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Synthesis and Molecular Recognition of Phosphatidylinositol-3-methylenephosphate

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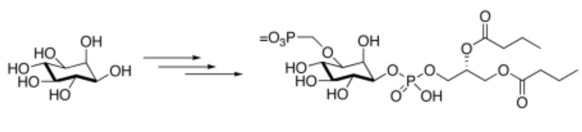
Abstract



Phosphatidylinositol-3-phosphate (PtdIns(3)P) is spatial regulator of vesicular trafficking and other vital cellular processes. We describe the asymmetric total synthesis of a metabolically stabilized analogue, phosphatidylinositol-3-methylenephosphate (PtdIns(3)MP) from a differentially-protected *myo*-inositol. NMR studies of PtdIns(3)MP bound to the ^{15}N-labelled FYVE domain showed significant ^{1}H and ^{15}N chemical shift changes relative to the unliganded protein.

Phosphoinositide (PtdInsP$_n$) signaling networks are dynamically modulated by proteins with lipid recognition motifs as well as kinase, phosphatase, and phospholipase enzymatic activities. In particular, the 3-phosphorylated PtdInsP$_n$ lipids have been implicated as activators of protein kinase C isoforms, and are messengers in cellular signal cascades pertinent to inflammation, cell proliferation, transformation, protein kinesis, and cytoskeletal assembly.[1–3] PtdIns(3)P is produced by the action of phosphoinositide-3-kinase (PI 3-K)[1,4] on PtdIns, and its interactions with cognate binding proteins, kinase and phosphatases are important in cell physiology. PtdIns (3)P specifically binds FYVE domains[5–7] and is involved in phagocytosis,[8] membrane trafficking and protein sorting.[9] PX domains also recognize PtdIns(3)P, and spatiotemporal changes mediate important aspects of cell respiration.[10] The myotubularin-related (MTMR) protein family[11] is comprised of PtdIns(3)P phosphatases that contribute to lipid remodeling and are mutated in genetic diseases.[12]

To gain deeper insights into these biological pathways, selective reagents that can interfere with ligand binding, inhibit enzyme activity, and activate protein mediated-lipid signaling are needed.[13] We recently described a general approach to the synthesis of methylphosphonate, (monofluoromethyl)phosphonate, and phosphorothioate analogues of PtdIns(3)P.[14] These metabolically-stabilized ligands were recognized by ^{15}N-labeled FYVE and PX domains, and were also substrates for PIKfyve, a 5-kinase required for the formation of multivesicular bodies. We now introduce a modified synthetic route that provides access to a stabilized

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methylenephosphonate analogue, PtdIns(3)MP, which retains the inositol 3-oxygen as well as the dianionic head group. In this modification, a methylene bridge was inserted between the oxygen of the inositol moiety and the phosphate head group. An similar approach was also used to generate alkoxymethylene phosphonate containing geranylgeranyl protein transferase inhibitors[15] and antiviral drugs,[16] including anti-HIV phosphorylated nucleoside analogues.[17,18] We have also used this approach to synthesize potent analogues of lysophosphatidic acid and phosphatidic acid, and these results will be presented in due course. Herein we describe the asymmetric total synthesis of the methylenephosphonate analogue of PtdIns(3)P, and we illustrate the binding of PtdIns(3)MP to the FYVE domain.

The synthetic strategy employed the simple and elegant protection scheme of Bruzik,[19] in which the 1-position of *myo*-inositol (**1**) was silylated with the TBDPS group, the phosphomonoester 3-positon was protected as a benzoate group, and all remaining hydroxyl groups were protected as methoxymethyl (MOM)-ethers. Thus, 1-*O*-(*tert*-butyl-diphenylsilyl)-2,4,5,6-*O*-*tetrakis*-(methoxymethylene)-*myo*-inositol (**2**) was synthesized from *myo*-inositol in six steps. Installation of the methylenephosphonate moiety required the preparation (Scheme 1) of dimethyl phosphonomethyltriflate (**5**) following the literature route.[20,21] Reaction of paraformaldehyde with dimethyl phosphite gave hydroxymethyl phosphonate **4**, which was converted to triflate **5** using 2,6-lutidine as the base, and was employed without further purification.

The alkoxide of protected inositide **2** (*n*-BuLi[15], −78°C) was alkylated with triflate **5** in 64% yield (Scheme 2). Use of NaH or *t*-BuOK as bases did not significantly improve the yield and resulted in greater decomposition of the starting material. The TBDPS group was removed by treating intermediate **6** with t-$BuNH_4F{\cdot}H_2O$. The resulting alcohol **7** was coupled with the di-butanoylglyceryl phosphoramidite[14] in the presence of 1*H*-tetrazole, followed by mild oxidation with n-$BuNIO_4$[14] to give fully protected PtdIns(3)MP (**8**). The removal of phosphate and hydroxyl protecting groups of **8** was accomplished under strictly anhydrous conditions with 20 eq of fresh TMSBr (CH_2Cl_2, rt, 1 h). After concentration *in vacuo*, the residue was dissolved in a 90% aq. CH_3OH and stirred for 40 min to hydrolyze the silyl phosphate esters. We found that under these conditions, not only were the phosphates deprotected, but all MOM groups were also removed. After complete evaporation of the organic solvent *in vacuo*, the crude compound was dissolved in water and passed through a short column of acidic Dowex ion-exchange resin to yield final product in >98% purity.

In endosomal membranes, PtdIns(3)P is specifically recognized by a number of protein binding partners including FYVE and PX domains. We next investigated the interactions of human EEA1 FYVE and yeast Vam7 PX domains with PtdIns(3)MP by NMR spectroscopy. Significant changes were observed in ^1H and ^{15}N resonances in the FYVE domain when titrating in dibutanoyl PtdIns(3)MP (**9**) (Figure 1a). These perturbations were of reduced magnitude, but paralleled the chemical shift changes apparent in the complex of the FYVE domain with dibutanoyl-PtdIns(3)P (Figure 1b). Thus, the PtdIns(3)MP analogue and native lipid are accommodated by the same binding pocket consisting of four Arg and two His residues of the FYVE domain. Based on ^1H and ^{15}N chemical shift changes the FYVE domain affinity for PtdIns(3)MP was calculated to be 3.8 ± 0.5 mM (see Supplementary Figure 2 in Supporting Information). To put this in perspective, the local concentration of PtdIns(3)MP in early endosomal membranes is quite high (~ 200 μM)[22] and the FYVE – dibutanoyl-PtdIns(3)P affinity is 135 μM under similar experimental conditions.[23] A similar experiment with the PX domain showed a much weaker binding without significant chemical shift changes. Addition of up to 11.3 mM (57-fold excess) of PtdIns(3)MP to a 0.2 mM PX domain sample induced no noticeable resonance changes. For comparison, the K_d of the PX domain for dibutanoyl-PtdIns(3)P under these conditions is approximately 300 μM (M. Cheever, T. Kutateladze, M. Overduin, unpublished results). This result may be attributed to the difference in the binding modes for the two complexes. PtdIns(3)P inserts its 3-phosphate between two loops and an a-helix of the PX domain.[24] However, in the case of the FYVE domain, PtdIns(3)P occupies a shallow pocket in a side-on orientation.[25] Apparently the extended methylenephosphonate group is too bulky to be accommodated in the PX domain binding pocket. Thus, PtdIns(3)MP is the first analogue of PtdIns(3)P that shows discrimination in its protein-ligand interactions, suggesting that it could be useful in selectively perturbing distinct PtdIns(3)P signaling pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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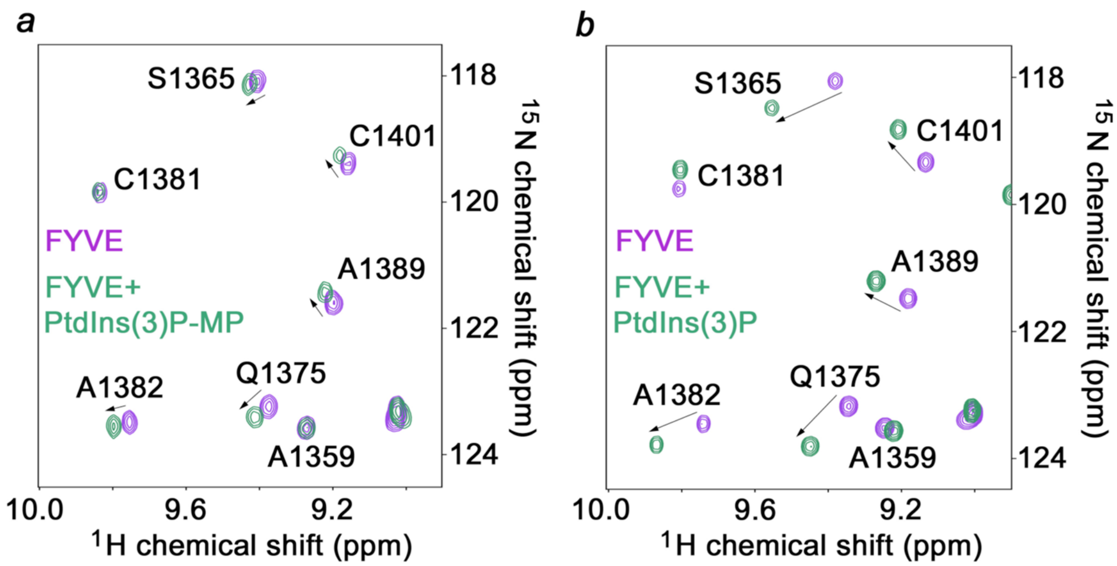
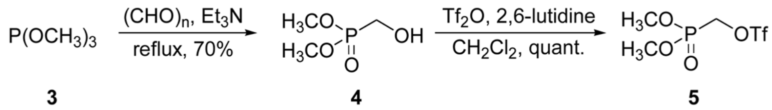


Figure 1.
Binding of PtdIns(3)P and PtdIns(3)MP to the FYVE domain. ^{1}H-^{15}N Heteronuclear single quantum coherence (HSQC) NMR spectra of 0.2 mM EEA1 FYVE domain before and after addition of (**a**) dibutanoyl-PtdIns(3)MP (**9**) and (**b**) dibutanoyl-PtdIns(3)P.



Scheme 1.
Synthesis of dimethyl phosphonomethyltriflate (**5**).

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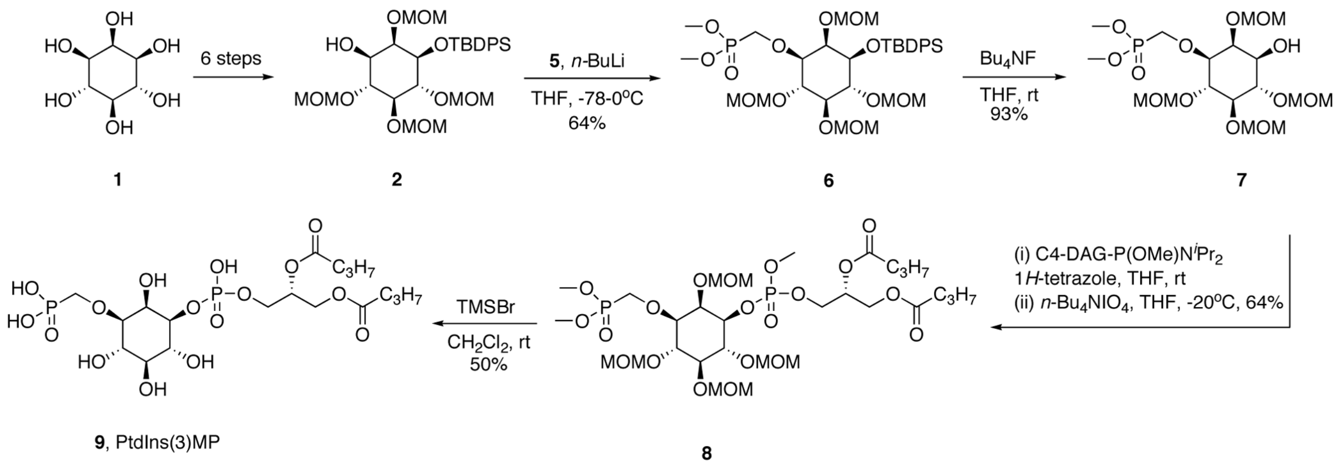

Scheme 2.
Synthesis of PtdIns(3)MP (**9**).