Synthesis and characterization of non-hydrolysable diphosphoinositol polyphosphate second messengers

Mingxuan Wu[a], **Barbara E. Dul**[a,‡], **Alexandra J. Trevisan**[a,‡], and **Dorothea Fiedler**[a]

Dorothea Fiedler: dfiedler@princeton.edu

Department of Chemistry, Princeton University, Washington Rd., 85 Princeton, NJ, 08544, USA. Tel: +1 609 258 1025

Abstract

The diphosphoinositol polyphosphates (PP-IPs) are a central group of eukaryotic second messengers. They regulate numerous processes, including cellular energy homeostasis and adaptation to environmental stresses. To date, most of the molecular details in PP-IP signalling have remained elusive, due to a lack of appropriate methods and reagents. Here we describe the expedient synthesis of methylene-bisphosphonate PP-IP analogues. Their characterization revealed that the analogues exhibit significant stability and mimic their natural counterparts very well. This was further confirmed in two independent biochemical assays, in which our analogues potently inhibited phosphorylation of the protein kinase Akt and hydrolytic activity of the Ddp1 phosphohydrolase. The non-hydrolysable PP-IPs thus emerge as important tools and hold great promise for a variety of applications.

Introduction

Cellular decision-making is governed by the concerted actions of signalling proteins and small molecule second messengers. Among the eukaryotic second messengers, the diphosphoinositol polyphosphates (PP-IPs) constitute an important class. These molecules have captured the attention of both chemists and biologists, because of their unique chemical structure and their ability to mediate a plethora of interesting phenotypes.[1]

The PP-IPs are a group of highly phosphorylated signalling molecules, based on the *myo*-inositol scaffold, that contain one or two high-energy pyrophosphate groups. These phosphate groups are installed in a biosynthetic pathway that is well conserved from yeast to mammals (a simplified pathway diagram is shown in Figure 1).[1]

The successive phosphorylation reactions are carried out by a set of dedicated small molecule kinases. Genetic perturbation of these kinases in yeast revealed important functions for PP-IPs in many cellular processes, including telomere maintenance,[2] response to oxidative stress,[3] and nutrient sensing.[4] Mice that lack the kinase Ip6k1 (inositol hexakisphosphate kinase 1) are not able to produce sufficient amounts of 5PP-IP$_5$ – the best characterized PP-IP to date – in various tissues (Figure 1).[5] These mice exhibit severe defects in insulin secretion, increased peripheral insulin sensitivity, and resistance to age and diet-induced obesity.[5, 6] While these phenotypes are truly remarkable, the underlying molecular mechanisms have remained enigmatic.

[‡]These authors contributed equally to this work.

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Traditionally, small diffusible messengers bind to particular target proteins to control their activity or localization. It is therefore commonly assumed that PP-IPs utilize an allosteric mechanism to regulate protein function. However, only a handful of targets have been identified to date, and in some cases the biological relevance is not clear.[4, 5b, 7] An alternative signalling mechanism for the PP-IPs involves the transfer of the high-energy β-phosphate group onto a phospho-serine residue, yielding a pyrophosphorylated protein.[8] But due to technical challenges, it has only been possible to identify pyrophosphorylated proteins in biochemical assays and not from complex cell lysates.[9] Overall, progress in decoding PP-IP signalling has been hindered by a lack of suitable methods and reagents. As current approaches rely on standard biochemical and genetic techniques, there is a pressing need for chemical tools that can help to decipher the discrete PP-IP signalling functions.

Here we report the synthesis and characterization of a number of non-hydrolysable methylene-bisphosphonate PP-IP analogues (Figure 2). We demonstrate that the analogues are good mimics of the natural counterpart with regard to their conformation in solution and their protein binding properties. Our analogues thus represent an important set of mechanistic probes that will be of great use for the inositol signalling community, and we highlight a number of possible applications.

Results and discussion

Expedient synthesis of PP-IP analogues

The methylene-bisphosphonate moiety has widely been used as a stable substitute for diphosphate groups; an important example is AMPPCP (β,γ-methyleneadenosine 5′-triphosphate), a non-hydrolysable analogue of ATP.[10] Among the PP-IP family members, the $5PP-IP_5$ messenger is the most prominent one and we therefore decided to focus on the synthesis of the non-hydrolysable methylene-bisphosphonate $5PCP-IP_5$ (**2**, Figure 2) using the synthetic route outlined in Scheme 1.[11] The protected *myo*-inositol **5** can be obtained from *myo*-inositol in three steps.[12] The bisphosphonate group was then installed in the 5-position, to provide intermediate **6a** in good yield. Removal of the benzoyland acetonide protecting groups furnished the pentaol **7b**, which was subsequently phosphitylated and oxidized (**8b**). The phosphate groups were unveiled as the free phosphates via hydrogenation in the presence of $NaHCO_3$, and $5PCP-IP_5$ (**2**) was isolated as the sodium salt. Compared to the synthesis of the natural molecule $5PP-IP_5$,[13] the non-hydrolysable analogue is accessible in good quantity and high purity, while requiring fewer synthetic steps.

Another PP-IP family member is $5PP-IP_4$ (Figure 1), a molecule that has been linked to telomere maintenance and DNA damage repair.[2] The corresponding bisphosphonate analogue $5PCP-IP_4$ (**3**) was synthesized using a slightly modified route: Removal of the acetonide groups from **6a** was followed by phosphitylation, and oxidation (**8a**). Subsequent hydrogenation afforded a diphosphoinositol phosphate analogue that is benzoyl-protected in the 2-position (**4**). The benzoyl group was cleaved using aqueous ammonia and yielded the non-hydrolysable analogue $5PCP-IP_4$ (**3**). Both $5PCP-IP_4$ (**3**) and $5PCP-IP_5$ (**2**) showed no signs of decomposition in aqueous solution at neutral pH, after 40 days at room temperature.

$5PP-IP_5$ and $5PCP-IP_5$ exhibit the same pH dependent conformational change

The pKa's of a pyrophosphate group differ slightly from those of a bisphosphonate group,[14] and it was therefore important to determine that $5PP-IP_5$ (**1**), and the non-hydrolysable analogue $5PCP-IP_5$ (**2**), exhibit the same properties in solution. Since the conformation of the inositol ring depends on the ionization state of the individual phosphate groups, ^1H and ^{31}P NMR titration curves can be used to compare the solution properties of **1** and **2**.[14]

To do so, 5PP-IP$_5$ (**1**) was synthesized according to a modified literature procedure (Scheme S1),[13, 16] and both 5PP-IP$_5$ and 5PCP-IP$_5$ were titrated in D_2O (containing 140mM K^+, 10mM Na^+, and 1mM Mg^{2+} to mimic cellular metal cation content) at room temperature. Figure 3 depicts a subset of the ^1H NMR titration data of the two molecules (for all data see Figures S1 and S2). The ring inversion, which converts 5 equatorial/1 axial substituents to 1 equatorial/5 axial substituents, occurred around pD 8.8 for both molecules.[17] While these data do not provide a direct measurement of the pK_a values for **1** and **2**, the titration curves highlight the similarity of the overall ionization state of the two molecules.[18]

5PCP-IP$_5$ exhibited much higher stability compared to 5PP-IP$_5$. Even at a low or high pD (2.0 or 13.0), no detectable decomposition occurred after 40 days at room temperature. The non-hydrolysable PP-IP analogues will therefore be of great use to characterize the physicochemical properties of the PP-IP messengers. Furthermore, our lab is interested in the rich metal-coordination chemistry of these molecules.[19] The presence of highly Lewis acidic metal cations, such as Mg^{2+} and Fe^{3+}, promotes hydrolysis of the PP-IPs, but our bisphosphonate analogues circumvent this problem and can be used as surrogates to delineate the metal-binding properties of PP-IP molecules.

PP-IP analogues potently inhibit Akt phosphorylation in vitro

It is thought that PP-IPs can control protein activity or localization via allosteric regulation.[4, 5b, 7] If 5PCP-IP$_5$ indeed closely mimics 5PP-IP$_5$, it should exert the same allosteric control over a given protein binding partner.

Recently, it was reported that 5PP-IP$_5$ binds to the pleckstrin homology (PH) domain of the protein kinase Akt (also known as protein kinase B).[5b] This binding event was proposed to stabilize Akt in an inactive conformation, which precludes Akt from becoming phosphorylated on threonine 308 by the upstream kinase PDK1 (3-phosphoinositide-dependent protein kinase, Figure 4a). We tested the inhibitory activity of both 5PP-IP$_5$ and 5PCP-IP$_5$ in a biochemical assay, using purified inactive Akt and activated PDK1. The two kinases were incubated in the presence of varying concentrations of 5PP-IP$_5$ or 5PCP-IP$_5$ and inhibition of Akt phosphorylation at threonine 308 was monitored using a phosphospecific antibody (Figure 4b). As previously reported, 5PP-IP$_5$ potently inhibited Akt phosphorylation. Under our assay conditions, 5PP-IP$_5$ displayed an IC$_{50}$ of 217 nM.[20] Similarly, 5PCP-IP$_5$ closely resembled its natural counterpart with an IC$_{50}$ of 129 nM. These data illustrate that changing the pyrophosphate moiety to a methylene-bisphosphonate group did not significantly alter the affinity of the small molecule for a known protein binding partner. Consequently, it should be feasible to utilize 5PCP-IP$_5$ as an affinity reagent to identify PP-IP protein binding partners.

From a synthetic perspective, the 2-position of 5PCP-IP$_5$ could most easily be derivatized for attachment to a solid support. To determine how important the phosphate group in the 2-position is for the binding interaction with Akt, we evaluated the analogues 4PCP-IP$_5$ (**3**) and 2Bz-4PCP-IP$_5$ (**4**), which contain a hydroxyl group or a benzoyl group in the 2-position, respectively. Remarkably, those structural changes did not diminish their affinity for Akt (Figure 4b).[21] Encouraged by these results, we are currently exploring a number of attachment strategies for these molecules.

PP-IP analogues inhibit the Ddp1 phosphohydrolase in vitro

We next sought to validate the non-hydrolysable analogues in a different biochemical assay, but examples of PP-IP binding proteins in the literature are scarce.[4, 5b, 7] Since the enzymes involved in PP-IP metabolism must bind to their substrates, we chose to investigate the binding of our analogues to the yeast polyphosphate phosphatase Ddp1. Ddp1 hydrolyses

diadenosine polyphosphates, inorganic polyphosphates, and a subset of PP-IPs.[22] A recent report from Saiardi and co-workers demonstrated that Ddp1 exhibited highly varied activity towards different PP-IP isomers: While 1PP-IP$_5$ - a PP-IP family member with the pyrophosphate group in the 1-position - was completely hydrolysed to IP$_6$ after 30 minutes at 37 °C, 5PP-IP$_5$ showed no signs of hydrolysis under the same conditions.[22c] We therefore reasoned that 5PP-IP$_5$ could be a competitive inhibitor for Ddp1 when used in combination with other Ddp1 substrates, such as diadenosine pentakisphosphate (Ap$_5$A).

Ddp1 was expressed and purified using an N-terminal polyhistidine tag (His). When His-Ddp1 was incubated with Ap$_5$A, it displayed robust phosphohydrolase activity, as determined by HPLC analysis of the reaction products (Figure 5a).[23] Next, varying concentrations of 5PP-IP$_5$ were added to the Ap$_5$A hydrolysis reaction. As expected, 5PP-IP$_5$ acts as a competitive inhibitor with an IC$_{50}$ of 2.5 μM (Figure 5b).[24] Testing of 5PCP-IP$_5$ and 5PCP-IP$_4$ revealed very similar inhibition profiles (Figures 5b and S4), which corroborates that the bisphosphonate moiety is a suitable mimic for the pyrophosphate group, as it is bound by the protein with similar affinity. Interestingly, 2Bz-5PCP-IP$_4$ exhibited the highest potency with an IC$_{50}$ value in the submicromolar range (0.16 μM). As was also observed for Akt, the benzoyl substituent in the 2-position does not lower the binding affinity; in fact, it made the molecule significantly more potent. Possible explanations for the increased potency include a favourable interaction of the benzoyl group with the protein, and/or changes in solvation of the unbound 2Bz-5PCP-IP$_4$, which may decrease its ground-state stabilization compared to the other analogues.

Based on its high potency, 2Bz-5PCP-IP$_4$ may become useful to inhibit Ddp1 activity in cell lysates to prevent hydrolysis of Ddp1 substrates, thereby facilitating their identification and analysis. Overall, the Ddp1 inhibition studies paralleled our observations from the Akt assay, further validating that the bisphosphonate analogues are adequate surrogates for the natural molecules.

Conclusions

Even though the PP-IPs were discovered almost 20 years ago, we are just beginning to appreciate their complex functions as central regulators of cell homeostasis. This gap in our understanding is due to the dearth of reagents available to study PP-IP function in vivo and in vitro. The PP-IP analogues described in this paper hold great promise for filling several of these gaps. We have shown that the bisphosphonate group adequately mimics the pyrophosphate group in PP-IPs, while providing easier synthetic accessibility and increased stability. The non-hydrolysable analogues can therefore be developed into affinity reagents for the systematic identification of PP-IP interacting partners. Subsequently, the increased stability of the analogues will be advantageous for co-crystallizations of PP-IPs with their binding partners.

The bisphosphonate analogues will also be highly informative with respect to the other PP-IP signalling mechanism, protein pyrophosphorylation. Our compounds are not able to transfer their β-phosphoryl group onto protein substrates and provide a facile means to distinguish the two signalling mechanisms in biochemical assays. Progress on using the analogues as mechanistic probes will be described in due course.

Until now there has been a reliance on genetic and molecular biology techniques to study PP-IP function. A major focus, however, should be on the molecules themselves. We believe that the non-hydrolysable PP-IPs describe in this paper will help to shift this imbalance, and will provide valuable insight into the biological functions of PP-IP molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Notes and references

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11. We have also considered the incorporation of a difluoromethylene-bisphosphonate (PCF_2P) group, because the PCF_2P group more closely resembles the pK_a values of the pyrophosphate group.10a However, the PCF_2P analogues posed a significant synthetic challenge, and we ultimately focused our efforts on the methylene-bisphosphonates. We acknowledge that the differences in the pK_a's of 5PP-IP_5 versus 5PCP-IP_5 could be an issue, but, as our subsequent biochemical studies show,

these differences in pK_a do not appear to be a significant limitation in the two examples highlighted.

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16. An HPLC purification step before the final deprotection proved to be essential to obtain 5PP-IP$_5$ in the desired purity.

17. The assignments of conformations was based on previous reports, coupling constant analysis, ^{1}H-^{1}H COSY, and ^{1}H-^{31}P HSQC (see Supporting Information).

18. Interestingly, there is a noticeable difference in the chemical shifts of H$_{4/6}$ for **1** versus **2** at basic pH. We believe that subtle changes in Mg^{2+} coordination may be responsible for this, as the difference in chemical shift is less pronounced when the NMR spectra were recorded in the absence of Mg^{2+} (see Supporting Information).

19. Nothing is known about the coordination chemistry of PP-IPs. For studies on the metal-coordination properties of IP$_5$ and IP$_6$ see: Veiga N, Torres J, Dominguez S, Mederos A, Irvine RF, Diaz A, Kremer C. J. Inorg. Biochem. 2006; 100:1800. [PubMed: 16920196] Torres J, Dominguez S, Cerda MF, Obal G, Mederos A, Irvine RF, Diaz A, Kremer C. J. Inorg. Biochem. 2005; 99:828. [PubMed: 15708805] Veiga N, Torres J, Godage HY, Riley AM, Domínguez S, Potter BV, Díaz A, Kremer C. J. Biol. Inorg. Chem. 2009; 14:1001. [PubMed: 19415348]

20. The published IC$_{50}$ value is 20 nM. Presumably differences in buffer composition and/or incubation times are responsible for this discrepancy.

21. 2Bz-5PCP-IP$_4$ appeared to be the most potent. This trend was paralleled in subsequent experiments using the yeast Ddp1 phosphohydrolase and is discussed in the the following section.

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23. We ruled out the possibility that the reaction product IP$_6$ may be responsible for this inhibition, as the IC$_{50}$ value for IP$_6$ in this reaction is more than ten times higher (data not shown).

24. As a control, we confirmed that Ap$_5$A showed no signs of hydrolysis when incubated with His-Ddp1EE/AQ, a catalytically inactive form of Ddp1 (Figure S3)
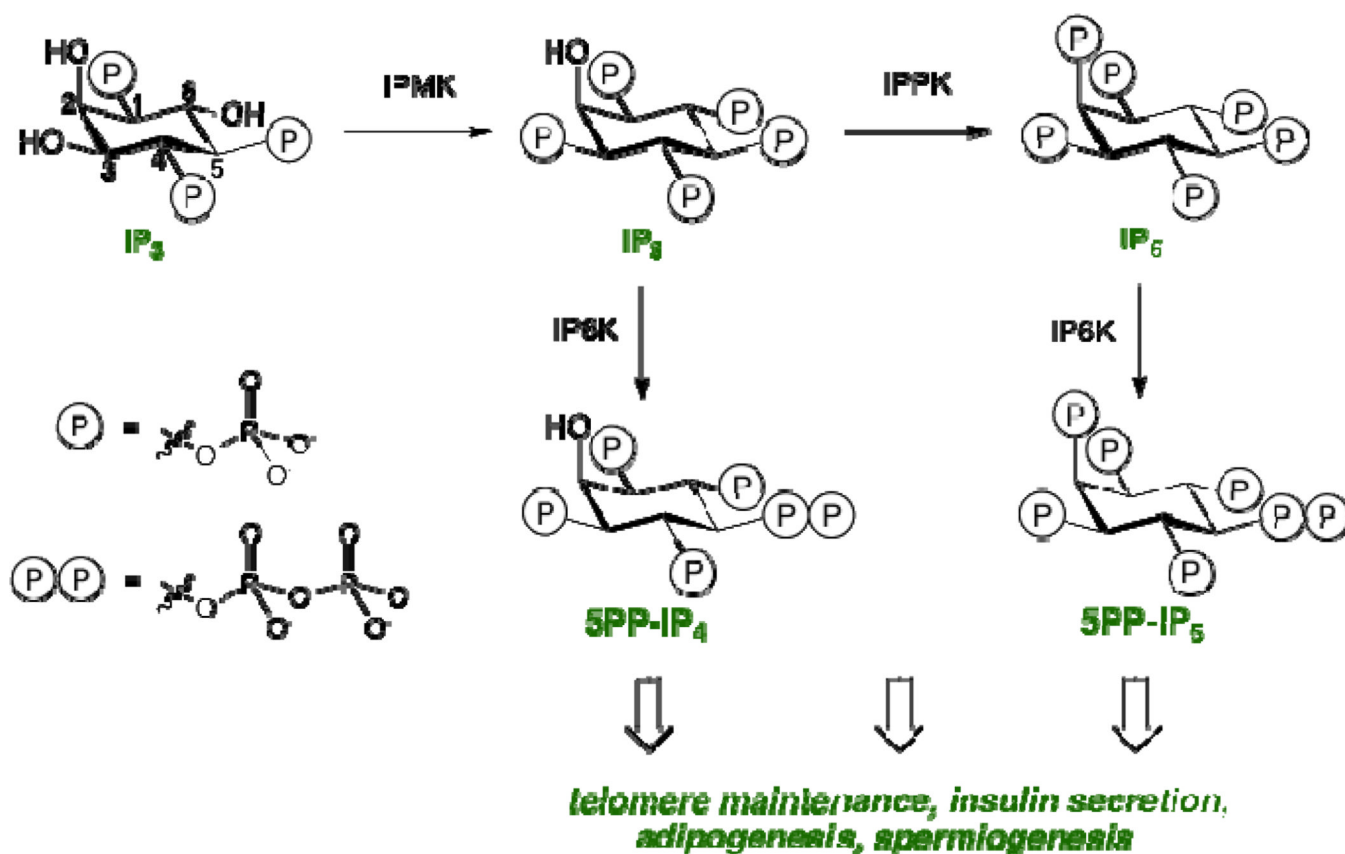
Figure 1.
The diphosphoinsitol polyphosphate biosynthetic pathway, in abbreviated form. Phosphorylation of inositol 1,4,5-triphosphate (IP_3) by inositol multikinase (IPMK) and inositol pentakisphosphate 2-kinase (IPPK) results in formation of inositol pentakisphosphate (IP_5) and inositol hexakisphosphate (IP_6). Both IP_5 and IP_6 are substrates for IP6K (inositol hexakisphosphate kinase) to yield the diphosphoinositol polyphosphates 5PP-IP_4 and 5PP-IP_5, respectively. The numbering of the ring positions is indicated in the IP_3 structure.
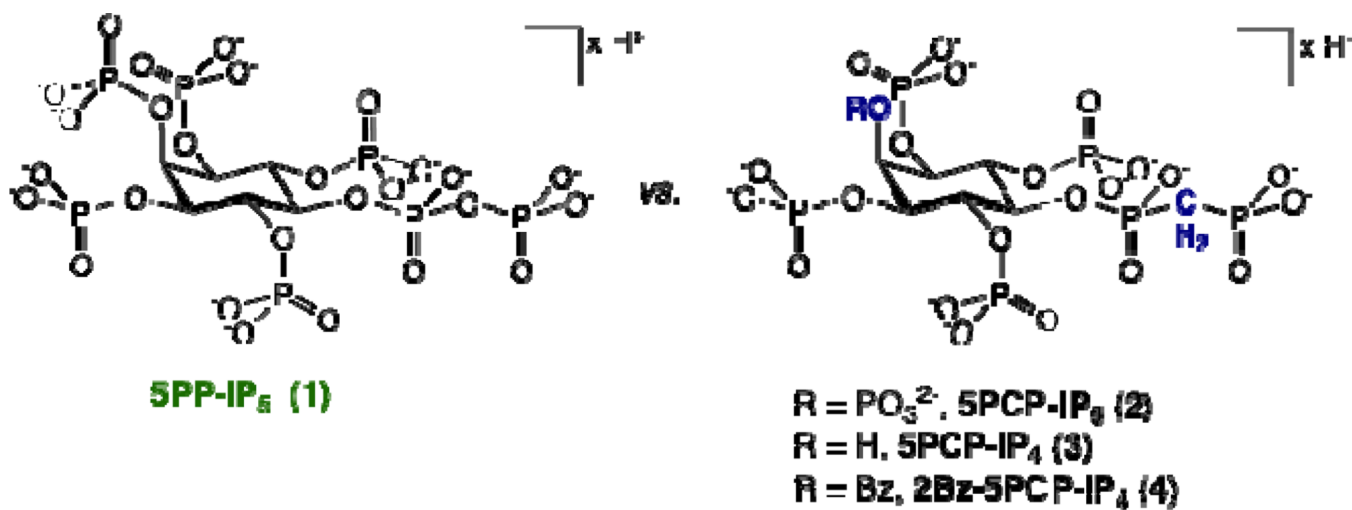
Figure 2.
Comparison of the naturally occurring signalling molecule 5PP-IP$_5$ (**1**) with the analogues described in this study.
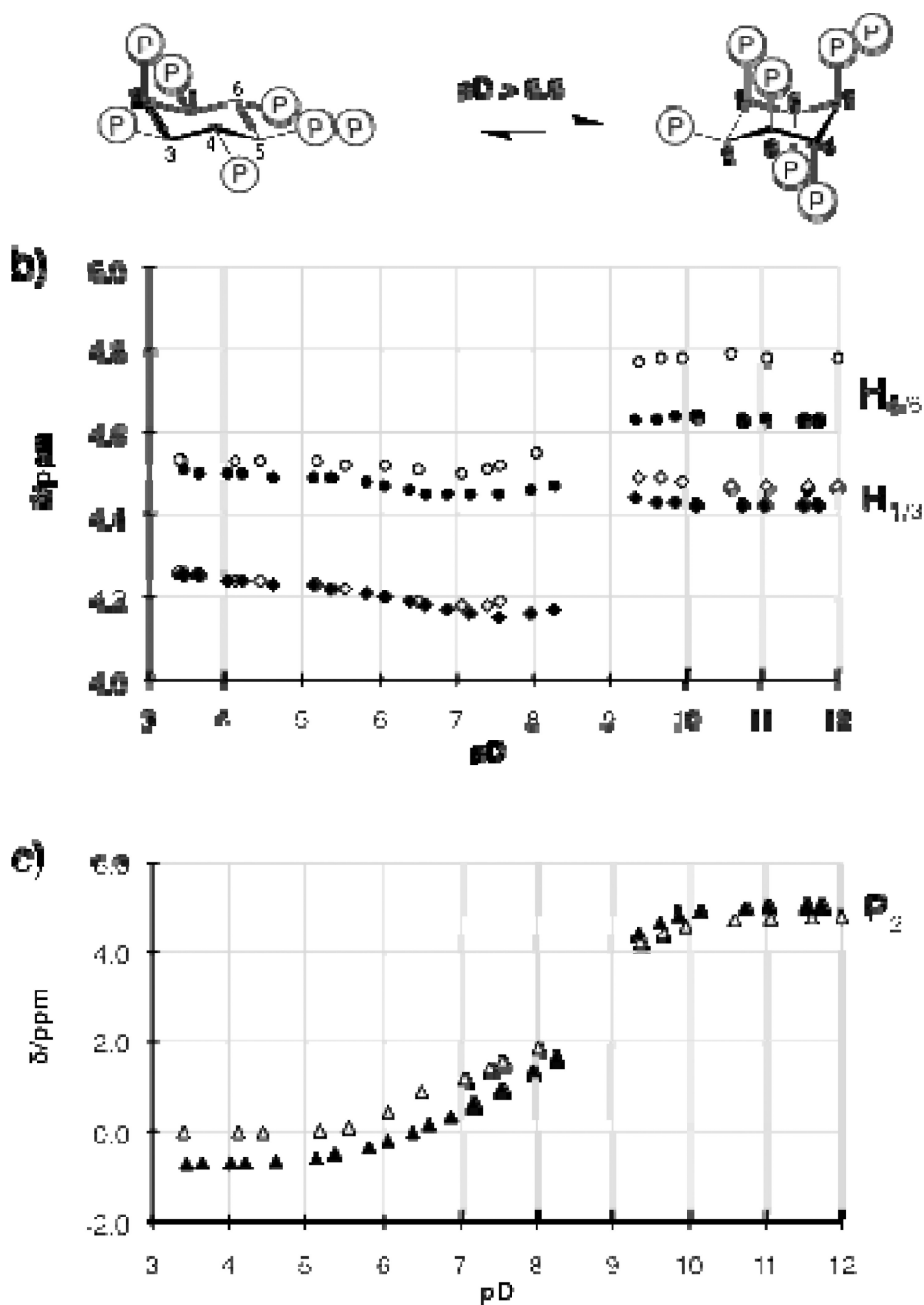
Figure 3.
a) 5PP-IP$_5$ and 5PCP-IP$_5$ undergo a conformational change around pD 8.8. b) ^{1}H NMR titration curves for 5PP-IP$_5$ (hollow circles for H$_{4/6}$ and hollow diamonds for H$_{1/3}$) and 5PCP-IP$_5$ (filled circles for H$_{4/6}$ and filled diamonds for H$_{1/3}$). Between pD 8.4 and pD 9.2 peaks could not be assigned due to severe broadening of the resonances (see Figure S1).c) ^{31}P NMR titrations curves for 5PP-IP$_5$ (hollow triangles for P$_2$) and 5PCP-IP$_5$ (filled triangles for P$_2$).
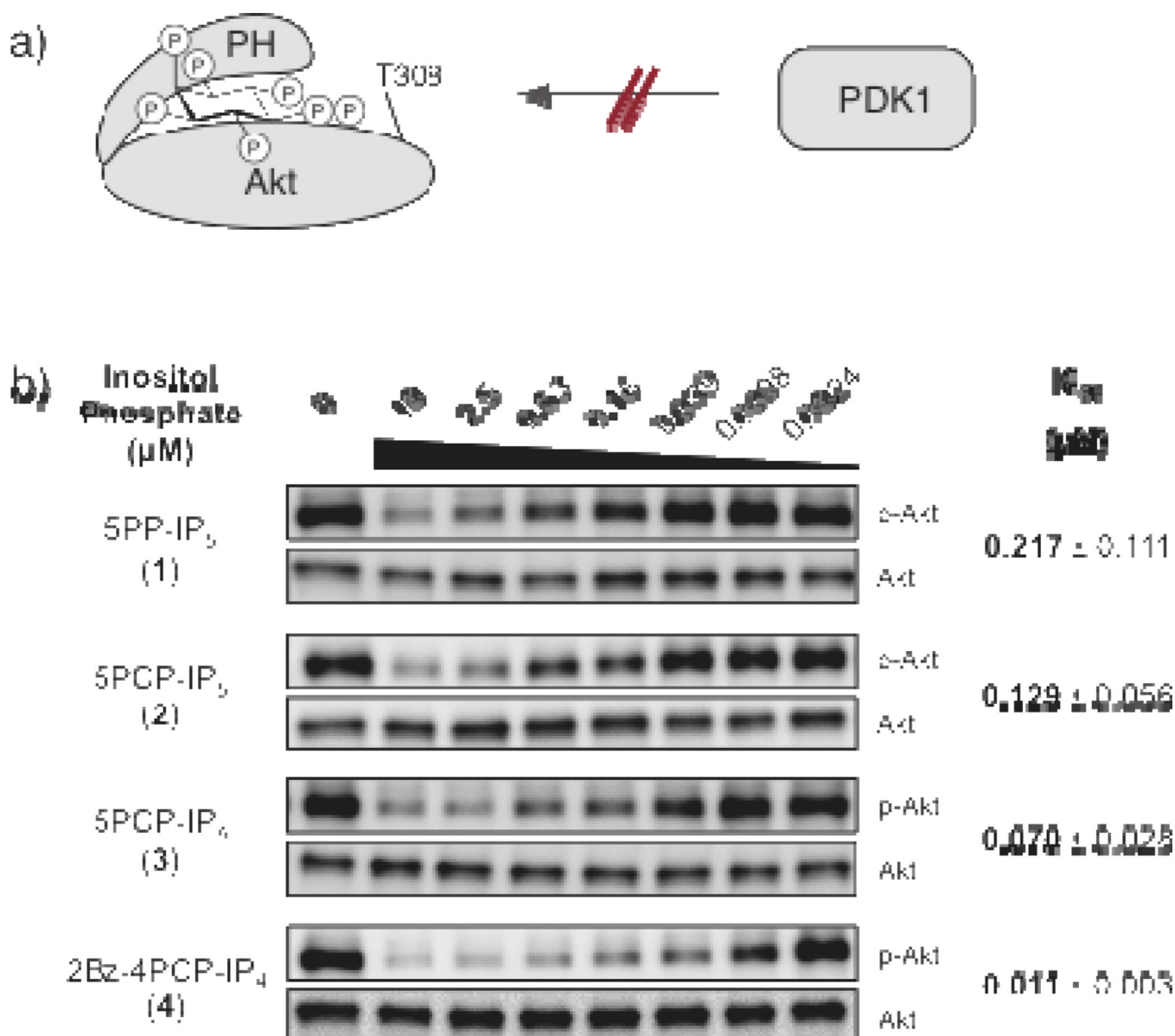
Figure 4.
a) Binding of 5PP-IP$_5$ is proposed to stabilize Akt in an inactive conformation that cannot become phosphorylated by PDK1. b) Western-blots of Akt inhibition experiments. Akt phosphorylation at threonine 308 (p-Akt) was measured using a phosphospecific antibody. A Western-blot for total Akt was used as a loading control. IC$_{50}$ values were determined in three independent experiments, and the errors are indicated.
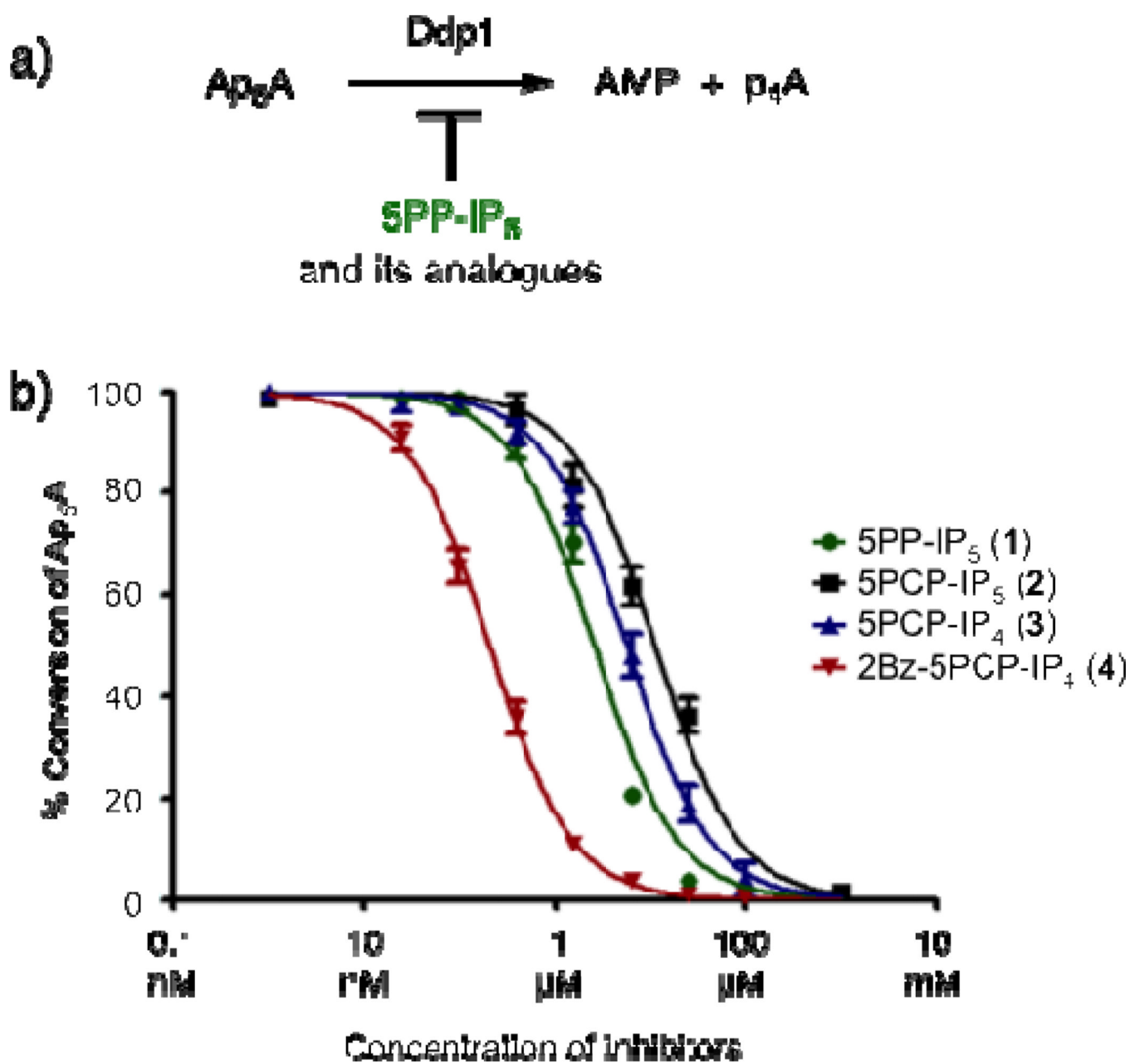
Figure 5.
a) Ddp1 hydrolyzes diadenosine pentakisphosphate (Ap$_5$A) to adenosine monophosphate (AMP) and adenosine tetrakisphosphate (p$_4$A), which is subsequently further degraded. b) Ddp1 mediated Ap$_5$A hydrolysis is inhibited by 5PP-IP$_5$ and the analogues. IC$_{50}$ values for 5PP-IP$_5$, 5PCP-IP$_5$, 5PCP-IP$_4$ and 2Bz-5PCP-IP$_4$ were determined in three independent experiments and the inhibition curves are shown (see also Figure S4).
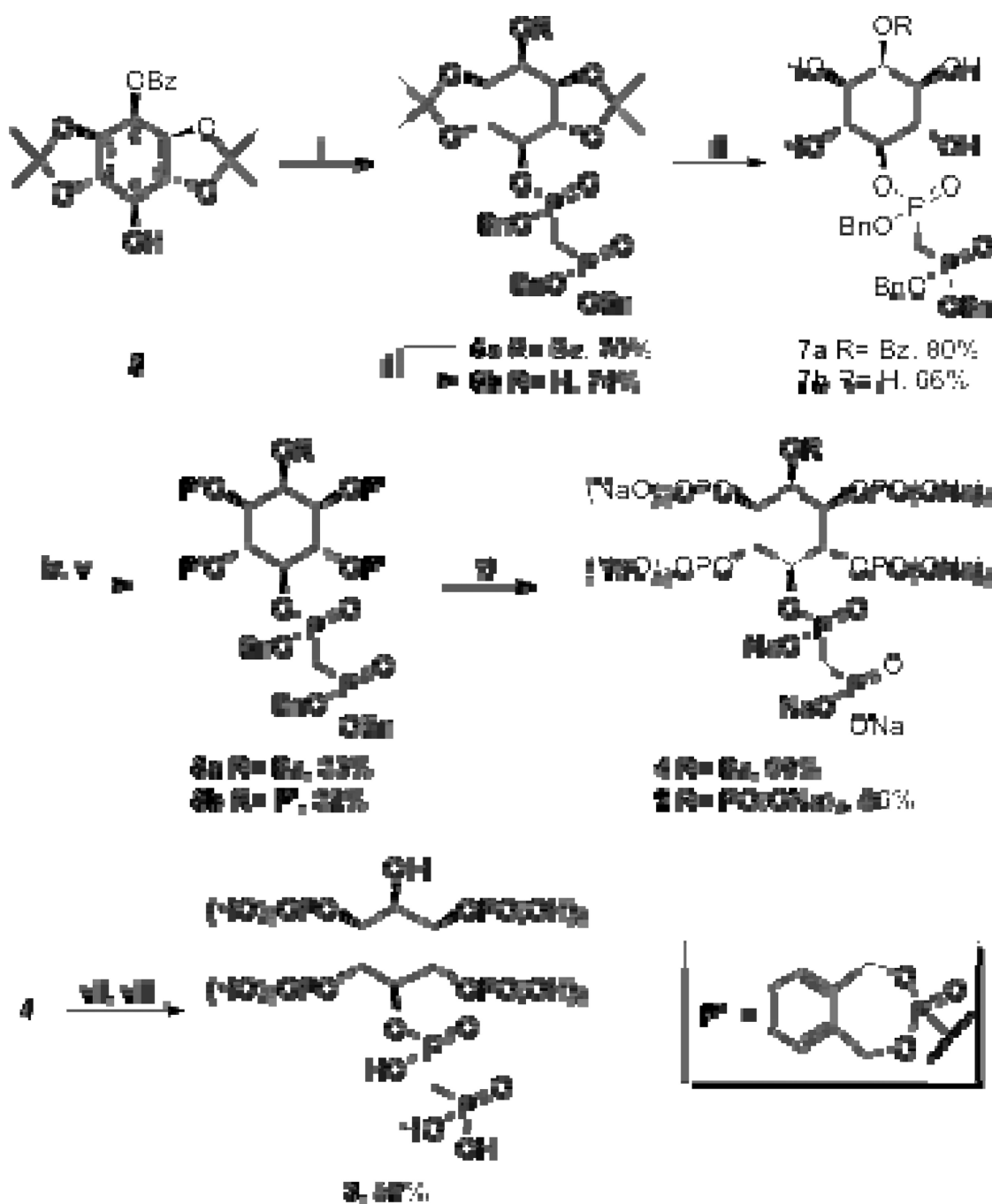
Scheme 1.
Synthesis of non-hydrolysable PP-IP analogues. Reagents and conditions: (i) (bis-benzyloxyphosphoryl-methyl)phosphoryl chloride, KHMDS, THF, −78 °C to rt, overnight; (ii) NaOMe, MeOH, rt, overnight; (iii) H_2O, p-TsOH, acetone, overnight; (iv) N,N-diethyl-1,5-dihydro-2,4,3-benzodioxaphosphepin-3-amine, $1H$-tetrazole, CH_3CN, 0 °C to rt, 36 h; (v) mCPBA, CH_3CN, 0 °C to rt, 3 h; (vi) H2, Pd black, $NaHCO_3$, t-BuOH/H_2O, rt, overnight; (vii) Conc. aq. NH_3, rt, 4 d; (viii) Dowex-H^+.