] and activates a plethora of downstream cellular processes.[4] These signaling events include cell proliferation and transformation,[5] cell shape and motility,[6] and insulin action and alteration of glucose transport.[7] PtdIns(3,4,5)P_3-regulated signaling can be modulated by the lipid 3-phosphatase PTEN[8] and SH2 domain-containing inositol 5-phosphatases SHIP1 and SHIP2.[9] Indeed, PTEN and SHIP1/2 are crucial regulators of PI 3-kinase signaling in T-lymphocytes,[10] drug-discovery targets in type 2 diabetes[11] and intimately involved in signaling networks for a myriad of other disease states.[12]

A metabolically stabilized (ms) analogue of PtdIns(3,4,5)P_3 that resists lipid 3- and 5-phosphatases would have numerous applications in understanding the role of PtdIns(3,4,5)P_3 in cell physiology. The ms-PtdIns(3,4,5)P_3 analogues could separate the activation of signal transduction from the degradation of the signal by phosphatase action in cells. This chemical biology approach to the dissection of the PI 3-K pathway would be complementary to the use of siRNA knockdowns or genetic knockouts for PTEN and SHIP.

We recently described the preparation and activity of the 3-phosphatase-resistant, metabolically stabilized analogues **1** and **2** (Scheme 1) of PtdIns(3,4,5)P_3, which were both stable to degradation by PTEN and acted as inhibitors of PTEN.[13] One of these analogues incorporated a single phosphorothioate (PT) substituent, and an additional study examined the chemistry and biological activity of the 3,4,5-trisphosphorothioate analogue (**3**) of PtdIns(3,4,5)P_3.[14] Phosphorothioates are important phosphomimetics that show greatly reduced rates of enzyme-mediated hydrolysis.[15] However, the replacement of P=O by P=S also affects the pK_a of the phosphate and removes a H-bond acceptor.[16,17] Indeed, we demonstrated that PtdIns(3)PT had reduced binding activity for cognate PtdIns(3)P-selective FYVE and PX domain binding proteins; this could be attributed to reduced H-bonding.[18] We also observed that a PT analogue of PtdIns(5)P was a long-lived agonist for chromatin remodeling,[19] and a tris(PT) cyclopentyl analogue[20] of Ins(1,4,5)P_3 was a long-lived agonist activities for

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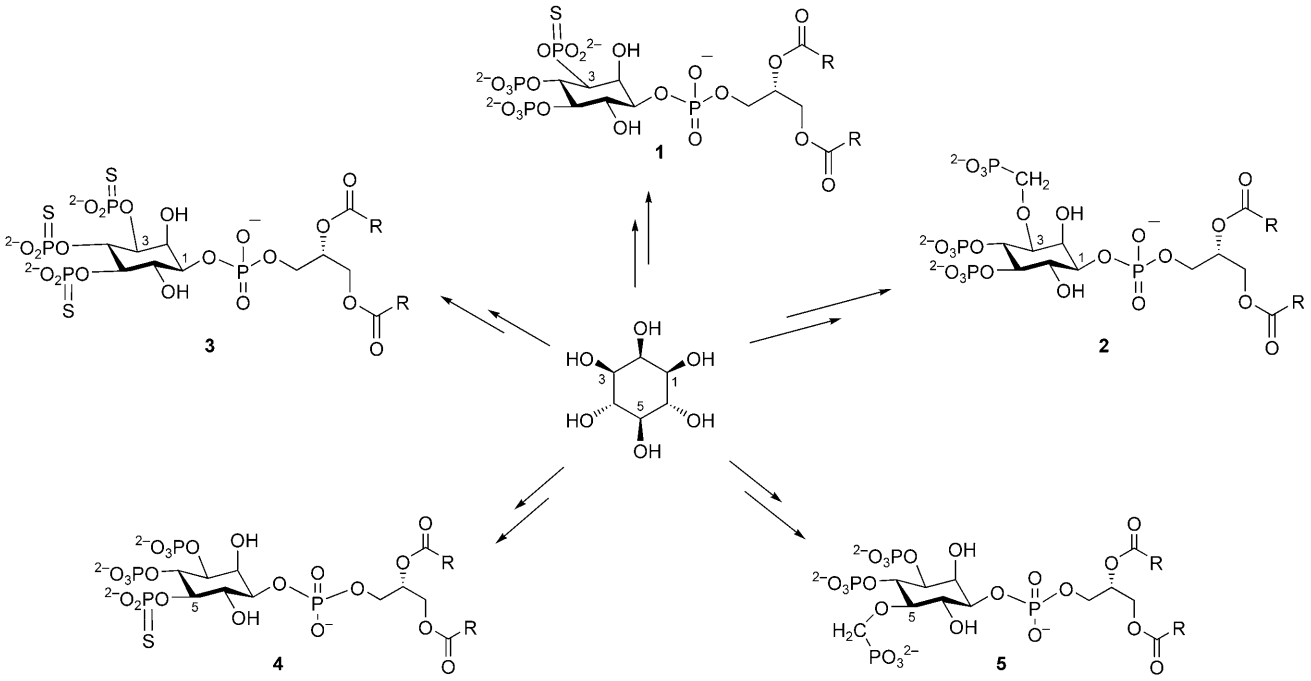
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.200900433: complete experimental details for synthesis and characterization of new compounds with chemical structures, inhibition of PTEN by metabolically stabilized analogues, Akt activation by analogues.



Scheme 1. Analogues of phosphatidylinositol 3,4,5-trisphosphate synthesized from D-*myo*-inositol.

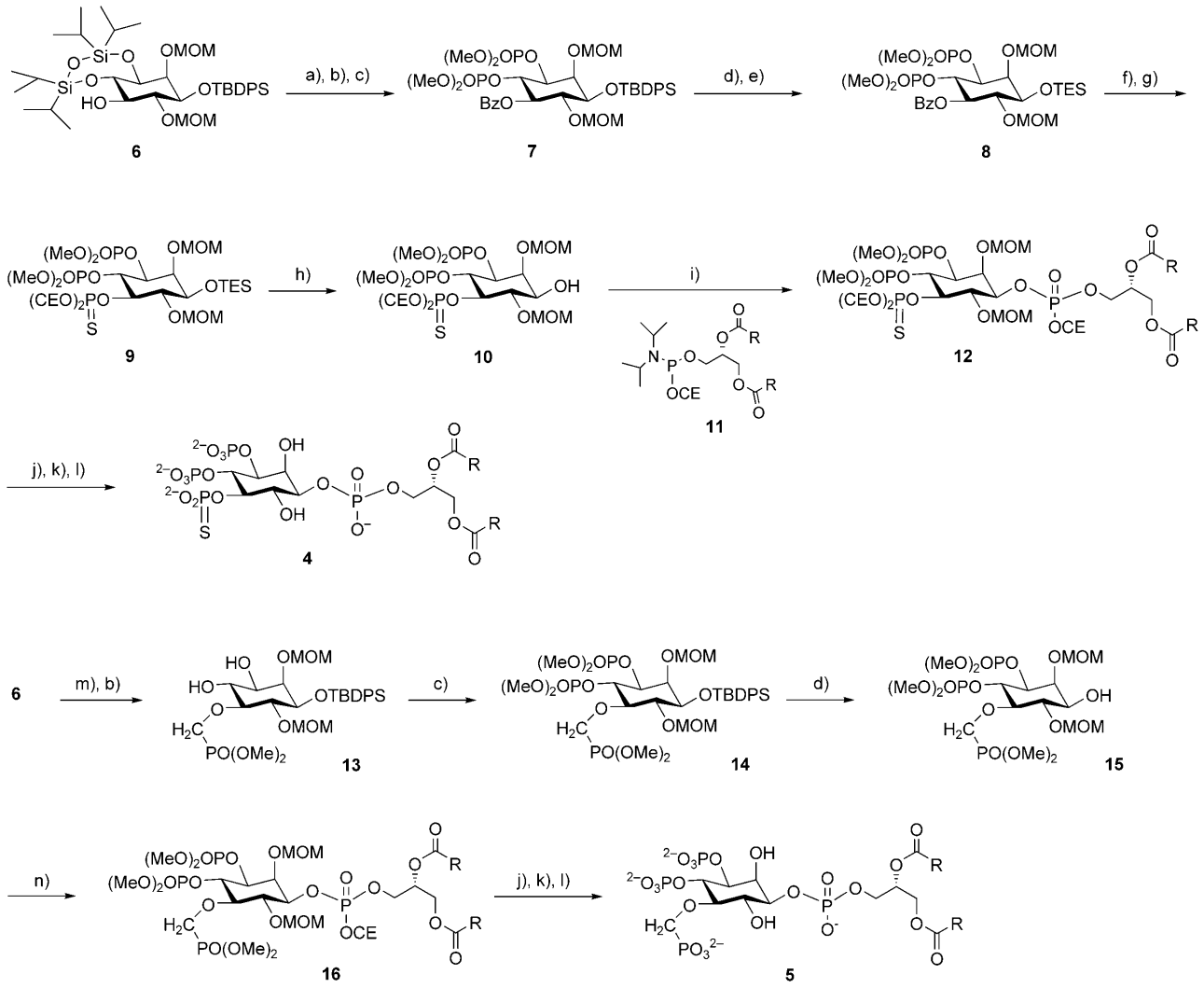
the IP_3 receptor. We hypothesized that 5-PT and 5-MP analogues of PtdIns(3,4,5)P_3 could be either antagonists or long-lived PtdIns(3,4,5)P_3 agonists, as they would be more slowly dephosphorylated by the 5-phosphatase SHIP and could potentially block the normal receptor-mediated signaling involving PtdIns(3,4,5)P_3. We also wished to test whether the 5-PT and 5-MP modifications could alter the processing of PtdIns-(3,4,5)P_3 by the 3-phosphatase PTEN.

Herein, we report the total asymmetric synthesis and characterization of the two 5-stabilized PtdIns(3,4,5)P_3 analogues **4** and **5** (Scheme 1). First, we investigated the relative binding affinities of the 3-, 5-, and 3,4,5-stabilized PtdIns(3,4,5)P_3 analogues to Grp1 PH domain using NMR titration methods. Second, we examined the relative efficiency of inhibition of SHIP1, SHIP2, and PTEN by these analogues, often with unexpected results. The reduced processing of the analogues as substrates in an enzyme and modification-selective fashion was also studied. Third, we explored the effects of the exogenously delivered 3-, 5-, and 3,4,5-stabilized PtdIns(3,4,5)P_3 analogues on complement factor C5a-mediated polarization and migration of wild-type murine neutrophils. Finally, the long-lived agonist activities of the 5-PT (**4**) and 5-MP (**5**) analogues in mimicking insulin action in sodium transport in A6 cells were compared to previous data for the 3-PT (**1**), 3-MP (**2**), and 3,4,5-PT_3 (**3**) analogues.[13,14]

Results and Discussion

Chemistry and synthesis

The PTEN resistant analogues, 3-PT-PtdIns(3,4,5)P_3 (**1**),[13] 3-MP-PtdIns(3,4,5)P_3 (**2**),[13] and 3,4,5-PT-PtdIns(3,4,5)P_3 (**3**)[14] have been synthesized previously. The synthesis of 5-ms-PtdIns-(3,4,5)P_3 required selective protection of the 5-OH and the use of enantiomerically pure *myo*-inositol derivatives. A number of strategies have been developed for the synthesis of optically pure phosphoinositides, either using the enantiopure natural precursors, such as D-glucose[21] or quinic acid.[22] Kinetic resolution or desymmetrization by enantioselective enzymatic acylation, nonenzymatic phosphorylation of *myo*-inositol derivatives,[23-25] and the separation of diastereomeric derivatives of *myo*-inositol with chiral auxiliaries are also commonly used.[13,26-28] The syntheses of **4** and **5** are illustrated in Scheme 2; complete step-by-step synthetic details and structures may be found in the Supporting Information. As with our previous syntheses of stabilized PtdIns(3,4,5)P_3 analogues,[13,14] we first prepared the 3,4,5-unprotected inositol using methods developed by Bruzik:[27,28] 1) acetalization of inositol with D-camphor dimethyl acetal, 2) selective silylation of 1-OH, 3) removal of the camphor ketal, 4) selective benzoylation of 3,4,5-OH, 5) complete protection of the hydroxyl groups, and 6) debenzoylation. Next, the appropriately protected 5-PT intermediate **10**, which bears a free 1-hydroxyl group for introduction of the diacylglyceryl-bearing phosphodiester, was prepared in 14 steps after 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (TIPDS)-bis-silylation, benzoylation, desilylation, and phosphorylation, as depicted in Scheme 2 (see the Supporting Information for complete experimental details). In the same way, the appropriately protected 5-MP intermediate **15**, equipped with a free 1-hydroxyl group, was synthesized from *myo*-inositol in 11 steps (Scheme 2; see the Supporting Information for complete experimental details) in close analogy to the synthesis[13] of 3-MP-PtdIns(3,4,5)P_3 (**2**).



Scheme 2. Synthesis of 5-PT and 5-MP analogues. a) BzCl, 4-dimethylamino pyridine, Py; b) tetrabutyl ammonium fluoride (TBAF), THF; c) dimethyl *N*,*N*-diisopropyl-phosphoramidite, 1*H*-tetrazole, *meta*-chloroperbenzoic acid; d) TBAF, DMF; e) triethylchlorosilane, imidazole; f) diisobutylaluminum hydride, CH_2Cl_2; g) $(iPr)_2NP(OCH_2CH_2CN)_2$, 1*H*-tetrazole, phenylacetyl disulfide; h) NH_4F, MeOH; i) 1*H*-tetrazole, CH_2Cl_2, RT, *t*BuOOH; j) triethylamine, BSTFA, CH_3CN; k) trimethylsilyl bromide/CH_2Cl_2 (2:3), RT; l) MeOH; m) $(MeO)_2POCH_2OTf$, *n*BuLi, hexamethyl phosphoramide; n) **11**, 1*H*-tetrazole, CH_2Cl_2, RT, *t*BuOOH. CE = cyanoethyl; MOM = methoxymethyl ether; R = C_3H_7, C_7H_{15}, $C_{15}H_{31}$, $C_{17}H_{33}$.

The phosphoroamidates **11** were prepared from 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite with diacylglycerols bearing dibutanoyl, dioctanoyl, or dihexadecanoyl chains,[13,18] and coupled with 5-PT intermediate **10** or with 5-MP intermediate **15**, in the presence of 1*H*-tetrazole. Phosphodiesters were produced by *t*-BuOOH oxidation, and no overoxidation of the 5-PT group was observed. The cyanoethyl groups were removed by using triethylamine plus bis(trimethylsilyl)trifluoroacetamide (BSTFA) in anhydrous acetonitrile.[13,14] Addition of BSTFA prevents the phosphorothioate anion from undergoing re-alkylation. Protocols (see Supporting Information) analogous to those for the 3-MP and 3-PT-PtdIns(3,4,5)P_3 analogue syntheses[13] provided the desired analogues 5-PT-PtdIns(3,4,5)P_3 (**4**) and 5-MP-PtdIns(3,4,5)P_3 (**5**)[13,14] in the acid form. The final products **4** and **5** were converted to the sodium salts by ion-exchange chromatography (Dowex 50@×8 200 Na^+ exchange resin) and stored lyophilized at −80 °C prior to use in biological experiments.

High-affinity binding to Grp1 PH

In mammalian cells, PtdIns(3,4,5)P_3 is transiently accumulated in plasma membranes following activation of PI 3-kinase.[29] PtdIns(3,4,5)P_3 is specifically recognized by the pleckstrin homology (PH) domain-containing proteins such as general receptor for phosphoinositides 1 (GRP1).[30,31] To test the biological activity of the PtdIns(3,4,5)P_3 analogues, we investigated their interactions with GRP1 PH domain by NMR spectroscopy (Figure 1 and Figure S1). The GRP1 PH domain recognizes all five PtdIns(3,4,5)P_3 analogues. The chemical-shift changes observed in ^1H,^{15}N HSQC spectra of the ^{15}N-labeled GRP1 PH domain indicate that the binding of 3-PT-PtdIns(3,4,5)P_3 (**1**)

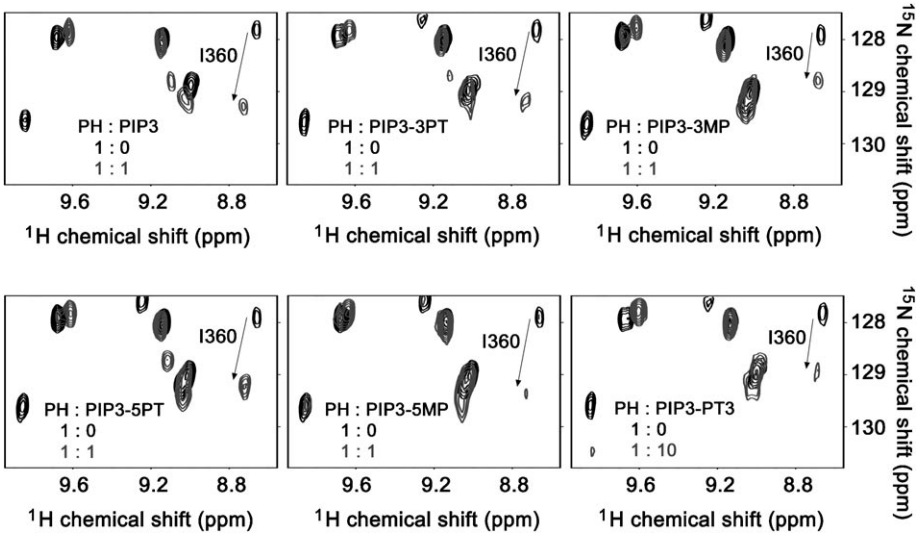


Figure 1. PtdIns(3,4,5)P_3 analogues are recognized by the PH domain of GRP1. Superimposed ^{1}H,^{15}N HSQC spectra of 0.2 mM ^{15}N-labeled GRP1 PH domain collected before (black) and after (gray) addition of indicated diC$_4$-PtdIns-(3,4,5)P_3 analogues. The chemical shift changes for the amide group of Ile360 are indicated by arrows for comparison.

and 5-PT-PtdIns(3,4,5)P_3 (**4**) is nearly equivalent to the binding of unmodified PtdIns(3,4,5)P_3. Figure S1 shows an overlay of the ^{1}H,^{15}N HSQC spectra of GRP1 PH collected as three dibutanoyl lipids were added (unmodified PtdIns(3,4,5)P_3, 3-PT-PtdIns-(3,4,5)P_3 (**1**), and 3,4,5-PT$_3$-PtdIns(3,4,5)P_3 (**3**)). The slow exchange regime on the NMR timescale in all three experiments suggests a robust lipid–protein interaction. Taken together, these data indicate that the binding affinity decreases in the order PtdIns(3,4,5)$P_3 \geq$ 3-PT-PtdIns(3,4,5)P_3 (**1**) $\approx$ 5-PT-PtdIns-(3,4,5)P_3 (**4**) $>$ 3-MP-PtdIns(3,4,5)P_3 (**2**) $\approx$ 5-MP-PtdIns(3,4,5)P_3 (**5**) $>$ 3,4,5-PT$_3$-PtdIns(3,4,5)P_3 (**3**). It is important to note that, while the phosphorothioate is considered isosteric with the phosphate, the methylene phosphonate is a methylene-extended analogue of the phosphate, rather than an isostere. Thus, these synthetic analogues can effectively substitute for PtdIns(3,4,5)P_3 in biochemical and biological assays while retaining affinity for PtdIns(3,4,5)P_3 binding sites.

Stabilized analogues are poor substrates for SHIP2 and PTEN

Incubation of 100 μM of diC$_8$-5-MP (**5**), diC$_8$-5-PT (**4**), or diC$_8$-3,4,5-PT$_3$ (**3**) with SHIP2 for 10 min resulted in no phosphate release, thus confirming the metabolic stability of these 5-stabilized analogues to the 5-phosphatase activity (Figure 2). In contrast, both the 3-MP (**2**) and 3-PT (**1**) substrates were at least partially dephosphorylated by SHIP2; this shows that the 5-phosphatase activity was capable of removing an unstabilized phosphomonoester in the analogues containing a 3-stabilized group.

In contrast, none of the five analogues was processed by PTEN during the 10 min incubation period (Figure 3). The absence of phosphate release confirms the metabolic stability of these analogues to the 3-phosphatase activity of PTEN, but is somewhat unexpected. One would have expected that 5-stabilization should not completely abrogate 3-phosphate removal from an unstabilized position. An alternative explanation is that the chemical modifications in 5-position of the 5-ms analogues are not well tolerated in the enzyme active site, and simply result in poor enzyme–substrate binding rather than binding with reduced turnover.

Competitive inhibition of SHIP1, SHIP2 and PTEN

We chose to work with Ins(1,3,4,5)P_4 as substrate in order to use relatively low substrate concentrations, taking advantage of the available tritium-labeled tracer and a well-characterized assay[32] (see Experimental Section). The alternative malachite green phosphate-release assay used to examine the substrate ability of the analogues required much higher substrate concentrations (50–100 μM).

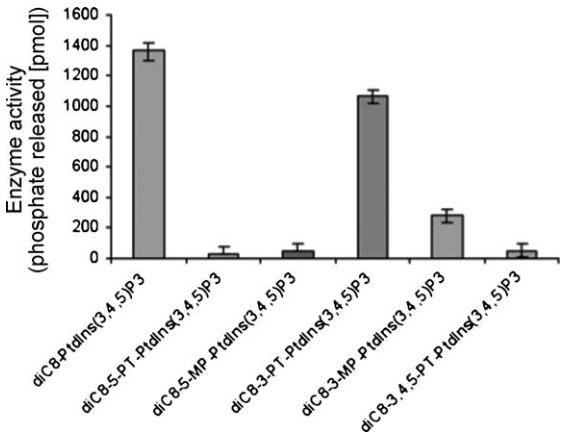


Figure 2. Phosphate release from metabolically stabilized analogues by SHIP2 in the presence of 200 μM PtdSer,[49] as detected by malachite green assay. Analogues, including the positive control diC$_8$-PtdIns(3,4,5)P_3, were added at 100 μM. The final enzyme concentrations were 3.4 μg mL^{-1}, and incubation was for 10 min at 37 °C. Enzyme activity is expressed as pmol of phosphate released (means of triplicates ± SD).

The new 5-stabilized PtdIns(3,4,5)P_3 analogues as well as the previously described 3-stabilized PtdIns(3,4,5)P_3 analogues[13] and 3,4,5-tris(phosphorothioate) analogue[14] of PtdIns(3,4,5)P_3 were examined as potential inhibitors of the 5-phosphatase and 3-phosphatase activities. Figure 4A summarizes the comparative inhibition of the 5-phosphatase activity of recombinant SHIP1, with 0.3 μM tritium-labeled Ins(1,3,4,5)P_4 as the substrate and each of the diC$_8$ analogues at 10 μM. DiC$_8$-

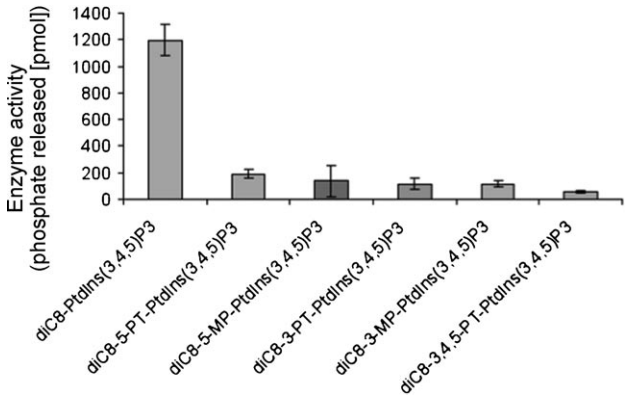


Figure 3. Phosphate release from metabolically stabilized analogues by PTEN in the presence of 200 μM PtdSer,[49] as detected by malachite green assay. Analogues, including the positive control diC_8-PtdIns(3,4,5)P_3, were added at 100 μM at 37 °C. The final enzyme concentration was 0.125 μg mL^{-1} and incubation time was 10 min. Enzyme activity is as described in the legend for Figure 2.

PtdIns(3,4,5)P_3 itself did not show any competitive inhibition of SHIP1 activity under these conditions, but each of the analogues significantly reduced SHIP1 activity. Interestingly, the most potent analogues in this assay were diC_8-5-MP (**5**), diC_8-3-PT (**1**), and diC_8-3,4,5-PT_3 (**3**). In contrast to the situation with SHIP1, little or no inhibition of SHIP2 activity was observed for any of the analogues under the same incubation conditions (Figure 4 B). This most likely reflects the difference in affinity of the substrate Ins(1,3,4,5)P_4 for SHIP1 and SHIP2.[32]

Evaluation of the five stabilized analogues for inhibition of PTEN 3-phosphatase activity is shown in Figure S2, for which 0.3 μM tritium-labeled Ins(1,3,4,5)P_4 as the substrate and 10 μM of each of the diC_8 analogues were employed. Only the 3,4,5-PT_3 analogue (**3**) appeared to inhibit PTEN 3-phosphatase activity with this substrate.

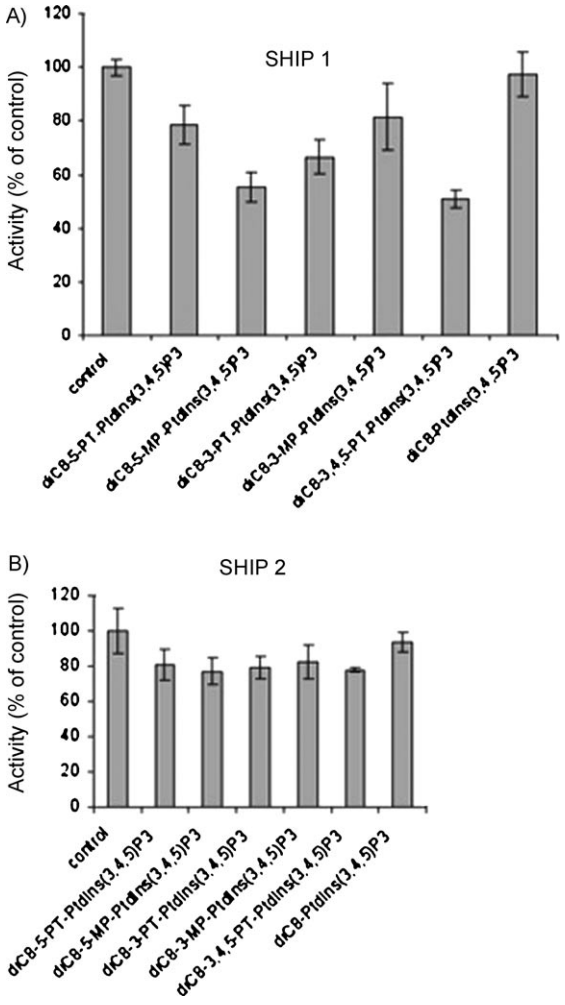


Figure 4. Inhibition of the 5-phosphatase activity of A) recombinant SHIP1 at 4.9 μg mL^{-1} and B) recombinant SHIP2 at 11.3 μg mL^{-1}, with 0.3 μM tritium-labeled Ins(1,3,4,5)P_4 as the substrate and each of the diC_8 analogues at 10 μM. Analogues were preincubated for 5 min in the presence of enzyme at 4 °C. The mixture was then mixed with the labeled substrate and incubated for 10 min at 37 °C. The 100% control refers to the activity in the presence of water, while each value below 100% represents the activity that was reached in the presence of analogue (mean value ± SD).

Neutrophil polarity and motility

Chemotaxis is a crucially important cellular response implicated in physiological activities.[33-36] Chemotactic stimulation of cells results in a complex sequence of events: increased actin organization, cell-shape changes, and the development of polarity.[37,38] The selective translocation of PtdIns(3,4,5)P_3 to the plasma membrane at the leading edge leads to cytoskeleton reorganization and pseudopod formation,[39] so the localized accumulation of PtdIns(3,4,5)P_3 was deemed to be the key event directing the recruitment and activation of the signaling components required for cell polarization and chemotaxis.[40,41] The amoeba *Dictyostelium discoideum* and mammalian neutrophils are commonly used as representative chemotactic cells. In *D. discoideum*, PI(3)Ks and PTEN control cell polarization and chemotaxis by regulating spatially localized PtdIns(3,4,5)P_3 accumulation.[42,43] In neutrophils, both PI3Kγ and SHIP1 play critical roles in polarization and motility;[36,44] SHIP1 restricts the localization of PtdIns(3,4,5)P_3 accumulation, and thereby governs the cell polarization required for proper motility and chemotaxis.[36] Thus, we examined the effects of metabolically stabilized PtdIns(3,4,5)P_3 analogues in wild-type neutrophils by using C5a as chemoattractant, which could in principle occur by a possible interaction with SHIP1 or with Akt or both. As shown in Figure 5, the polarization of wild-type neutrophils was greatly impaired by exogenous addition of each of the five PtdIns(3,4,5)P_3 analogues. The 3- and 5-ms-PtdIns(3,4,5)P_3 analogues might bind the SHIP1 as an inhibitor or antagonist, thus the ability of SHIP1 to govern PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 accumulation at the leading edge of chemotaxing neutrophils is decreased, the formation of the pseudopod is retarded.

To determine if the metabolically stabilized analogues might be acting at the level of Akt activation, mouse peritoneal neutrophils were purified, stimulated, and lysed as described.[36] The lysates were separated by 10% SDS-PAGE and immunoblotted with phospho-specific antibodies against Akt pS473 and pan-Akt. As shown in Figure S3, none of the five ana-

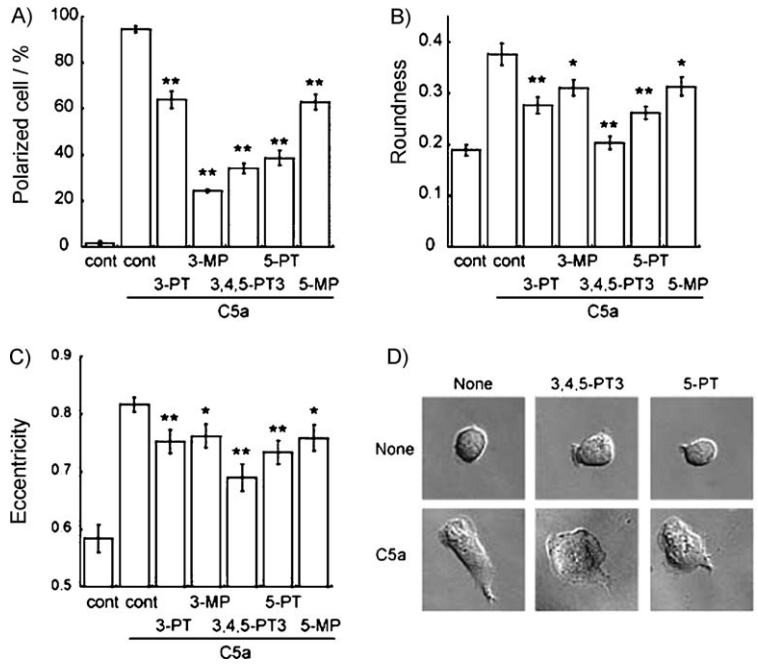


Figure 5. Effects of metabolically stabilized PtdIns(3,4,5)P_3 analogues on cell polarization. Mouse neutrophils (4×10^5) were preincubated with or without metabolically stabilized PtdIns(3,4,5)P_3, followed by the addition of C5a and a further incubation for 15 min. Subsequently, cells were stained with fluorescent phalloidin, and the percentage of A) polarized cells, B) roundness, or C) eccentricity was determined as described in the Experimental Section. Higher values of roundness and eccentricity indicate that the cell shape is further from a circle and that the cell is more polarized. All data are presented as the mean $\pm$ SD. Significant differences in each parameter from cells treated with C5a in the absence of a PtdIns(3,4,5)P_3 analogue are indicated by asterisks: *: $p<0.05$, **: $p<0.01$ versus control (histone H1 carrier) (Student t-test). D) Selected micrographs of inactivated (top) or C5a-activiated (bottom) neutrophils treated with no ligand or one of the two most potent ligands, 5-PT-PtdIns(3,4,5)P_3 or 3,4,5-PT_3-PtdIns(3,4,5)P_3.

logues activated Akt phosphorylation, and the two analogues tested, 3-PT and 3,4,5-PT3, failed to inhibit C5a-stimulated Akt phosphorylation.

We had hypothesized that the 5-stabilized analogues would be able to prevent the physiological production of PtdIns-(3,4)P_2 that is, at least in part, produced by the action of SHIP1 on PtdIns(3,4,5)P_3. In other words, by interfering with SHIP1 activity, we had envisaged that the exogenous addition of an analogue such as diC_8-3,4,5-PT_3-PtdIns(3,4,5)P_3 could qualitatively phenocopy the effect of the absence of SHIP1 in altering neutrophil polarization[36] (see Figure 5). In the end, however, this desired result was not achieved.

Sodium transport

To test the function of these analogues, we used A6 cell monolayers, a renal epithelium model that expresses epithelial sodium channels (ENaC), in which carrier-mediated intracellular delivery[45] of PtdIns(3,4,5)P_3 activates sodium transport.[46] ENaC activity is the rate-limiting step of the sodium transport and is stimulated by insulin.[47] DiC_{16}-PtdIns(3,4,5)P_3 is an early mediator of the insulin-stimulated sodium transport in A6 cells. Thus, we compared the effect of the unmodified diC_{16}-PtdIns-(3,4,5)P_3 with diC_{16}-5-PT-PtdIns(3,4,5)P_3 (**4**) and diC_{16}-5-MP-PtdIns(3,4,5)P_3 (**5**) on sodium transport across confluent monolayers of A6 cells. As shown in Figure S4, apical addition of analogues **4** and **5** increased sodium transport. The 5-ms analogues **4** and **5** also mimicked the activity of unstabilized PtdIns(3,4,5)P_3. It is worth noting that there was no significant variation in the resistance of the monolayers of A6 cells upon addition of the PtdIns(3,4,5)P_3 analogues tested in this study up to 60 min. Moreover, addition of amiloride to the apical bathing medium completely inhibited the current stimulated by either insulin or PtdIns(3,4,5)P_3.[46] We propose that these analogues might have other applications as pharmacological tools to probe role of PtdIns(3,4,5)P_3 in a cellular context.

Conclusions

Two 5-phosphatase-resistant analogues of PtdIns-(3,4,5)P_3 were synthesized and characterized in biochemical and biological contexts. First, the modification of a single phosphate of PtdIns(3,4,5)P_3 resulted in retention of binding to the PtdIns(3,4,5)P_3-specific GRP1 PH domain, with varying relative affinities. Second, modest inhibition of the action of SHIP1 phosphatase activity on Ins(1,3,4,5)P_4 was evident, and less marked inhibition of SHIP2 or PTEN dephosphorylation of Ins(1,3,4,5)P_4 by any of the analogues was observed. Third, while none of the five phosphatase-resistant analogues released phosphate when incubated with the 3-phosphatase PTEN, the 3-stabilized analogues **1** and **2** were partially dephosphorylated by the 5-phosphatase activity. Fourth, each of the analogues severely impaired complement factor C5a-mediated polarization and migration of murine neutrophils, with 3-MP (**2**) showing the greatest effect on polarization and 3,4,5-PT_3 (**3**) leading to the greatest decrease in eccentricity. Finally, the new 5-stabilized analogues **4** and **5**, similar to the previously tested analogues **1**, **2**, and **3**, both activated sodium transport in A6 cells.

We had originally envisaged using these new analogues as phosphatase-resistant mimics of PtdIns(3,4,5)P_3 to selectively manipulate cell responses. Unfortunately, the complexity of differential interactions of the analogues with different PtdIns-(3,4,5)P_3 targets, for example, Akt, GRP1, and PDK1, rendered this expectation unrealistic in a cellular context.

Experimental Section

Chemical syntheses: The synthesis of the 3-metabolically stabilized PtdIns(3,4,5)P_3 analogues **1**, **2** and 3,4,5-PT-PtdIns(3,4,5)P_3 (**3**) have been previously published. The 5-stabilized PtdIns(3,4,5)P_3 analogues **4** and **5** were synthesized in a similar way, and full details for their preparation and characterization can be found in the Supporting Information.

Protein expression and purification: The DNA fragment encoding residues 261–385 of the PH domain of human GRP1 was cloned in

pRSET A vector (Invitrogen). The ^{15}N-labeled protein was expressed in *E. coli* Rosetta in minimal medium supplemented with $^{15}NH_4Cl$ (Cambridge Isotope). Bacteria were harvested by centrifugation after induction with isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 mM) and lysed in a French Press. The His_6 fusion protein was purified on a Talon resin column (Clontech Laboratories, Inc.). The His tag was cleaved with EKMax (Invitrogen). The protein was further purified by ion-exchange chromatography on a HiTrap SP HP column (Amersham) in Bis-Tris buffer (pH 6.5), and concentrated in Millipore concentrators.

NMR spectroscopy and titration of PtdIns(3,4,5)P_3 analogues: NMR spectra were recorded at 25°C on a Varian INOVA 500 MHz spectrometer. The ^1H,^{15}N HSQC spectra of 0.2 mM uniformly ^{15}N-labeled PH domain were collected while dibutanoyl (C_4)-PtdIns-(3,4,5)P_3 analogues were added stepwise. (See also Figure S1).

Expression of human SHIP1 and SHIP2 enzymes. The SHIP2 catalytic domain was expressed as a glutathione-S-transferase (GST) fusion protein and expressed in bacteria as reported.[49] The cDNA encoding human SHIP1 was subcloned in pTrHis bacterial expression vector (this construct was provided by, Dr Len Stephens, Babraham, UK) and expressed as His-tagged fusion protein.[50] Briefly, an overnight culture of bacteria (0.4 L) was twofold diluted the next day to reach an OD_{600} of 0.6 and induced by IPTG for 5 h at 20°C. The crude lysate was taken up in Tris-HCl (50 mM, pH 7.5) containing 0.1% Triton X-100, NaCl (300 mM), 10% glycerol, $MgCl_2$ (5 mM), and complete protease inhibitors (Roche). The enzyme was applied to a Ni-NTA agarose column (Qiagen), washed, and eluted with imidazole (100 mM) in the same buffer. GST-PTEN was expressed and purified as reported.[49]

Determination of SHIP1, SHIP2, and PTEN phosphatase activities: SHIP1 and SHIP2 phosphatase activities were determined in the presence of [^3H]-Ins(1,3,4,5)P_4 (0.3 μM), and Dowex columns was used to isolate the dephosphorylated product.[51] When the substrate specificity of SHIP2 and PTEN was addressed with the various analogues, the malachite phosphate release assay was used as described.[49] The enzyme activity data are shown as means of triplicates ± standard deviation. (See Figure S2 for PTEN inhibition.)

Western blot for Akt activation: Mouse peritoneal neutrophils were purified, stimulated, and lysed as described.[36] Lysates were separated by 10% SDS-PAGE, followed by immunoblotting with phospho-specific antibodies against Akt pS473 and pan-Akt (Cell Signaling Technology). (See Figure S3).

Neutrophil morphology: Mouse neutrophils (4×10^5) were seeded onto glass coverslips. Cells were allowed to attach for 10 min at room temperature, incubated at 37°C with or without diC_{16}-PtdIns-(3,4,5)P_3 analogues (10 μM) and carrier (1.5 μM histone H1) for 30 min, and stimulated with complement factor 5a (C5a, 5 nM). The reaction was stopped by fixing the cells in 4% paraformaldehyde. The fixed cells were washed and incubated for 1 h in PBS containing Alexa Fluor 488 phalloidin (0.2 U mL^{-1}; Molecular Probes), 0.1% lysophosphatidylcholine (Nacalai, Kyoto, Japan), and 1% BSA. Cell shape was determined from confocal images of fluorescently labeled cells. Cells with distinctive, F-actin-rich leading edges were qualified as polarized ($n>300$ cells). The perimeter (x), area (y), eccentricity, and radial standard deviation (radial S.D.) were determined for each cell ($n>43$) by using the analysis functions of IPLab software (Scanalytics, Fairfax, VA). The formula used to determine roundness (R) was $R=1-4\pi yx^{-2}$. Low values for eccentricity, radial S.D. and roundness indicate a lack of polarization.

Experimental determination and evaluation of sodium transport (I_{Na^+} [μA cm^{-2}]): Briefly, A6 cells were subcultured onto 24 mm Millicell inserts (Millipore) for 10 days and, the day before the experiment, incubated overnight in a serum-free 260 mosmol per kg H_2O amphibian Ringer solution. diC_{16}-PtdIns(3,4,5)P_3, analogues **4** and **5** was complexed by histone H1 carrier (50 μM) and then added to the apical side of the monolayer. The results were compared with insulin basolateral stimulation (100 nM) and control (histone H1 alone). This experiment is representative of three independent experiments. (See Figure S4.)

Acknowledgements

We thank the NIH (NS 29632 to G.D.P. and GM 071424 to T.G.K.). The research of T.S. was supported by a Grant-in-Aid for Creative Scientific Research and a Grant for Young Scientists (A) from JSPS and MEXT. C.E. was supported by a grant from the Interuniversity Attraction Poles Programme (P6/28)—Belgium State—Belgium Science Policy and by the Fonds de la Recherche Scientifique Médicale. We thank Colette Moreau for her help in conducting the inositol phosphatase assays.

Keywords: asymmetric synthesis · enzyme inhibition · inositol lipid · metabolic stability · phosphatases · sodium transport

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Received: August 30, 2009
Published online on January 5, 2010

