

Tethered phytic acid as a probe for measuring phytase activity

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Abstract—A novel approach for measuring phytase activity is presented. We have developed a new chromophoric substrate analog of phytic acid, 5-*O*-[6-(benzoylamino)hexyl]-*D*-*myo*-inositol-1,2,3,4,6-pentakisphosphate that permits direct measurement of the phosphate ester bond-cleavage reaction using HPLC. This compound, along with its dephosphorylated T-phosphatidylinositol intermediates, are quantified using reversed phase chromatography with UV detection.
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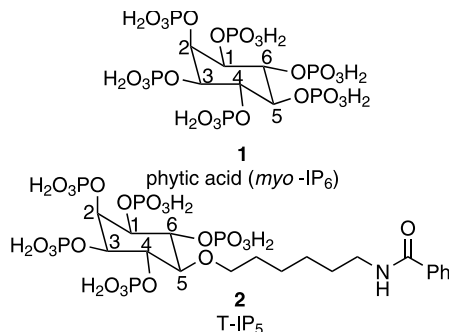
The majority of phosphorous (P) in farm animal feed grains is present as a mixed salt of *myo*-inositol hexakisphosphate,¹ more commonly referred to as phytic acid (*myo*-IP₆ (**1**)). Because grain-consuming swine and poultry do not produce phytase, *myo*-IP₆ (**1**) is largely unavailable as a phosphorus-containing nutrient source and thus excreted in waste in high concentrations.^{1,2} Such manures are applied to cropland to enhance soil fertility or as a means of disposal. Little information exists regarding the process of how *myo*-IP₆ (**1**) is transformed into a crop-available nutrient. Recent experimental evidence suggests that bacterial phytase plays an important role in these (bio)chemical transformations.³

Phytase assays conducted on bacterial cell wall-free lysate or whole cell lysate routinely call for addition of

myo-IP₆ (**1**) to buffered cell lysate and subsequent measurement of the released orthophosphate (ortho-P) by colorimetric analysis.^{4,5}

While conducting similar experiments, we have observed a substantial ortho-P release from bacterial cell lysate resulting from 'cell free' phosphatase-mediated hydrolysis (e.g., exopolyphosphatase, non-specific acid phosphatases) of cell-associated phosphate compounds including accumulated (intracellular) polyphosphate. Interestingly, bacteria, archaea and fungi are all capable of producing polyphosphate.^{6,7} It would appear that the ortho-P release assay is not a specific measure of phytase activity in lysed cell preparations and its use under these conditions could result in either an exaggerated estimate of phytase activity or possibly even a false positive test result.

Phytases catalyze the sequential hydrolysis of *myo*-IP₆ (**1**), forming ortho-P and a series of partially dephosphorylated phosphoric esters of *myo*-inositol.⁸ In some cases, hydrolysis may go to completion yielding the parent compound *myo*-inositol.⁸ Based on biochemical properties and amino acid sequence alignment, Oh et al.⁹ have categorized phytases into two major classes, the histidine acid phytases (containing the PhyA, PhyB, and PhyC groups), to which most of the bacterial and fungal phytases belong, and the alkaline phytases (PhyD). The phytate-degrading enzyme, 3-phytase (*myo*-inositol hexakisphosphate 3-phosphohydrolase, EC 3.1.3.8; PhyA and PhyB groups) hydrolyzes *myo*-IP₆ (**1**) preferentially at the C-3 position, while 6-phytase

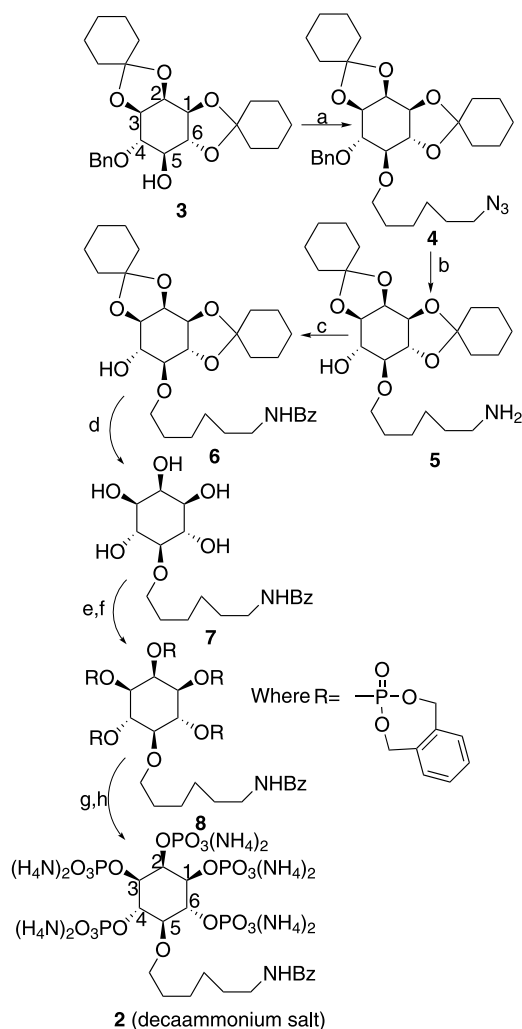


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(*myo*-inositol hexakisphosphate 6-phosphohydrolase, EC 3.1.3.26; PhyC) hydrolyzes *myo*-IP₆ (**1**) preferentially at the C-6 position.⁸

Our overall goal is to develop a specific and sensitive quantitative enzyme assay capable of measuring phytase activity in either cell culture filtrate, cell-lysate preparations, or soils using HPLC with UV detection. Development of a method for determining soil phytase activity is critical to understanding the fate of *myo*-IP₆ (**1**) in soil and water–sediment environments. As pointed out recently by Turner et al.,¹⁰ ‘... there has been little research on phytase in soils and waters because no artificial substrates are presently available that can be used for convenient measurement of activity’. We now report the synthesis of 5-*O*-[6-(benzoylamino)hexyl]-*D*-*myo*-inositol-1,2,3,4,6-pentakisphosphate, i.e. T-IP₅ (**2**) (Scheme 1) where T represents the tethered chromophore, for use in the development of a phytase assay. We chose 4-*O*-benzyl-1,6:2,3-di-*O*-cyclohexylidene-*myo*-inositol (**3**) as the starting point for our synthesis since it is a convenient intermediate that allows for ether derivatization at the 5-position (phytic acid numbering).¹¹ An ether linkage would be hydrolytically stable and resistant to phosphatase activity. We also felt that derivatization at the 5-position would be desirable for two reasons. First, it positions the linker ‘*meta*’ to the initial site of reaction, the 3-position, which should minimize the chances that the linker (plus chromophore) would interfere with the preferential active site of a 3-phytase. Second, the placement of the linker in the meso-plane (the 5-position) simplifies the stereochemical issues.

Following the literature procedure,¹² we were able to prepare 100 g of the pure isomer **3** with flash chromatography followed by recrystallization. The protected *myo*-inositol **3** was alkylated at the open 5-position using the mesylate of 6-azidohexan-1-ol¹³ and sodium hydride in DMF. It was found that moderate heat was necessary to ensure a complete reaction. The resinous product **4** was isolated in 55% yield. Simple hydrogenation of this material over 10% Pd/C reduced the azide to the amine with concomitant removal of the benzyl group at the 6-position affording a 55% yield of **5**. Selective benzoylation of the amino group in **5** was accomplished with benzoyl cyanide¹⁴ in dichloromethane giving a 77% yield of **6**. When **6** was heated in an acetic acid/water mixture at 100 °C for 45 min, the 1,6:2,3-cyclohexylidene groups were selectively removed, giving the 5-*O*-derivatized *myo*-inositol **7** in 94% yield. Initial attempts to multiphosphorylate **7** by reacting the lithium salt of **7** with tetrabenzyl pyrophosphate^{15,16} were unsuccessful. It has been reported that multi-phosphorylation of inositol derivatives can be problematic.^{17,18} Recent procedures using phosphoramidites for multi-phosphorylation of inositol derivatives offered alternatives.^{19–21} Through the reaction of **7** with *N,N*-diethyl-1,5-dihydro-2,4,3-benzodioxaphosphepin-3-amine²² in the presence of tetrazole, followed by oxidation with *m*-CPBA, we were able to prepare **8**, in 88% yield. The ³¹P NMR of **8** showed three singlets in a 2:1:2 ratio, consistent with a molecule containing a meso-plane and equilibration of



Scheme 1. Synthesis of T-IP₅ (**2**). Reagents and conditions: (a) MsO(CH₂)₆N₃ (2.5 equiv), NaH (2 equiv), DMF, 55 °C, 16 h (97%); (b) 10% Pd/C, THF–MeOH (1:1), H₂, 50 psi, 8 h (55%); (c) BzCN (1.1 equiv), CH₂Cl₂, 16 h, rt (76.6%); (d) HOAc, H₂O, 100 °C, 24 h (94%); (e) *N,N*-diethyl-1,5-dihydro-2,4,3-benzodioxaphosphepin-3-amine (7 equiv), tetrazole (10.5 equiv), MeCN, 24 h; (f) 11 equiv MCPBA, –50 to 25 °C (88%, two steps); (g) 5% Pd/C, MeOH, THF, H₂O, H₂, 50 psi; (h) 10 equiv NH₃ (95.8%, two steps).

chair conformations. These results are in contrast to those reported for 2-*O*-(6-carbobenzoyloxyamino)hexyl-perbenzyl phytate (with the linker esterified to the phosphate group at the C-2 position) where the ³¹P NMR indicated that the cyclohexane ring was twisted, reportedly due to steric crowding, and each non-equivalent phosphorous gave rise to a singlet.¹⁹ Removal of the protecting groups from **8** was accomplished by hydrogenation over 5% Pd/C, giving **2** as a penta-dihydrogen phosphate, which was then directly, without isolation, converted to the decaammonium salt **2** via treatment with ammonium hydroxide. The overall yield for these last two steps was 96%. The ³¹P NMR for **2** consisted of three singlets at 0.56, 1.19, and 3.12 ppm (integration 2:1:2), consistent with the phosphates at C-1 and C-3 being equivalent, as with the phosphates at C-4 and C-6, and with the singlet at 1.19 ppm (integration one) attributable to the C-2 meso-plane phosphate group. The ¹H NMR spectrum confirmed the structural

components, that is, linker, benzamido, and ring methines, for structure **2**. Further definitive evidence for **2** was obtained from the mass spectrum. Though a negative ion Maldi mass spectrum did give an $[M-H]^-$ ion at m/z 782, determination of an exact mass was not possible. However, the exact mass of 782.0168 (predicted 782.0183), consistent with the desired product **2** anion, was achieved by negative ion electrospray.

The 3-phytase assay consisted of 3 mL 0.2 M glucine-HCl buffer (pH 2.6) solution containing 0.27 U of *Aspergillus ficuum* 3-phytase (EC 3.1.3.8, Sigma) and 1.2 mM T-IP₅ (**2**) (ammonium salt form). Similarly, the 6-phytase assay consisted of 3 mL 0.2 M sodium acetate buffer (pH 5.2) solution containing 0.27 U of wheat 6-phytase (EC 3.1.3.26, Sigma) and 1.2 mM T-IP₅ (**2**). Control assays were set up in a similar manner but without phytase. Reactions were carried out in closed tinted reaction vessels at an incubation temperature of 37 °C (3-phytase) or 50 °C (6-phytase) with continuous stirring. Aliquot samples of the 3-phytase reaction mixture were taken ($t = 0, 10, 20, 30, 40, 150, 300$, and 1440 min) for HPLC²³ and/or ortho-P colorimetric²⁴ analysis. Aliquot samples of the 6-phytase reaction mixture were taken at $t = 0, 1, 2.5$, and 6 h for HPLC²³ analysis.

The chromatographic analysis of the products formed from the 3- and 6-phytase catalyzed reaction of T-IP₅ (**2**) was performed on an analytical HPLC system using a reversed phase column, buffered tetrabutylammonium hydroxide eluent²³ and a PEEK tubing system throughout to minimize adsorption of the T-IP₅ (**2**). Since *myo*-IP₆ (**1**) readily adsorbs to stainless steel surfaces,²³ it seemed plausible that T-IP₅ (**2**) would also. Compounds were quantified by the external standards method.

T-*myo*-inositol pentakisphosphate (T-IP₅ (**2**); $t_R = 8.5$ min) was dephosphorylated in the presence of 3-phytase as evidenced by appearance of T-*myo*-inositol phosphate intermediates (Fig. 1) and concomitant release of ortho-P (Fig. 2a). In the initial reaction, 3-phytase removed a P group, presumably from the 3-position, yielding T-*myo*-inositol tetrakisphosphate (T-IP₄). Based on hydrophobicity considerations (i.e., a decrease in the number of P groups on T-*myo*-inositol results in fewer lipophilic tetrabutylammonium counterion groups associated with the molecule), we have tentatively as-

signed T-IP₄ the retention time of 7.7 min in the HPLC chromatogram (Fig. 1). HPLC analysis of the time course experiment revealed that as T-IP₅ (**2**) and T-IP₄ concentrations diminished, a new T-phosphatidylinositol intermediate with a retention time of 5.8 min increased, reaching a maximum of 0.857 mM following 2.5 h of incubation (Figs. 1 and 2b). We have tentatively identified this intermediate as T-IP₃ based on the observed (simultaneous) decrease in the concentration of T-IP₅ (**2**) and T-IP₄ along with the concomitant release of 2.57 nM ortho-P (Fig. 2a). The loss of two P groups from T-IP₅ (**2**) (starting concentration 1.2 mM), yielding T-IP₃, would result in a release of 2.4 mM ortho-P. HPLC analysis of reaction intermediates also shows that as the concentration of T-IP₃ decreased, the amounts of two T-phosphatidylinositol intermediates, with retention times of 3.9 and 4.3 min (shoulder on 3.9 min), increased (Fig. 1). Based on the results of a recent biochemical characterization study of five fungal phytases (three isolated from *Aspergillus* species), which revealed that the P group located at (five) equatorial position of *myo*-IP₆ (**1**) was preferentially removed with a tendency for accumulation of *myo*-inositol 2-monophosphate (P-group in axial position)²⁵ along with our observations that the concentration of T-*myo*-inositol (**7**) (0.21 mM) formed (Fig. 3), and the amounts of T-IP₃ remaining (0.67 mM) and of ortho-P released (3.28 mM) after 5 h, we designated the intermediate with the retention time of 3.9 min as T-*myo*-inositol 2-monophosphate (T-IP₁). Further investigation will be required to confirm the identity of the T-phosphatidylinositol intermediates.

Accumulation of the phosphatidylinositol intermediate, T-IP₃ (Figs. 1 and 2b) indicates that removal of a phosphate group from T-IP₃ to give T-IP₂ is a rate-controlling step for the 3-phytase catalyzed conversion of T-IP₅ (**2**). Ullah and Phillippy²⁶ observed a similar dephosphorylation pattern while working with *myo*-IP₆ (**1**) and immobilized *A. ficuum* 3-phytase. In their experiments, *myo*-IP₄ tended to accumulate, indicating that dephosphorylation of *myo*-IP₄ and concomitant production of *myo*-IP₃ was a rate-controlling step. Subsequent to observing nearly complete conversion of T-IP₅ (**2**) (1.2 mM) to T-*myo*-inositol (**7**) (0.96 mM) within 24 h (Fig. 3), we did observe a slight buildup of T-IP₁ (Fig. 1). By comparison, *myo*-IP₆ (**1**) was converted to

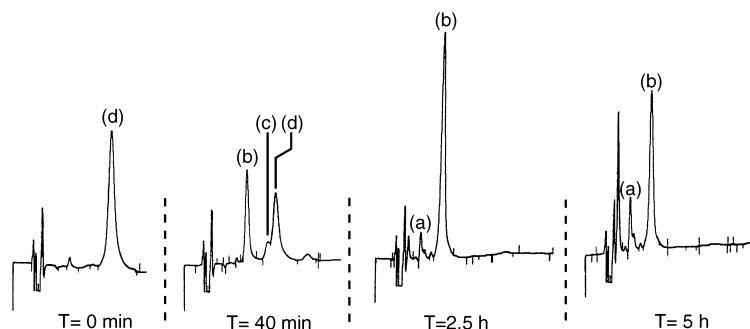


Figure 1. Separation of T-IP₅ (**2**) and the phosphatidylinositol intermediates, T-IP₄, T-IP₃, and T-IP₁, produced during the 3-phytase catalyzed dephosphorylation of T-IP₅, was achieved by reversed phase ion pair chromatography. Where (a) = T-IP₁ (t_R 3.9 min); (b) = T-IP₃ (t_R 5.8 min); (c) = T-IP₄ (t_R 7.7 min), and (d) = T-IP₅ (**2**) (t_R 8.5 min).

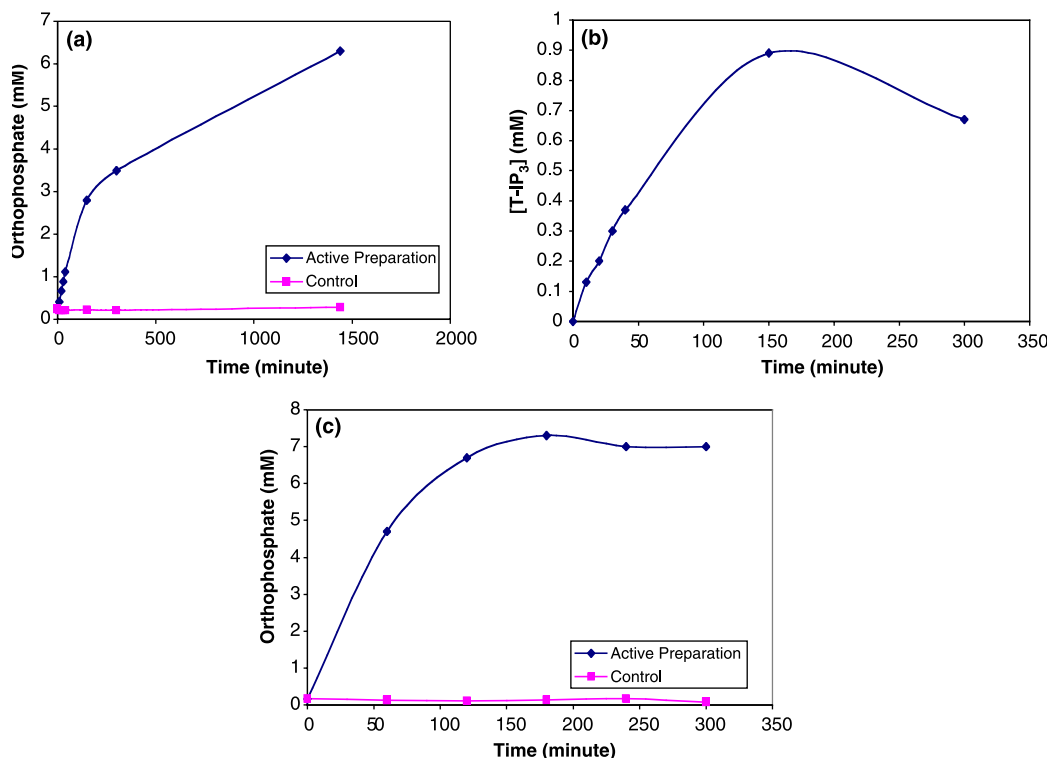


Figure 2. Dephosphorylation kinetic studies. (a) Progress curve showing release of ortho-P during the 3-phytase catalyzed dephosphorylation of T-IP₅ (2). (b) Progress curve showing accumulation of T-IP₃ subsequent to its disappearance. The concentration of T-IP₃ was determined by using T-IP₅ (2) as the standard. (c) Progress curve showing ortho-P release for 3-phytase catalyzed dephosphorylation of *myo*-IP₆ (1) (initial concentration 1.2 mM)—included for comparative purposes.

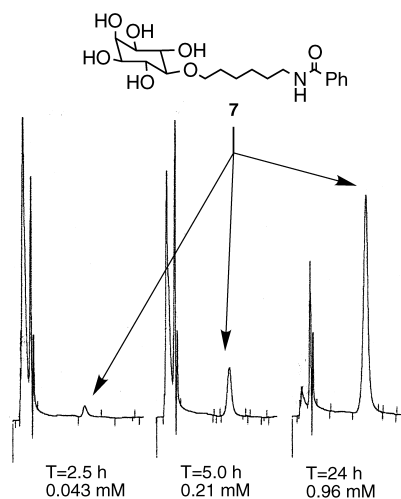


Figure 3. HPLC chromatograms illustrating the appearance of T-*myo*-inositol (7) from the 3-phytase dephosphorylation assay of T-IP₅ (2). T-*myo*-inositol was separated on a PRP-1 reversed phase column using MeOH–H₂O. The concentration of the T-*myo*-inositol (7) was determined by use of an authentic standard.

myo-inositol (based on ortho-P analysis) within 3 h (Fig. 2c). The complete conversion of T-IP₅ (2) and *myo*-IP₆ (1) to the T-*myo*-inositol and *myo*-inositol, respectively, was not anticipated because phosphohydrolases classified as 3-phytase generally do not catalyze the removal of the phosphate group from *myo*-inositol 2-monophosphate, leaving this compound as an end product.²⁵ However, Ullah and Phillippy²⁶ have reported that a 3-

phytase isolated from *A. ficuum* is capable of converting *myo*-inositol 2-monophosphate to *myo*-inositol, albeit at an extremely slow rate. It is also possible that conversion of *myo*-inositol 2-monophosphate to *myo*-inositol in our assays was catalyzed by a phosphatase (i.e., the *A. ficuum* 3-phytase preparation from Sigma may have contained some phosphatase). In addition to producing an extracellular 3-phytase, *A. ficuum* is known to produce extracellular phosphomonoesterases capable of dephosphorylating *myo*-inositol 2-monophosphate.²⁶ These phosphomonoesterases apparently do not exhibit activity toward *myo*-IP₆ (1).²⁷ Following 24 h of incubation, there was no discernable reduction in T-IP₅ (2) concentration in the control reaction as determined by HPLC analysis. Furthermore, there was no indication that the amide bond, which secures the benzamido chromophore to the linker, was hydrolyzed. In the unlikely event that the amide bond were to be cleaved during an assay, the product, benzoic acid would be readily quantifiable by HPLC. No benzoic acid was detected during the course of the reaction of the 3-phytase with T-IP₅ (2).

The T-IP₅ (2) probe can also serve as a substrate for 6-phytase (Fig. 4). Based on the dephosphorylation pattern, 6-phytase catalyzed phosphorus removal from T-IP₅ ($t_R = 11.2$ min) appears to result in the formation of T-IP₄ ($t_R = 8.6$ min) with the subsequent buildup of T-IP₃ ($t_R = 6.9$ min). In the control reaction, following 6 h of incubation, there was no discernable reduction in T-IP₅ (2) concentration as determined by HPLC anal-

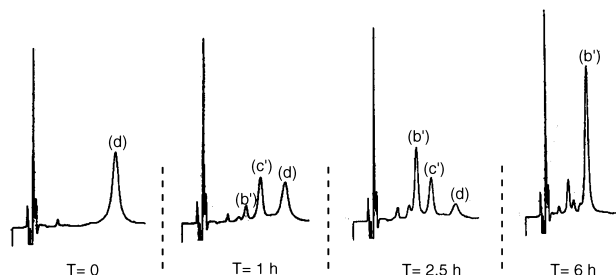


Figure 4. Separation of tethered (T)-IP₅ (**2**) and the phosphatidylinositol intermediates produced during the 6-phytase catalyzed dephosphorylation of T-IP₅ was achieved by reversed phase ion pair chromatography. Where (b') = T-IP₃ (*t_R* 6.9 min); (c') = T-IP₄ (*t_R* 8.6 min), and (d) = T-IP₅ (**2**) (*t_R* 11.2 min). Note that primes are used, since T-IP₃ and T-IP₄ may be different isomeric forms of the compounds referred to in Figure 1. The ratio of the MeOH–H₂O component of the mobile phase was 57:43.

ysis. Once again, there was no indication that the amide bond, securing the benzamido chromophore to the linker, was hydrolyzed. T-*myo*-inositol was not detected in the 6-phytase reaction mixture (data not shown).

In summary, we have synthesized a novel tethered phytic acid probe that can serve as a chromophoric substrate for phytase. Dephosphorylation of T-IP₅ (**2**) is readily quantified using reversed phase HPLC with UV detection. Detection of the T-phosphatidylinositol intermediates, T-IP₄ and T-IP₃, in an HPLC analysis of assay samples containing uncharacterized phosphohydrolases would be indicative of 3-phytase or possibly 6-phytase. It is important to realize, however, bacterial cells contain numerous phosphohydrolases with overlapping substrate specificities.²⁸ We cannot yet rule out that non-specific phosphohydrolases may be able to dephosphorylate T-IP₅ (**2**), but if this were the case, we suspect the dephosphorylation would be at a much slower rate, suggesting that reaction kinetics could also play a useful role in identifying a 3- or 6-phytase.

We are currently exploring the derivatization of T-*myo*-inositol pentakisphosphate (T-IP₅ (**2**)) with fluorescent markers (at the amine position of the linker) for probing the surface of minerals (e.g., goethite) or perhaps for use in biological studies where phytate presence has important biological implications.²⁹

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Supplementary data

Selected characterization data for compounds **4**, **6**, **7**, **8**, and **2** along with HPLC protocol. The supplementary data is available online with the paper in ScienceDirect. Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bmcl.2005.04.009](https://doi.org/10.1016/j.bmcl.2005.04.009).

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