

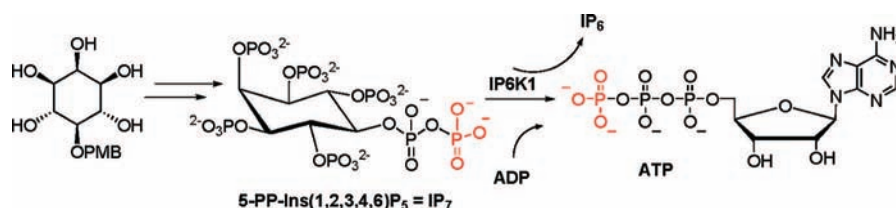
A Scalable Synthesis of the IP₇ Isomer,
5-PP-Ins(1,2,3,4,6)P₅Honglu Zhang,[†] James Thompson,[‡] and Glenn D. Prestwich^{*,†}

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ABSTRACT



The phosphorylated inositol diphosphates, including the diphosphoinositol pentakisphosphate regioisomers, play critical roles in signal transduction and cellular regulation. In particular, the IP₇ isomer 5-PP-Ins(1,2,3,4,6)P₅ is implicated in a nonenzymatic phosphate transfer converting a protein serine phosphate residue to a serine diphosphate. A scalable, practical new synthesis of 5-PP-Ins(1,2,3,4,6)P₅ is described that also allows access to a variety of IP₇ and IP₈ regioisomers. The identity of the synthetic 5-PP-Ins(1,2,3,4,6)P₅ was validated using IP6K1 to catalyze the conversion of IP₇ + ADP to ATP + IP₆.

Inositides, particularly inositol phosphates and phosphatidylinositol lipids, are crucially important cell signaling molecules that are linked to a series of signaling events.^{1–3} These signaling events include ion-channel function,^{4,5} vesicle trafficking,⁶ apoptosis,^{7,8} transcriptional regulation,⁹ motility,¹⁰ cell proliferation, and transformation.¹¹ Inositol

1,4,5-trisphosphate (IP₃) is a ubiquitous second messenger, which couples agonist stimulation of a wide variety of receptors to the mobilization of intracellular calcium.¹² The more highly phosphorylated inositides, particularly the “omnipotent” inositol hexakisphosphate¹³ and phosphorylated inositol diphosphates (commonly called pyrophosphates in the biological literature), play critical roles in signal transduction and cellular regulation.^{2,6}

In 1993, two inositol diphosphates, diphosphoinositol pentakisphosphate (5-PP-Ins(1,2,3,4,6)P₅, or IP₇) and bis-diphosphoinositol tetrakisphosphate (PP₂–InsP₄, or IP₈), were purified from *Dictyostelium*.^{6,14} The diphosphate bond of the 5-PP-Ins(1,2,3,4,6)P₅ has a calculated phosphorylation potential that equals or exceeds that of ATP,^{6,15} suggesting that it could serve a similar function. 5-PP-Ins(1,2,3,4,6)P₅ is biosynthesized from inositol hexakisphosphate (IP₆) by a

[†] The University of Utah.[‡] Echelon Biosciences.(1) McLaughlin, S.; Murray, D. *Nature* **2005**, *438*, 605–611.(2) Irvine, R. F.; Schell, M. J. *Nat. Rev. Mol. Cell. Biol.* **2001**, *2*, 327–338.(3) Toker, A.; Cantley, L. C. *Nature* **1997**, *387*, 673–676.(4) Chuang, H. H.; Prescott, E. D.; Kong, H.; Shields, S.; Jordt, S. E.; Basbaum, A. I.; Chao, M. V.; Julius, D. *Nature* **2001**, *411*, 957–962.(5) Rohacs, T.; Lopes, C. M.; Michailidis, I.; Logothetis, D. E. *Nat. Neurosci.* **2005**, *8*, 626–634.(6) Bennett, M.; Onnebo, S. M.; Azevedo, C.; Saiardi, A. *Cell. Mol. Life Sci.* **2006**, *63*, 552–564.(7) Morrison, B. H.; Bauer, J. A.; Kalvakolanu, D. V.; Lindner, D. J. *J. Biol. Chem.* **2001**, *276*, 24965–24970.(8) Nagata, E.; Luo, H. R.; Saiardi, A.; Bae, B. I.; Suzuki, N.; Snyder, S. H. *J. Biol. Chem.* **2005**, *280*, 1634–1640.(9) Shears, S. B. *Bioessays* **2000**, *22*, 786–789.(10) Pinal, N.; Goberdhan, D. C.; Collinson, L.; Fujita, Y.; Cox, I. M.; Wilson, C.; Pichaud, F. *Curr. Biol.* **2006**, *16*, 140–9.(11) Cantley, L. C.; Auger, K. R.; Carpenter, C.; Duckworth, B.; Graziani, A.; Kapeller, R.; Soltoff, S. *Cell* **1991**, *64*, 281–302.(12) Berridge, M. J.; Lipp, P.; Bootman, M. D. *Nat. Rev. Mol. Cell. Biol.* **2000**, *1*, 11–21.(13) Shears, S. B. *Cell. Signalling* **2001**, *13*, 151–158.(14) Bhandari, R.; Chakraborty, A.; Snyder, S. H. *Cell. Metab.* **2007**, *5*, 321–323.(15) Saiardi, A.; Bhandari, R.; Resnick, A. C.; Snowman, A. M.; Snyder, S. H. *Science* **2004**, *306*, 2101–2105.

family of three inositol hexakisphosphate kinases (InsP6Ks).^{16–18} Inositol hexakisphosphate kinase-2 (InsP6K2), one of the InsP6Ks, was found to be an important physiologic mediator of cell death. More recent studies revealed that IP₇ plays an important role in regulation of insulin secretion.^{19–22} Recently, fluorometric detection of IP₇ in the presence of IP₆ and lower IP_n congeners was achieved by the Matile laboratory using a synthetic multifunctional pore.²³ A different regioisomer of IP₇ is produced by yeast Vip1 and is necessary for Pho81 cyclin-dependent kinase (CDK) inhibition of the cyclin–CDK complex Pho80–Pho85, thereby regulating an important metabolic network.^{24,25} Although first identified²⁴ as 4(6)-PP-InsP₅, this was recently revised²⁶ to 1(3)-PP-InsP₅, a result that has been independently validated and extended by synthesis and assay of the enantiopure 1-PP and 3-PP-InsP₅ isomers.²⁷

The reversible phosphorylation of proteins regulates nearly every aspect of cell physiology.²⁸ Phosphorylation and dephosphorylation, catalyzed by protein kinases²⁹ and protein phosphatases, are depicted in Figure 1. Some 30% of the proteins encoded by the human genome are phosphorylated, and abnormal phosphorylation is now recognized as a cause or a consequence of many human pathologies. As a result, protein kinases are already the second largest group of drug targets after G-protein-coupled receptors, and they account for 20–30% of the drug discovery programs of many companies.³⁰

Unlike ATP, however, the inositol diphosphate IP₇ appears to phosphorylate serine phosphate residues nonenzymatically (Figure 1).³¹ 5-PP-Ins(1,2,3,4,6)P₅ has been demonstrated to phosphorylate a variety of Ser-rich protein targets in yeast and mammals.¹⁵ The resulting 5-PP-Ins(1,2,3,4,6)P₅-phosphorylated peptides are more acid-labile and more resistant to phosphatases, suggesting that a protein diphosphate bond had been formed (Figure 1).³¹ Moreover, only the Ser-rich

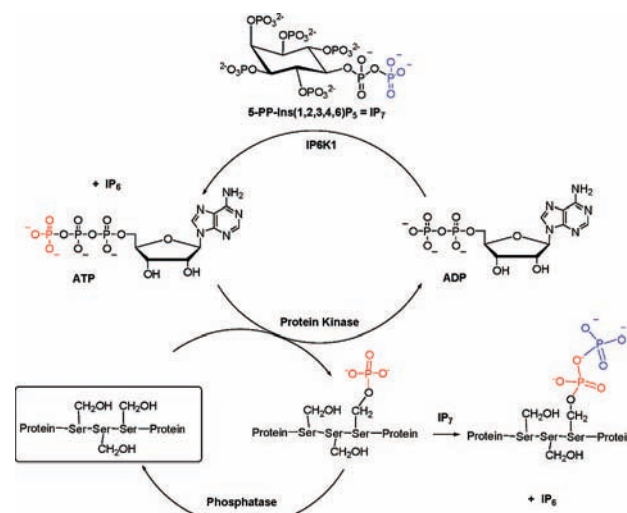
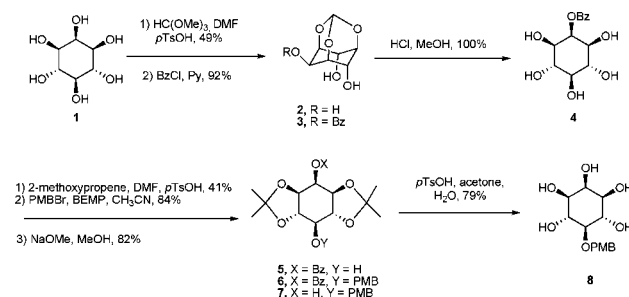


Figure 1. Reversible protein phosphorylation and pyrophosphorylation mechanisms.

regions of target proteins that had been previously phosphorylated by a protein kinase were substrates, strongly implicating Ser-PP, a serine diphosphate (pyrophosphate), as the product of the nonenzymatic diphosphorylation. This diphosphorylation may represent a novel mode of signaling,³¹ but its study in many cases is limited by the availability of 5-PP-Ins(1,2,3,4,6)P₅ and a family of chemical and biological tools to probe the structure and function of diphosphorylated proteins. To address this unmet need, we describe herein a scalable and efficient new method for the synthesis of 5-PP-Ins(1,2,3,4,6)P₅ based on modifications of previous inosite syntheses by the laboratories of Prestwich³¹ and Falck.^{32,33}

The three key problems in the synthesis of any IP₇ stereoisomer are (i) the method of stereoselective introduction of the protected 5-diphosphate, (ii) the stability of the protected diphosphorylated intermediate(s), and (iii) the complete removal of protecting groups under mild conditions with minimal degradation of the desired IP₇. We selected the synthetic route as shown in Scheme 1 to prepare the key intermediate **8**. It is important to note that the starting materials, intermediates, and final products are all formally *meso* compounds, since the 5-diphosphate will be positioned

Scheme 1. Synthesis of Advanced Intermediate **8**



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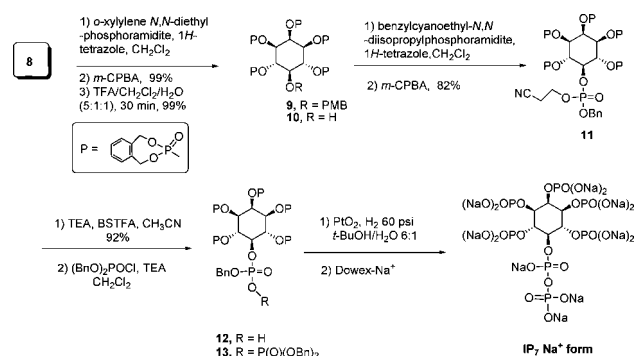
on the C-2–C-5 plane of symmetry. Following known procedures, *myo*-inositol 1,3,5-orthoformate **2**, available in one step from *myo*-inositol,³⁴ was selectively benzoylated at 2-OH with benzoyl chloride.³⁵ Hydrolysis of orthoformate **2** in 4 M HCl/MeOH afforded benzoate **4**,³⁵ which was converted to the acetonide **5** as a mixture with other isomers.³⁶ Compound **5** was easily separated from other isomers by flash chromatography. Introduction of the 4-methoxybenzyl ether was unexpectedly problematic. Under standard benzylation conditions (NaH or *n*BuLi, 4-MeOBnCl or 4-MeOBnBr, THF or DMF) or phase-transfer catalysis conditions (4-MeOBnCl, Bu₄NHSO₄, aqueous NaOH), starting material was converted to complex product mixtures. Nonetheless, reaction of **5** with 4-methoxybenzyl bromide in the presence of the hindered organic base, 2-*tert*-butylimino-2-diethylamino-1,3-dimethylperhydro-1,3,2-diazaphosphorine (BEMP),³⁷ in CH₃CN afforded the fully protected inositol **6** in 84% yield. Treatment of benzoate **6** with NaOMe gave 2-OH inositol **7** in 82% yield, and hydrolysis of the acetonides with *p*TsOH in acetone/H₂O gave the 5-PMB-protected inositol **8** in 79% yield. Since **8** was poorly soluble in EtOAc and CH₂Cl₂, purification was accomplished simply by trituration of the white solid with these two solvents.

With the advanced intermediate 5-PMB inositol in hand, different phosphoramidites coupling with compound **8** would generate different phosphates. Considering the stability of the IP₇, dimethyl phosphate and diethyl phosphate could not be used as protecting groups because the use of TMSBr to remove these protecting groups could cause decomposition of the diphosphate moiety. The dibenzyl phosphate^{32,33} and *o*-xylylene phosphate are the best candidates because they are readily removed by hydrogenolysis. In addition, since the *o*-xylylene phosphate is sterically smaller than dibenzyl phosphate, we expected that the *o*-xylylene phosphate intermediate **13** would be more stable in this highly sterically congested intermediate. Thus, the pentakis(*o*-xylylene phosphate) **9** was prepared in 99% yield by global phosphorylation of pentaol **8** with *o*-xylylene *N,N*-diethylphosphoramidite and 1*H*-tetrazole in CH₂Cl₂. The 4-methoxybenzyl functional group was removed with TFA/CH₂Cl₂/H₂O (5:1:1, v/v) to furnish alcohol **10**.

Here again, our route diverges from previous methods. The unsymmetrical phosphoramidite, benzyl-2-cyanoethyl *N,N*-diisopropylphosphoramidite, was prepared in 86% yield from commercial 2-cyanoethyl *N,N*-diisopropyl-chlorophosphoramidite and benzyl alcohol in the presence of Hunig's base. Reaction of **10** with this phosphoramidite in the presence of 1*H*-tetrazole, followed by *m*-CPBA oxidation,

generated the differentially protected hexakisphosphate **11**. Selective removal of the cyanoethyl group under mild conditions (TEA/BSTFA/CH₃CN)^{38–40} and purification by Dowex-H⁺ chromatography afforded pure monophosphoric acid **12** in 92% yield (Scheme 2). After treatment of **12** (1

Scheme 2. Global Phosphorylation and Final Conversion to 5-PP-Ins(1,2,3,4,6)P₅



equiv) with triethylamine (2 equiv) in anhydrous CH₂Cl₂ at 0 °C followed by dibenzylphosphoryl chloride (2 equiv) for 2 h at rt, solvents were removed to stop the reaction and give a crude product. The crude residue **13** was then deprotected by stirring an aqueous *t*-butanol solution under H₂ (60 psi) with PtO₂ for 4 h. After removal of the catalyst and concentration, the residue was dissolved in water and washed with EtOAc and CH₂Cl₂. The aqueous solution was concentrated and purified through ion exchange chromatography (Dowex 50W × 8 – 200 Na⁺ exchange resin) by elution with water to afford the 5-PP-Ins(1,2,3,4,6)P₅ sodium form in 81% yield as white solid. The ³¹P NMR spectrum of 5-PP-Ins(1,2,3,4,6)P₅ in D₂O displayed a characteristic pyrophosphate peak, at –9.40 to –10.20 ppm, integrating at a 2.0:5.04 ratio relative to the monophosphate resonances between 0.2 and 1.2 ppm (Figure 2). The complexity of the signals reflects a distribution of sodium salt and protonated species each with characteristic chemical shifts. Similarly, the key feature of the complex proton spectrum is the 1.0:4.88 ratio of peaks for H-5:H1-4, H6. Both ¹H NMR and ³¹P NMR were consistent with the 5-PP-Ins(1,2,3,4,6)P₅ isolated from *Polysphondylum*.⁴¹

In this new synthetic route, intermediate **5** could be readily prepared in 10 g amounts in four steps, including removal of stereoisomers, in 18% yield from inexpensive commercially available materials. With this known material, the final 5-PP-InsP₅ was prepared in 33% overall yield for seven steps. Importantly, the *o*-xylylene phosphate **13** was more stable than the globally benzyl-protected 5-PP-InsP₅ inter-

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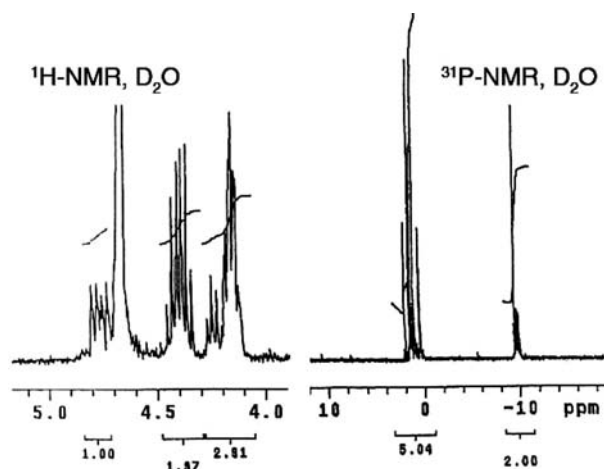


Figure 2. ^1H NMR and ^{31}P NMR of 5-PP-InsP₅.

mediate, which only gave a 25% yield for the final three steps described by us previously.³¹

To validate the biological activity of the synthetic 5-PP-Ins(1,2,3,4,6)P₅, we used it as a substrate to phosphorylate ADP. Inositol hexakisphosphate kinase 1 (IP6K1) not only catalyzes the transfer of a phosphate from ATP to IP₆ yielding 5-PP-Ins(1,2,3,4,6)P₅ plus ADP but also readily catalyzes the reverse reaction in which a phosphate is transferred from 5-PP-Ins(1,2,3,4,6)P₅ to ADP yielding ATP and IP₆.⁴² Thus, synthetic 5-PP-Ins(1,2,3,4,6)P₅ was incubated with ADP and recombinant IP6K1, and ATP synthase activity was monitored using the activity of the enzyme luciferase. An increase in ATP, resulting from its production from 5-PP-Ins(1,2,3,4,6)P₅ plus ADP, was quantified by measuring the luciferase-catalyzed conversion of luciferol to oxyluciferol (Figure 3). Synthetic 5-PP-Ins(1,2,3,4,6)P₅ may be used as a substrate for ATP synthesis in a reaction catalyzed by IP6K1.

To ensure IP₇ was not directly phosphorylating ADP to ATP without the enzyme IP6K1, the luciferase reaction was repeated without the addition of the enzyme IP6K1. In this

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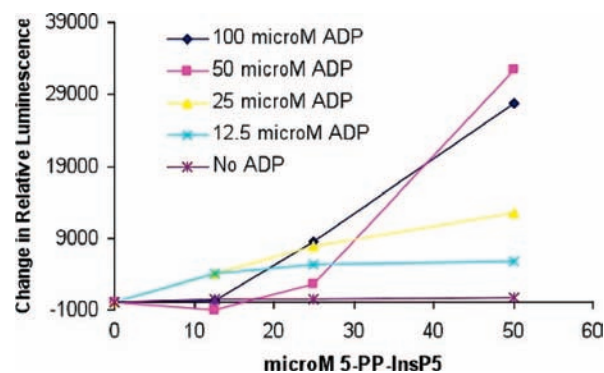


Figure 3. Effects of the concentration of ADP and synthetic 5-PP-Ins(1,2,3,4,6)P₅ on ATP production by IP6K1.

case, no increase in luciferase activity was observed, indicating IP6K1 is indeed required for ATP synthase activity (Supporting Information, Figures 1 and 2). Additionally, incubation of 5-PP-Ins(1,2,3,4,6)P₅ with ATP and the enzyme luciferase results in decreased conversion of luciferin to oxyluciferin indicating 5-PP-Ins(1,2,3,4,6)P₅ may inhibit the luciferase enzyme (Supporting Information, Figure 1).

In conclusion, we have developed an efficient and scalable method for the synthesis of the important IP₇ regioisomer 5-PP-Ins(1,2,3,4,6)P₅, which will meet the demand of cell signaling scientists in the studies of protein pyrophosphorylation, exocytosis in pancreatic beta cells, and other key cellular processes. In addition, this new synthetic approach method can be adapted to the chemical synthesis of a variety of regioisomers of IP₈, the bisdiphosphoinositol tetrakisphosphates (PP₂–InsP₄), which have recognized importance in cell signaling research.

Acknowledgment. We thank the NIH for financial support (NS 29632 to GDP).

Supporting Information Available: Experimental details for synthesis, characterization of new compounds, and experimental methods for IP6K1-catalyzed reaction figures for enzyme-free experiment. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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