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Synthesis of phosphatase-resistant analogues of phytic acid ($InsP_6$)

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Abstract—Phytic acid is enzymatically catabolized to numerous inositol polyphosphates and subsequently to *myo*-inositol and inorganic phosphate. These conversions are important in energy metabolism, metabolic regulation, and signal transduction pathways. A concise synthesis of phosphatase-resistant analogues of phytic acid ($InsP_6$) is described in which the P-1 phosphate has been replaced by phosphorothioate, phosphoroselenate, or methylphosphonate moieties. In addition, a new synthesis of P-1 tethered aminoalkyl $InsP_6$ and the corresponding affinity resin are described.

Phytic acid is the major storage form of phosphorus in cereals, legumes, and oilseeds. It serves several physiological functions and also significantly influences the functional and nutritional properties of cereals, legumes, and oilseeds by forming complexes with proteins and minerals.[1] The chemical description of phytic acid is *myo*-inositol 1,2,3,4,5,6-hexakis-dihydrogen phosphate, or Ins(1,2,3,4,5,6)P_6 (also abbreviated as $InsP_6$ or IP_6). Phytic acid is also found in animal cells. However, the primary function of these compounds in animal cells is not to serve as a storage form of phosphorus or *myo*-inositol. Rather, their major role is in transmembrane signaling and mobilization of calcium from intracellular reserves. Similarly, in plant cells, $InsP_6$ regulates a Ca^{2+} ion channel in guard cells.[2] Therefore, these *myo*-inositol phosphates can be used as enzyme substrates for metabolic investigation or as enzyme inhibitors and therefore potentially as drugs.[3] Phytic acid has been shown to exert an antineoplastic effect in animal models of both colon and breast carcinomas.[4] The presence of undigested IP_6 in the colon may protect against the development of colonic carcinoma.[1,5]

$InsP_6$ also functions as an extracellular neurotransmitter. High specific activity binding sites for $InsP_6$ have been identified in cerebellar membranes and in the anterior pituitary.[6] Moreover, $InsP_6$ is the substrate for IP6K enzymes,[7] which generate the high-energy diphosphate-containing $InsP_7$ and $InsP_8$ species that have been implicated in signaling and in protein phosphorylation.[8–10] The complexity of the signal transduction mechanism has triggered renewed interest in the chemical synthesis of $InsP_6$ and its new analogues. In order to gain further insight into the biological pathways, the availability of *myo*-inositol phosphates in which a particular phosphate was replaced with a phosphatase-resistant moiety would be highly advantageous. Phytase is acid phosphatase that efficiently and selectively cleaves the ester bond of phytate (*myo*-inositol hexakisphosphate) to *myo*-$InsP_n$ ($n = 0$–5) and inorganic phosphate.[11] Phytases belong to the family of histidine acid phosphatases, a subclass of phosphatases, and all family members involve in the formation of a phosphohistidine intermediate during the phosphoryl transfer reaction.[12] Since phytases from different sources exhibit a variety of regioselective phosphatase activities, we elected to modify one particular site, the P-1 position, to generate a metabolically stabilized phosphatase-resistant analog of $InsP_6$.

Phosphorothioate[13–15] and methylphosphonate[16,17] analogues of inositol phosphates ($InsP_n$) and phosphorylated phosphatidylinositols ($PtdInsP_n$) analogues have been synthesized and used as probes of cellular signal transduction. Although phosphoroselenoate analogues of oligonucleotides have been prepared and investigated widely,[18,19] the corresponding $InsP_n$ and $PtdInsP_n$ analogues have not yet been reported. As part of our ongoing program to prepare metabolically stabilized $InsP_n$

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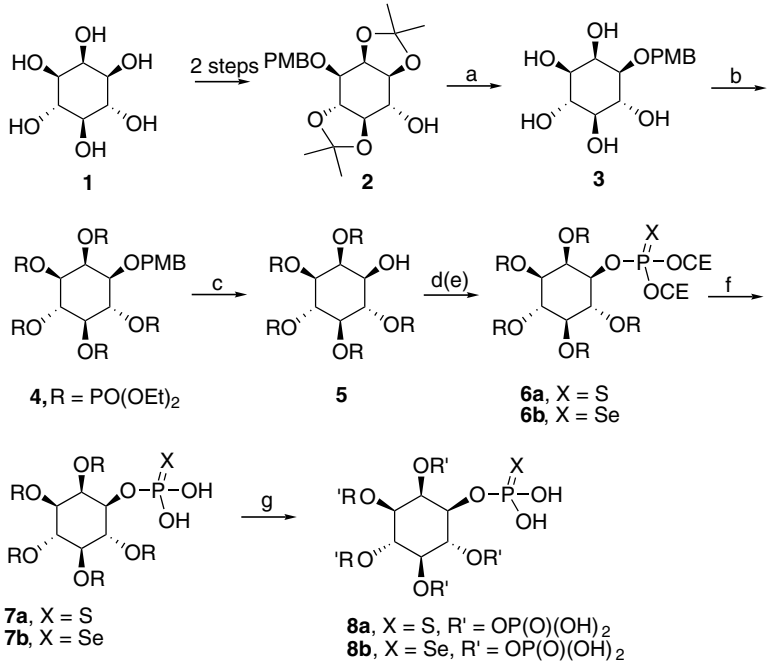
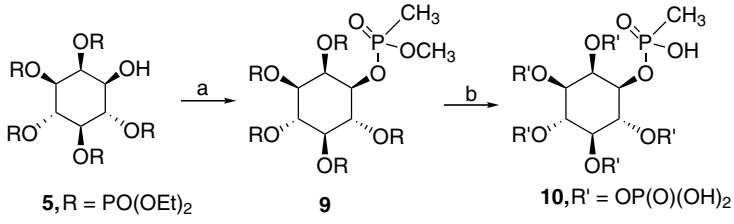
and PtdInsP_n analogues, we present herein the synthesis of the P-1 phosphorothioate(selenoate) and methylphosphonate analogues of InsP_6 as phytase-resistant analogues.

A suitably protected derivative of *myo*-inositol was required for the synthesis of phosphorothioate(selenoate) and methylphosphonate InsP_6 analogues. The starting material, D,L-1,2;4,5-bis-*O*-isopropylidene-3-(*p*-methoxybenzyl)-*myo*-inositol **2**, was obtained by a known procedure from *myo*-inositol.[20–22] Hydrolysis of the acetone ketal in 10% *p*-TsOH-methanol overnight gave the racemic 1(3)-PMB protected inositol **3**.[22] Phosphitylation of the pentaol **3** with diethyl chlorophosphite in the presence of diisopropylethylamine (DIPEA), followed by oxidation with hydrogen peroxide, gave the protected inositol pentakisphosphate **4** in 63% yield.[23] Initial attempts at phosphorylation using diethyl(methyl) chlorophosphate catalyzed by various bases were unsuccessful. The failure may arise from the steric effect of the five protected phosphate groups. Moreover, we could not employ benzyl phosphate as protecting groups because hydrogenolytic removal would be incompatible with the phosphorothioate or phosphoroselenoate groups (Schemes 1 and 2).

The 1-position PMB protective group was removed by $(NH_4)_2Ce(NO_3)_6$ in wet acetonitrile.[22] The cyanoethyl ester protective group was employed to introduce phosphorothioate and phosphoroselenoate groups at the 1-position. Alkyl esters of phosphorothioate are extremely stable compared to phosphate esters. Although the commonly used reagents TMSBr or TMSI can remove alkyl esters in phosphates, this reaction fails with phosphorothioates.[24] Therefore, cyanoethyl ester (CE) groups were selected for the phosphorothioate and phosphoroselenoate syntheses, as these protecting groups could be readily removed under mild basic conditions. Thus, the 1-hydroxyl inositol **5** was phosphorylated with di(2-cyanoethyl)phosphorochloridite[25] in the presence of DIPEA, and followed by oxidation with elemental sulfur in pyridine provided phosphorothioate **6a**.[26] Correspondingly, oxidation with KSeCN afforded phosphoroselenoate **6b** in good yield.[27] The CE groups were removed prior to deprotection of ethyl esters of phosphate using *tert*-butylamine in the presence



Scheme 1. Synthesis of phosphorothioate and phosphoroselenoate analogues of InsP_6. Reagents and conditions: (a) *p*-TsOH, CH_3OH, 90%; (b) $ClP(OEt)_2$, DIPEA; then H_2O_2, 63%; (c) $(NH_4)_2Ce(NO_3)_6$, CH_3CN, H_2O, 84%; (d) $ClP(OC_2H_4CN)_2$, DIPEA; then S_8, Pyridine, 90%; (e) $ClP(OC_2H_4CN)_2$, DIPEA; then KSeCN, 90%; (f) *t*-$BuNH_2$, BSA, CH_3CN, 87%; (g) TMSBr, CH_3CN; then CH_3OH/H_2O, 100%.



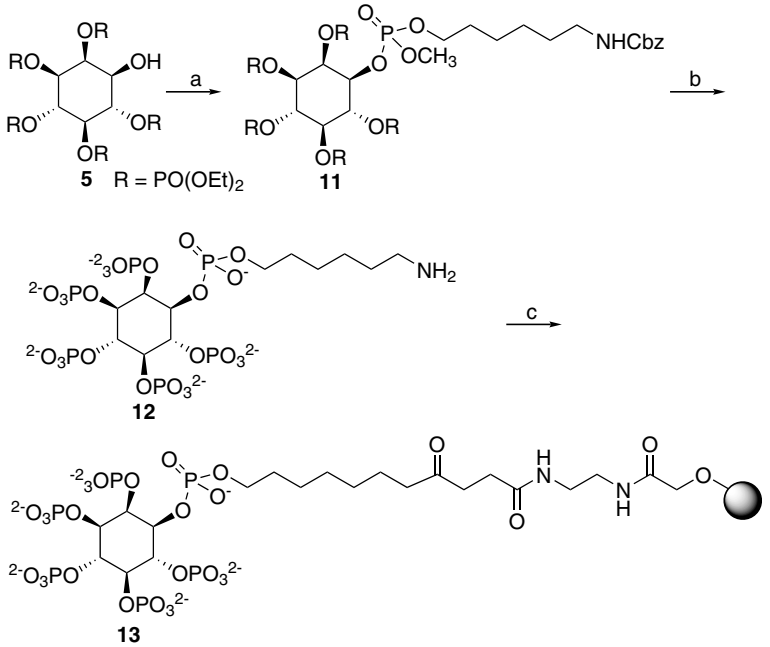
Scheme 2. Synthesis of methylphosphonate analogue of InsP_6. Reagents and conditions: (a) $CH_3P(O)(OCH_3)Cl$, triethylamine, CH_2Cl_2, 56%; (b) TMSBr, CH_3CN; CH_3OH/H_2O, 93%.

of bistrimethylsilane acetamide (BSA). The presence of BSA is essential to completely remove all of the CE groups, since this reaction is reversible and the reaction is driven to completion by trapping the silyl ethers. Finally, the TMSBr removed all the ethyl ester groups and afforded the final racemic 1-phosphorothioate and 1-phosphoroselenoate $InsP_6$ analogues.

The phosphonochloridates derived from simple phosphonic acid alkyl esters are reliable and accessible reagents for the phosphonylation of alcohols to prepare phosphonates.[28,29] The methyl phosphonyl chloride was readily prepared from dimethyl methylphosphonate by chlorination with PCl_5 at rt.[30] The secondary alcohol **5** was phosphonylated with methyl methylphosphonyl chloride in the presence of triethylamine to give good yield (56%) of the protected 1-methylphosphonate. Among several bases examined, triethylamine gave the best results. TMSBr successfully deprotected the fully protected inositol **9** in one step, and hydrolysis provided the final product **10**.[39]

We have reported previously the preparation of a P-1 tethered aminoalkyl $InsP_6$ derivative for use in purification and affinity labeling of $InsP_6$ binding proteins and IP6K.[6,22,31] We now report a revised route for the synthesis of P-1 tethered $InsP_6$ derivatives. Immobilized $InsP_6$ was prepared by attaching a terminal alkyl amino function at the 1-phosphate moiety to Affi-Gel 10, an active ester functionalized agarose bead. The starting material for this synthesis is 2,3,4,5,6-penta-*O*-diethylphosphate-*myo*-inositol **5**. Using phosphoroamidite chemistry (Scheme 3), *N*-Cbz-protected aminoalkyl methyl (*N*,*N*-diisopropylamino)phosphate was prepared from methoxyl (*N*,*N*-diisopropylamino)chlorophosphine and *N*-Cbz-6-aminohexanol.[22,32] The reagent was used to phosphitylate the pentaphosphate intermediate **5**, promoted by 1-*H*-tetrazole. Oxidation of the intermediate phosphites to the phosphates was accomplished in the presence of the *N*-Cbz group by using a slight excess of *m*-chloroperbenzoic acid at −40 °C and then at 0 °C. The remaining oxidizing reagent was destroyed by addition of aqueous sodium sulfite to the cold solution.

Removal of the ethyl protecting groups was accomplished using TMSBr.[33,34] The most frequently used method for cleavage of Cbz group is catalytic hydrogenation.[35] There are some examples in which TMSBr can also deprotect Cbz groups in the presence of trifluoroacetic acid and thioanisole at rt.[36,37] In this case, we found that neat TMSBr deprotected the Cbz group and ethyl phosphates simultaneously. The fully deprotected tethered IP_6 analogue was thus obtained quantitatively by using neat TMSBr overnight, followed by hydrolysis. We have observed in other reactions that TMSBr can remove the Cbz group without the addition of a protic acid; this may be due to increased electrophilicity of the solvent-free TMSBr in the reaction mixture. This simultaneous deprotection avoids hydrogenolysis, which requires use of heavy metal catalysts. Since $InsP_6$ strongly binds many mono-, di-, and trivalent metal cations forming insoluble complexes,[38] this avoids metal ion contamination of the final product. Therefore, the TMSBr deprotection of the Cbz group in analogues of $InsP_6$ provides a new and efficient route for preparing tethered $InsP_6$ analogues. As reported, the tethered $InsP_6$ amine **12** was coupled to the *N*-hydroxysuccinimide (NHS) activated ester resin, Affi-Gel 10, to afford the corresponding affinity matrix.[31] The $InsP_6$ modified matrix **13** was prepared in water using excess $NaHCO_3$, by reacting the activated Affi-Gel 10 resin with the ω-amino-substituted $InsP_6$ analogue at 4 °C overnight.



Scheme 3. Synthesis of P-1 tethered aminoalkyl $InsP_6$ and the corresponding affinity resin. Reagents and conditions: (a) $CH_3OP(i\text{-}Pr_2N)(OC_6H_{12}NHCbz)$, 1*H*-tetrazole, CH_2Cl_2; then MCPBA, 56%; (b) TMSBr, CH_3CN; then CH_3OH/H_2O; (c) $NaHCO_3$, H_2O, Affi-Gel 10 beads.

In summary, we have developed a novel and efficient approach to the synthesis of 1-phosphorothioate and phosphoroselenoate-$InsP_5$ and 1-methylphosphonate-$InsP_5$ as phosphatase-resistant analogues of $InsP_6$. This strategy was also employed in an improved route to P-1 tethered IP_6. The metabolically stabilized $InsP_6$ compounds reported herein now allow examination of their roles in cell signaling, and biological applications of these analogues will be presented in due course.

Acknowledgments

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39. Typical procedure: 1-(Methylphosphonate)-1,2,4,5,6-pentakisphosphate-*myo*-inositol, **10**. The phosphate **9** (40 mg, 0.042 mmol) in 5 mL flask was dried in vacuo. Anhydrous TMS bromide (1.0 mL) and CH_3CN (0.5 mL) were added into the flask. The solution was stirred at rt for 1 day. TMS bromide and volatile products were evaporated under high vacuum during 6 h. The residue was dissolved in $MeOH/H_2O$ (95%, 1.0 mL) and stirred for 30 min at rt. The solution was thoroughly concentrated for an additional 3 h under high vacuum. The crude product was purified on anion-exchange column (Q-Sepharose Fast Flow) and eluted with 0.01–1.0 M ammonium acetate solution. The appropriate fractions were combined and adjusted the pH 8.0 by diluted ammonium hydroxide, and then freeze-dried to provide the pure product as a white ammonium salt (31 mg, 0.037 mmol, 88%). ^1H NMR (CD_3OD): δ 4.55 (m, 1H), 4.18 (m, 4H), 4.16 (m, 1H), 1.34 (d, $J = 16.8$ Hz, 3H), 2.10–2.22 (m, 2H), 2.00 (m, 1H), 1.80 (m, 1H). ^{31}P NMR (CD_3OD): δ 29.69 (s, 1P), 5.45 (s, 1P), 4.69 (s, 3P), 4.36 (s, 1P). MS (ESI) *m/z* 680.9 (M^++Na, $C_7H_{20}NaO_{23}P_6$).