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Synthesis and Biological Activity of Phosphatidylinositol 3, 4, 5-Trisphosphorothioate

Honglu Zhang^a, Yong Xu^a, Nicolas Markadieu^b, Renaud Beauwens^b, Christophe Erneux^c, and Glenn D. Prestwich^a

^aDepartment of Medicinal Chemistry, The University of Utah, 419 Wakara Way, Suite 205, Salt Lake City, Utah 84108-1257 USA

^bDepartment of Cell Physiology, Université Libre de Bruxelles, Campus Erasme, Route de Lennik 808, 1070 Bruxelles, Belgium.

^cInstitut de Recherche Interdisciplinaire (IRIBHM), Université Libre de Bruxelles, Campus Erasme, Route de Lennik 808, 1070 Bruxelles, Belgium.

Abstract

Metabolically-stabilized analogues of PtdIns(3,4,5)P₃ have shown long-lived agonist activity for cellular events mediated by this phosphoinositide. We describe an efficient method for the total asymmetric synthesis of the trisphosphorothioate (PT) analogue of PtdIns(3,4,5)P₃. Intracellular delivery of dipalmitoyl PtdIns(3,4,5)PT₃ mimicked insulin in activating sodium transport in A6 cells.

Phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P₃) is a crucially important second messenger found ubiquitously in higher eukaryotic cells.¹ The activation of Class I PI 3-K and subsequent production of PtdIns(3,4,5)P₃ is an important cell signaling event that has been causally linked to the activation of variety of downstream cellular processes.² These signaling events include cell proliferation and transformation,³ cell shape and motility,⁴ and the action of insulin in altering glucose transport.⁵ PtdIns(3,4,5)P₃-regulated signaling is governed both by its formation by PI 3-K and its conversion into phosphatidylinositol 4,5- and 3,4-bisphosphates by the 3-phosphatase PTEN⁶ or the 5-phosphatase SHIP⁷, respectively. Control of PI 3-K and PtdIns(3,4,5)P₃ levels have emerged as important targets⁸ in cardiovascular disease, allergy and autoimmune disease,⁹ cancer, and diabetes.⁸

In addition to regulation of PtdIns(3,4,5)P₃ production, catabolism by the phosphatases PTEN and SHIP have emerged as crucial processes to control PtdIns(3,4,5)P₃-dependent signaling (Scheme 1). The availability of affinity probes for isolation and characterization of PtdInsP_n binding proteins, and the availability of tools for real-time localization of PtdInsP_ns in cells are providing a continuous influx of new information on the roles of phosphoinositides in cellular processes.¹⁰ However, many questions regarding the specific effects of PtdIns(3,4,5)P₃ remain unanswered due to its transient nature in the cell.

We recently described the preparation and activity of 3-phosphatase-resistant, metabolically-stabilized (ms) analogues of PtdIns(3,4,5)P₃ that was both stable to degradation by PTEN and acted as an inhibitor of PTEN.¹¹ One of these analogues incorporated a single

Phone: +1-801-585-9051. Fax: +1-801-585-9053. Email: gprestwich@pharm.utah.edu.

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phosphorothioate (PT) substituent. Phosphorothioates are important phosphomimetics that show greatly reduced rates of enzyme-mediated hydrolysis.¹² However, the replacement of P=O by P=S also affects the pKa of the phosphate and removes a H-bond acceptor.^{13,14} Indeed, we demonstrated that PtdIns(3)PT had reduced binding activity for cognate PtdIns(3) P-selective FYVE and PX domain binding proteins, which was attributable to reduced H-bonding.¹⁵ We hypothesized that a 3,4,5-tris(PT) analogue of PtdIns(3,4,5)P₃ could be either an antagonist or a long-lived agonist in the related signaling pathway, because it would be more slowly dephosphorylated by both PTEN and SHIP, and could potentially block the normal receptor-mediated signaling involving PtdIns(3,4,5)P₃. We also observed that a PT analogue of PtdIns(5)P¹⁶ and a tris(PT) cyclopentyl analogue¹⁷ of Ins(1,4,5)P₃ had agonistic activities and long biological half-lives. We now describe the first asymmetric total synthesis of a PtdIns(3,4,5)PT₃, a trisphosphorothioate analogue of PtdIns(3,4,5)P₃ that is resistant to hydrolysis by both PTEN and SHIP. In addition, we document the ability of these analogues to effect in sodium transport in A6 cell monolayers, an amphibian renal epithelium model expressing the epithelial sodium channels.¹⁸

Starting from 3,4,5-benzoyl inositol **1**^{19,20}, the fully protected inositol intermediate **2** was obtained by reaction of diol **1** with MOMCl and DIPEA at 50 °C (Scheme 2). Treatment of compound **2** with 1.0 M NaOMe in methanol-THF gave the desired triol **3** in 85% yield, which was then phosphorylated and oxidized with elemental sulfur to yield the corresponding phosphorothioate **4** in 28% yield.¹⁵ Use of the HF•Py complex in THF-pyridine solution selectively removed the TBDPS ether. This deprotection reaction was very slow, and required supplemental addition of HF•Py after 3 days reaction, and three weeks to give key intermediate **5** in low yield.¹⁵

The phosphoramidite **6a**¹⁵ was then coupled with inositol **5** in the presence of 1*H*-tetrazole to yield the phosphite intermediate, which was oxidized utilizing *t*-BuOOH to give protected phosphorothioate **7a** as shown in Scheme 2. The cyanoethyl groups (CE) on the phosphorothioate were removed by using triethylamine (TEA) plus bis(trimethylsilyl) trifluoroacetamide (BSTFA) in anhydrous acetonitrile. BSTFA was added to prevent the phosphorothioate anion from undergoing re-alkylation. The cleavage of the *O*-silyl derivatives was achieved by aqueous hydrolysis at neutral pH to give the MOM ether-protected intermediate. The MOM groups were removed using ethanethiol and BF₃•Et₂O at rt to give the final product **8a**.^{15,21}

Although this synthetic route afforded the desired tris(PT) **8a**, a more efficient synthetic approach was deemed necessary. The disadvantages included the low yield of oxidation with elemental sulfur, and the low yield resulting from a TBDPS deprotection protocol that required several weeks. The sulfur-pyridine reaction conditions to convert the phosphite esters to the phosphorothioates¹⁵ resulted in decomposition of the six cyanoethyl groups (CE) under basic conditions. We thus chose to replace the TBDPS group with TES, because TES is still sterically bulky but can be easily removed under milder conditions. Also, phenylacetyl disulfide^{11,22} was selected as an alternative to elemental sulfur, and this reagent can be employed under neutral conditions to minimize destruction of the CE protecting groups.

The TES ether **9** was thus obtained in high yield in two steps; first, deprotection of the TBDPS ether **2** with solid TBAF in DMF for 3 h, followed by reaction with TESCl and imidazole at room temperature.¹¹ Next, removal of the three benzoyl groups of TES-ether **9** sodium methoxide gave only a low yield of triol **10**, accompanied by partial TES cleavage. As an alternative, DIBAL-H/−78 °C was employed for benzoyl removal,¹¹ because the TES and MOM groups were stable to these conditions; compound **10** was obtained in 81% yield. Then, triol **10** was converted to the protected tris(PT) in a two-step, one pot reaction. Phosphitylation with 1*H*-tetrazole and bis(2-cyanoethoxy) (diisopropylamino)phosphine was followed by

oxidation of the resulting phosphite ester with phenylacetyl disulfide in 30 min, giving the desired phosphorothioate **11** in 72% yield.¹¹ Since the cyanoethyl groups were labile under basic conditions, we chose the weakly acidic reagent NH₄F for deprotection of the TES. After reaction with NH₄F in MeOH for 3 h the key intermediate **5** was obtained 90% yield as a single product (Scheme 3).¹¹ The modifications introduced herein gave the protected inositol intermediate more efficiently and in higher overall yield.

Condensation of **5** with each of the freshly prepared phosphoramidites **6b–6d** in the presence of 1*H*-tetrazole and *t*-BuOOH oxidation gave rise to the fully protected phosphatidylinositol analogues **7b–7d**.¹¹ The cyanoethyl groups in intermediates **7b–7d** were removed by using triethylamine and BSTFA in anhydrous acetonitrile. After 24 h, we removed the solvents to afford a residue, of which the ¹H NMR showed that the cyanoethyl groups were eliminated and the hydroxy groups were protected with TMS. Thus, BSTFA effectively prevents the phosphorothioate anions from undergoing realkylation. The TMS protected intermediates also provided us a method to remove the methyl and MOM group; that is, TMSBr could be added directly to the residue, and this led to complete removal of the methyl and MOM groups to give products **13b–13d**.¹¹ In our previous synthesis¹⁵ and in work by the Bruzik laboratory,²⁰ the TMS-esters **12** were generally converted to the ammonium salts. Then, reaction of the ammonium salts with TMSBr frequently gave only partial cleavage of the MOM ethers. However, we observed that the MOM groups were completely removed by direct exposure of the TMS-esters **12** to TMSBr in CH₂Cl₂.^{23,24} This is very convenient for the purification of the final product, because all the reaction reagents are readily removed under vacuum. Finally, methanol hydrolysis of the fully TMS protected intermediates **13b–13d** gave the final PtdIns (3,4,5)PT₃ analogues **8b–8d** in their acid forms (Scheme 3).

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 delivery²⁵ of PtdIns(3,4,5)P₃ activates GLUT4 translocation to the plasma membrane⁵ and sodium transport.²⁶ ENaC activity is the rate-limiting step of the sodium transport and is stimulated by insulin.²⁷ DiC₁₆-PtdIns(3,4,5)P₃ is an early mediator of the insulin-stimulated sodium transport in A6 cells. Thus, we compared the effect of the unmodified diC₁₆-PtdIns(3,4,5)P₃ with diC₁₆-PtdIns(3,4,5)PT₃ **8d** on sodium transport across confluent monolayers of A6 cells. As shown in Figure 1, apical addition of analogue **8d** increased sodium transport.²⁸ The 3,4,5-tris(PT) analogue **8d** also mimicked the activity unstabilized PtdIns(3,4,5)P₃. The lag time observed between PtdIns(3,4,5)PT₃ addition and the final effect on sodium transport revealed that the rate of intracellular delivery of this analogue is faster than that of unstabilized PtdIns(3,4,5)P₃. We propose that these PtdIns(3,4,5)PT₃ analogues may have other applications as pharmacological tools to probe role of PtdIns(3,4,5)P₃ in a cellular context.

Supplementary Material

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Acknowledgments

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28. Experimental determination and evaluation of sodium transport (I_{Na^+} , $\mu A/cm^2$). Briefly, A6 cells were subcultured onto 24-mm Millicell inserts (Millipore, Bedford, MA) for 10 days and the day before the experiment, incubated overnight in a serum-free 260 mosmol/kg H₂O amphibian Ringer solution. DiC₁₆-PtdIns(3,4,5)P₃, analogue **8d** was complexed by histone H1 carrier (50 μM) and then added to the apical side of the monolayer. Results were compared with insulin basolateral stimulation (100 nM) and control (histone H1 alone). This experiment is representative of three independent experiments.

Figure 1. Stimulation of A6 cell monolayers. Experiment and evaluation of sodium transport (I_{Na^+} , $\mu A/cm^2$) was carried out as described.²⁶ Briefly, A6 cells were subcultured onto 24-mm Millicell inserts (Millipore, Bedford, MA) for 10 days and the day before the experiment, incubated overnight in a serum-free 260 mosmol/kg H₂O amphibian Ringer solution. DiC_{16} -PtdIns(3,4,5)P₃, diC_{16} -PtdIns(3,4,5)PT₃ **8d** (50 μM) was complexed by histone H1 carrier (50 μM) and then added to the apical side of the monolayer. Results were compared with insulin basolateral stimulation (100 nM) and control (histone H1 alone).

Scheme 1.
Pathways for PtdIns(3,4,5)P₃ biosynthesis and catabolism.

Scheme 2.

Reagents and conditions: (a) MOMCl, DIPEA, DMF, 50 °C, 75%; (b) NaOMe, CH₃OH/THF, 85%; (c) Bis(2-cyanoethoxy)(diisopropylamino)phosphine, 1*H*-tetrazole; S, CS₂/Py, 28%; (d) HF•Py, THF/Py, 3 weeks, 53%; (e) 1*H*-tetrazole, CH₂Cl₂; (f) *t*-BuOOH, 48%; (g) TEA, BSTFA, CH₃CN; (h) NH₄OAc, H₂O; (i) EtSH, BF₃•Et₂O, 88%.

Scheme 3.

Reagents and conditions: (a) TBAF•3H₂O, DMF, 86%; (b) TESCl, imidazole, CH₂Cl₂, 87%; (c) DIBAL-H, CH₂Cl₂, -78 °C, 81%; (d) Bis(2-cyanoethoxy)(diisopropylamino)phosphine, 1*H*-tetrazole; phenylacetyl disulfide, 72%; (e) NH₄F, MeOH, 90%; (f) 1*H*-tetrazole, CH₂Cl₂, rt; *t*-BuOOH; (g) TEA, BSTFA, CH₃CN; (h) TMSBr/CH₂Cl₂ (2:3), rt; (i) MeOH.