Synthetic tools for studying the chemical biology of InsP₈†

Andrew M. Riley, a Huanchen Wang, b Stephen B. Shears b and Barry V. L. Potter* ac

To synthesise stabilised mimics of InsP₈, the most phosphorylated inositol phosphate signalling molecule in Nature, we replaced its two diphosphate (PP) groups with either phosphonoacetate (PA) or methylenebisphosphonate (PCP) groups. Utility of the PA and PCP analogues was verified by structural and biochemical analyses of their interactions with enzymes of InsP₈ metabolism.

The myo-inositol phosphates (InsPs) are a family of intracellular signalling molecules containing combinatorial arrangements of monophosphate (P) and diphosphate (PP) groups arranged around the hexahydroxy cyclohexane ring of myo-inositol (Ins). There is much current interest in the specialised chemistry and biology of the diphosphoinositol polyphosphates (PP-InsPs, inositol pyrophosphates); recent research has highlighted the central roles that PP-InsPs play in cellular and organismic homeostasis in all eukaryotes. For example, PP-InsPs regulate DNA repair, immunity and metabolic homeostasis.

The most studied of the PP-InsPs are 5-InsP₇, 1-InsP₇ and InsP₈ (Fig. 1), which are formed from InsP₆, by InsP₆ kinases (IP₆Ks) and diphosphoinositol pentakisphosphate kinases (PPIP₅Ks). The diphosphate groups that are produced by IP₆K and PPIP₅K are hydrolysed by a family of PP-InsP diphosphohydrolases (DIPPs), leaving monophosphate groups and liberating inorganic orthophosphate (Pi).

Evidence has been obtained that the PP-InsPs non-enzymatically pyrophosphorylate a range of target proteins. It has also been reported that 5-InsP₇ produces a separate range of cellular effects by interacting with pleckstrin homology (PH) domains of proteins. Additionally, in response to viral invasion, 1-InsP₇ was recently shown to stimulate phosphorylation of IRF3, an activator of interferon transcription. No independent function for InsP₆ has, however, yet been shown. Nevertheless, in mammalian cells, levels of InsP₈ are regulated in a stimulus-dependent fashion, increasing several-fold following osmotic stress or thermal challenge, whereas bioenergetic stress decreases InsP₈ concentrations. Indeed, InsP₈ is the only PP-InsP so far shown to exhibit such acute, stimulus-dependent changes in

Fig. 1 (a) Biosynthesis of diphosphoinositol polyphosphates from myo-inositol hexakisphosphate (InsP₆). IP₆K, inositol hexakisphosphate 5-kinase; PPIP₅K, diphosphoinositol pentakisphosphate kinase; DIPP, diphosphoinositol polyphosphate phosphohydrolase; (b) structures of synthetic α-phosphonoacetic acid ester (PA) analogue 1 and methylenebisphosphonate (PCP) analogue 2.

† Electronic supplementary information (ESI) available: Data deposition: atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 5BYA and 5BYB). See DOI: 10.1039/c5cc05017k
its levels. Such responses are typically hallmarks of a signalling event, in which the concentration-dependent influence of a messenger upon a target protein (a receptor) is transduced into a biological response. It is therefore reasonable to consider the existence of \( \text{InsP}_8 \) "receptors".

The structure of mammalian \( \text{InsP}_8 \) has been identified\(^{14,15}\) as \(1,5[\text{PP}]_2\)-\(\text{InsP}_4\) (Fig. 1) and a chemical synthesis\(^{16}\) has provided isomerically pure material in the amounts required for detailed biological studies with cell extracts. However, in such experiments, the PP-\(\text{InsP}_n\)s are rapidly metabolised by phosphatases. Therefore, there is a need for synthetic \(\text{InsP}_8\)-based probes, especially stabilised analogues in which the labile diphasate (PP) is replaced with mimics more resistant to chemical and enzymatic degradation. Such compounds could be used to screen for \(\text{InsP}_8\) receptors in cellular or tissue lysates and may have greater potential for further synthetic elaboration than \(\text{InsP}_8\) itself. They may also be useful as mechanistic probes because they cannot substitute for the ability of \(\text{InsP}_8\) to transfer its levels. Such responses are typically hallmarks of a signalling event, in which the concentration-dependent influence of a messenger upon a target protein (a receptor) is transduced into a biological response. It is therefore reasonable to consider the existence of \(\text{InsP}_8\) "receptors".

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**Scheme 1** Synthesis of 1 and 2. Reagents and conditions: (a) \(\text{EDAC}, \text{CH}_2\text{Cl}_2\); (b) \(\text{H}_2, \text{Pd(OH)}_2/C, \text{MeOH, H}_2\text{O}\); (c) \(\text{tmsBr, CH}_2\text{Cl}_2\); (d) \(\text{TMSBr, CH}_2\text{Cl}_2\); (e) [EtO]_2\(\text{PO}\text{CH}_2\text{PO}(\text{O})(\text{OEt})\text{Cl, DIPEA, CH}_2\text{Cl}_2\); (f) [EtO]_2\(\text{PO}\text{CH}_2\text{PO}(\text{O})(\text{OEt})\text{Cl, DIPEA, CH}_2\text{Cl}_2\); (g) \(\text{H}_2, \text{Pd(OH)}_2/C, \text{MeOH, THF, H}_2\text{O, AcOH, TEAB, aqueous triethylammonium bicarbonate; Bn, benzyl. Yields are shown in respect of each step. Stereogenic phosphorus atoms are indicated by an asterisk.}
Removal of the benzyl protecting groups from 9 followed by phosphitylation and oxidation gave fully protected 10, again as a mixture of four diastereoisomers. The NMR spectra of 10 were highly complex, with at least 30 lines in the 31P NMR spectrum (see ESI†). However, on global deprotection with TMSBr followed by cleavage of silyl esters with methanol, the stereogenic centres at phosphorus were abolished, at last revealing the expected pattern of NMR signals for 1,5- [PCP]2-InsP4 (2), which was isolated as the triethylammonium salt after ion-exchange chromatography.

When we incubated either InsP8 analogue 1 or 2 with DIPP, we did not detect any Pi release. Next, we examined the interactions of 1 and 2 with the highly specific kinase domain of human PPIP5K2 (PPIP5K2KD). We have previously found that the reaction catalysed by PPIP5K2KD is reversible in vitro, yielding ATP when PPIP5K2KD is incubated with ADP and InsP8.21 Detection of the generated ATP with luciferin/luciferase provides a sensitive assay for the reverse kinase reaction.17,21,22 No ATP was detected using 1 or 2 in these assays (data not shown).

Previously,15 we obtained enzyme-product complexes by soaking InsP8 into crystals of PPIP5K2KD containing ADP. In the present work, we soaked product analogues 1 and 2 into similarly prepared crystals of PPIP5K2KD. X-ray analyses showed that both compounds bound to the catalytic site of PPIP5K2KD (Fig. 2). Significantly, the PA-containing analogue 1 was also observed in a second binding site, the function of which was previously demonstrated to enhance capture of substrate from the bulk phase.22 In this respect, compound 2 more closely mimics natural InsP8, which was also found to occupy exclusively the catalytic site.15,16 This should not be taken to mean that InsP8 and 2 do not also bind to the capture site but that, presumably, they occupy it only transiently. Thus, the PA compound 1 provides additional insights into a subsequent step of the catalytic cycle; the transfer of newly-formed InsP8 to the capture site prior to release. Notably, the 1-PA group of 1 is solvent-exposed and has no interactions with the capture/release site. This is consistent with the requirement that the site must bind both substrate (without 1-PP) and product (with 1-PP); it also suggests a suitable attachment point for reporter groups in probes designed to target this site.

In conclusion, we report the syntheses of two stabilised analogues of InsP8: 1,5-[PA]2-InsP4 (1) and 1,5- [PCP]2-InsP4 (2). Our observation that the PA analogue 1 occupies the capture/release site in PPIP5K2 reveals how InsP8 mimics can give insight into different enzyme states within the overall catalytic cycle. Stabilised mimics will also be useful for identifying receptors within cell lysates. Since these two new probes might interact with receptors in subtly different ways, the use of both may increase opportunities to capture InsP8 receptors. The differing and complementary interactions of 1 and 2 with PPIPK2 (Fig. 2) illustrate this point.

Cellular levels of InsP8 are subject to stimulus-dependent regulation, yet it remains an orphan signal; no specific InsP8 receptor has yet been identified. Nevertheless, InsP8, which possesses the most crowded array of phosphate groups in Nature, demands from the cell a significant investment in energy to sustain its levels against a backdrop of high ongoing turnover. With recent advances in delivering PP-InsPs (and hence PP-InsP mimics) into cells,23 it should now become possible to screen for biological effects of InsP8, and InsP8 mimics, in intact cells.

B.V.L.P. is a Wellcome Trust Senior Investigator (Grant 101010). This research was also supported by the Intramural

Fig. 2 Refined 2Fα–Fα maps contoured at 1.0 σ for both compounds (top) and simulated annealing omit maps (Fα–Fc, bottom panels) contoured at 2.0 σ for 1,5- [PA]2-InsP4 (1) and 3.0 for 1,5- [PCP]2-InsP4 (2). Compounds are shown as stick models. Carbon atoms are shown in cyan (1) or green (2), oxygen atoms in red, nitrogen atoms in blue, and phosphorus atoms in orange. The protein is shown as a surface representation.
Notes and references

† The required starting material (−)-3 was found to have the opposite specific rotation to that previously reported for 1→2,3,4,6-tetra-O-benzyl-inositol.20 The correct absolute configuration for (−)-3 was determined in the present work (see ESI† for details).

‡ Although the acyl esters in 1 will be susceptible to hydrolytic cleavage at high pH, the 31P NMR spectrum of a solution of the triethylammonium salt of 1 in D2O was unchanged after >1 year at 4 °C.

¶ We found that the nature and pattern of protecting groups strongly influenced the outcome of the reaction. Phosphonylation of less sterically hindered substrates at either O-1 or O-5 was high-yielding under these conditions.

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