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A Scalable Synthesis of the IP_7 Isomer, 5-PP-Ins(1,2,3,4,6)P_5

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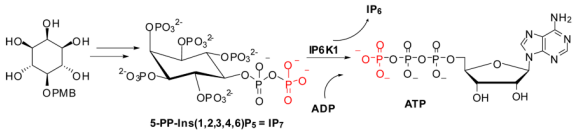
Abstract



The phosphorylated inositol diphosphates, including the diphosphoinositol pentakisphosphate regioisomers, play critical roles in signal-transduction and cellular regulation. In particular, the IP_7 isomer 5-PP-Ins(1,2,3,4,6)P_5 is implicated in a non-enzymatic 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)P_5 is described that also allows access to a variety of IP_7 and IP_8 regioisomers. The identity of the synthetic 5-PP-Ins(1,2,3,4,6)P_5 was validated using IP6K1 to catalyze the conversion of IP_7 + ADP to ATP + IP_6.

Inositides, particulary 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 (IP_3) 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 hexakisphosphate[13] 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)P_5, or IP_7) and bisdiphosphoinositol tetrakisphosphate (PP_2-$InsP_4$, or IP_8), were purified from *Dictyostelium*.[6,14] The diphosphate bond of the 5-PP-Ins(1,2,3,4,6)P_5 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)P_5 is biosynthesized from inositol hexakisphosphate (IP_6) by a 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 IP_7 plays an important role in regulation of insulin secretion.[19–22] Recently, fluorometric detection of IP_7 in the presence of IP_6 and lower IP_n congeners was achieved by the Matile lab using a synthetic multifunctional pore.[23] A different regioisomer of IP_7 is produced by yeast Vip1 and is

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Supporting Information Available: Experimental details for synthesis, characterization of new compounds; 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.

necessary for Pho81 cyclic-dependent kinase (CDK) inhibition of the the cyclin-CDK complex Pho80-Pho85, thereby regulating an important metabolic network.[24,25] Although first identified[24] as 4(6)-PP-InsP_5, this was recently revised[26] to 1(3)-PP-InsP_5, a result that has been independently validated and extended by synthesis and assay of the enantiopure 1-PP and 3-PP-InsP_5 isomers.[27]

The reversible phosphorylation of proteins regulates nearly every aspect of cell physiology.[28] Phosphorylation and dephosphorylation, catalyzed by protein kinases[29] and protein phosphatases, is 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 IP_7 appears to phosphorylate serine phosphate residues non-enzymatically (Figure 1).[31] 5-PP-Ins(1,2,3,4,6)P_5 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)P_5-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 regions of target proteins that had been previously phosphorylated by a protein kinase were substrates, strongly implicated Ser-PP, a serine diphosphate (pyrophosphate), as the product of the non-enzymatic diphosphorylation. This diphosphorylation may represent a novel mode of signaling.[31] This diphosphorylation may represent a new fundamental signaling mechanism, but its study in many cases is limited by the availability of 5-PP-Ins(1,2,3,4,6)P_5 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)P_5 based on modifications of previous inositide syntheses by the laboratories of Prestwich[31] and Falck.[32,33]

The three key problems in the synthesis of any IP_7 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 IP_7. 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 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, Bu_4NHSO_4, 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 CH_3CN afforded the fully protected inositol **6** in 84% yield. Treatment benzoate **6** with NaOMe gave 2-OH inositol **7** in 82% yield, and hydrolysis of the acetonides with *p*TsOH in acetone/H_2O, gave the 5-PMB protected inositol **8** in 79% yield. Since **8** was poorly soluble in EtOAc and CH_2Cl_2, 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 IP_7, 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 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 CH_2Cl_2. The 4-methoxybenzyl functional group was removed with TFA/CH_2Cl_2/H_2O (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/CH_3CN)[38–40] and purification by Dowex-H^+ chromatography afforded pure monophosphoric acid **12** in 92% yield (Scheme 2). After treatment of **12** (1 equiv) with triethylamine (2 equiv) in anhydrous CH_2Cl_2 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 H_2 (60 psi) with PtO_2 for 4 h. After removal of the catalyst and concentration, the residue was dissolved in water and washed with EtOAc and CH_2Cl_2. 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)P_5 sodium form in 81% yield as white solid. The ^{31}P NMR spectrum of 5-PP-Ins(1,2,3,4,6)P_5 in D_2O 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 ^{31}P NMR were consistent with the 5-PP-Ins(1,2,3,4,6)P_5 isolated from *Polysphondylium*.[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-InsP_5 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-InsP_5 intermediate, 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)P_5, 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 IP_6 yielding 5-PP-Ins(1,2,3,4,6)P_5 plus ADP, but readily catalyzes the reverse reaction in which a phosphate is transferred from 5-PP-Ins (1,2,3,4,6)P_5 to ADP yielding ATP and IP_6.[42] Thus, synthetic 5-PP-Ins(1,2,3,4,6)P_5 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)P_5 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)P_5 may be used as a substrate for ATP synthesis in a reaction catalyzed by IP6K1.

To ensure IP_7 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 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)P_5 with ATP and the enzyme luciferase results in decreased conversion of luciferin to oxyluciferin indicating 5-PP-Ins(1,2,3,4,6)P_5 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 IP_7 regioisomer 5-PP-Ins(1,2,3,4,6)P_5, 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 IP_8, the bisdiphosphoinositol tetrakisphosphates (PP_2-InsP_4), which have recognized importance in cell signaling research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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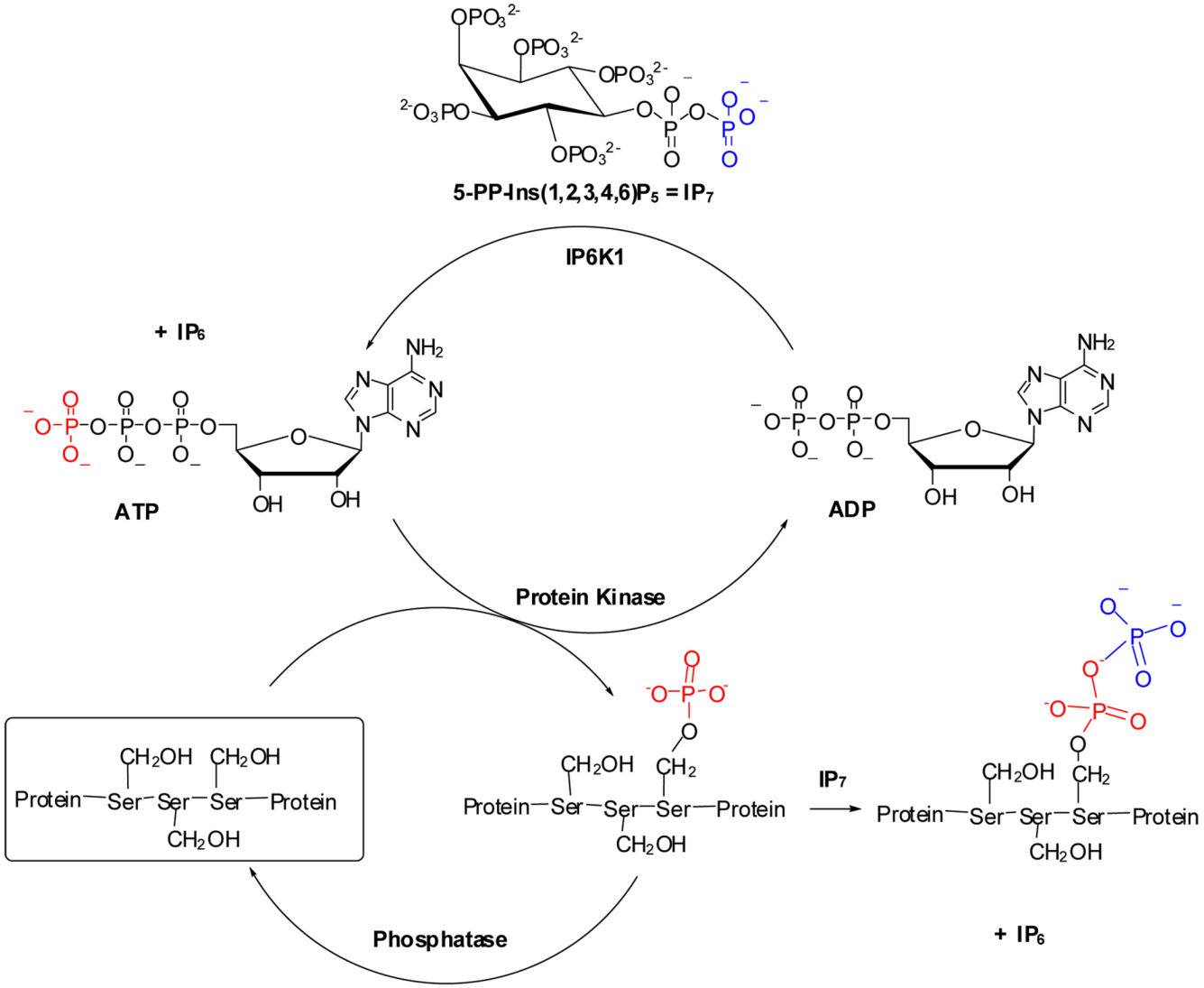


Figure 1.
Reversible protein phosphorylation and pyrophosphorylation mechanism.

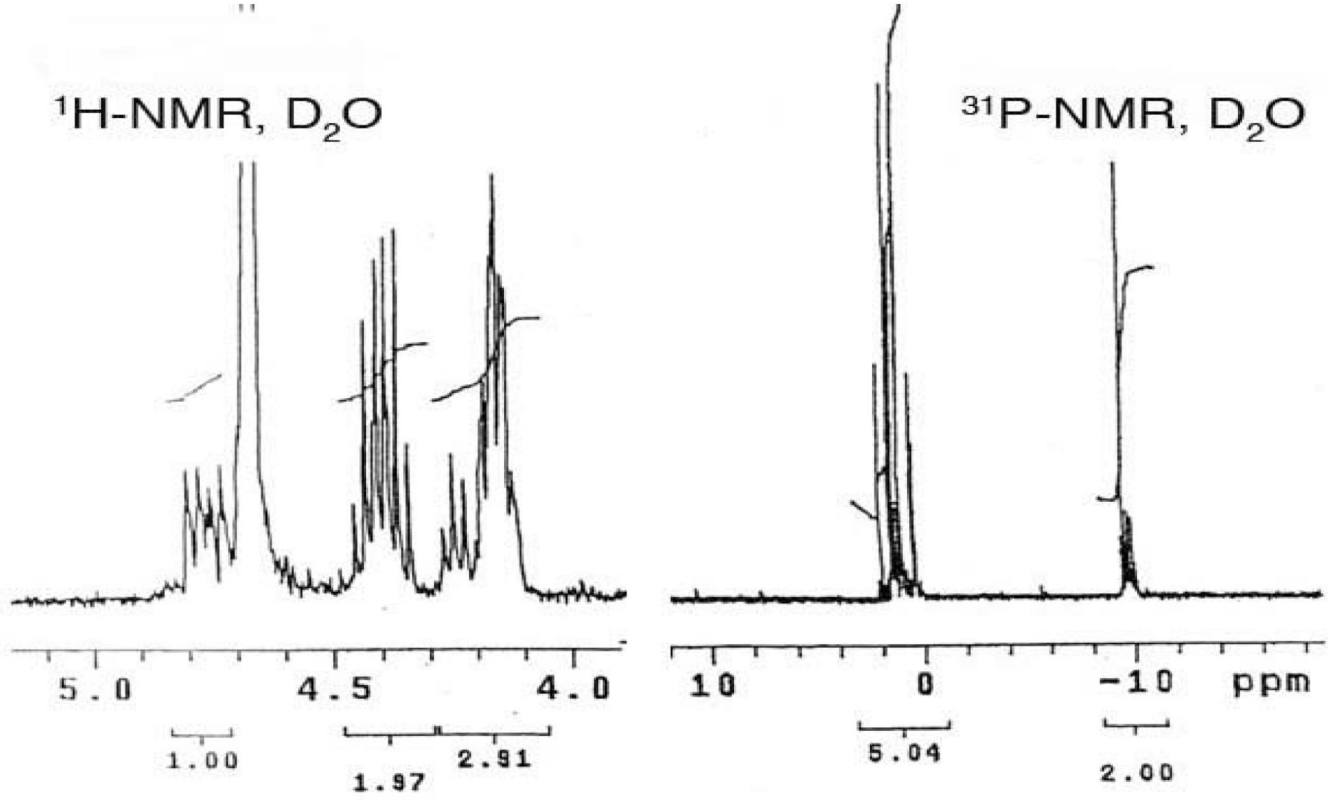


Figure 2.
^{1}H NMR and ^{31}P NMR of 5-PP-InsP_5.

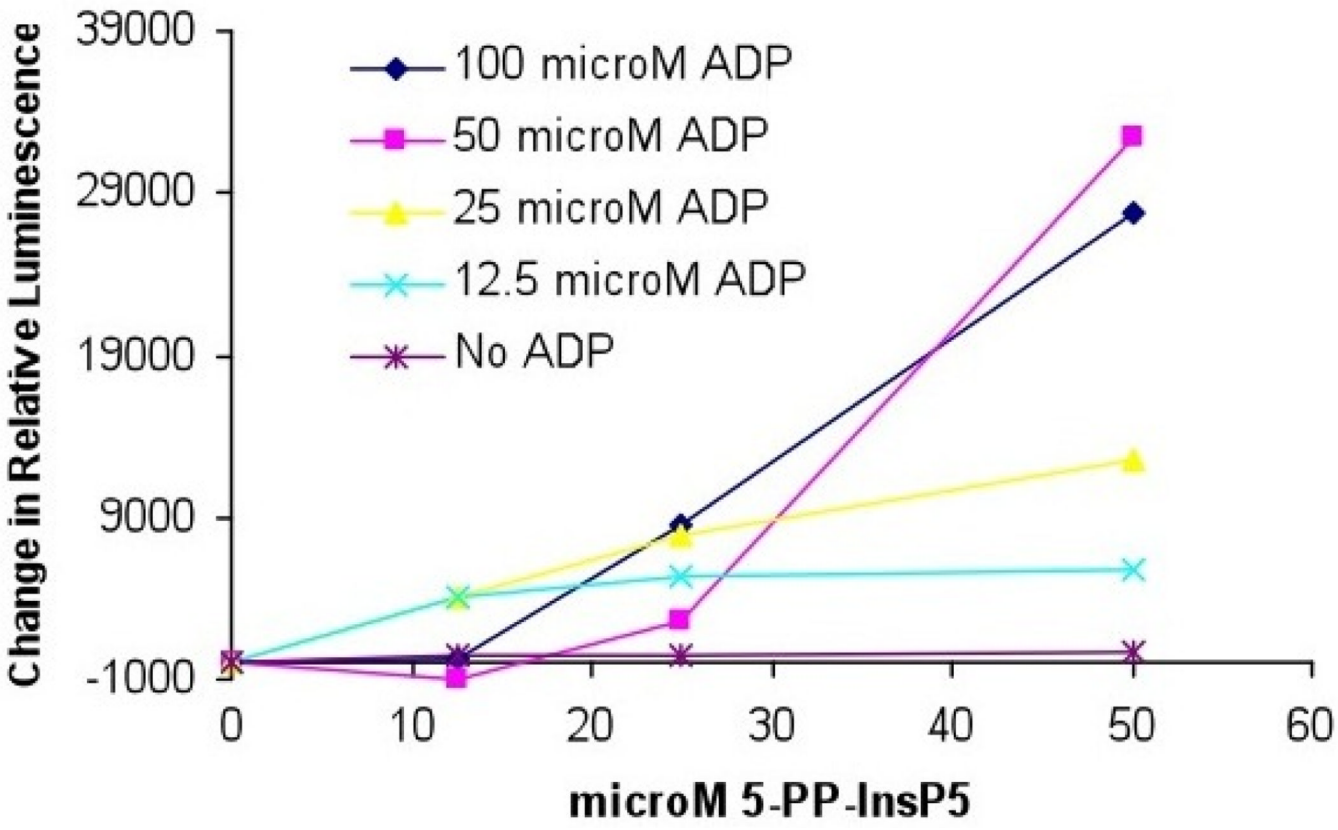
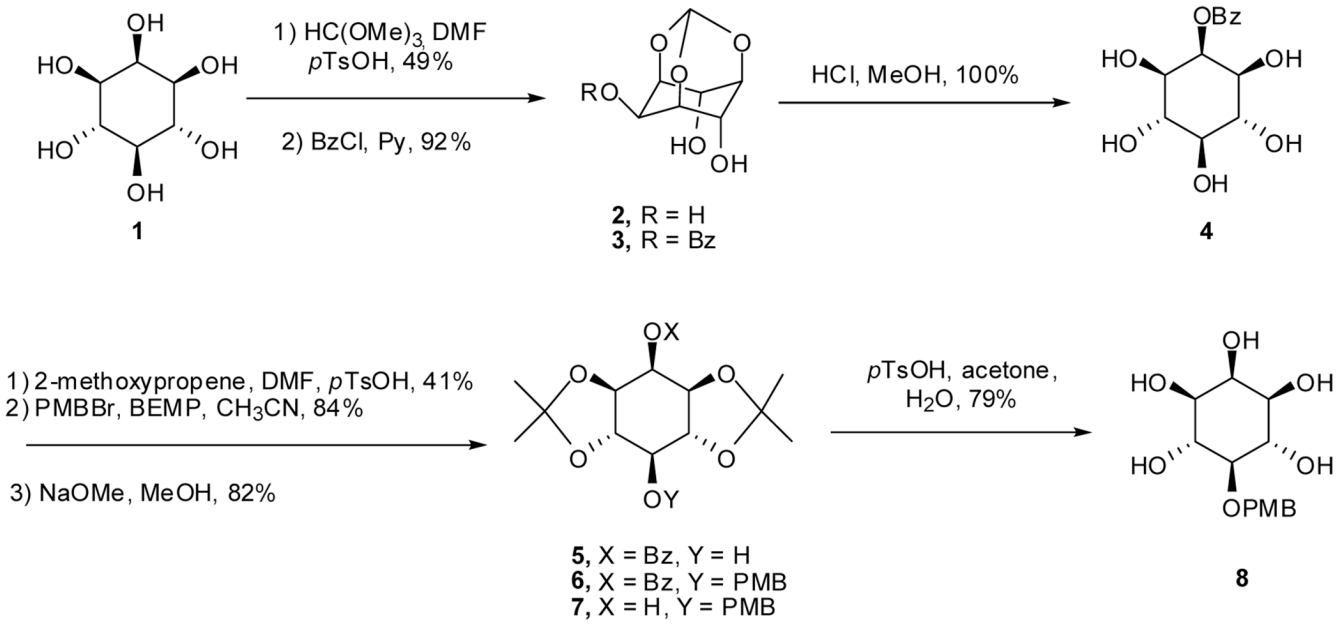
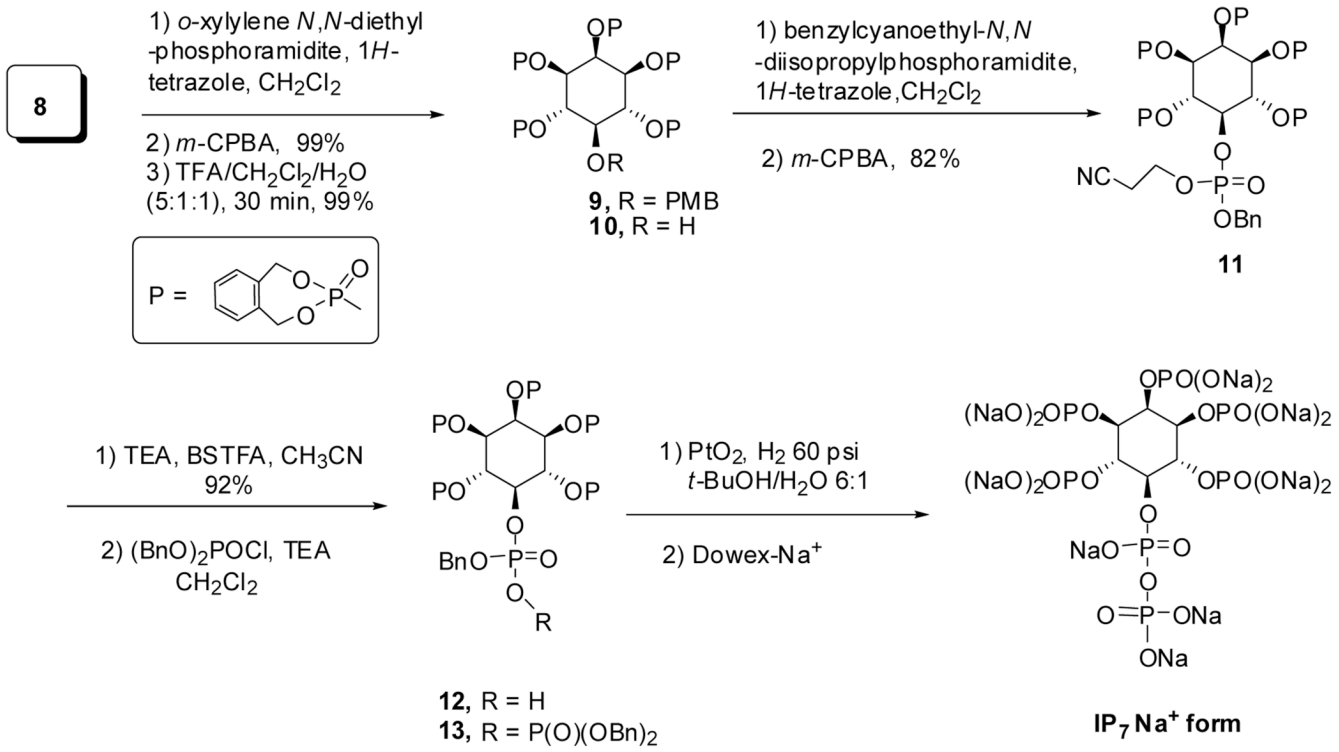


Figure 3.
Effects of the concentration of ADP and synthetic 5-PP-Ins(1,2,3,4,6)P_5 on ATP production by IP6K1.



Scheme 1.
Synthesis of advanced intermediate **8**



Scheme 2.
Global phosphorylation and final conversion to 5-PP-Ins(1,2,3,4,6)P_5