SUPPLEMENTARY INFORMATION

Determination of *neo*- and D-*chiro*-inositol hexakisphosphate in soils by solution ^{31}P NMR spectroscopy

Benjamin L. Turner, Alexander W. Cheesman, H. Yasmin Godage, Andrew M. Riley, Barry V.L. Potter

Summary

There are 13 pages of supplementary information, which includes detailed information on methods, two tables, and a figure showing solution ^{31}P NMR spectra of two soils.

General methods for synthesis of *neo*- and D-*chiro*-inositol hexakisphosphate

D-Pinitol was purchased from New Zealand Pharmaceuticals Ltd. Anhydrous acetonitrile and dichloromethane were purchased from Fisher Scientific (Loughborough, UK) and used without further purification. All other chemicals were purchased from either Aldrich Chemical Co. (Gillingham, UK) or Alfa Aesar (Heysham, UK) and were of commercial quality. Bis(cyanoethyl)(*N*,*N*-diisopropylamino)phosphine was synthesised by a modification of the method of Uhlmann and Engels (*1*) and stored under nitrogen at –20 °C for several months without decomposition. *neo*-Inositol was synthesized as previously reported (*2*) in five steps from *myo*-inositol. D-*chiro*-Inositol was obtained by *O*-demethylation of D-pinitol with 55% hydroiodic acid under reflux, followed by crystallisation of product by dilution of the reaction mixture with ethanol and the subsequent recrystallisation from ethanol/water, $[\alpha]_D^{20} = +64°$ (*c* = 1, H_2O), literature $[\alpha]_D^{20} = +63.2°$ (*c* = 1, H_2O) (ref. *3*); ^{1}H NMR (400 MHz, D_2O) δ 3.43–3.47 (2H, m, C-3-H and C-4-H), 3.59–3.64 (2H, m, C-2-H and C-5-H), 3.89 (2H, br s, C-1-H and C-6-H); ^{13}C NMR (100.6 MHz, D_2O) δ_C 70.4 (d, C-2 and C-5), 71.6 (d, C-1 and C-6), 72.7 (d, C-3 and C-4).

All target polyphosphates were purified by ion exchange chromatography on Q-Sepharose Fast Flow using a Pharmacia Biotech Gradifrac system with a P-1 pump, eluting with gradients of triethylammonium bicarbonate (TEAB) buffer and using H_2O of MilliQ quality. 2 M TEAB was prepared by bubbling carbon dioxide gas into 2 M triethylamine solution. Phosphate containing fractions were identified using a modification of the Briggs phosphate test (*4*) and the target polyphosphates were accurately quantified using the Ames phosphate assay (*5*).

^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on a Varian Mercury VX machine at 400 MHz (^{1}H), 100 MHz (^{13}C) or 162 MHz (^{31}P). NMR spectral assignments, where given, are based on (^{1}H-^{1}H) COSY, (^{1}H-^{13}C) HMQC, (^{1}H-^{13}C) or (^{1}H-^{31}P) HMBC, 135DEPT and PENDANT experiments.

Chemical shifts (δ) were determined in parts per million (ppm) relative to tetramethylsilane (TMS) as an internal standard. ^{31}P chemical shifts were measured in ppm relative to external 85% phosphoric acid (H_3PO_4). Coupling constants (*J*) were recorded to the nearest 0.1 Hz. Electrospray (ES) HRMS data were recorded with a Bruker micrOTOF-Focus instrument. Elemental analyses were performed by the Microanalysis Service, University of Bath. Melting points (mp) were determined using a Stanford Research Systems Optimelt MPA100 and are uncorrected. Optical rotations were measured using an Optical Activity Ltd. AA-10 polarimeter using a 5 cm cell.

Analytical information for D-*chiro*-inositol 1,2,3,4,5,6-hexakisphosphate

$[\alpha]_D^{20}$ = +9° (*c* = 1, Methanol), literature $[\alpha]_D^{20}$ for L-*chiro*-inositol 1,2,3,4,5,6-hexakisphosphate = – 24.4° (*c* = 1.17, H_2O) (ref. *6*); ^{31}P NMR (109.4 MHz, H-decoupled, D_2O) δ –1.70 (2P, s, C-1-P and C-6-P), –0.99 (2P, s C-2-P and C-5-P), –0.41 (2P, s, C-3-P and C-4-P); ^{1}H NMR (400 MHz, D_2O) δ 4.31 (4H, m, C-2-H, C-3-H, C-4-H and C-5-H), 4.60 (2H, d, $J_{P\text{-}H}$ = 9.0 Hz, C-1-H and C-6-H); ^{13}C NMR (100.6 MHz, D_2O) δ_C 72.7 (m, C-1 and C-6), 72.9, 76.5 (2 × m, C-2 and C-5, C-3 and C-4); HRMS calculated for $C_6H_{17}O_{24}P_6$ ($[M - H]^-$) 658.8536, found 658.8541.

Synthesis of *neo*-inositol 1,2,3,4,5,6-hexakisphosphate

Bis(cyanoethyl)(*N*,*N*-diisopropylamino)phosphine (1.61 g, 6.00 mmol) was added to a stirred suspension of *neo*-inositol (90.1 mg, 0.50 mmol) (ref. *2*) and 5-phenyltetrazole (877.1 mg, 6.00 mmol) in anhydrous dichloromethane and acetonitrile (1:1, 20 mL) under an argon atmosphere. Stirring continued for 4 days at room temperature. The reaction mixture was cooled to –40 °C and *tert*-butyl hydroperoxide (70%, 0.87 mL, 6.0 mmol) was added portion-wise while stirring. The mixture was allowed to warm to room temperature and the stirring was continued for a further 1 h.

After evaporation of the solvent under reduced pressure, water (50 mL) was added to the crude residue and the mixture was filtered. The remaining solid was further washed with water and methanol to afford the pure 1,2,3,4,5,6-hexakis-*O*-[bis(cyanoethyloxy)phosphoryl] *neo*-inositol (252 mg, 39%) as a white solid. [m.p.158–159 °C (methanol/dichloromethane); ^{31}P NMR (161.9 MHz, H-decoupled, $(CD_3)_2CO$ + one drop of D_2O) δ –3.94 (2P, s, C-2-P and C-5-P), –2.74 (4P, s, C-1-P, C-3-P, C-4-P and C-6-P); ^{1}H NMR (400 MHz, $(CD_3)_2CO$ + one drop of D_2O) δ 3.03 to 3.08 (24H, m, $OCH_2C\underline{H}_2CN$), 4.46 to 4.56 (24H, m, $OC\underline{H}_2CH_2CN$), 5.16 to 5.20 (4H, m, $J_{P\text{-}H}$ = 8.1 Hz, C-1-H, C-3-H, C-4-H and C-6-H), 5.50 (2H, d, $J_{P\text{-}H}$ = 8.9 Hz, C-2-H and C-5-H); ^{13}C NMR (100.6 MHz, $(CD_3)_2CO$ + one drop of D_2O) δ_C 19.1, 19.1, 19.2, 19.2 (4 × t, 12 × $OCH_2\underline{C}H_2CN$), 63.6, 63.7, 63.7, 63.9, 64.0 (5 × t, 12 × $O\underline{C}H_2CH_2CN$), 72.8 (m, C-1, C-3, C-4 and C-6), 76.1 (m, C-2 and C-5), 117.7, 117.8, 117.9 (3 × s, 12 × $OCH_2CH_2\underline{C}N$); Elemental analysis, calculated for $C_{42}H_{54}N_{12}O_{24}P_6$ (1296.79): C, 38.90%; H, 4.20%; N, 12.96%; measured: C, 38.60%; H, 4.12%; N, 12.70%.]

To a suspension of 1,2,3,4,5,6-hexakis-*O*-[bis(cyanoethyloxy)phosphoryl] *neo*-inositol (240 mg, 0.19 mmol) in a Pyrex pressure tube, concentrated aqueous ammonia solution (15 mL) was added and the mixture was heated at 60 °C for 20 h. After evaporation of the solution under vacuum, the residue was purified by ion exchange chromatography on Q Sepharose Fast Flow resin eluting with a gradient of aqueous triethylammonium bicarbonate (TEAB; 0 to 2.0 mol L^{-1}) to afford the pure triethyl ammonium salt of *neo*-inositol 1,2,3,4,5,6-hexakisphosphate (203 mg, 94%) as a hygroscopic white solid (*6*, *7*). [^{31}P NMR (161.9 MHz, H-decoupled, D_2O, pH adjusted to 1 with DCl) δ –0.75 (2P, s, C-2-P and C-5-P), –0.03 (4P, s, C-1-P, C-3-P, C-4-P and C-6-P); ^{1}H NMR (400 MHz, D_2O, pH adjusted to 1 with DCl) δ 4.40 (4H, m, $J_{P\text{-}H}$ = 7.8 Hz, C-1-H, C-3-H, C-4-H and C-6-H), 4.86 (2H, d, $J_{P\text{-}H}$ = 9.4 Hz, C-2-H and C-5-H); ^{13}C NMR (100.6 MHz, D_2O, pH

adjusted to 1 with DCl) δ_C 71.9 (m, C-1 and C-3, C-4 and C-6), 75.1 (m, C-2 and C-5); HRMS calculated for $C_6H_{17}O_{24}P_6$ ($[M - H]^-$) 658.8536, found 658.8541.]

Hypobromite oxidation

For authentic compounds, 1 mL of sample was diluted to 4 mL with deionized water and adjusted to pH ~14 by adding 1.2 mL of 10 M NaOH. Pure bromine (0.1 mL, cooled in an ice bath) was added and the mixture was allowed to stand for 1 h at room temperature. The solution was then boiled for 5 min on a hot plate, allowed to cool, and then acidified to pH < 3 with ~ 2 mL of 6 M HCl. This step was accompanied by a color change from yellow to orange. The solution was heated again for 5 min to dispel excess bromine (accompanied by a color change from orange to yellow) and the pH was increased to 8.5 by adding 1 M NaOH dropwise. This final step was accompanied by a color change from yellow to colorless.

For soil NaOH–EDTA extracts, 10 mL of extract was placed in a glass beaker and adjusted to pH ~14 by adding 1 mL of 10 M NaOH. Ice-cold bromine (0.2 mL) was added and the solution was treated as described above.

Brominated samples (both authentic compounds and soil extracts) at pH 8.5 were diluted to 20 mL with deionized water and phosphates were precipitated by adding 5 mL of 50% ethanol and 10 mL of 10% barium acetate. The mixture was heated gently for 10 minutes and then allowed to stand overnight at room temperature. The samples were centrifuged (1500 *g*, 15 min), the supernatant discarded, the precipitate washed in 50% ethanol, and the phosphates re-suspended in 20 mL of cation exchange resin (Amberlite IR120, hydrogen form; Sigma-Aldrich, St Louis, MI) in an equal volume of deionized water. This procedure was repeated (precipitation and resuspension) and the pH was adjusted to > 13 with 1 M NaOH. For authentic compounds

the volume was reduced to ~ 2 mL for analysis directly by solution ^{31}P NMR spectroscopy. For soil extracts, solutions were frozen, lyophilized, and ground to a fine powder.

Solution ^{31}P NMR spectroscopy

For authentic compounds, 0.9 mL of sample was mixed with 0.2 mL D_2O and 0.9 mL of 1 M NaOH–100 mM EDTA solution. For soil extracts, lyophilized powder was suspended in 0.3 mL D_2O and 2.7 mL of 1 M NaOH–100 mM EDTA. Samples were vortexed (1 min) and transferred to a 10 mm NMR tube. Soil extracts were filtered (1 µm GF-B filter, Whatman) prior to analysis. The inclusion of EDTA in the re-dissolved solution ensures well-resolved spectra following hypobromite oxidation (*8*).

Spectra were acquired on a Bruker Avance 500 Console with a Magnex 11.75 T/51mm Magnet and a 10-mm BBO Probe. Spectra acquisition was carried out at a stabilized 25°C with a calibrated (~30°) pulse, a zgig pulse program, a 0.58 s acquisition time, and a 2 s T_1 delay. These parameters yield quantitative spectra based on literature reports (e.g., *9*). Although a 90° pulse could reduce run time for a given signal to noise ratio, it also requires inversion recovery experiments to be conducted for every sample to optimize interpulse delay times. Our approach, with a 30° tip angle and a conservative delay time of 2.64 s, produces quantitative spectra and is the logical approach when analyzing a number of samples likely to vary in their T_1 constants if one does not have access to a large amount of instrument time. This is reflected in the widespread use of such parameters in the NMR literature. By selecting such parameters we avoided the need to determine sample-specific T_1 constants that would otherwise negate any time saving from running experiments with 90° tip angles and smaller numbers of scans. Further, the benefit of any reduced run time would have a negligible influence on measured signals, given

that the degradation of compounds such as RNA is complete within minutes in the NaOH-EDTA solution (*10*).

The number of scans varied from ~1000 for authentic compounds to ~30,000 for soil extracts to ensure acceptable signal to noise ratios. Spectra were processed in NMR Utility Transform Software (NUTS) version 1.0.1 (Acorn NMR Inc., Livermore, CA). Spectra were first plotted with a line broadening of 5 Hz and chemical shifts of signals were determined in parts per million (ppm) relative to methylene diphosphonic acid (MDP) in a co-axial insert set as δ = 17.10 ppm after its comparison to an external orthophosphoric acid standard (85%) set as $\delta = 0$ ppm. Signals were subsequently adjusted using the chemical shift of *scyllo*-inositol hexakisphosphate (4.03 ppm) to facilitate signal identification among spectra. Signals were assigned to phosphorus compounds based on literature reports of model compounds spiked in NaOH–EDTA soil extracts (*10*). Signal areas were calculated by integration and deconvolution was performed on the region between δ = 7.5 and 3 ppm to separate orthophosphate from phosphomonoesters and to quantify signals from inositol phosphates. Finally, spectra were plotted with 1 Hz line broadening to show fine resolution in the phosphomonoester region.

Lower-order inositol phosphates

As all other organic phosphorus compounds are destroyed by hypobromite oxidation (*8*), we assume that phosphomonoesters in brominated extracts approximate the total inositol phosphate pool. In addition to the hexakisphosphates, the total inositol phosphate pool potentially includes pentakis-, tetrakis-, tris-, bis, and monophosphates. The hexakisphosphates resist hypobromite oxidation, although the extent to which this applies to the lower-order esters is unknown. However, resistance presumably declines from pentakisphosphates to monophosphates,

suggesting that most of the lower-order esters resisting bromination are penta- and tetrakisphosphates.

The proportion of lower-order inositol phosphates resisting bromination was calculated by subtracting the total IP_6 (i.e., all four stereoisomeric forms) from the total inositol phosphate in brominated extracts (i.e., the phosphomonoesters resisting bromination). Lower-order inositol phosphates in the three soils constituted between 14.2 and 16.4% of the total extracted P (mean 15.5 ± 1.2%), which represented 17.7–20.6% of the total organic P (mean 19.6 ± 1.7%) (Table S2). As a proportion of the total inositol phosphate, lower-order esters constituted between 27.9 and 29.5% (mean 28.6 ± 0.8%) (Table S2). Thus, about a fifth of the soil organic phosphorus and a quarter of the total inositol phosphate occurred as lower-order inositol phosphates. These values may be slight overestimates given the possibility that the unidentified signal at $\delta = 4.3$ ppm was increased by hypobromite oxidation and might not represent an inositol phosphate. Given the quantitative importance of the lower-order inositol phosphates in these soils, further work is now required to enable their identification in soil extracts and to determine the extent to which the various esters and stereoisomeric forms resist hypobromite oxidation.

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Table S1. Location and properties of three soils from the Falkland Islands used in this study.

Property	Soil 1 (East 50)	Soil 2 (West 18)	Soil 3 (West 22)
Site name	Brenton Loch	Fow Bay	Roy Cove
Latitude (S)	51° 46′ 35″	51° 50′ 30″	51° 33′ 06″
Longitude (W)	59° 03′ 56″	60° 03′ 49″	60° 23′ 02″
Vegetation	*Cortaderia pilosa* plus *Baccharis magellanica*	*Cortaderia pilosa*	Reseeded pasture
Total C (g C kg^{-1})	149	232	106
Total N (g N kg^{-1})	11.2	15.7	8.2
C:N	13.3	14.8	12.9
pH (water)	5.17	5.15	5.40
Total P (mg P kg^{-1})	1107	885	753
NaOH–EDTA extractable P[a] (mg P kg^{-1})	1001 (90%)	851 (96%)	674 (90%)

[a] Values in parentheses are the proportion (%) of the total phosphorus recovered in NaOH–EDTA.

Table S2. Summary of inositol phosphates in NaOH–EDTA extracts of three soils from the Falkland Islands determined by hypobromite oxidation and solution ^{31}P NMR spectroscopy. Average values are the mean ± standard deviation of the three soils. SA, spectral area, P_o; organic phosphorus; IP, inositol phosphate.

Soil	Total IP[a]	Total inositol hexakisphosphate[b]		Lower inositol phosphate esters[c]		
	—% SA—	—% SA—	—% P_o—	—% SA—	—% P_o—	—% total IP—
East 50	57.0	41.1	53.6	15.9	20.6	27.9
West 18	55.7	39.3	47.4	16.4	20.6	29.5
West 22	50.2	36.0	45.6	14.2	17.7	28.3
Average	54.3 ± 3.6	38.8 ± 2.6	48.9 ± 4.2	15.5 ± 1.2	19.6 ± 1.7	28.6 ± 0.8

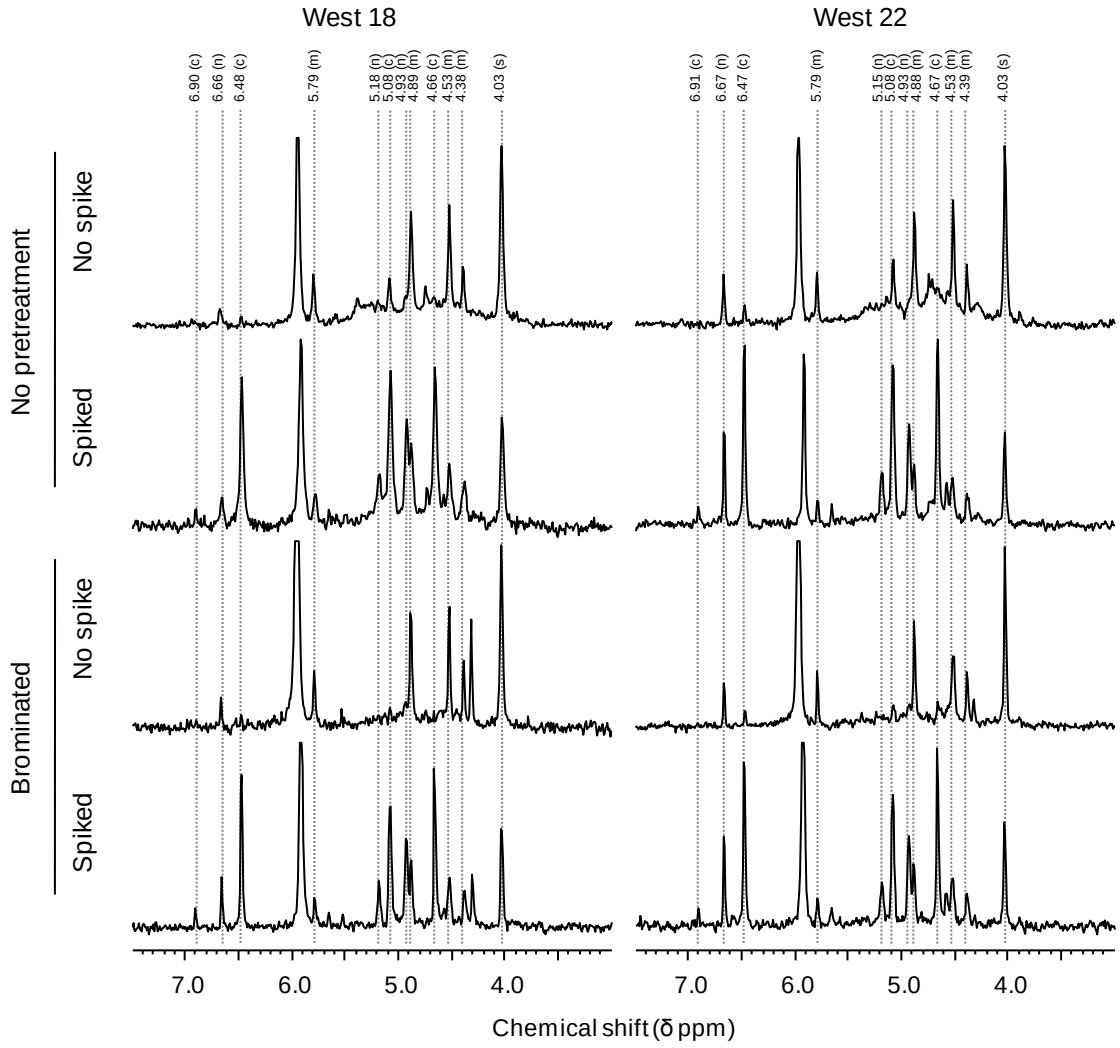
[a] Phosphomonoesters resisting hypobromite oxidation (from Table 2 in the main manuscript).

[b] Total inositol hexakisphosphate in all four stereoisomeric forms (from Table 3 in the main manuscript).

[c] Potentially including inositol pentakis-, tetrakis-, tris-, bis-, and monophosphates, although likely to be mainly the pentakis- and tetrakisphosphates.

Figure S1. Solution ^{31}P NMR spectra of NaOH–EDTA extracts of two soils from the Falkland Islands showing the phosphomonoester region in detail (δ = 3.0 to 7.5 ppm). The following treatments are shown (from top to bottom): untreated, untreated and spiked with a mixture of *neo*- and D-*chiro*-inositol hexakisphosphate (IP_6), pretreated by hypobromite oxidation, pretreated with hypobromite oxidation and spiked with a mixture of *neo*- and D -*chiro*-IP_6. Signal assignments are from the untreated spiked sample and assigned to *myo* (m), *scyllo* (s), D-*chiro* (c), or *neo* (n) IP_6 stereoisomers. Inorganic orthophosphate is the large signal close to δ = 6.00 ppm. Spectra are plotted with 1 Hz line broadening and referenced to the chemical shift of *scyllo*-IP_6 in spectrum A (δ = 4.03 ppm). The signal at 4.58 ppm from the two axial phosphates of the 4-equatorial/2-axial conformer of *neo*-IP_6 were small in the spike solution and were not detectable in soil extracts.

Figure S1


West 18
West 22
6.90 (c)
6.66 (n)
6.48 (c)
5.79 (m)
5.18 (n)
5.08 (c)
4.93 (n)
4.89 (m)
4.66 (c)
4.53 (m)
4.38 (m)
4.03 (s)
6.91 (c)
6.67 (n)
6.47 (c)
5.79 (m)
5.15 (n)
5.08 (c)
4.93 (n)
4.88 (m)
4.67 (c)
4.53 (m)
4.39 (m)
4.03 (s)
No pretreatment
Brominated
No spike
Spiked
No spike
Spiked
7.0
6.0
5.0
4.0
Chemical shift (δ ppm)