Angewandte
Communications

Second Messenger Signaling

DOI: 10.1002/anie.201402905

Elucidating Diphosphoinositol Polyphosphate Function with Nonhydrolyzable Analogues**

*Mingxuan Wu, Lucy S. Chong, Samanta Capolicchio, Henning J. Jessen, Adam C. Resnick, and Dorothea Fiedler**

***Abstract:** The diphosphoinositol polyphosphates (PP-IPs) represent a novel class of high-energy phosphate-containing messengers which control a wide variety of cellular processes. It is thought that PP-IPs exert their pleiotropic effects as allosteric regulators and through pyrophosphorylation of protein substrates. However, most details of PP-IP signaling have remained elusive because of a paucity of suitable tools. We describe the synthesis of PP-IP bisphosphonate analogues (PCP-IPs), which are resistant to chemical and biochemical degradation. While the two regioisomers 1PCP-IP$_5$ and 5PCP-IP$_5$ inhibited Akt phosphorylation with similar potencies, 1PCP-IP$_5$ was much more effective at inhibiting its cognate phosphatase hDIPP1. Furthermore, the PCP analogues inhibit protein pyrophosphorylation because of their inability to transfer the β-phosphoryl group, and thus enable the distinction between PP-IP signaling mechanisms. As such, the PCP analogues will find widespread applications for the structural and biochemical characterization of PP-IP signaling properties.*

Second messengers are integral components of cellular signaling pathways and are essential for maintaining cell homeostasis. The chemical structure of small-molecule second messengers varies widely and ranges from simple ions or gases to more elaborate molecules such as cyclic adenosine monophosphate and lipid phosphoinositides. Within the phosphoinositide family, further complexity is attained through the controlled generation of closely related compounds, which differ with respect to the number and position of phosphate groups.[1] Despite the structural similarity among phosphoinositides, their interactions with protein-binding partners, and thus their signaling properties, can be very distinct.[2]

Hydrolysis of the glycerophospholipid backbone of the phosphoinositides provides access to a group of water-soluble inositol polyphosphates, exemplified by the calcium ion releasing factor inositol 1,4,5-trisphosphate (IP$_3$). Consecutive phosphorylation of IP$_3$ by cytosolic and nuclear inositol kinases leads to the formation of fully phosphorylated inositol hexakisphosphate (IP$_6$). IP$_6$ can be converted further to form the diphosphoinositol polyphosphate second messengers (PP-IPs, Figure 1 a), which contain one or two high-energy

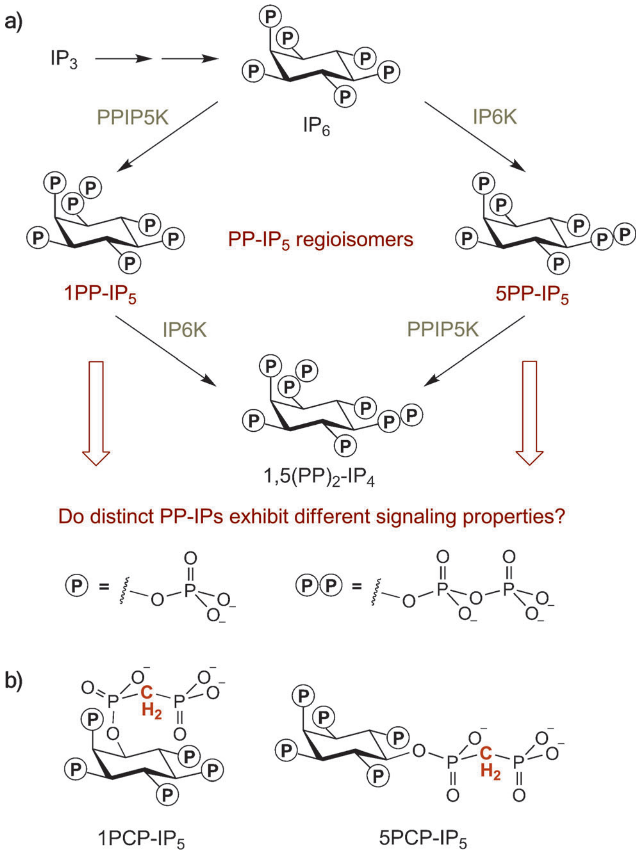


Figure 1. Abbreviated pathway for diphosphoinositol polyphosphate (PP-IP) biosynthesis in mammalian cells. Phosphorylation of inositol-1,4,5-trisphosphate (IP$_3$) by inositol multikinase and inositol pentakisphosphate 2-kinase produces inositol hexakisphosphate (IP$_6$). Inositol hexakisphosphate kinase (IP6K) and PPIP5 kinase (PPIP5K) further convert IP$_6$ into the pyrophosphate-containing messengers 5PP-IP$_5$, 1PP-IP$_5$, and 1,5(PP)$_2$-IP$_4$.

---

[*] M. Wu, Dr. D. Fiedler
Department of Chemistry, Princeton University
Washington Rd, Princeton, NJ 08544 (USA)
E-mail: dfiedler@princeton.edu

L. S. Chong, Dr. A. C. Resnick
Colket Translational Research Bldg
The Children's Hospital of Philadelphia
3501 Civic Center Blvd, Philadelphia, PA 19104 (USA)

S. Capolicchio, Dr. H. J. Jessen
Department of Chemistry, University of Zürich
Winterthurerstrasse 190, 8057 Zürich (Switzerland)

[**] We would like to thank the Muir, Doyle, MacMillan, and Sorensen research groups for use of their chemicals and instruments. We are grateful to Dr. Barbara E. Dul for assistance with hDIPP1 cloning, and to Dr. Mohammad R. Seyedsayamdost for insightful discussions. Financial support for D.F. and M.W. was provided by Princeton University and the NIH (R00 GM087306. D.F. is a Kimmel Scholar and a Rita Allen Scholar. A.C.R. gratefully acknowledges the NSF (CAREER Grant No. MCB-1253809).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201402905.

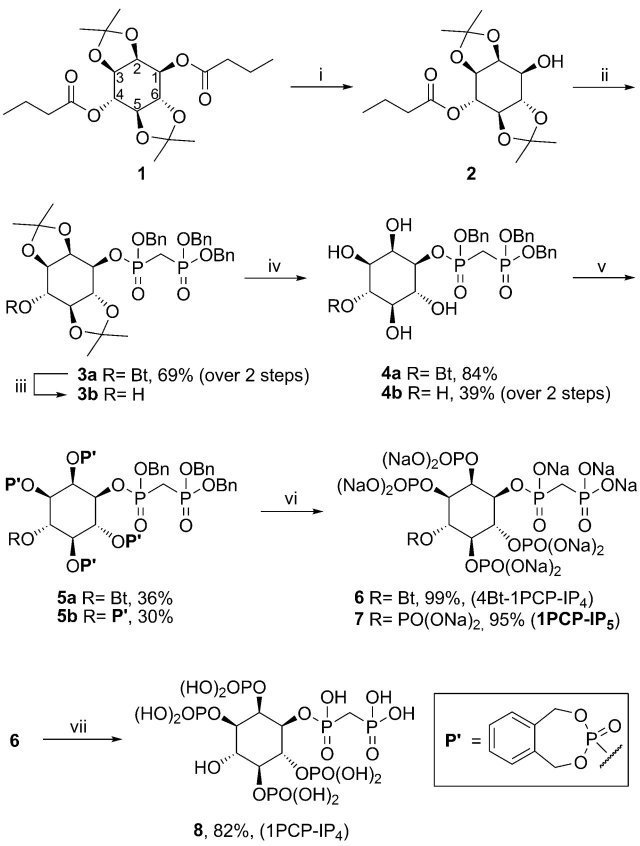
diphosphate groups.[3] Deletion of the PP-IP biosynthetic genes in yeast points to regulatory functions of these molecules in telomere maintenance,[4] chromatin remodeling,[5] energy homeostasis,[6] and nutrient signaling.[7] In mammals, the PP-IPs play a crucial role in insulin signaling[8] and body-weight regulation.[9] Interestingly, PP-IPs have been demonstrated to function through distinct modes of action, either allosterically regulating protein activity or by post-translational pyrophosphorylation of protein substrates.[3,6,7] However, in many cases the direct connection of a phenotype to a specific isomeric form of the PP-IPs, or the identification of the regulatory mechanism, have not been possible. This gap in knowledge can be attributed to a lack of tools to interrogate PP-IP signaling biochemically and in vivo.

Our research group previously reported the synthesis of a nonhydrolyzable methylene-bisphosphonate analogue of 5PP-IP_5, 5PCP-IP_5 (Figure 1 b), and showed that it closely mimicked the physicochemical and biochemical properties of its natural counterpart.[10] We now describe the synthesis of a nonhydrolyzable analogue of 1PP-IP_5, a regioisomer of 5PP-IP_5 (Figure 1 a). Evaluation of the PCP-IP analogues in a range of biochemical assays showcases their utility for the characterization of PP-IP–protein interactions, and for differentiating between the two PP-IP signaling mechanisms.

The synthesis of the methylene-bisphosphonate analogue of 1PP-IP_5 commenced with enantiomerically pure, commercially available inositol derivative **1** (Scheme 1). The 1-butyryl group was selectively removed by treatment with DIPEA in methanol to yield **2**.[11] The methylene-bisphosphonate moiety was then coupled to the hydroxy group to provide **3a** in good yield. After cleavage of the 4-butyryl group, the ketals of **3b** were hydrolyzed in wet TFA/MeOH/CH_2Cl_2. The resulting pentol (**4b**) was phosphitylated and oxidized. Subsequent hydrogenolysis of **5b** in the presence of sodium bicarbonate afforded 1PCP-IP_5 (**7**) as the sodium salt in high purity. The only difference between 1PCP-IP_5 and 1PP-IP_5 is the replacement of the oxygen atom in the phosphoanhydride linkage by a methylene group, as characterized by the apparent triplet at 27.0 ppm in the ^{13}C NMR spectrum of **7**.

From intermediate **3a**, a modified synthetic route was used to synthesize 1PCP-IP_4 (**8**), in which the 4-butyryl group was removed after phosphitylation, oxidation, and hydrogenolysis. Although no biological relevance has been ascribed to 1PP-IP_4, compound **8** can be used to define the contribution of the 4-phosphate group in PP-IP–protein interactions.

With these compounds in hand, we wanted to determine how well 1PCP-IP_5 resembled 1PP-IP_5 in a biochemical assay. The pleckstrin homology (PH) domain of the protein kinase Akt is one of the few protein binding partners characterized for 5PP-IP_5. It was proposed that the binding of 5PP-IP_5 stabilizes Akt in an inactive conformation, which prevents its phosphorylation at threonine 308 by PDK1[9b] (3-phosphoinositide-dependent protein kinase, Figure 2 a). Since we formerly used this inhibitory effect to highlight the similarity between 5PP-IP_5 and 5PCP-IP_5,[10] we sought to compare the IC_{50} values of 1PP-IP_5 and 1PCP-IP_5 in Akt inhibition. Furthermore, we wondered what the differential selectivity of the Akt PH domain towards the isomeric analogues 1PCP-IP_5 and 5PCP-IP_5 would be.



Scheme 1. Synthetic route to methylene-bisphosphonate analogues 1PCP-IP_5 and 1PCP-IP_4. Reagents and conditions: i) DIPEA, MeOH, 36 °C, 14 h; ii) (bisbenzyloxyphosphorylmethyl) phosphoryl chloride, DBU, 1*H*-tetrazole, CH_2Cl_2, 0 °C to RT, overnight; iii) DIBAL, CH_2Cl_2, −78 °C, 6 min; iv) TFA/MeOH/CH_2Cl_2, 0 °C, 3 h; v) *N*,*N*-diethyl-1,5-dihydro-2,4,3-benzodioxaphosphepin-3-amine, 5Ph-1*H*-tetrazole, CH_2Cl_2, 0 °C to RT, 18 h, then mCPBA, −78 °C to RT, 3 h; vi) H_2, Pd black, $NaHCO_3$, *t*BuOH/H_2O, RT, overnight; vii) Conc. aq NH_3, RT, 4 days, then Dowex-H^+. Bn = benzyl, DIPEA = *N*,*N*-diisopropylethylamine, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, DIBAL = diisobutylaluminum hydride, TFA = trifluoroacetic acid, mCPBA = meta-chloroperbenzoic acid.

Inactive Akt and activated PDK1 were incubated in the presence of various concentrations of PP-IPs or PCP-IPs, and inhibition of Akt phosphorylation was monitored with a phosphospecific antibody. Both 1PCP-IP_5 and 1PP-IP_5 (prepared by chemical synthesis[12]) were potent inhibitors (Figure 2 b), thus illustrating that replacing the pyrophosphate group at the 1-position with the methylene-bisphosphonate moiety did not significantly alter the biochemical activity (Figure 2 b; see also Figure S1 in the Supporting Information). In addition, we tested 1PCP-IP_4 and 4Bt-1PCP-IP_4, which also inhibited PDK1-mediated phosphorylation, albeit with slightly lowered potency (Figure 2 b). Given our previous results—in which we determined the IC_{50} values of 5PP-IP_5 and 5PCP-IP_5 to be 217 nM and 129 nM, respectively—we conclude that the PH domain of Akt is quite promiscuous towards the PP-IP messengers.[13]

To further validate the lack of selectivity of the Akt PH domain towards the PP-IP regioisomers, we tested their inhibition in the presence of PIP_3 (phosphatidylinositol 3,4,5-

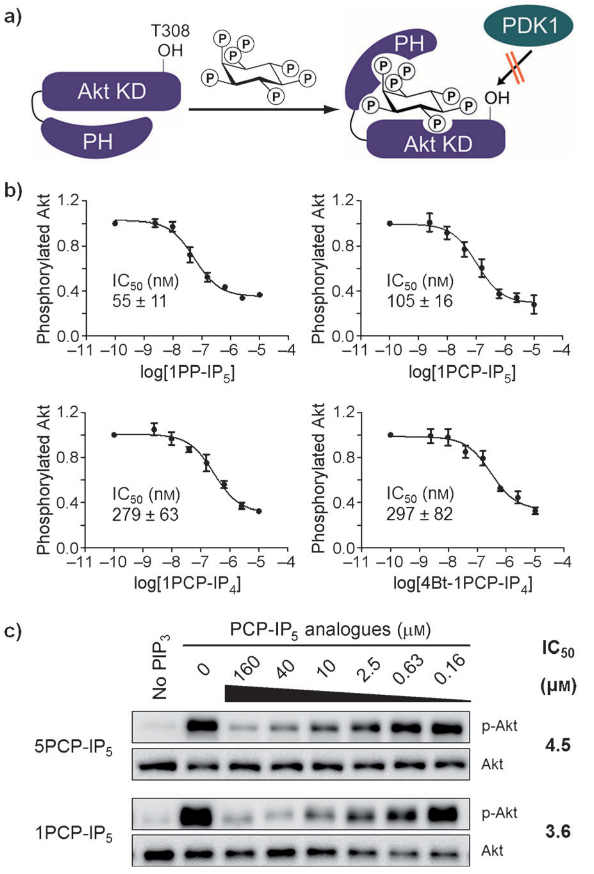


Figure 2. Inhibitory effect of 1PP-IP$_5$ and PCP-IPs on Akt phosphorylation. a) Binding of PP-IPs is proposed to stabilize Akt in a conformation that is not phosphorylated by PDK1 (PH = pleckstrin homology domain; KD = kinase domain). b) IC$_{50}$ curves for in vitro inhibition of Akt phosphorylation (at threonine 308) by PCP analogues. IC$_{50}$ values were determined in three independent experiments, and errors are indicated. c) Western blots of Akt inhibition in the presence of PIP$_3$. Western blots for total Akt were used as a loading control.

trisphosphate), an endogenous stimulator of Akt activation.[14,15] When Akt, PIP$_3$, and PCP-IPs were incubated with PDK1, PIP$_3$-induced Akt phosphorylation was inhibited by both 1PCP-IP$_5$ and 5PCP-IP$_5$. In accordance with our prior experiment, the inhibitory effects of 5PCP-IP$_5$ versus 1PCP-IP$_5$ were almost identical (Figure 2c). Low specificity of the Akt PH domain towards the isomeric PP-IPs was recently also reported by Shears and co-workers.[16] It is possible that the Akt PH domain is quite malleable—as has been observed for PH domain/ligand interactions of isomeric forms of inositol pentakisphosphate (IP$_5$)[17]—and can accommodate the PCP-IP isomers in different orientations. Taken together, these results demonstrate that we are just beginning to understand how the PP-IPs modulate the PI3K/Akt signaling pathway. Parsing out the subtleties in PP-IP–protein interactions will be an important endeavor for connecting PP-IP metabolism with this central growth factor signaling pathway.

We next evaluated the PCP bisphosphonate analogues as inhibitors for the PP-IP-metabolizing diphosphoinositol polyphosphate phosphohydrolases (DIPPs).[18] The hydrolytic activity of DIPPs varies among the PP-IP molecules, and 1PP-IP$_5$ is consumed faster than the 5PP-IP$_5$ regioisomer.[19] We therefore anticipated distinct inhibitory activities of the nonhydrolyzable analogues. Because DIPPs also hydrolyze diadenosine polyphosphates, such as diadenosine pentakisphosphate (Ap$_5$A),[18b,20] we used a competitive inhibition assay to assess our analogues (Figure 3a). Human DIPP1 (hDIPP1) was preincubated with 1PCP-IP$_5$ or 5PCP-IP$_5$, followed by addition of Ap$_5$A, and hydrolysis of Ap$_5$A was monitored. Determination of the initial reaction rates at various Ap$_5$A concentrations confirmed that the PCP analogues indeed acted as competitive inhibitors (Figure 3b; see also Figure S4 in the Supporting Information). Strikingly, the inhibition constant (K_i) for 1PCP-IP$_5$ was about 80-fold lower than the K_i value for 5PCP-IP$_5$ (Table 1).[21] This observation is in agreement with recent reports that 1PP-IP$_5$ is the preferred substrate for mammalian and yeast DIPPs, rather than 5PP-IP$_5$.[19] To further confirm the substrate preference under our assay conditions, we determined the K_i values for 5PP-IP$_5$ and 1PP-IP$_5$. The K_i value for 5PP-IP$_5$ was compa-

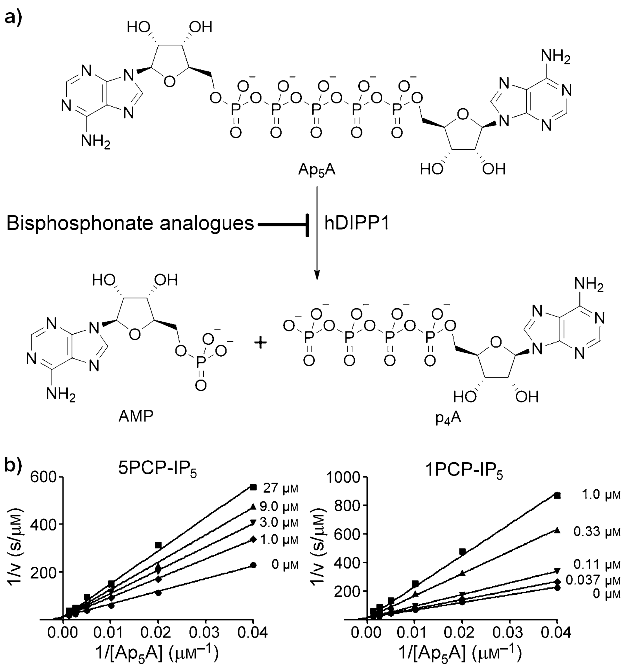


Figure 3. Inhibition of hDIPP1-mediated hydrolysis of diadenosine pentakisphosphate (Ap$_5$A) by PP-IPs and PCP-IPs. a) hDIPP1 hydrolyzes Ap$_5$A to adenosine monophosphate (AMP) and adenosine tetrakisphosphate (p$_4$A), which can be monitored by HPLC. b) Inhibition of Ap$_5$A hydrolysis at various concentrations of 5PCP-IP$_5$ or 1PCP-IP$_5$. Data are presented as Lineweaver–Burk plots, and K_i values were determined by nonlinear regression.

Table 1: Summary of K_i values for PP-IPs and PCP-IPs for inhibiting Ap$_5$A hydrolysis by hDIPP1.

Compound	K_i [µM]	Compound	K_i [µM]
1PCP-IP$_5$	0.11	5PCP-IP$_5$	8.4
1PP-IP$_5$	15	5PP-IP$_5$	8.5
1PCP-IP$_4$	0.027	IP$_6$	17
4Bt-1PCP-IP$_4$	0.023		

rable to that of 5PCP-IP$_5$, which indicates that the hydrolysis of 5PP-IP$_5$ was too slow to be measured. The K_i value for 1PP-IP$_5$, in contrast, was much higher than that of nonhydrolyzable 5PCP-IP$_5$ (Table 1). Presumably, 1PP-IP$_5$ hydrolyzed during the preincubation with hDIPP1 and we effectively measured the K_i value for IP$_6$ instead (as was determined by independent measurement of the K_i value for IP$_6$). Unlike 1PP-IP$_5$, 1PCP-IP$_5$ cannot undergo hydrolysis and constitutes a significantly more potent inhibitor than the natural counterpart. Modification of the 4-position on the 1PCP molecules furnished even more potent compounds 1PCP-IP$_4$ ($K_i = 0.027\ \mu\text{M}$) and 4Bt-1PCP-IP$_4$ ($K_i = 0.023\ \mu\text{M}$). These molecules may prove useful in the future for the selective inhibition of DIPP activity in cell lysates.[22]

Evaluation of the PCP analogues for Akt and hDIPP1 inhibition emphasized their utility in determining the binding preferences of the isomeric PP-IP messengers. Nevertheless, we were curious if the PCP analogues could be harnessed for the study of protein pyrophosphorylation. The β-phosphoryl group of the PP-IPs can participate in trans-phosphorylation reactions as a result of its high free energy of hydrolysis.[23] Interestingly, the PP-IPs phosphorylate pre-existing phosphoserine residues non-enzymatically to generate pyrophosphoproteins.[6,24] While pyrophosphorylation was first described in 2007,[24a] several issues regarding its mechanism still exist. It is not clear how the β-phosphate group in PP-IPs is activated for nucleophilic attack, nor is it known whether a specific binding interaction between PP-IP and the protein substrate precedes the phosphoryl transfer reaction (Figure 4a). The latter question can be addressed with our PCP analogues. They can bind to the same sites as the natural molecules, but are not able to participate in phosphoryl transfer chemistry. Hence, the PCP analogues should act as inhibitors of pyrophosphorylation if this reaction depends on a specific binding event.

5PP-IP$_5$ is known to pyrophosphorylate Nsr1 (nuclear signal recognition 1), a nucleolar protein from *S. cerevisiae* involved in ribosome biogenesis.[24a] The non-enzymatic reaction can only proceed if Nsr1 has been "primed" through phosphorylation by casein kinase 2 (CK2), so that a phosphoserine residue on Nsr1 can undergo pyrophosphorylation. A fragment of Nsr1 (aa27–50) was shown to be a sufficient pyrophosphorylation substrate and we expressed this fragment in *E. coli* as the GST fusion. Co-expression of GST-Nsr1 with CK2 provided a phosphorylated (primed) Nsr1 fragment.[9a] We then exposed the two Nsr1 fragments to radiolabeled β[^{32}P]5PP-IP$_5$ for 20 min at 37 °C. Only the phosphorylated fragment was pyrophosphorylated by β-[^{32}P]5PP-IP$_5$, as seen by autoradiography (Figure 4b). When the pyrophosphorylation of the primed Nsr1 substrate was conducted in the presence of various concentrations of PCP-IPs, we observed dose-dependent inhibition of pyrophosphorylation. The inhibitory effect of the PCP-IPs strongly indicates that a specific binding site for PP-IPs exists on the Nsr1 fragment, and that a binding event precedes the phosphoryl-transfer reaction. Interestingly, both 1PCP-IP$_5$ and 5PCP-IP$_5$ inhibited pyrophosphorylation with similar potencies (IC_{50}(1PCP-IP$_5$) = 1.2 μM; IC_{50}(5PCP-IP$_5$) = 0.67 μM; see also Figure S7 in the Supporting Information).[25] We are currently investigating other pyrophosphorylation substrates to evaluate the specificity of the PP-IP regioisomers in pyrophosphorylation reactions.

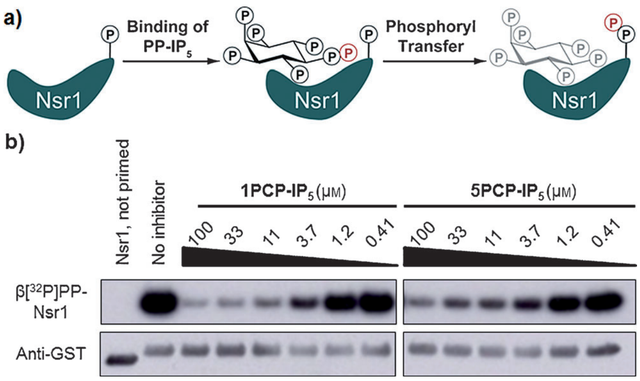


Figure 4. The PCP analogues inhibit pyrophosphorylation of Nsr1. a) Proposed mechanism for Nsr1 pyrophosphorylation by PP-IPs. b) Pyrophosphorylation of a pre-phosphorylated Nsr1 fragment is dose-dependently inhibited by PCP-IPs. GST-Nsr1 fragments, non-phosphorylated or pre-phosphorylated by CK2, were treated with β[^{32}P]5PP-IP$_5$ in the presence of various concentrations of PCP-IPs. Pyrophosphorylation was visualized using autoradiography and an anti-GST Western blot served as a loading control.

Given these results, we believe the bisphosphonate analogues constitute a unique mechanistic tool to distinguish between the two PP-IP signaling mechanisms. For proteins that are regulated by PP-IPs through an allosteric mechanism, the PCP analogues and the natural molecules will have the same effect (as seen for Akt inhibition). In contrast, a different result will be observed for protein pyrophosphorylation: The PCP analogues are inhibitors of the pyrophosphorylation reaction (Figure 5).

The PP-IPs are emerging as a central group of cellular messengers, yet their functions have remained poorly defined because of a dearth of reagents and methods. To this end, we have contributed a set of nonhydrolyzable analogues, which contain a bisphosphonate moiety in place of the pyrophosphate group. The analogues are accessed by chemical synthesis, which reduces the risk of biological contamination. Introduction of the stabilized bisphosphonate moiety minimizes chemical and biochemical degradation. These features make the bisphosphonate analogues attractive tools for the biochemical characterization of PP-IP–protein interactions. Considering the close structural similarity among these messengers, the determination of their nuanced binding preferences will greatly benefit from the availability of stable and pure compounds which are resistant to isomerization and decomposition pathways.

The enhanced stability of the PCP analogues also renders them well-suited for the interrogation of PP-IP biosynthesis and metabolism, which was exemplified by the increased potency we observed for 1PCP-IP$_5$ versus 5PCP-IP$_5$ towards hDIPP1. It will be interesting to determine the structural basis for this substrate preference and the PCP analogues will prove useful for cocrystallization experiments. Furthermore, much about feedback regulation within the biosynthetic pathway and the cellular turnover of PP-IPs remains to be elucidated, and our analogues can facilitate the detailed

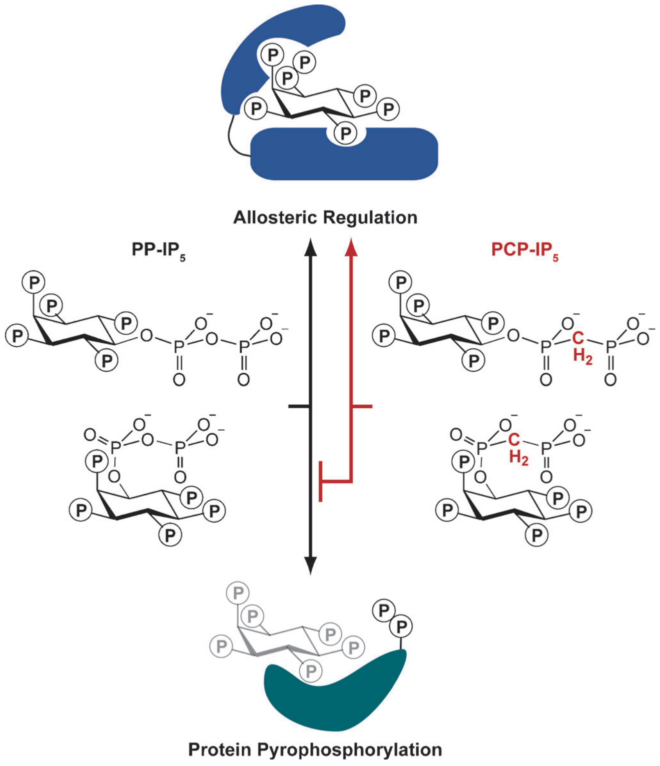


Figure 5. The nonhydrolyzable analogues can be used as mechanistic probes. PP-IPs and PCP-IPs will have the same biochemical effect if proteins are regulated by an allosteric mechanism. If the PP-IPs control protein function through pyrophosphorylation, however, the PP-IPs and PCP-IPs will have opposing effects.

biochemical analysis of the inositol kinases and phosphatases. While the current analogues are not cell permeable due to their high negative charge, strategies exist to overcome this limitation: The phosphate groups can be masked as esters to increase membrane permeability;[26] endogenous esterases can subsequently remove these ester groups to release the original PP-IP messengers.

Finally, the bisphosphonate molecules are not able to participate in phosphoryl transfer reactions and are potent inhibitors of protein pyrophosphorylation. In a biochemical setting, the PCP analogues can therefore be used to clearly distinguish between the allosteric and pyrophosphorylation signaling mechanisms of PP-IPs. As such, the molecules describe herein will aid to decipher the signaling properties of these poorly understood second messengers and thereby promote this newly developing field. By making these tools available to research groups across disciplines, we can begin to detail the complex functions of PP-IPs in healthy and diseased states.

Received: February 28, 2014
Revised: April 25, 2014

Keywords: diphosphoinositol polyphosphate · mechanistic probe · nonhydrolyzable analogues · second messengers · phosphorylation

[1] a) M. A. Lemmon, *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 99; b) T. G. Kutateladze, *Nat. Chem. Biol.* **2010**, *6*, 507.

[2] a) S. K. Dove, R. C. Piper, R. K. McEwen, J. W. Yu, M. C. King, D. C. Hughes, J. Thuring, A. B. Holmes, F. T. Cooke, R. H. Michell, P. J. Parker, M. A. Lemmon, *EMBO J.* **2004**, *23*, 1922; b) N. Tamura, M. Oku, M. Ito, N. N. Noda, F. Inagaki, Y. Sakai, *J. Cell Biol.* **2013**, *202*, 685.

[3] a) A. Chakraborty, S. Kim, S. H. Snyder, *Sci. Signaling* **2011**, *4*, re1; b) A. Saiardi, *Subcell. Biochem.* **2012**, *59*, 413; c) M. S. C. Wilson, T. M. Livermore, A. Saiardi, *Biochem. J.* **2013**, *452*, 369; d) T. Wundenberg, G. W. Mayr, *Biol. Chem.* **2012**, *393*, 979; e) J. P. Monserrate, J. D. York, *Curr. Opin. Cell Biol.* **2010**, *22*, 365; f) S. B. Shears, *Mol. Pharmacol.* **2009**, *76*, 236.

[4] a) A. Saiardi, A. C. Resnick, A. M. Snowman, B. Wendland, S. H. Snyder, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 1911; b) S. J. York, B. N. Armbruster, P. Greenwell, T. D. Petes, J. D. York, *J. Biol. Chem.* **2005**, *280*, 4264.

[5] J. Worley, X. Luo, A. P. Capaldi, *Cell Rep.* **2013**, *3*, 1476.

[6] Z. Szijgyarto, A. Garedew, C. Azevedo, A. Saiardi, *Science* **2011**, *334*, 802.

[7] a) Y.-S. Lee, S. Mulugu, J. D. York, E. K. O'Shea, *Science* **2007**, *316*, 109; b) Y.-S. Lee, K. Huang, F. A. Quiocho, E. K. O'Shea, *Nat. Chem. Biol.* **2008**, *4*, 25.

[8] a) C. Illies, J. Gromada, R. Fiume, B. Leibiger, J. Yu, K. Juhl, S.-N. Yang, D. K. Barma, J. R. Falck, A. Saiardi, C. J. Barker, P.-O. Berggren, *Science* **2007**, *318*, 1299; b) J. Kamimura, K. Wakui, H. Kadowaki, Y. Watanabe, K. Miyake, N. Harada, M. Sakamoto, A. Kinoshita, K. Yoshiura, T. Ohta, T. Kishino, M. Ishikawa, M. Kasuga, Y. Fukushima, N. Niikawa, N. Matsumoto, *J. Hum. Genet.* **2004**, *49*, 360.

[9] a) R. Bhandari, K. R. Juluri, A. C. Resnick, S. H. Snyder, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 2349; b) A. Chakraborty, M. A. Koldobskiy, N. T. Bello, M. Maxwell, J. J. Potter, K. R. Juluri, D. Maag, S. Kim, A. S. Huang, M. J. Dailey, M. Saleh, A. M. Snowman, T. H. Moran, E. Mezey, S. H. Snyder, *Cell* **2010**, *143*, 897; c) E. K. Speliotes, C. J. Willer, S. I. Berndt, K. L. Monda, G. Thorleifsson, A. U. Jackson, H. L. Allen, C. M. Lindgren, J. a. Luan, R. Maegi, J. C. Randall, S. Vedantam, T. W. Winkler, L. Qi, T. Workalemahu, I. M. Heid, V. Steinthorsdottir, H. M. Stringham, M. N. Weedon, E. Wheeler, A. R. Wood, T. Ferreira, R. J. Weyant, A. V. Segre, K. Estrada, L. Liang, J. Nemesh, J.-H. Park, S. Gustafsson, T. O. Kilpelaenen, J. Yang, N. Bouatia-Naji, T. Esko, M. F. Feitosa, Z. Kutalik, M. Mangino, S. Raychaudhuri, A. Scherag, A. V. Smith, R. Welch, J. H. Zhao, K. K. Aben, D. M. Absher, N. Amin, A. L. Dixon, E. Fisher, N. L. Glazer, M. E. Goddard, N. L. Heard-Costa, V. Hoesel, J.-J. Hottenga, A. Johansson, T. Johnson, S. Ketkar, C. Lamina, S. Li, M. F. Moffatt, R. H. Myers, N. Narisu, J. R. B. Perry, M. J. Peters, M. Preuss, S. Ripatti, F. Rivadeneira, C. Sandholt, L. J. Scott, N. J. Timpson, J. P. Tyrer, S. van Wingerden, R. M. Watanabe, C. C. White, F. Wiklund, C. Barlassina, D. I. Chasman, M. N. Cooper, J.-O. Jansson, R. W. Lawrence, N. Pellikka, I. Prokopenko, J. Shi, E. Thiering, H. Alavere, M. T. S. Alibrandi, P. Almgren, A. M. Arnold, T. Aspelund, L. D. Atwood, B. Balkau, A. J. Balmforth, A. J. Bennett, Y. Ben-Shlomo, R. N. Bergman, S. Bergmann, H. Biebermann, A. I. F. Blakemore, T. Boes, L. L. Bonnycastle, S. R. Bornstein, M. J. Brown, T. A. Buchanan, et al., *Nat. Genet.* **2010**, *42*, 937.

[10] M. Wu, B. E. Dul, A. J. Trevisan, D. Fiedler, *Chem. Sci.* **2013**, *4*, 405.

[11] M. Mentel, V. Laketa, D. Subramanian, H. Gillandt, C. Schultz, *Angew. Chem.* **2011**, *123*, 3895; *Angew. Chem. Int. Ed.* **2011**, *50*, 3811.

[12] S. Capolicchio, D. T. Thakor, A. Linden, H. J. Jessen, *Angew. Chem.* **2013**, *125*, 7050; *Angew. Chem. Int. Ed.* **2013**, *52*, 6912.

[13] In addition, we tested the inhibitory effect of IP_3 (inositol 1,4,5-trisphosphate) on Akt phosphorylation. We found that the IC_{50} value for IP_3 was significantly higher ($IC_{50} = 1.31\ \mu\text{M}$) than for the PP-IPs, thus indicating a preference of the PH domain towards the highly phosphorylated inositol phosphates (see Figure S2 in the Supporting Information).

[14] V. Calleja, D. Alcor, M. Laguerre, J. Park, B. Vojnovic, B. A. Hemmings, J. Downward, P. J. Parker, B. Larijani, *PLoS Biol.* **2007**, *5*, 780.

[15] a) D. Stokoe, L. R. Stephens, T. Copeland, P. R. J. Gaffney, C. B. Reese, G. F. Painter, A. B. Holmes, F. McCormick, P. T. Hawkins, *Science* **1997**, *277*, 567; b) D. R. Alessi, S. R. James, C. P. Downes, A. B. Holmes, P. R. J. Gaffney, C. B. Reese, P. Cohen, *Curr. Biol.* **1997**, *7*, 261.

[16] N. A. Gokhale, A. Zaremba, A. K. Janoshazi, J. D. Weaver, S. B. Shears, *Biochem. J.* **2013**, *453*, 413.

[17] a) S. G. Jackson, Y. Zhang, R. J. Haslam, M. S. Junop, *BMC Struct. Biol.* **2007**, *7*, 80; b) S. G. Jackson, S. Al-Saigh, C. Schultz, M. S. Junop, *BMC Struct. Biol.* **2011**, *11*, 11.

[18] a) S. T. Safrany, J. J. Caffrey, X. N. Yang, M. E. Bembenek, M. B. Moyer, W. A. Burkhart, S. B. Shears, *EMBO J.* **1998**, *17*, 6599; b) S. T. Safrany, S. W. Ingram, J. L. Cartwright, J. R. Falck, A. G. McLennan, L. D. Barnes, S. B. Shears, *J. Biol. Chem.* **1999**, *274*, 21735.

[19] a) A. Lonetti, Z. Szijgyarto, D. Bosch, O. Loss, C. Azevedo, A. Saiardi, *J. Biol. Chem.* **2011**, *286*, 31966; b) R. S. Kilari, J. D. Weaver, S. B. Shears, S. T. Safrany, *FEBS Lett.* **2013**, *587*, 3464.

[20] J. L. Cartwright, A. G. McLennan, *J. Biol. Chem.* **1999**, *274*, 8604.

[21] We also tested the activity of $1PCP\text{-}IP_5$ and $5PCP\text{-}IP_5$ against hDIPP1 in a buffer from Ref. [19b]. We obtained K_i values of 9.3 nM for $1PCP\text{-}IP_5$ and 1.1 nM for $5PCP\text{-}IP_5$, thereby illustrating that $1PCP\text{-}IP_5$ remains the preferred substrate for hDIPP1 under different buffer conditions (see Figure S5 in the Supporting Information).

[22] Remarkably, we observed no signs of degradation when $1PCP\text{-}IP_5$ or $5PCP\text{-}IP_5$ were incubated with a yeast cell lysate for 18 h at 37 °C (see Figure S6 in the Supporting Information).

[23] a) L. Stephens, T. Radenberg, U. Thiel, G. Vogel, K. H. Khoo, A. Dell, T. R. Jackson, P. T. Hawkins, G. W. Mayr, *J. Biol. Chem.* **1993**, *268*, 4009; b) A. Saiardi, R. Bhandari, A. C. Resnick, A. M. Snowman, S. H. Snyder, *Science* **2004**, *306*, 2101; c) C. E. Hand, J. F. Honek, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 183.

[24] a) R. Bhandari, A. Saiardi, Y. Ahmadibeni, A. M. Snowman, A. C. Resnick, T. Z. Kristiansen, H. Molina, A. Pandey, J. K. Werner, Jr., K. R. Juluri, Y. Xu, G. D. Prestwich, K. Parang, S. H. Snyder, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 15305; b) C. Azevedo, A. Burton, E. Ruiz-Mateos, M. Marsh, A. Saiardi, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 21161.

[25] Interestingly, $5PP\text{-}IP_5$ also showed similar potency ($IC_{50} = 0.74\ \mu\text{M}$; see Figures S7 and S8 in the Supporting Information). We are currently investigating the kinetic parameters for this phosphoryl transfer reaction.

[26] a) H. J. Jessen, T. Schulz, J. Balzarini, C. Meier, *Angew. Chem.* **2008**, *120*, 8847; *Angew. Chem. Int. Ed.* **2008**, *47*, 8719; b) D. Subramanian, V. Laketa, R. Müller, C. Tischer, S. Zarbakhsh, R. Pepperkok, C. Schultz, *Nat. Chem. Biol.* **2010**, *6*, 324; c) M. P. Wymann, C. Schultz, *ChemBioChem* **2012**, *13*, 2022.