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Synthesis of a Tethered *myo*-Inositol (1,3,4,5,6)Pentakisphosphate (IP5) Derivative as a Probe for Biological Studies

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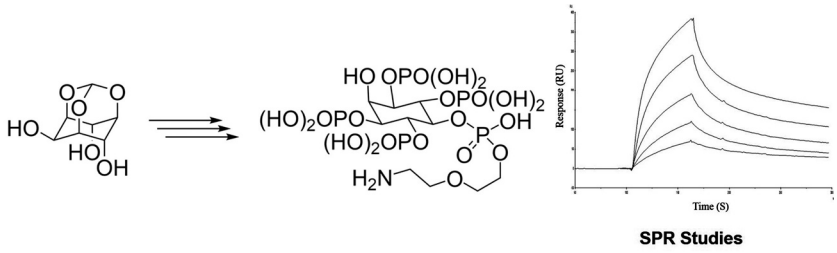
This paper is dedicated to Prof. Steven Ley on the occasion of his 70th birthday and in recognition of his pioneering synthesis of the GPI anchor.



SPR Studies

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Abstract There is sufficient evidence to suggest that *myo*-inositol pentakisphosphate is a vital intermediate species in higher inositol phosphate metabolism, however, its biological roles and physiological function in cells remain uncertain. A tethered *myo*-inositol pentakisphosphate (IP5) derivative with a terminal amine group is synthesised allowing facilitated immobilisation onto M-270 magnetic Dynabeads for pull-down experiments and biosensor chip preparation for surface plasmon resonance studies. The probes are validated by both pull-down and surface plasmon resonance (SPR) studies of the known binding protein GRP-1 (general receptor for phosphoinositides 1), and furthermore by SPR studies of protein kinase B (PKB or AKT) binding.

Key words protecting groups, proteins, total synthesis, regioselectivity, carbohydrates, phosphates, bioorganic chemistry, hydrogenation

The family of inositol compounds has an extraordinary range of biological functions and only slight changes in their level of phosphorylation can have huge effects on their biological roles.[1,2] Since the initial discovery of *myo*-inositol (1,4,5)triphosphate (IP3) and its role in the regulation of Ca^{2+} signalling pathways,[3] the function of both inositol phosphates (IPs) and their lipid counterparts (phosphatidylinositol phosphates, phosphoinositides or simply PIPs) has become a widely studied topic in the scientific community. Although a great deal is now known about the structure, binding interactions and disease association of phosphoinositides, gaps in the understanding of these incredible molecules still remain.[4]

Of the six possible structural isomers of *myo*-inositol pentakisphosphate, I(1,3,4,5,6)P_5 (**IP5**, Figure 1) was the first to be characterised from natural sources.[5] For the most part it is a stable cell constituent with turnover times of many hours or even days in mammalian cells.[6] However, it can occasionally exhibit rapid changes in concentration in response to stimuli.[7] IP5 has been linked with many cellular activities including chromatin remodelling,[8] regulation of Ca^{2+} channels,[9] apoptosis[10] and viral assembly.[11] Interestingly, there is growing evidence supporting the fact that IP5 acts as a regulator of important phosphoinositide interactions.[4] In addition, IP5 has been shown to mediate the Wnt/β-catenin signalling pathway through canonical signalling.[12] These combined findings place IP5 as a major regulator of disease related pathways in biological systems.

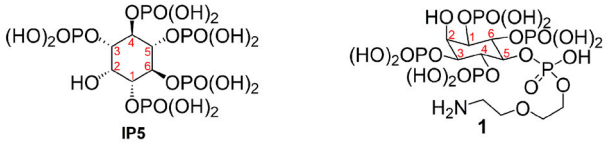


Figure 1 Structure of the most naturally abundant form of *myo*-inositol pentakisphosphate (**IP5**) with the carbons numbered 1–6 (in red) and the structure of the target molecule **1** – a functionalised **IP5** molecule with a tether and terminal amine group for further manipulation

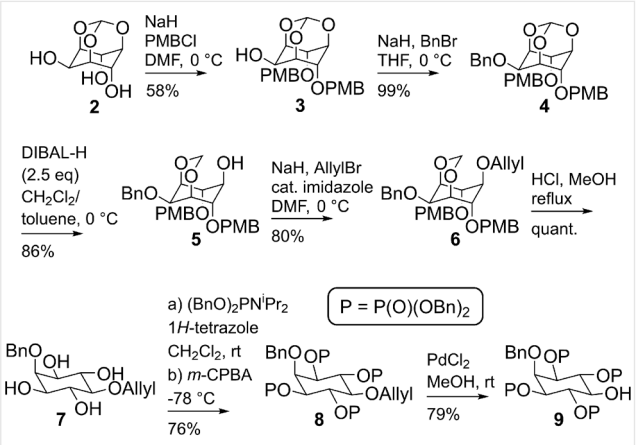
Synthetic chemistry has been a useful tool in assisting biological researchers to further evaluate the properties of these inositol-based molecules.[4] One synthetic approach is to modify the compound of interest with a tethered chain allowing it to be covalently attached to reporter groups,[2,13] other small molecules,[14] or beads and surfaces.[2] Previously, we have synthesised all eight members of the PIP family as simplified lipid analogues and also with terminal amino groups.[15] Conjugation through this amine onto affinity ma-

trices allowed us to identify binding proteins for PI(3,4,5)P_3, PI(4,5)P_2, P(3,5)P_2 and PI(3)P from the colorectal carcinoma cell line LIM1215.[16]

The advantage when designing chemical probes to mimic the PIPs is that coordination can take place on the lipid chain away from the main head group. The inositol sugar acts as the main anchoring point for most protein interactions, although often the membrane environment plays a significant role in the affinity of the head group for the target protein. The only option when designing tethered chemical probes of inositol phosphates is to connect the reporter group directly to the inositol ring.[17] The syntheses of tethered IP5 compounds have been previously explored. Ozaki[18] as well as Potter[14] have synthesised substituted derivatives from the C-2 oxygen. However, although the syntheses have been reported, only Potter proved that their probe could be used for biological studies (although mainly for studying IP3 rather than IP5).[14] In addition, the importance of the axial hydroxy group at the C-2 position is not fully understood. To simplify the synthetic effort, it is also beneficial to attach the tether via the oxygens at C-2 or C-5 as this preserves a plane of symmetry through the compound, allowing a *meso* structure to be maintained.

Here we report the synthesis of an amino-modified IP5 analogue, **1**, with the tether linked through the phosphate at the C-5 position, allowing immobilisation onto solid support/affinity matrices for biological investigation. The probe was validated by both pull-down and surface plasmon resonance (SPR) studies of the known binding protein GRP-1 (general receptor for phosphoinositides 1), and furthermore by SPR studies of protein kinase B (PKB or AKT) binding.

Selective protection of both axial hydroxy groups of orthoformate **2**[19] gave the mono alcohol **3** in good yield (Scheme 1).[20] Benzylation at the C-2 alcohol afforded **4** in good yield. This protection of the axial alcohol was carried through to the end of the synthesis. The orthoformate **4** was then reductively cleaved, selectively, with diisobutylaluminum hydride (DIBAL-H) to expose the C-5 positioned hydroxy moiety, a method previously developed by our group.[15,21]

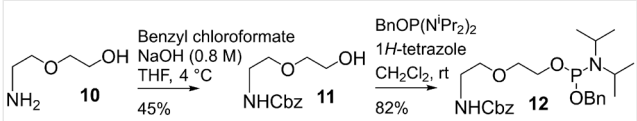


Scheme 1 Synthesis of intermediates en route to tethered IP5 derivative **1**

The free C-5 alcohol of the acetal **5** was subsequently protected with an allyl group to give the fully protected inositol **6**, as confirmed by the distinctive signals of the allyl group between δ 5–6 in the ^{1}H NMR spectrum. The allyl group was used as it can survive the strongly acidic conditions required for the deprotection of the acetal and removal of the *p*-methoxybenzyl (PMB) groups. This acid treatment produced the tetraol **7**, a vital intermediate, in quantitative yield.

Phosphorylation of **7** using standard phosphoramidite chemistry conditions followed by oxidation as previously developed in the group[15] proceeded as expected (Scheme 1), giving two singlets in the ^{31}P NMR spectrum at δ 1.59 and δ –2.00, each representing two equivalent phosphorus atoms. The next step required the removal of the allyl group, which initially proved to be problematic, as regular deprotection conditions, such as the use of Wilkinson's catalyst, were unsuccessful; the very slight acidic nature of this method resulted in some dephosphorylation. However, conditions using palladium(II) chloride as the catalyst in methanol seemed to be an effective and simple, as well as a very mild approach to this deprotection step giving the alcohol **9** in 79% yield.[22] This transformation was monitored by signals in the ^{31}P NMR spectrum shifting to δ –0.43 and δ –1.96.

There are various factors, such as length, polarity and stability, that needed to be considered when selecting the most appropriate linker for these experiments.[23] A medium length ethylene glycol derived structure, functionalised with a terminal amine for later conjugation, was used due to its capability of retaining rigidity. The replacement of carbon atoms, in an alkyl chain, with oxygen atoms reduces the lipophilicity and thus improves the water solubility of the final compound. The linker was synthesised via the protection of the commercially available 2-aminoethoxyethanol **10** with a benzyloxycarbonyl (Cbz) group followed by coupling with a diphosphoramidite under inert conditions to give the tethered phosphoramidite **12** (confirmed by the presence of a single ^{31}P NMR signal at δ 147.7) in 82% yield (Scheme 2).

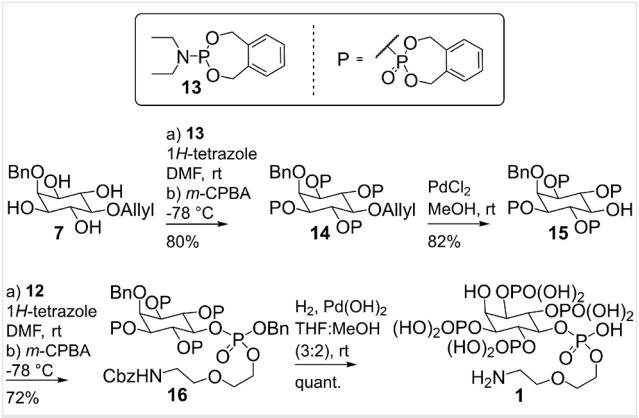


Scheme 2 Synthesis of phosphoramidite tether **12**

Coupling of this freshly made phosphoramidite to the now exposed C-5 alcohol of tetraphosphate **9** did not go according to plan; no reaction was observed. It seems that the limitations of this reaction were the result of the sheer

amount of steric hindrance owing to the surrounding benzyl phosphate groups. Thus, an alternative phosphoramidite **13** was prepared.[18,24]

Phosphorylation of tetraol **7** with phosphoramidite **13** in the presence of 1*H*-tetrazole gave the tetraphosphorylated inositol **14** in 80% yield (Scheme 3). Subsequent deallylation using palladium(II) chloride gave alcohol **15** that demonstrated downfield shifts of the ^{31}P NMR resonances. Coupling of the phosphoramidite **12** with alcohol **15** gave the fully protected IP5 precursor **16** in good yield. Traditional analysis by ^{1}H NMR spectroscopy of these materials was difficult owing to the intense signals of the benzyl and xylyl protons, however, ^{31}P NMR spectroscopy revealed five signals at δ –1.45, δ –1.53, δ –1.60, δ –2.14 and δ –2.64 representing the five now non-equivalent phosphorus atoms, due to the chiral phosphorus at C-5. A peak in the HRMS of 1412.2718 (1412.2712 calculated for $C_{64}H_{68}NO_{24}P_5Na$ [M + Na]$^+$) was also observed, further supporting the identity of the product **16**.



Scheme 3 Synthesis of tethered IP5 derivative **1**

Finally, the fully protected precursor **16** was then globally deprotected by hydrogenolysis (at a pressure of 10 bar) in the presence of palladium hydroxide to give the desired product **1**. All phosphate benzyl esters, the C-2 benzyl ether and the benzyloxycarbonyl protecting groups had been removed to reveal the IP5 structure with a C-5 position linker group, culminating in a free amine to attach to reporter molecules, beads and surfaces. All the resonances in the ^{31}P NMR spectrum were shifted upfield [δ 4.22 (2 P), δ 3.01 (2 P) and δ 0.44 (1 P)], consistent with deprotection of the phosphate groups. HRMS identification of *m*/*z* 665.9587 (665.9562 calculated for $C_{10}H_{25}NO_{22}P_5$ [M – H]$^-$) and other characterisations confirmed that the desired structure **1** was obtained.[25]

We have previously immobilised the ω-amino PIP derivatives onto affinity beads and surfaces.[15] It was hoped that this technique could be applied in the field of IPs, firstly using the terminal amino IP5 derivative **1** to couple onto beads for pull-down experiments. In addition, the compound could also be functionalised with biotin for subsequent attachment onto chips for biosensor experiments. By performing these biological evaluation techniques, potential new binding partners, their affinities and specificities, and a further understanding of the role and function of IP5 in the cell might be discovered.

We previously used Affi-Gel beads, however, preliminary studies showed that coupling to the Affi-Gel beads with the much more polar and charged amino-IP5 compound **1** was not as efficient as with the more lipophilic ω-amino PIP derivatives. After testing a number of alternatives, magnetic Dynabeads (M-270 carboxylic acid) were found to couple with the amino-IP5 efficiently and facilitate quick and effective washing (see the Supporting Information for further details).

Before affinity pull-downs from complex mixtures of proteins, such as cell extracts, it was important to confirm that the beads were working as expected. It is well known that **IP5** competes with a number of PIPs for the binding of the Pleckstrin homology domain (PH domain) of GRP (general receptor for phosphoinositides 1),[26] and is known to bind to protein kinase B (PKB or AKT). The PH domains of cytohesin-3/general receptor of phosphoinositides 1 (GRP1)(O43739: 264–381) and RAC-alpha serine/threonine-protein kinase (AKT1)(P31749: 5–108) were produced as glutathione S-transferase (GST) fusion proteins. Transformations were carried out using BL21 competent cells and the PH domains in pGEXT2TK as previously described.[16a] GST fusion proteins were purified using glutathione agarose beads followed by micropreparative size-exclusion chromatography with a Superose 12 column (HR10/30) equilibrated in phosphate-buffered saline (PBS) and connected to an AKTA purifier system. Protein purity was analysed using Coomassie-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and protein identity was confirmed using LC–MS/MS analysis. The GST-GRP PH domain was diluted in PBS and was separately incubated with blank beads and IP5 beads. The beads were then washed three times with PBS and heated in SDS-PAGE reduction buffer at 95 °C for 10 minutes to extract any protein from the beads; the protein was then separated by SDS-PAGE (Figure 2,A).

The IP5 beads show a distinct band corresponding to the GRP protein and the blank beads show very little presence of the same band. This indicates that the functionalised IP5 beads are distinctly different from the blank beads and are specifically binding to and pulling down GRP. This not only acts as further validation that the amino-IP5 compound is indeed tethered to the beads, but also suggests that the inositol ring is on the surface of the bead and enables binding to the protein. Another possible purification procedure would be to elute the proteins from the IP5-affinity beads with free IP5. In the absence of ligand elution, we have validated the IP5 binding using an IP5 biosensor chip (Figure 2,B).

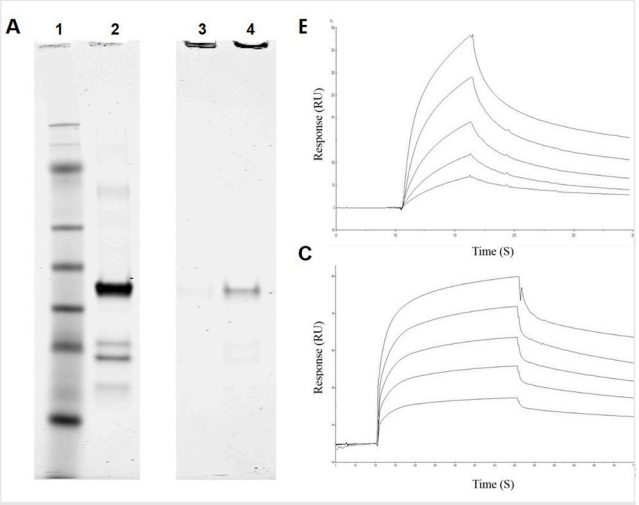


Figure 2 (A) Gel electrophoresis (SDS-PAGE) display of the products from the pull-down of GST-GRP-PH domain to validate IP5 beads using MES buffer. Lanes: (1) marker, (2) crude GST-GRP-PH domain, (3) pull-down with blank beads, (4) pull-down with IP5 beads. (B) Biosensor analysis of an IP5-derivatised biosensor chip with GRP-PH domain at varying concentrations (2.5 μM, 1.25 μM, 625 nM, 312 nM and 156 nm); 1:1 Langmuir analysis gives an approximate KD of 520 nM. (C) Biosensor analysis of an IP5-derivatised biosensor chip with AKT-2-PH domain at varying concentrations (2.5 μM, 1.25 μM, 625 nM, 312 nM and 156 nm); 1:1 Langmuir analysis gives an approximate KD of 80 nM

To further validate the amino-IP5 compound, and to develop a method for analysing the specificity and affinity of IP5 for any proteins in proteomics experiments, the IP5 compound was attached to a chip for biosensor studies. The amino-IP5 compound was coupled with an *N*-hydroxysuccinimide (NHS) activated biotin reporter group and purified by anion exchange column chromatography using a linear gradient of ammonium bicarbonate (0–2 M). The purified biotinylated IP5 was then immobilised directly onto a neutravidin-derivatised sensor surface.[27] Injection of varying concentrations of GRP (2.5 μM, 1.25 μM, 625 nM, 312 nM and 156 nM) across the immobilised IP5 chip, with a neutravidin channel as a control, provided the binding curves shown in Figure 2,B.

This kinetic analysis determined that the dissociation constant (KD) of GRP for the IP5 compound was approximately 520 nM. In addition the same studies for another known IP5 binding protein, AKT, were performed and gave a dissociation constant of around 80 nM (Figure 2,C), being comparable to literature values.[28]

In conclusion, a cross-disciplinary study using organic synthesis as an important tool to explore the biological significance of IP5 analogues has been performed. A novel tethered IP5 derivative was successfully synthesised containing a terminal amino group on the tether to allow immobilisation onto solid supports to form IP5 chemical probes for biological evaluation. The synthesis was achieved from *myo*-inositol via a number of synthetic steps including regioselective protection and deprotection, phosphite coupling (and oxidation), followed by hydrogenolytic global deprotection chemistry. The use of an orthoformate group in the key intermediate **4** provides an opportunity to differentiate the six positions on the inositol ring and can also be used in a similar fashion for the synthesis of other members of the inositol phosphate family.

The amino-IP5 compound was successfully conjugated to magnetic Dynabeads to form a probe for pull-down experiments. Its ability to bind to proteins of interest was validated with a known binding protein. The specific binding of purified GRP and AKT with IP5 was confirmed using a biosensor technique which also validated the capacity of the IP5 probe. These initial results provide an excellent platform for further biological study.

Acknowledgment

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Supporting Information

Supporting information for this article is available online at http://dx.doi.org/10.1055/s-0035-1560381.

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(25) **5-*O*-Amino Ethoxyethyl-phosphate-*myo*-inositol-1,3,4,6-tetraphosphate (1)**
To a solution of the fully protected derivative **16** (10 mg, 72 µmol) dissolved in MeOH–THF (2:3, 2 mL) was added $Pd(OH)_2$ (10 mg). The mixture was reacted under H_2 (10 bar pressure, in a Buchi hydrogenation vessel) and was allowed to stir vigorously at r.t. for 48 h. The mixture was filtered twice through Celite and the filtrate was lyophilised to give the desired product **1** (5 mg, quant.) as a white powder.
IR (neat): 3500–3000 (br), 1579, 1409, 1353, 1087, 973 cm^{-1}. ^1H NMR (500 MHz, D_2O): δ = 4.60–4.40 (m, 5 H), 4.25 (br s, 1 H), 4.14 (br s, 2 H), 3.88–3.81 (m, 4 H), 3.76–3.64 (m, 2 H), 3.14 (br s, 2 H). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 76.7, 76.2, 75.5, 73.0, 71.6, 69.9, 66.3, 66.0, 60.4, 39.2. ^{31}P NMR (202 MHz, $CDCl_3$): δ = 4.22 (2 P), 3.01 (2 P), 0.44 (1 P). HRMS (ESI): *m*/*z* [M – H]$^-$ calcd for $C_{10}H_{25}NO_{22}P_5$: 665.9562; found: 665.9587; *m*/*z* [M – 2 H]$^{2-}$ calcd for $C_{10}H_{24}NO_{22}P_5$: 332.4745; found: 332.4755.
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