Simple synthesis of $^{32}$P-labelled inositol hexakisphosphates for study of phosphate transformations

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Abstract

**Background and aims** In many soils inositol hexakisphosphate in its various forms is as abundant as inorganic phosphate. The organismal and geochemical processes that exchange phosphate between inositol hexakisphosphate and other pools of soil phosphate are poorly defined, as are the organisms and enzymes involved. We rationalized that simple enzymic synthesis of inositol hexakisphosphate labeled with $^{32}$P would greatly enable study of transformation of soil inositol phosphates when combined with robust HPLC separations of different inositol phosphates.

**Methods** We employed the enzyme inositol pentakisphosphate 2-kinase, IP5 2-K, to transfer phosphate from $[^{γ-32}P]$ATP to axial hydroxyl(s) of myo-, neo- and 1D-chiro-inositol phosphate substrates.

**Results** $^{32}$P-labeled inositol phosphates were separated by anion exchange HPLC with phosphate eluents. Additional HPLC methods were developed to allow facile separation of myo-, neo-, 1D-chiro- and scyllo-inositol hexakisphosphate on acid gradients.

**Conclusions** We developed enzymic approaches that allow the synthesis of labeled myo-inositol 1,$^{[32}P]$2,3,4,5,6-hexakisphosphate; neo-inositol 1,$^{[32}P]$2,3,4,$^{[32}P]$5,6–hexakisphosphate and 1D-chiro-inositol $^{[32}P]$1,2,3,4,5,$^{[32}P]$6-hexakisphosphate. Additionally, we describe HPLC separations of all inositol hexakisphosphates yet identified in soils, using a collection of soil inositol phosphates described in the seminal historic studies of Cosgrove, Tate and coworkers. Our study will enable others to perform radiotracer experiments to analyze fluxes of phosphate to/from inositol hexakisphosphates in different soils.

**Keywords** Phytate · neo- · 1D-chiro- · myo- · scyllo-inositol hexakisphosphate · Phosphate

**Abbreviations**

HEPES 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid

HPLC High pressure liquid chromatography

i.d. Internal diameter
Introduction

In consideration of the different forms of inositol hexakisphosphate identified in soils: 1D-chiro-, myo-, neo- and scyllo- ((Anderson 1964; Anderson and Malcolm 1974; Baker 1974, cited in Turner et al. 2002; Cosgrove 1962, 1963, 1966, 1969a, b; Cosgrove and Tate 1963; Halstead and Anderson 1970; L’Annunziata 1975; L’Annunziata and Fuller 1971; L’Annunziata et al. 1972; reviewed, Cosgrove 1980); Irving and Cosgrove 1982), it remains unclear what the biotic or abiotic origins of D-chiro-, neo- and scyllo-inositol phosphates are (L’Annunziata 2007; Turner and Richardson 2004; Turner et al. 2002). A limited number of studies have shown biotic contribution to the epimerization of unsubstituted inositols (Cosgrove 1969b; L’Annunziata 1975; L’Annunziata and Gonzalez 1977 (reviewed L’Annunziata 2007)), or have shown that chemical, and hence, geochemical, epimerization of myo-inositol pentakisphosphate is possible (Cosgrove 1972). It is clear from the foregoing that studies of soil phosphate transformations, particularly those arising from input of myo-inositol hexakisphosphate from plant sources, would be greatly enabled by the provision of 32P or 33P-labelled myo-inositol hexakisphosphate, and, indeed, of other inositol hexakisphosphates. Such materials would, with established extraction and separation techniques, allow facile determination of the exchange of phosphate between organic and inorganic pools and, with appropriate separation techniques, would allow study of transformations of different isomers of inositol hexakisphosphate. With labelled inositol hexakisphosphates and an increasing literature on the ‘pathways’ of myo-inositol hexakisphosphate degradation by phytases of different classes; cysteine phytase, histidine acid phytase, purple-acid phytase, β-propeller phytase (Konietzny and Greiner 2002), it would be possible to begin to describe ‘pathways’ of inositol hexakisphosphate turnover in soils and the contribution of different organisms to that turnover.

With these thoughts in mind, we have sought to synthesize 32P-labeled inositol hexakisphosphates by enzymic means. We have taken opportunity of a recombinant inositol pentakisphosphate 2-kinase (IP5 2-K, also known as IPK1) characterized (Banos-Sanz et al. 2012; Gonzalez et al. 2010; Gosein and Miller 2013; Sweetman et al. 2006). This enzyme transfers the gamma-phosphate) from ATP to the sole axial hydroxyl on carbon 2 of myo-inositol phosphates, and is believed to be the enzyme responsible for synthesis of myo-inositol hexakisphosphate in all kingdoms that make this molecule. We reasoned that the enzyme might be capable of transferring phosphate from ATP to the axial hydroxyl(s) of other inositols bearing equatorial phosphates.

The structures of the different inositol ‘parents’ of the inositol phosphates used in this study are shown in Fig. 1. The rules for numbering of carbon atoms, and hence of inositol phosphates bearing phosphate substituents on particular carbons are given in the IUPAC-IUB rules (IUPAC-IUB 1973, 1977). For myo-inositol phosphates, only, a relaxation of the rules (NC-IUB 1989) allows numbering of carbons by the D- (1D) or L- (1L) nomenclature, e.g. to assist in delineating metabolic sequences. The 1D- and 1L-numbering of myo-inositol is shown in Fig. 1. Supplemental Fig. 1 shows symmetry aspects of the ‘parent’ inositols of the myo-, neo-, 1D-chiro- and scyllo-inositol phosphates discussed hereafter, while Supplemental Fig. 2 shows the structures of the substrates and products obtained therefrom.

For the assistance of researchers wishing to adopt these methods, we provide (Table 1) a summary of the methodologies used with an indication of their applicability and a reference to prior use of the method. We also list the figures in this manuscript in which the method is applied.

Methods

Inositol phosphate substrates

The synthesis of neo-inositol 1,3,4,6-tetrasphosphate (neo-Ins(1,3,4,6)P₄), 1D-chiro-inositol 1,3,4,6-tetrasphosphate (1D-chiro-Ins(1,3,4,6)P₄), 1D-chiro-inositol 2,3,4,5-tetrasphosphate (1D-chiro-Ins(2,3,4,5)P₄), myo-inositol 1,3,4,5,6-pentakisphosphate (myo-Ins(1,3,4,5,6)P₄), scyllo-inositol pentakisphosphate and of neo- and 1D-chiro inositol hexakisphosphates was described (Diogenous 1999; Liu et al. 1999; Liu et al. 2001; Godage et al. 2013; Riley et al. 2006; Turner et al. 2012). Myo-inositol hexakisphosphate was obtained from Merck Millipore (Product No. 407125).
Radioisotopes

ATP, [γ-^32P]-3000 Ci mmol\(^{-1}\) was obtained from PerkinElmer.

Cloning and expression of AtIPK1

*Arabidopsis thaliana* IPK1 (AGI number): At5G42810, was cloned from *Arabidopsis thaliana* Col-0 cDNA with forward primer ATGGAGATGATTTTGGAGGAGAA and reverse primer ATGGTCTAGAAAGCTTTAGCTG (vector specific sequence in italics) using Phusion High Fidelity Polymerase (Thermo Scientific). Purified product (Wizard SV Gel and PCR Cleanup System, Promega) was inserted into pOPINF linearized with HindIII and KpnI (Berrow et al. 2007) by ligation independent cloning using InFusion HD enzyme kit (Clontech). The vector adds a N-terminal hexahistidine tag to the recombinant protein. Recombinant plasmid was transformed into *E. coli* Rosetta (DE3) (Novagen) and protein production induced from an overnight culture grown in LB containing 0.5% (w/v) glucose and ampicillin by transfer to LB containing 0.5 mM IPTG and ampicillin with further growth for 7 h at 25 °C.

Protein purification

AtIPK1 was purified according to (Banos-Sanz et al. 2012).

Enzyme assays

For assays without radiolabel, inositol phosphate-dependent conversion of ATP to ADP was followed by reverse-phase ion pair HPLC and subsequent detection of nucleotides at 260 nm (Caddick et al. 2008). Briefly, 1 µg enzyme was incubated with 500 µM inositol phosphate and 50 µM ATP in 20 mM HEPES, pH 7.3, 1 mM MgCl\(_2\) for 2 h at 25 °C, the volume of the assay was 20 µL. Reactions were terminated by the addition of 1 µL of conc. HCl, followed after 5 min on ice by the addition of 50 µL water. Aliquots (50 µL) were analysed by HPLC.
| Subject                                      | Method                                              | Comment                                                                                                                                                                                                 | Description/Use                                                                 | Reference                             |
|----------------------------------------------|-----------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------|---------------------------------------|
| Stereochemistry of inositol phosphates       | A comprehensive review of inositol (phosphate) chemistry and stereochemistry                         |                                                                                                                                                    | Fig. 1                                                                          | Thomas et al. 2016                   |
| Source of inositol phosphates                | Complete synthesis or extracted from soils          | The soil extracted inositol phosphates were the kind gift of Alan Richardson (CSIRO) from the personal stocks of the late Dennis Cosgrove, or were provided by Max Tate (The University of Adelaide) | Figs. 1, 2, 3, 4, 5, and 6; Suppl. Fig. 1                                        | Diogenous 1999; Liu et al. 1999, 2001; Riley et al. 2006; Turner et al. 2012; Godage et al. 2013; collected works of Cosgrove, Tate and others (reviewed this manuscript) |
| Source of synthetic enzyme                   | Non-radioactive verification of enzyme specificity |                                                                                                                                                    | Figs. 2, 3, 4, 5, and 6; Suppl. Fig. 1                                           | Sweetman et al. 2006                 |
| Production of $^{32}$P-labeled inositol phosphates | Reverse-phase ion-pair chromatography with UV detection of nucleotides                               | Used to titrate enzyme concentration before use of radiolabel                                                                                | Fig. 2                                                                          | Caddick et al. 2008                  |
| Substrate specificity/product profiles of different phytases | Anion-exchange chromatography on Partisphere SAX columns with online detection by Cerenkov counting | Used to separate/verify reaction products                                                                                                          | Fig. 3                                                                          | Brearley and Hanke 1996a, b; Nagy et al. 2009; Stephens 1990                   |
| Non radioactive detection of inositol phosphates on acid gradients with UV detection | Anion exchange chromatography on CarboPac PA200 columns with post-column addition of ferric nitrate | Can be used to separate/purify particular isomers                                                                                               | Fig. 4                                                                          | Stentz et al. 2014                   |
| Non radioactive detection of pentakisphosphates that can be generated from soil inositol hexakisphosphates | Anion exchange chromatography on CarboPac PA200 columns with post-column addition of ferric nitrate | A particularly robust separation method for higher inositol phosphates using volatile (HCl) or non-volatile (methanesulfonic) acid eluents. Could be combined with Cerenkov counting for radiolabeled inositol phosphates | Fig. 5                                                                          | Phillippy and Bland 1988; Blaabjerg et al. 2010                               |
| The structures of substrates and products of AtIP5 2 K | Anion exchange chromatography on CarboPac PA200 columns with post-column addition of ferric nitrate | A particularly robust separation method for higher inositol phosphates using volatile (HCl) or non-volatile (methanesulfonic) acid eluents. Could be combined with Cerenkov counting for radiolabeled inositol phosphates | Fig. 6                                                                          | This manuscript                      |
For assays with radiolabel, ATP-dependent conversion of inositol phosphates to higher (more phosphorylated) species was followed by anion-exchange HPLC with on-line detection of $^{32}$P. Briefly, 1 μg enzyme was incubated with 500 μM inositol phosphate and 5 μM ATP in the presence of 0.37 MBq [$^{32}$P]ATP in 20 mM HEPES, pH 7.3, 1 mM MgCl$_2$ for 1 h at 25 °C, the volume of the assay was 20 μL. The reaction products were diluted with water and approximately 1–5% of the products were analysed by HPLC.

HPLC separation of inositol phosphates

Radiolabeled inositol phosphates were separated by anion exchange HPLC on Partisphere SAX columns eluted at a flow rate of 1 mL min$^{-1}$ with a gradient derived by mixing solvent from reservoirs containing (A) water and (B) 1.25 M (NH$_4$)$_2$HPO$_4$, adjusted to pH 3.8 with H$_3$PO$_4$, according to the following schedule: time (min), % B: 0, 0; 5, 0; 65, 100. Radioactivity was detected by Cerenkov counting in a Radiomatic A500 Series Flo Detector (Canberra Packard, Pangbourne, Bucks, UK) fitted with a 0.5 mL flow cell using an integration interval of 12 s (Hanke et al. 2012).

Non-labelled inositol phosphates were resolved by anion exchange HPLC on a 250 × 3 mm i.d. CarboPac PA200 column (Dionex UK, Ltd) and guard column 50 × 3 mm of the same material, eluted at a flow rate of 0.4 mL min$^{-1}$ with a gradient derived by mixing solvent from reservoirs containing (A) water and (B) 1.25 M (NH$_4$)$_2$HPO$_4$, adjusted to pH 3.8 with H$_3$PO$_4$, according to the following schedule: time (min), % B: 0; 0; 5; 0; 65, 100. Radioactivity was detected by Cerenkov counting in a Radiomatic A500 Series Flo Detector (Canberra Packard, Pangbourne, Bucks, UK) fitted with a 0.5 mL flow cell using an integration interval of 12 s (Hanke et al. 2012).

HPLC separation of nucleotides

Aliquots of the products of enzyme assays were analysed according to (Caddick et al. 2008).

Nomenclature

For the purpose of this article, the term ‘Ins’ with prefix 1D-chiro-, myo-, neo- or scylo- is used as an abbreviation of the described inositol phosphate. Hence, neo-inositol 1,3,4,6-tetraisphosphate is abbreviated neo-Ins(1,3,4,6)$P_4$. It should be noted, however, that the numbering of phosphate substituents (of the carbon atoms to which they are attached) is not necessarily the same for different stereoisomers of inositol. The reader is referred to Shears and Turner (2007) for a concise description of terminology and to (Thomas et al. 2016) for a comprehensive review of inositol and inositol phosphate nomenclature and terminology.

Results

AtIP5 2-K can be used to synthesize a range of inositol epimer epimers, verifiable by non-radioactive assay of inositol phosphate production

We have previously described the use of AtIP5 2-K to synthesize myo-Ins(1,$^{32}$P)2,3,4,5,6$P_6$ from [$^{32}$P]ATP and myo-Ins(1,3,4,5,6)$P_5$ (Nagy et al. 2009). Here, we have further examined the ability of AtIP5-2 K to phosphorylate myo-Ins(1,3,4,6)$P_4$. We did so, not only because myo-Ins(1,3,4,6)$P_4$ possesses an axial 2-OH, but also because of the availability of the neo-inositol epimer, neo-Ins(1,3,4,6)$P_4$ (Diogenous 1999). This inositol phosphate shares the plane of symmetry that bisects the myo-Ins(1,3,4,6)$P_4$ molecule between C2 and C5, but also possesses a second axial hydroxy1 on C5 which creates a C$_2$-axis of rotational symmetry that bisects the C1-C6 bond and the C3-C4 bond (Supplementary Figure 1). The consequence of this is that single phosphorylation of C2 generates the same product as phosphorylation of C5, while in contrast phosphorylation of C2 of myo-Ins(1,3,4,6)$P_4$ is not equivalent to phosphorylation of C5.

We incubated AtIP5 2-K with 500 μM inositol phosphate and 50 μM ATP. The products were resolved by reverse-phase ion-pair HPLC with detection of nucleotides at 260 nm (Fig. 2). Peak areas were integrated and the % of the nucleotide converted to ADP was calculated. A control incubation without inositol phosphate confirmed that AtIP5 2-K is not a phosphatase, the 1.3% of nucleotide recovered as ADP is typical of the level of contamination of commercial ATP with ADP (Fig. 2a). Inclusion of myo-Ins(1,3,4,6)$P_4$ increased ADP production, 12.1% of total nucleotide was recovered as ADP, without production of AMP (Fig. 2b), whereas for the physiological substrate myo-Ins(1,3,4,5,6)$P_4$, included at 50 μM, 26% of nucleotide was recovered as ADP (Fig. 2c). Clearly, myo-Ins(1,3,4,6)$P_4$ is a substrate, albeit a poorer one than myo-Ins(1,3,4,5,6)$P_5$. In contrast, neo-Ins(1,3,4,6)$P_4$...
was a strong substrate with ADP production at 90.8% (Fig. 2d).

Similarly, inclusion of 1D-chiro-Ins(2,3,4,5)P₄ at 500 μM resulted in 92% conversion of ATP to ADP (Fig. 2e), while 1D-chiro-Ins(1,3,4,6)P₄ whose two hydroxyls, on C2 and C5 are equatorial (Supplementary Figure 1) was not a substrate for AtIP5 2-K, with ADP representing 1.5% of the total nucleotide (Fig. 2f).

These results confirm the utility of neo-Ins(1,3,4,6)P₄ and 1D-chiro-Ins(2,3,4,5)P₄ as substrates from which higher neo- and 1D-chiro-inositol phosphates can be synthesized with AtIP5 2-K. They further confirm the exclusive phosphorylation of axial hydroxyls by this enzyme (Gonzalez et al. 2010; Sweetman et al. 2006).

We additionally tested racemic mixtures of 1D/L-neo-Ins(1,2,4)P₃ and 1D/L-neo-Ins(1,3,4)P₃ as substrates in extended (16 h) incubations at 500 μM concentration with 50 μM ATP. We did not observe production of ADP, thus these molecules are not substrates despite possessing one and two axial hydroxyls at C5, and C2 and C5 respectively (data not shown).

AtIP5 2-K can be used to synthesize a range of ³²P-labeled inositol phosphates

Having established that neo-Ins(1,3,4,6)P₄ and 1D-chiro-Ins(2,3,4,5)P₄ are novel substrates of AtIP5 2 K, we performed enzyme assays to produce ³²P-labelled inositol phosphates. The substrate concentrations used were 500 μM inositol phosphate and 5 μM ATP. Reactions were terminated and the products spiked with additional ATP to allow online tandem UV-radioactivity monitoring of the chromatography (Fig. 3). We included myo-Ins(1,3,4,5,6)P₅ as the canonical substrate and observed (Fig. 3a) in addition to a major peak of myo-InsP₅ eluting at a retention time of 55 min, a small peak of unidentified material eluting at 38.8 min, a major peak of unreacted ATP at 25.2 min and a peak of inorganic phosphate at 14.6 min. We note that others have reported impurities in commercial [³²P]ATP that elute on Partisphere SAX columns with similar chromatographic mobility to myo-InsP₅ (Stephens 1990).

Provision of neo-Ins(1,3,4,6)P₄ as substrate yielded (Fig. 3b) a major peak of label with the chromatographic property of InsP₅ at 46.4 min and a more polar (highly charged peak) with the chromatographic property of an InsP₆. This peak eluted at 56.4 min, slightly after myo-InsP₆. The trace also showed peaks of assumed impurity, ATP and inorganic phosphate. Considering the structure of neo-Ins(1,3,4,6)P₄ and its C₂ axis of rotational symmetry (Supplemental Figs. 1, 2), the two axial hydroxyls are superposable so there is only one possible InsP₅ product. IUPAC conventions recommend the naming of substituents by the lowest numbered locants,
hence single phosphorylation of one axial hydroxyl of neo-Ins(1,3,4,6)P₄ yields neo-Ins(1,2,3,4,6)P₅ = neo-

Ins(1,3,4,5,6)P₅ with preferred use of the former name. The addition of ³²P to an unlabeled substrate therefore yields neo-Ins(1,[³²P]2,3,4,6)P₅. The elution of a second more polar peak, we assume to represent the double phosphorylation of neo-Ins(1,3,4,6)P₄ to give neo-

Ins(1,[³²P]2,3,4,[³²P]5,6)P₆.

We also tested 1D-chiro-Ins(1,3,4,6)P₄ and 1D-chiro-Ins(2,3,4,5)P₄ as co-substrates with [³²P]ATP (Fig. 3c, d). Significantly, the former, which bears equatorial hydroxyls on C2 and C5, and lacks axial hydroxyls (Fig. 1; Supplemental Figs. 1, 2), was not a substrate and yielded peaks of unreacted ATP, inorganic phosphate and the assumed contaminant with retention time 39 min (Fig. 3c). In contrast, 1D-chiro-Ins(2,3,4,5)P₄, like neo-Ins(1,3,4,6)P₄, yielded ³²P-labeled peaks with the chromatographic mobility of InsP₅, retention time 48 min; and InsP₆, retention time 59.5 min (Fig. 3d). Comparison of the retention time of peaks of InsP₅ and InsP₆ products (Fig. 3a–d) reveals that the InsP₅ and InsP₆ products of different epimers of inositol (tetrakisphosphate) are chromatographically distinct. Again, 1D-chiro-Ins(2,3,4,5)P₄, another substrate with two axial hydroxyls, yielded products which, relative to the substrate, were singly and doubly phosphorylated by AtIP5 2-K. Moreover, 1D-chiro-Ins(2,3,4,5)P₄ possesses a C₂-axis of symmetry, here bisecting the C1-C6 and C3-C4 bonds, which superposes C1 and C2 substituents. Consequently, a common InsP₅ product is generated from phosphorylation of either axial hydroxyl and the product is 1D-chiro-Ins(1,2,3,4,5)P₅, here 1D-chiro-Ins([³²P]1,2,3,4,5)P₅. The InsP₆ product is 1D-chiro-Ins([³²P]1,2,3,4,5,[³²P]6)P₆.

Different phytases yield characteristic product profiles from ³²P-labeled myo-inositol hexakisphosphate

By way of illustration of how different phytases yield different product profiles from the same substrate, we show (Fig. 4) the products of progressive dephosphorylation of myo-Ins([³²P]1,2,3,4,5,6)P₆ by histidine acid phytases of fungal and bacterial origin. We chose Aspergillus ficuum phytase, a D-3 phytase (data of Fig. 4c), where D-3 signifies the position of attack on myo-InsP₆ and HD is one of the canonical motifs, D (aspartate) being a proton donor that activates a water molecule that is responsible for the cleavage of scissile phosphate. The other phytase (data of Fig. 4b) is another histidine acid phytase, but is the archetype of a recently
described HAE subclass where E (glutamate) is the likely proton donor. This enzyme generates three resolvable InsP₅ products from myo-InsP₆ (Stentz et al. 2014). These data reveal the utility of inositol hexakisphosphate labeled on the axial 2-position for study of phytases in vitro. Clearly, they indicate the potential utility of such compounds and such chromatography for study of soil processes.

Using the post-column complexation method (Phillipps and Bland 1988) and a CarboPac PA-200 column eluted with a gradient of HCl, we were able to resolve in order of increasing retention time, neo-InsP₆, muco-InsP₆, 1D-chiro-InsP₆, myo-InsP₆ and scylo-InsP₆ (Fig. 5a), all samples obtained from the laboratory of the late Dennis Cosgrove. We assume that the muco-InsP₆ was that made by chemical phosphorylation of the inositol (Cosgrove 1975). The gradient shows a strongly sloping baseline arising from the acid eluent and this UV

Separation of inositol hexakisphosphates identified in soils

Using the post-column complexation method (Phillipps and Bland 1988) and a CarboPac PA-200 column eluted with a gradient of HCl, we were able to resolve in order of increasing retention time, neo-InsP₆, muco-InsP₆, 1D-chiro-InsP₆, myo-InsP₆ and scylo-InsP₆ (Fig. 5a), all samples obtained from the laboratory of the late Dennis Cosgrove. We assume that the muco-InsP₆ was that made by chemical phosphorylation of the inositol (Cosgrove 1975). The gradient shows a strongly sloping baseline arising from the acid eluent and this UV
absorbance can be negated when using methanesulfonic acid as the eluent (Blaabjerg et al. 2010); however, at least up to 0.6 M, methanesulfonic acid was not a strong enough eluent to elute scyllo-InsP₆ from this column.

Separation of neo-inositol phosphates

Methanesulfonic acid was, however, suitable for separation of neo-InsPs: neo-InsP₄s, neo-InsP₅s and neo-InsP₆ (Fig. 5b), again, these compounds were likely produced by chemical phosphorylation of neo-inositol with sodium trimetaphosphate (Cosgrove 1969a). Irving (1980) reported that neo-InsP₆ was a good substrate of both the Pseudomonas (sp. unknown) bacterium (SB₂) phytase of Cosgrove (Cosgrove et al. 1970) and A. ficuum phytase (Irving and Cosgrove 1974). We note that there are nine possible neo-InsP₄s, comprised of three pairs of enantiomers and three meso-compounds and there are three possible neo-InsP₅s (Thomas et al. 2016). Of the neo-InsP₅s, two: 1L-neo-Ins(1,2,3,4,5)P₅ = 1D-neo-Ins(1,2,3,5,6)P₅ and 1D-neo-Ins(1,2,3,4,5)P₅ = 1L-neo-Ins(1,2,3,5,6)P₅ are a pair of enantiomers, the third is the meso-compound neo-Ins(1,2,3,4,6)P₅. In the absence of chiral HPLC methods for separating enantiomers of inositol phosphates, the separation of two peaks of neo-InsP₅ from the Cosgrove samples (Fig. 5b) is all that is achievable, but nevertheless could be diagnostic in studies of neo-InsP₆ transformation in soils.

Separation of inositol pentakisphosphates; the initial products of phytase action on inositol hexakisphosphate

Finally, we show (Fig. 6) the separation of a variety of InsP₅s including the four separable peaks of myo-InsP₅, identified in Fig. 6b by the position of the single hydroxyl; the two enantiomeric pairs 1D-1/3-OH and 1D-4/6-OH, indicated [1/3-OH] and [4/6-OH] on the figure, cannot be separated into individual enantiomers on non-chiral HPLC. Figure 6b also shows the single scyllo-InsP₅ and several InsP₅s present in a 1L-chiro-InsP₅ sample. There are three possible 1L-chiro-InsP₅s and, similarly, three possible 1D-chiro-InsP₅s. Minor chiro-InsP₅ peaks with common retention times were observed in the 1D-chiro-InsP₆ sample (Fig. 6a) and the 1L-chiro-InsP₅ sample (Fig. 6b), with two of the three co-eluting precisely with peaks in a 1L-chiro-InsP₅ sample (Fig. 6a). Clearly, this column/eluent combination has great resolving power for all the InsP₅s expected of soil samples.

Discussion

Inositol phosphates are major components of total soil phosphate and commonly the dominant organic phosphates in these environments (Turner et al. 2002). Despite their abundance, the origins of neo-, 1D-chiro- and scyllo-inositol hexakisphosphates in soils are poorly defined. It is plausible that they derive from the considerable inputs to soil of plant-derived myo-inositol
hexakisphosphate, representing up to several percent of seed dry weight (Raboy 2003). It has been estimated that the sequestration of phosphorus in the myo-inositol hexakisphosphate, phytate, reserves of cropped organs of plants is equivalent to the per annum application of phosphorus as fertilizer to soils (Lott et al. 2000). It is remarkable therefore that we do not know, how plant-derived phytate is, likely, converted to other forms (epimers) of inositol phosphates, nor whether the processes are organismal or geochemical.

The epimerization of unsubstituted inositols by unsterilized soil is well described (L’Annunziata and Gonzalez 1977) and pathways by which myo-inositol and scyllo-inositol are converted have been engineered in Bacillus subtilis (Kang et al. 2017; Tanaka et al. 2013; Yamaoka et al. 2011). Similarly, Geobacillus kaustophilus HTA426, has three dehydrogenases that are capable of acting as epimerases to interconvert myo-, scyllo-, and 1D-chiro-inositols (Yoshida et al. 2012). The American cockroach, Periplaneta americana, was reported to possess epimerase activity producing neo-inositol. (Higgs et al. 1973). Plants have the ability to epimerize a number of methylated inositols, reviewed (Thomas et al. 2016). These studies seem to suggest that epimerase activity is restricted to inositols lacking phosphate substituents. It is intriguing therefore that scyllo-phosphoinositides were detected in barley (Narasimhan et al. 1997) and that highly phosphorylated inositol phosphates and diposphoinositol phosphates containing neo-rather than myo-inositol are the major form of inositol phosphate in Entamoeba histolytica (Martin et al. 2000), despite earlier suggestion to the contrary (Martin et al. 1993).

Because the axial 2-phosphate is the last phosphate added in the synthesis of myo-inositol hexakisphosphate in plants (Brearley and Hanke 1996) and Dictyostelium discoideum (Stephens and Irvine 1990) it is possible to use the enzyme catalyzing this step to make myo-inositol 1,3,4,5,6-hexakisphosphate. Moreover, because most phytases of plant, bacterial or fungal origin do not remove this phosphate until very late in the sequence of myo-inositol hexakisphosphate degradation, if at all (Konietzny and Greiner 2002), the $^{32}$P label will be retained in successive myo-InsP$_5$, InsP$_4$, InsP$_3$ and InsP$_2$ products of dephosphorylation. Consequently, addition of $^{32}$P-labeled myo-InsP$_5$ to soils will, by simple chromatography using the methods elaborated here, allow researchers to study myo-inositol hexakisphosphate turnover in their soil of choice.

From a practical perspective, the use of a high energy $\beta$ emitter such as $^{32}$P allows facile detection of radioactivity in column eluates by on-line Cerenkov counting, obviating the requirement for the addition of scintillation fluid. The use of flow-detectors therefore allows for simple collection of radiolabelled fractions, albeit in high salt concentrations required to elute highly polar inositol phosphates. For phosphate eluents, it is a simple exercise to desalt the collected fractions on Dowex AG1 X8 resin with volatile ammonium formate / formic acid mixtures, subsequently removed by freeze-drying (Stephens 1990; Brearley et al. 1997), while for HCl eluents the HCl can be removed directly by freeze-drying.

From a diagnostic perspective, because different phytases produce different myo-InsP$_5$ products (after all, this is the explanation of their classification e.g. as D3, Aspergillus; D6, E.coli or D5, lily pollen alkaline phytases (Konietzny and Greiner 2002)), simple analysis of products at the level of myo-InsP$_5$ will allow identification of the likely class of enzyme predominantly responsible for initial degradation of myo-InsP$_5$ in different soils. Indeed, it will be fascinating to correlate myo-inositol hexakisphosphate degradation products with metagenomic characterization of phytase and microorganism abundance in different soils of the sort recently described (Neal et al. 2017).

In consideration of the routes of degradation of other inositol hexakisphosphates, the other labeled isomers that we describe will be of particular value. Perhaps unsurprisingly, we note the seminal work of Cosgrove (1969a, 1970) and Irving and Cosgrove (1971) in characterization of products of dephosphorylation of myo-inositol-, scyllo-inositol-, and 1D-chiroinositol hexakisphosphate by a bacterial phytase, and similar studies of wheat phytase by Lim and Tate (1971, 1973) following the methods of Tomlinson and Ballou (1962). These works, reviewed (Irving 1980), show that the axial phosphates of myo- and 1D-chiro-inositol hexakisphosphates are the last to be removed by plant, bacterial and fungal phytases where tested. Similar conclusions can be drawn for fungal phytase action on 1L-chiro-inositol hexakisphosphate (Adelt et al. 2003). With these observations in mind, the use of the labeled InsP$_5$ and InsP$_6$ species and the powerful separation approaches identified in the foregoing should allow for facile assessment of the exchange of phosphate between different inositol phosphates in soil contexts.
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Author contributions HW, BVLP, AMR and CAB designed the study. All authors discussed the results, HW and CAB wrote the manuscript with input from other coauthors.

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