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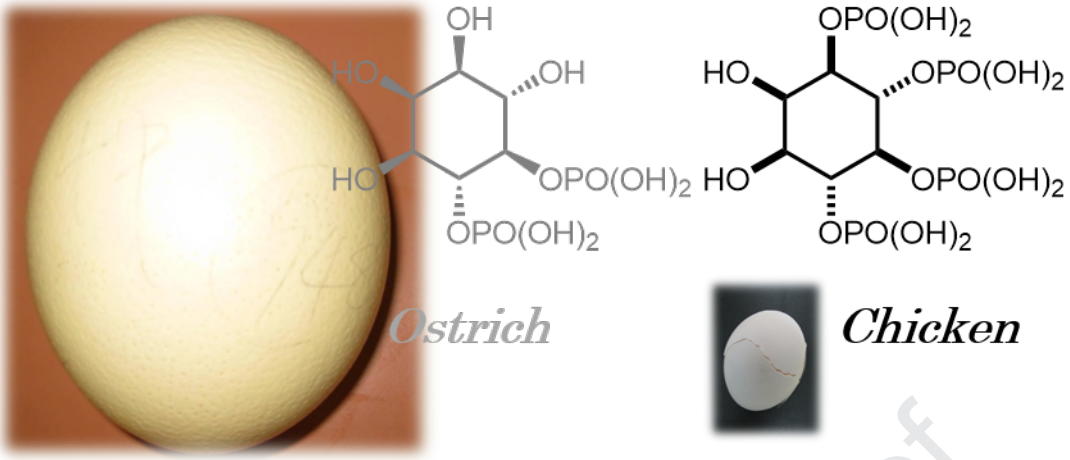

Inositol tetrakisphosphate from chicken eggshell

Taku Ito,[a] Hajime Itokawa,[a] Takanori Miyaki,[a] Miho Kamimura,[a] Mariko Hamano,[a] Masaya Nakata,[a] and Yoko Saikawa[a]*

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Abstract: Unlike our previous study that identified D-*myo*-inositol 4,5-bisphosphate (Ins(4,5)P_2, 1) from ostrich eggshell, the compound *myo*-inositol 1,4,5,6-tetrakisphosphate (Ins(1,4,5,6)P_4, 2) was isolated as an almost racemic mixture from the internal region of chicken eggshell. Furthermore, 2, 2-[^{2}H]-2, and amorphous $CaCO_3$ were prepared as tools for assessment about transformation of $CaCO_3$ during bone formation.

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Keywords: inositol phosphate • eggshell • labeled compound • amorphous calcium carbonate

1. Introduction

Approximately 250,000 tons of chicken eggshell waste is produced annually worldwide after the yolk and albumen are processed for food. About 80% of the eggshells are simply burned and the rest are used as fertilizer and supplements, or in investigations of new applications.[1,2] Eggshell calcium, in particular, exhibits a unique feature as a calcium supplement which is easier to digest than pure calcium carbonate, as demonstrated by reports involving piglet feeding experiments[3] and suppression of bone loss in mice after ovariectomy by feeding eggshells to mice.[4] These results are not surprising since some eggshell calcium is resorbed by a chick embryo during its bone formation.[5] As a representative biomineral,[6] eggshell is regarded as a hybrid of inorganic ($CaCO_3$) and organic materials, although chicken eggshell consists of mostly $CaCO_3$ (97%).[7] The rest of eggshell components are mainly composed of proteins whose roles for biomineralization are known to be nucleation of $CaCO_3$ precipitation, promotion of faster mineralization, and regulation of the size of calcite crystals.[8] However, a key molecule which regulates the structure and composition of $CaCO_3$ for smooth dissolution as the chick embryo resorbs eggshell during embryogenesis was unknown. Therefore, we have focused on the eggshell components particularly in the internal region of eggshells where dissolution of $CaCO_3$ occurs. In our previous paper, we reported that D-*myo*-inositol 4,5-bisphosphate (Ins(4,5)P_2, **1**) was identified in ostrich eggshell and **1** stabilized amorphous calcium carbonate (ACC), a soluble polymorph of $CaCO_3$ (Figure 1).[9] In addition, **1** was turned out to be localized in an internal region of the ostrich eggshell, suggesting the organophosphate as a regulator of $CaCO_3$ polymorph to assist smooth dissolution of ostrich eggshell. To evaluate the contribution of the above organophosphate to resorption of calcium ion by the

embryo, we planned to feed the ACC prepared in the presence of the organophosphate to an embryo in shell-less culture, where an embryo is cultured in an artificial vessel and requires a calcium source.[5] Although large size of ostrich eggshells allowed chemical and analytical approaches, the shell-less culture of ostrich is experimentally difficult. Therefore, we planned to use chicken embryo. We examined the interior of chicken eggshell and identified *myo*-inositol 1,4,5,6-tetrakisphosphate (Ins(1,4,5,6)P_4, **2**) as a major organophosphate (Figure 1).

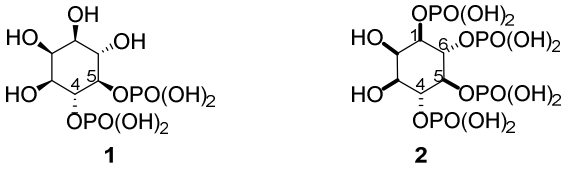


Fig. 1. D-*myo*-inositol 4,5-bisphosphate (Ins(4,5)P_2, **1**) and *myo*-inositol 1,4,5,6-tetrakisphosphate (Ins(1,4,5,6)P_4, **2**).

2. Results and Discussion

2.1. Identification of an organophosphate in chicken eggshell

The chicken eggshell components were extracted according to our previous report for ostrich eggshell.[9] The inside of the eggshell was extracted for one hour by adding 10% acetic acid into the eggshell. The acid etching dissolved $CaCO_3$ in the eggshell, thereby removing the attached outer shell membrane. The crude extract contained abundant calcium acetate, which could be removed by cation exchange chromatography. Treatment of the extract with weakly acidic IRC-76 partially removed the calcium ions to form white precipitates. Since phosphates strongly bind to calcium ion, the precipitates were expected to contain calcium organophosphates. Thus, the precipitates were filtered and subjected to a second cation exchange chromatography using strongly acidic SK1B resin. The ^{1}H NMR spectrum of the resulting yellow syrup contained doublets at around 2.0 ppm assigned to citric acid, and some polyol signals. Its ^{13}C NMR spectrum also contained major signals corresponding to citric acid and several peaks at 69–80 ppm. As expected, the ^{31}P NMR spectrum showed a major broad signal corresponding to phosphoric acid. This singlet signal, however, was slightly asymmetrical, suggesting that another minor phosphorus compound existed in the extract. To determine this, the ^{1}H-^{31}P HMBC spectrum was obtained, which revealed the existence of a minor phosphorus compound as four characteristic cross-peaks. ^{31}P-correlated ^{1}H signals were observed near 4 ppm that were similar to those of Ins(4,5)P_2. After continuous liquid-liquid extraction using diethyl ether to remove the abundant citric acid and phosphoric acid, anion exchange chromatography was performed with an Accell QMA Sep-pak, which was used to separate inositol phosphates.[10] The extracts were loaded on the Sep-pak cartridge, and successively washed with aqueous ammonium formate at different concentrations. The major component eluted with 0.3 M ammonium formate and was recovered after lyophilization. The ^{1}H NMR spectrum of the major component showed six signals at around 4 ppm and four signals that were correlated to

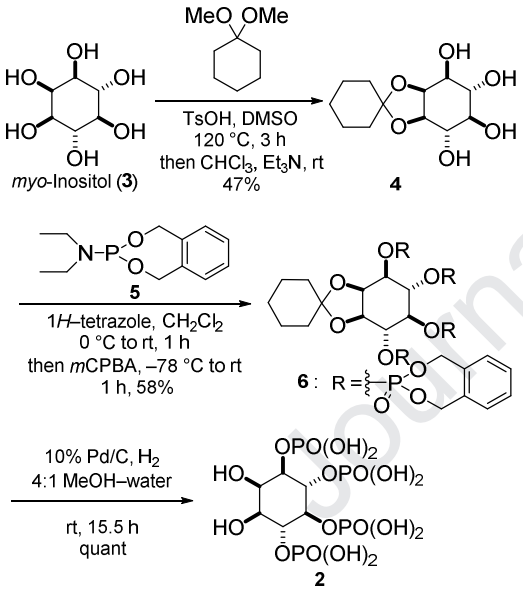
phosphorus by the above ^{1}H-^{31}P HMBC spectrum. A combination of DQF-COSY to analyze the coupling constants and HMBC to determine the carbon skeleton suggested that the structure of this component was *myo*-inositol 1,4,5,6-tetrakisphosphate (Ins(1,4,5,6)P_4, **2**)[11,12] whose structure was confirmed by synthetic methods (vide infra).[12] Curiously, the optical rotation of the isolated compound was nearly zero. The reported absolute value of the optical rotation of *myo*-inositol 1,4,5,6-tetrakisphosphate is small and is dependent on the pH of the solution; the maximum absolute value reported was 10.2 in aqueous NaOH solution.[13] However, that of the isolated **2** in the basic solution prepared by adding NaOH was -0.77.

Both enantiomers, D-*myo*-inositol 1,4,5,6-tetrakisphosphate ((−)-**2**)[14] and D-*myo*-inositol 3,4,5,6-tetrakisphosphate ((+)-**2**),[15] have been identified in mixtures of intracellular metabolic products of inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and inositol 1,3,4,5,6-pentakisphosphate (Ins(1,3,4,5,6,)P_5).[16] In these reports, 2-[^{3}H] inositol was pre-incubated with the cells and the labeled metabolites were separated and detected. In addition, the absolute configuration of the labeled **2** was determined by using enzymatic conversion of one enantiomer into Ins(1,3,4,5,6)P_5[14,15] or multistep degradation followed by enzymatic oxidation.[17] In spite of the subtle experiments, stereochemical complexities as seen in the present study were often reported. The enantiomeric intricacy of the previously-isolated metabolite **2** was rationalized by the following possibilities: acid-mediated interconversion of both enantiomers via phosphate migration in the purification process (e.g. during solid phase extraction using 0.5 M HCl),[17] multi-enzymatic phosphorylation-dephosphorylation process via Ins(1,4,5)P_3 or Ins(1,3,4,5,6)P_5,[16] or enzymatic direct conversion of enantiomers.[18] However, the reason why Ins(1,4,5,6)P_4 **2** was isolated as a racemic form in our case is not clear.

In regard to the other possibilities, we believe that (−)- and (+)-**2** in chicken eggshell are metabolites of Ins(1,3,4,5,6)P_5, which is the major component that binds to hemoglobin in the erythrocytes of birds, except for ostriches.[19]. Eggshell forms in the oviduct surrounded by the blood vessels; through the blood vessels, calcium ions[20] and cuticle pigments (the degradation products of blood erythrocytes)[21] are supplied for shell formation. Similar to these components, Ins(1,4,5,6)P_4 is expected to be supplied from the blood of the mother bird. It is also expected that both enantiomers **2** are supplied through dephosphorylation of Ins(1,3,4,5,6)P_5. We assume the reason for the difference in eggshell components (Ins(4,5)P_2 **1** from ostrich eggshell and Ins(1,4,5,6)P_4 **2** from chicken eggshell) as follows. Most avian erythrocytes have a large quantity of Ins(1,3,4,5,6)P_5[19] whereas inositol tetrakisphosphate, whose entire structure was unknown, was isolated as a major organophosphate from ostrich erythrocytes.[22] The difference in inositol phosphates between ostrich and chicken eggshells might stem from the difference in inositol phosphates in mother erythrocytes.

2.2. Synthesis of racemic Ins(1,4,5,6)P_4 2 and ACC preparation

As it turned out that Ins (1,4,5,6)P_4 **2** was the major organophosphate in chicken eggshell, we next forward to synthesis of **2** and preparation of ACC in the presence of **2** as a calcium source to be fed shell-less culture. Synthesis of *myo*-inositol 1,4,5,6-tetrakisphosphate has already been reported.[12] This synthetic method was modified to make it shorter and more efficient. A vicinal *cis*-diol moiety of *myo*-inositol (**3**) were converted selectively to an acetal with 1,1-dimethylcyclohexane and *p*-toluene sulfonic acid in DMSO.[23] When the crude mixture was treated with triethylamine and $CHCl_3$, a white precipitate formed. The precipitate was collected by filtration and washed with a mixture of $CHCl_3$, methanol, and water. The washings were concentrated to give the desired acetal **4**. Then, the other four hydroxy groups of acetal **4** were phosphorylated with freshly prepared amidite reagent **5** activated by tetrazole followed by *m*CPBA oxidation to give tetrakisphosphate **6** in 54% yield.[24] Finally, phosphate **6** was subjected to hydrogenation catalyzed by palladium on carbon to cleave the benzene dimethanol ester, and the resulting phosphoric acid acted as an acid catalyst to hydrolyze the acetal moiety to afford *myo*-inositol 1,4,5,6-tetrakis phosphate (**2**) as a free acid.[13]



Scheme 1. Synthesis of **2**.

In our previous report,[9] ACC preparation in the presence of Ins(4,5)P_2 **1** was successful in Tris-HCl buffer, according to literal method;[25] 100 mM $NaHCO_3$ with 2 mM of **1** in the buffer was mixed with 100 mM $CaCl_2$ were mixed to give white nano-sized precipitates. Similarly, we succeeded in preparing ACC by addition of Ins(1,4,5,6)P_4 **2** (2 mM) by same method as above; the ability to stabilize ACC was comparable to those of **1**. However, the method to prepare ACC required modification since the ACC prepared by this procedure would contain other ions such as sodium, ammonium, and chloride ion, the latter of which exerts a fatal toxic effect on chick embryos.[26] Thus, we adopted a method with bubbling CO_2 into $Ca(OH)_2$ solution. Through several investigation of conditions such as concentration of $Ca(OH)_2$ and timing of bubbling, the

ACC without the above ions was obtained by the following procedure; Ins(1,4,5,6)P_4 **2** was dissolved in water and bubbled CO_2 and then to the solution was added a cold solution of 13 mM $Ca(OH)_2$. The resulting white suspension was directly evaporated to give the ACC precipitates whose polymorph was determined by X-ray diffraction (XRD). As similar to the previously reported ACC, the peaks corresponding to calcite, the most stable polymorph of $CaCO_3$, was not observed (Fig. 2), indicating formation of the desired amorphos particles.

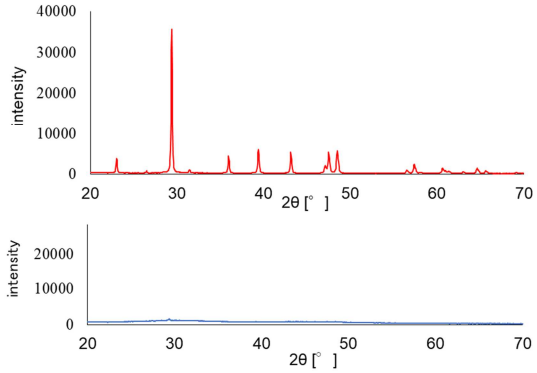
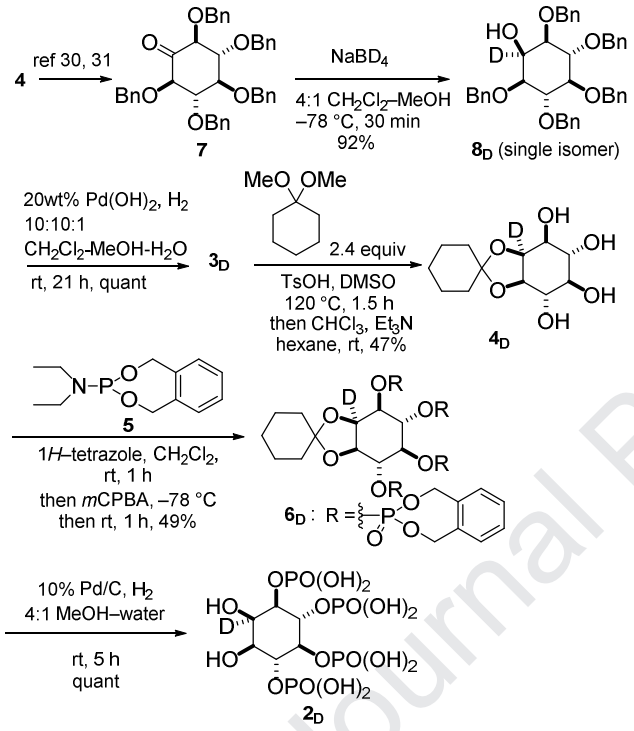


Fig. 2. XRD spectra of the $CaCO_3$ precipitates in absense (upper) and in presence (lower) of 0.1 mM **2**.

2.3. Synthesis of 2-[^{2}H]-Ins(1,4,5,6)P_4 $\mathbf{2_D}$

Since inositol phosphates are responsible for various physiological cellular responses,[16] our interest lies on whether the inositol phosphates in avian eggshell are resorbed by chick embryo concomitant with calcium resorption, and how the compounds are metabolized. As mentioned above, permeation of ^{3}H-labeled inositol into various cells followed by recovery of labeled metabolites were well investigated to examine metabolic pathway of inositol phosphates. These labeling studies have been replaced by LC-MS metabolomics analysis and ELISA (Enzyme-Linked Immunosorbent Assay) so far, probably due to limitation of radioisotopes usage. We envisioned mass-imaging analysis[9,27] and NMR method to detect the metabolite of **2** in shell-less culture, therefore ^{2}H-labeled **2** was designed. At first, we investigated the late-stage introduction of deuterium; such as deprotection of acetal **6** followed by conversion of the resulting 2,3-diol. However, any conversions after phosphorylation were unsuccessful. Thus, synthesis of **2** via 2-[^{2}H]-inositol ($\mathbf{3_D}$) was attempted (Scheme 2). The report for the synthesis of $\mathbf{3_D}$ involves deuteride reduction of *scyllo*-inosose which is prepared by microbiological oxidation of **3**,[28] but the direct reduction of *scyllo*-inosose provides a mixture of diastereomers.[29] As a practical synthesis of $\mathbf{3_D}$, the afore-mentioned acetal **4** was used and converted to benzyl *myo*-inosose **7** via transformation of functionalities with combination of the known procedures.[30,31] Reduction of the resulting benzyl *scyllo*-inosose **7** with $NaBD_4$ required a modification. In the reported procedure, $NaBH_4$ reduction was conducted at 0 ºC to give the protected *myo*-inositol with its epimer

in a ratio of ~4:1.[31] We performed $NaBD_4$ reduction at −78 ºC to provide $\mathbf{8_D}$ as a single isomer.[32] The following hydrogenation catalyzed by $Pd(OH)_2$ in a mixed solvent afforded $\mathbf{3_D}$ quantitavely. The obtained $\mathbf{3_D}$ was converted to 2-[^{2}H]-Ins(1,4,5,6)P_4 $\mathbf{2_D}$ by similar manner to the route for the synthesis of **2** (Scheme 1). In the acetalization, the amount of $\mathbf{3_D}$, much more costly than inositol (**3**), was reduced and the product was recovered as precipitates by addition of hexane. The cyclohexylidene $\mathbf{4_D}$ was phosphorylated with **5** and the resulting phosphate $\mathbf{6_D}$ was subjected to hydrogenation to give the desired $\mathbf{2_D}$.



Scheme 2. Synthesis of $\mathbf{2_D}$ via deuteride reduction.

3. Conclusion

In summary, we identified *myo*-inositol 1,4,5,6-tetrakisphosphate (**2**) as a racemic mixture from chicken eggshell. The difference in inositol phosphates between ostrich and chicken eggshells was revealed and the result implies that these inositol phosphates in avian eggshell might be supplied from erythrocytes of mother birds and that the inositol phosphate might be resorbed by chick embryo during dissolution of eggshell. Now that ACC prepared in presence of **2** is ready for assay and 2-[^{2}H]-*myo*-inositol 1,4,5,6-tetrakisphosphate ($\mathbf{2_D}$) is available, we will discuss contribution of inositol phosphates in avian eggshell during embryonic resorption of calcium ion in shell-less culture of chicken embryo, in near future.

4. Experimental

4.1. General

Optical rotations were measured on a P-2100 polarimeter (JASCO, Tokyo). IR spectra were recorded on an ALPHA FT-IR spectrometer (Bruker, Billerica, USA). The NMR spectra were recorded on an ECA-500 spectrometer (JEOL, Tokyo, Japan), an ECS-400 spectrometer (JEOL, Tokyo, Japan), and ECZ800R (JEOL, Tokyo, Japan) at ambient temperature. The high-resolution electron spray ionization (ESI) mass spectra were recorded on a LCT Premier XE (Waters, Milford, USA) by direct injection with solvent (50% MeCN–0.1% aqueous formic acid). X-ray diffraction (XRD) was measured on an AXS D8 ADVANCE X-ray powder diffractometer (Bruker, Billerica, USA) with Cu-Kα radiation at 40 kV and 40 mA.

4.2. Materials

Chicken eggshells: For chicken eggs, commercial ones were used. The eggs were cut at the broad pole by an eggshell cutter to form a hole of approximately 3 cm in diameter. After removal of yolk, albumen, and inner shell membrane by exfoliating, the eggshells with outer shell membrane were stored at 4 ºC until use.

4.3. Extraction and purification of organophosphate in chicken eggshell

The inside of the eggshell (2.04 kg, 410 eggshells) was filled with 10% aqueous acetic acid for 1 h at room temperature. The solution was filtered through filter paper (No. 5A, Kiriyama, Tokyo, Japan) under suction. Each filtrate from 25-30 eggshells were decalcified by cation exchange chromatography (Amberlite IRC-76, 400 mL, H^+ form, Organo, Tokyo, Japan) and the resin was filtered off and washed with water (2 L). The above resin treatment was repeated until all of the filtrate was treated. The filtrate and washings were combined and concentrated *in vacuo*. Then H_2O (3 L) was added to the residue, and white precipitates (1.77 g) were collected by centrifugal separation. The precipitates were decalcified by cation exchange resin (DIAION SK1B, 40 mL, H^+ form), and the resin was filtered off and washed with water. The filtrate and washings were combined and concentrated *in vacuo*. The residue (950 mg) was dissolved in H_2O (3 mL) and continuously extracted with diethyl ether for 14 days by using Soxhlet's apparatus. The H_2O layer was concentrated *in vacuo*, and the residue was obtained (76.6 mg). A part of the residue (18.1 mg) was subjected to anion exchange chromatography (Accell QMA SEP-PAK, HCO_2^- form, Waters, Milford, USA). The resin was successively washed with water (Fr. 1, 14.6 mL), 0.1 M aqueous NH_4HCO_2 (Fr. 2, pH 4.60, 7.3 mL), 0.2 M aqueous NH_4HCO_2 (Fr. 3, pH 4.78, 7.3 mL), 0.3 M aqueous NH_4HCO_2 (Fr. 4, pH 4.82, 7.3 mL), 0.4 M aqueous NH_4HCO_2 (Fr. 5, pH 4.73, 7.3 mL), and 1.0 M aqueous NH_4HCO_2 (Fr. 6, pH 4.75, 7.3 mL). Fr. 4 was lyophilized to give the residue (16.5 mg) which contained (±)-*myo*-inositol 1,4,5,6-tetrakisphosphate (**2**).

*4.3.1. Analytical data of (±)-myo-inositol 1,4,5,6-tetrakisphosphate (**2**)*: a colorless foam; $[\alpha]_D$ −0.77 (*c* 0.84, H_2O, sodium salt) (D-myo-inositol 1,4,5,6-tetrakisphosphate lit.[13] $[\alpha]_D$ −10.2 (*c* 2.46, H_2O, sodium salt); ^1H NMR (500 MHz, D_2O, DSS = 0.00 as an external standard): δ 3.76 (dd, *J* = 9.3, 2.5 Hz, 1H, H-3), 4.13 (ddd, *J* = 9.5, 9.5, 2.5 Hz, 1H, H-1), 4.17 (ddd, *J* = 9.5, 9.5, 9.3 Hz, 1H, H-5), 4.25 (br dd, 1H, H-2), 4.35 (ddd, *J* =9.3, 9.3,

9.0 Hz, 1H, H-4), 4.47 (ddd, *J* = 9.5, 9.5, 9.5 Hz, 1H, H-6); ^{13}C NMR (200 MHz, D_2O, DSS = 0.00 as an external standard): δ 72.8 (C3), 73.7 (C2), 77.2 (C1), 79.0 (C6), 79.7 (C4), 80.3 (C5); ^{31}P NMR (202 MHz, D_2O, H_3PO_4 = 0.00 as an external standard): δ −0.13 (P-1), 0.58 (P-6), 0.66 (P-4), 0.73 (P-5); ^{31}P chemical shifts were determined by ^{1}H-^{31}P HMBC.

4.4. Synthesis of (±)-Ins(1,4,5,6)P_4

*4.4.1. 1,2-O-Cyclohexylidene-myo-inositol (**4**).*[23b] A mixture of inositol (**3**) (1.00 g, 5.56 mmol), 1,1-dimethoxycyclohexane (0.42 mL, 2.8 mmol), TsOH•H_2O (25.7 mg, 0.132 mmol), and DMSO (1.1 mL) was stirred at 120 ºC for 3 h under Ar atmosphere. After cooling to rt, $CHCl_3$ (16.6 mL) and Et_3N (0.002 mL) were added with stirring to give precipitates. The precipitates was filtered off and the filtrate was concentrated. The resulting residue was suspended in 5:3:0.1 $CHCl_3$–MeOH–H_2O and the precipitates was filtered and washed to give **4** (0.337 g, 47%, based on 1,1-dimethoxycyclohexane); R_f = 0.73 (5:3:0.1 $CHCl_3$–MeOH–H_2O); ^{1}H NMR (400 MHz, D_2O, HOD = 4.79): δ 1.36–1.74 (m, 10H, cyclohexylidene), 3.23 (dd, *J* = 11.5, 9.6 Hz, 1H, H-5), 3.55 (dd, *J* = 11.5, 8.0 Hz, 1H, H-6), 3.62 (dd, *J* = 9.6, 9.6 Hz, 1H, H-4), 3.82 (dd, *J* = 9.6, 4.2 Hz, 1H, H-3), 4.02 (dd, *J* = 8.0, 4.8 Hz, 1H, H-1), 4.45 (dd, *J* = 4.8, 4.2 Hz, 1H, H-2).

*4.4.2. 2,3-O-Cyclohexylidene-1,4,5,6-tetrakis-O-(1,5-dihydro-3-oxido-2,4,3-benzodioxaphosphepin-3-yl) myo-inositol (**6**).* To a stirred solution of **4** (800 mg, 3.08 mmol) in dry CH_2Cl_2 (20.7 mL) were added at 0 ºC 1*H*-tetrazole (1.94 g, 27.7 mmol) and *N*,*N*-diethyl-1,5-dihydro-2,4,3-benzodioxaphosphepin-3-amine (**5**)[24] (3.99 mL, 18.5 mmol). After 1 h at rt, a solution of *m*CPBA (6.83 g, 27.7 mmol, contains *ca.* 30% water) in dry CH_2Cl_2 (31.9 mL) was added dropwise at –78 ºC before the mixture was warmed to rt and stirred for 1 h at rt. Then the mixture was diluted with CH_2Cl_2 and washed consecutively with aqueous $NaHSO_3$, saturated aqueous $NaHCO_3$, and saturated aqueous NaCl, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (230 g, 3:1 $CHCl_3$–acetone) followed by precipitation in EtOAc to give **6** (1.73 g, 58%) as white solids; R_f = 0.40 (2:1 EtOAc–benzene); mp: 205 ºC (dec); IR (KBr, ν_{max} cm^{-1}) 3483, 2938, 2898, 1462, 1371, 1294, 1225, 1020, 849, 733; ^{1}H NMR (400 MHz, $CDCl_3$, $CHCl_3$ = 7.26): δ 1.40–1.95 (m, 10H, C_6H_{10}), 4.35 (dd, *J* = 7.8, 4.6 Hz, 1H, H-3), 4.79 (dd, *J* = 4.6, 4.4 Hz, 1H, H-2), 4.87 (dd, *J* = 13.2, 11.6 Hz, 1H, CH_2Ph), 4.90 (ddd, *J* = 9.6, 9.4, 9.4 Hz , 1H, H-5), 4.94 (ddd, *J* = 9.4, 9.2, 7.8 Hz, 1H, H-4), 5.01 (overlapped, 1H, H-1), 4.99–5.21 (m, 7H, CH_2Ph), 5.33 (ddd, *J* = 9.4, 9.2, 9.2 Hz , 1H, H-6), 5.36 (dd, *J* = 13.6, 12.4 Hz, 1H, CH_2Ph), 5.45–5.72 (m, 7H, CH_2Ph), 7.29–7.43 (m, 16H, C_6H_4); ^{13}C NMR (125 MHz, $CDCl_3$, $CHCl_3$ = 77.00) δ 23.68, 24.78, 29.62, 35.30, 37.54, 68.62 (d, *J* = 6.5 Hz), 69.08 (d, *J* = 7.8 Hz), 69.14 (d, *J* = 7.3 Hz), 69.25 (d, *J* = 7.8 Hz), 69.36 (d, *J* = 8.0 Hz), 69.43 (d, *J* = 6.2 Hz), 69.50 (d, *J* = 7.8 Hz), 69.84 (d, *J* = 7.8 Hz), 73.74 (C-2), 74.19 (bd, C-1), 76.31 (C-3), 76.41 (bd, C-6), 77.00 (C-4), 79.11 (C-5), 112.49, 128.81, 128.99, 129.11 (3C), 129.20 (4C), 129.28, 129.36 (2C), 129.45 (3C), 129.51, 135.17, 135.21, 136.46 (2C), 135.60, 135.69, 135.74, 135.81; ^{31}P NMR (202 MHz, $CDCl_3$, external reference:

triphenyl phosphate = –17.3) δ –1.95, –4.16, –4.24, –4.99; HRMS (*m/z*): $[M+H]^+$ calcd. for $C_{44}H_{48}O_{18}P_4$, 989.1868; found 989.1869.

*4.4.3. 1,4,5,6-Tetrakis(dihydrogen phosphate) myo-inositol (**2**).*[13] To a stirred solution of **6** (19.9 mg, 0.0202 mmol) in 4:1 MeOH–H_2O (2.17 mL) was added at 0 ºC 10% Pd on carbon (26.9 mg). The mixture was vacuum degassed three times and refilled with Ar before replaced by H_2. After 15.5 h at rt, the mixture was filtered through filter paper under suction and the filtrate was concentrated *in vacuo* to afford **2** (quant); R_f = 0.00 (3:1 $CHCl_3$–acetone); ^{1}H NMR (500 MHz, D_2O, DSS = 0.00 as an external standard): δ 3.75 (dd, *J* = 9.3, 2.5 Hz, 1H, H-3), 4.12 (ddd, *J* = 9.5, 9.5, 2.5 Hz, 1H, H-1), 4.16 (ddd, *J* = 9.5, 9.5, 9.3 Hz, 1H, H-5), 4.25 (br dd, 1H, H-2), 4.34 (ddd, *J* =9.3, 9.3, 9.0 Hz, 1H, H-4), 4.46 (ddd, *J* = 9.5, 9.5, 9.5 Hz, 1H, H-6); ^{13}C NMR (125 MHz, D_2O, DSS = 0.00 as an external standard): δ 72.7 (C3), 73.6 (C2), 77.2 (C1), 79.0 (C6), 79.7 (C4), 80.3 (C5); ^{31}P NMR (202 MHz, D_2O, H_3PO_4 = 0.00 as an external standard): δ −0.13 (P-1), 0.58 (P-6), 0.58 (P-4), 0.65 (P-5).; ^{31}P chemical shifts were determined by ^{1}H-^{31}P HMBC.

4.5. Preparation of ACC in the presence of 2.

To a cooled (0 ºC), aqueous solution of **2** (0.40 mM in 50 mL water) was bubbled with CO_2 until saturation. Then a cooled solution of $Ca(OH)_2$ (13 mM in 150 mL water) was dropped into the above CO_2-filled solution over 0.5 min with stirring at 0 ºC. Then the suspension was filtered and the filtrate was dried *in vacuo* to give a white ACC powder. The obtained ACC powder was put into the well of 25 mm in diameter of glass sample holder and pressed flat with a glass slide. The XRDs were measured to determine the polymorph of the above sample; the intensity of calcite signal compared with those of control sample (calcite calcium carbonate) is assessed as it is inversely correlated with the content of ACC.

4.6. Synthesis of 2-[^{2}H]-Ins(1,4,5,6)P_4 2_D

*4.6.1. 1,2,3,4,5-Penta-O-benzyl-myo-inososе (**7**).*[31] Benzyl *scyllo*-inososе **7** was synthesized from cyclohexylidene 4 according to the known procedure.[30,31] **7**;[31] R_f = 0.33 (5:1 hexane–EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$, $CHCl_3$ = 7.26) δ 3.61 (t, *J* =9.2 Hz, 2H, H-4, H-6), 3.86 (t, *J* =9.2 Hz, 1H, H-5), 4.15 (d, *J* =9.2 Hz, 2H, H-1, H-3), 4.53 (d, *J* =11.2 Hz, 2H, CH_2Ph), 4.73–4.93 (m, 8H, CH_2Ph), 7.58–8.11 (m, 25H, C_6H_5).

*4.6.2. 1,3,4,5,6-Penta-O-benzyl-[2-^{2}H] myo-inositol (**8$_D$**).* Benzyl *scyllo*-inososе **7** (1.00 g, 1.59 mmol) was dissolved in 4:1 CH_2Cl_2–MeOH (79.6 mL). To this solution sodium borodeuteride (141 mg, 3.18 mmol) was added in one portion at –78 ºC and the reaction mixture was stirred at –78 ºC for 0.5 h. The reaction was quenched by adding saturated solution of ammonium chloride (1 mL). The resulting mixture was concentrated under reduced pressure and the resulting suspension was extracted with EtOAc. The extract was filtered through a pad of SiO_2 with EtOAc, and the filtrate was concentrated under reduced pressure. The residue was recrystallized in hexane to give **8$_D$** (918 mg, 92%) as colorless solids; R_f = 0.47 (2:1 hexane–EtOAc); mp

121.5–124.5 ºC; IR (KBr, ν_{max} cm^{-1}) 3453, 3223, 3088, 3065, 3032, 2920, 2864, 1497, 1454, 1356, 1151, 1133, 1067, 1030, 754, 721, 700.; ^{1}H NMR (500 MHz, $CDCl_3$, $CHCl_3$ = 7.26) δ 2.47 (s, 1H, OH) 3.38 (d, *J* =9.5 Hz, 2H, H-1, H-3), 3.45 (dd, *J* =9.5, 9.5 Hz, 1H, H-5), 4.00 (dd, *J* =9.5, 9.5 Hz, 2H, H-4, H-6), 4.68–4.91 (m, 10H, CH_2Ph), 7.24–7.35 (m, 25H, C_6H_5).; ^{13}C NMR (125 MHz, $CDCl_3$, $CHCl_3$ = 77.00) δ 66.94 (bt, C2), 72.72 (CH_2Ph), 72.72 (CH_2Ph), 75.93 (CH_2Ph), 75.93 (CH_2Ph), 75.93 (CH_2Ph), 79.69 (C1, C3), 81.17 (C4, C6), 83.13 (C5), 127.54 (Ph), 127.56 (Ph), 127.56 (Ph), 127.82 (Ph), 127.82 (Ph), 127.82 (Ph), 127.82 (Ph), 127.85 (Ph), 127.85 (Ph), 127.85 (Ph), 127.85 (Ph), 127.85 (Ph), 127.99 (Ph), 127.99 (Ph), 127.99 (Ph), 127.99 (Ph), 128.33 (Ph), 128.33 (Ph), 128.33 (Ph), 128.33 (Ph), 128.33 (Ph), 128.45 (Ph), 128.45 (Ph), 128.45 (Ph), 128.45 (Ph), 137.91 (Ph), 137.91 (Ph), 138.64 (Ph), 138.69 (Ph), 138.69 (Ph).; HRMS m/*z* $(M+Na)^+$ calcd for $C_{41}H_{41}DO_6Na$ 654.2942, found 654.2957.

*4.6.3. [2-^{2}H] myo-Inositol (**3$_D$**).*[29a] **8$_D$** (350 mg, 0.554 mmol) and 20 wt% $Pd(OH)_2$ (87.5 mg, 25 wt %) were suspended in 10:10:1 (v/v) CH_2Cl_2–MeOH–H_2O (8.0 mL). The suspension was stirred at rt under H_2 atmosphere for 22 h. The catalyst ($Pd(OH)_2$) was filtered off and the filtrate was evaporated to give **3$_D$**[29a] (105 mg, >100%) as white solids; R_f = 0.00 (3:1 hexane–EtOAc); ^{1}H NMR (400 MHz, D_2O, HOD = 4.79) δ 3.27 (dd, *J* =9.6, 9.6 Hz, 1H, H-5), 3.52 (d, *J* =9.6 Hz, 2H, H-1, H-3), 3.62 (dd, *J* =9.6, 9.6 Hz, 2H, H-4, H-6).

*4.6.4. 1,2-O-Cyclohexylidene-[2-^{2}H]myo-inositol (**4$_D$**).* A mixture of **3$_D$** (40.9 mg, 0.266 mmol), 1,1-dimethoxycyclohexane (0.0815 mL, 0.542 mmol), TsOH-H_2O (2.0 mg, 0.011 mmol), and DMSO (0.2 mL) was stirred at 120 ºC for 1.5 h under Ar atmosphere. After cooling to rt, $CHCl_3$ (1.35 mL), Et_3N (0.002 mL, 0.011 mmol), and hexane (5 ml) were added to give precipitates. Filtration of the suspension and washing with hexane afforded **4$_D$** (27.5 mg, 47%) as white solids; R_f = 0.73 (5:3:0.1 $CHCl_3$–MeOH–H_2O); mp 178.5–182.0 ºC; IR (KBr, ν_{max} cm^{-1}) 3376, 2935, 2859, 1447, 1366, 1279, 1253, 1231, 1165, 1151, 1121, 1067, 1014, 962, 927, 894, 750, 716, 603.; ^{1}H NMR (500 MHz, D_2O, TSP = 0.00) δ 1.39–1.76 (m, 10H, cyclohexylidene), 3.27 (dd, *J* = 10.0, 10.0 Hz, 1H, H-5), 3.58 (dd, *J* = 8.0, 10.0 Hz, 1H, H-6), 3.65 (dd, *J* = 10.0, 10.0 Hz, 1H, H-4), 3.85 (d, *J* = 10.0 Hz, 1H, H-3), 4.06 (d, *J* = 8.0 Hz, 1H, H-1).; ^{13}C NMR (125 MHz, D_2O, TSP = 0.00) δ 26.08, 26.39, 27.10, 37.43, 40.27, 72.34, 75.09, 75.47, 77.90, 78.12 (t, *J* = 22.6 Hz), 80.90, 114.31.; HRMS m/*z* $(M+H)^+$ calcd for $C_{12}H_{20}DO_6$ 262.1401, found 262.1380.

*4.6.5. 2,3-O-Cyclohexylidene-1,4,5,6-tetrakis-O-(1,5-dihydro-3-oxido-2,4,3-benzodioxaphosphepin-3-yl)-[2-^{2}H]myo-inositol (**6$_D$**).* To a solution of **4$_D$** (43.3 mg, 0.164 mmol) and 1*H*-tetrazole (104 mg, 1.48 mmol) in dry CH_2Cl_2 (1.5 mL) was added *N*,*N*-diethyl-1,5-dihydro-2,4,3-benzodioxaphosphepin-3-amine (**5**)[24] (0.21 mL, 0.98 mmol), and the mixture was stirred for 1 h at rt under Ar atmosphere. The mixture was cooled to –78 ºC, and a solution of *m*CPBA (258 mg, 1.48 mmol) in dry CH_2Cl_2 (2.6 mL) was added. Stirring was continued for 1 h at rt. Then the reaction mixture was diluted with CH_2Cl_2 (10 mL × 5) and washed consecutively with aqueous $NaHSO_3$ (10 mL × 5), saturated aqueous $NaHCO_3$ (10 mL × 5), and brine (10 mL × 5). After evaporation of CH_2Cl_2, the resulting oil was chromatographed on silica gel (16 g, 3:1 $CHCl_3$–acetone) to give

the mixture including **6$_D$**. The mixture was recrystallized in EtOAc to give **6$_D$** (79.6 mg, 49%) as colorless solids; R_f = 0.27 (3:1 $CHCl_3$–acetone); mp: >200 ºC (decomp.); IR (KBr, ν_{max} cm^{-1}) 3477, 3000, 2938, 2897, 2861, 1500, 1462, 1371, 1295, 1225, 1196, 1196, 1164, 1124, 1050, 1019, 944, 848, 732, 665, 623, 603, 561, 461, 431.; ^{1}H NMR (500 MHz, $CDCl_3$, $CHCl_3$ = 7.26) δ 1.38–1.97 (m, 10H, C_6H_{10}), 4.32 (d, *J* = 8.0 Hz, 1H, H-3), 4.87 (dd, *J* = 13.5, 7.5 Hz, 1H, CH_2Ph), 4.88 (ddd, *J* = 7.5, 7.5, 7.5 Hz , 1H, H-5), 4.96 (ddd, *J* = 7.5, 7.5, 7.5 Hz, 1H, H-4), 4.98 (overlapped, 1H, H-1) 4.93–5.21 (m, 7H, CH_2Ph), 5.34 (ddd, *J* = 7.5, 7.5, 7.5 Hz , 1H, H-6) 5.35 (dd, *J* = 13.5, 7.5 Hz, 1H, CH_2Ph), 5.45–5.72 (m, 7H, CH_2Ph), 7.30–7.42 (m, 16H, C_6H_4).; ^{13}C NMR (125 MHz, $CDCl_3$, $CDCl_3$ = 77.00) δ 23.72, 23.83, 24.82, 35.37, 37.57, 68.69 (d, *J* = 6.5 Hz), 69.17 (d, *J* = 6.6 Hz), 69.22 (d, *J* = 5.9 Hz), 69.32 (d, *J* = 7.8 Hz), 69.47 (d, *J* = 8.0 Hz), 69.50 (d, *J* = 6.1 Hz), 69.56 (d, *J* = 7.8 Hz), 69.92 (d, *J* = 7.1 Hz), 74.19 (br d, C-1), 76.23 (C-3), 76.14 (b, C-5, C-6), 79.36 (br d, C-4), 90.03 (br t, C-2), 112.60, 128.84, 129.02, 129.16, 129.16, 129.23, 129.23, 129.27, 129.27, 129.30,129.30, 129.35, 129.43,129.43, 129.51, 129.51, 129.59, 135.16, 135.21, 135.48, 135.51, 135.66, 135.73, 135.76, 135.86.; ^{31}P NMR (202 MHz, $CDCl_3$, external reference: triphenyl phosphate = –17.3) δ –1.98, –4.15, –4.24, –4.99.; HRMS m/*z* $(M+H)^+$ calcd for $C_{44}H_{48}DO_{18}P_4$ 990.1932, found 990.1910.

*4.6.6. 1,4,5,6-Tetrakis(dihydrogen phosphate)-[2-^{2}H]myo-inositol (**2$_D$**).* To a suspension of **6$_D$** (41.0 mg, 0.0414 mmol) in 4:1 MeOH–H_2O (3.97 mL) was added 10% Pd–C (49.2 mg) at 0 ºC under Ar atmosphere. The suspension was stirred at rt under H_2 atmosphere for 5 h. The catalyst (Pd–C) was filtered off and the filtrate was evaporated to give (±)-**2$_D$** (21.1 mg, 100%); R_f = 0.00 (3:1 $CHCl_3$–acetone); IR (KBr, ν_{max} cm^{-1}) 3424, 2934, 2378, 2345, 1721, 1688, 1630, 1384, 1152, 1041, 966, 825, 497.; ^{1}H NMR (500 MHz, D_2O, TSP = 0.00) δ 3.81 (d, *J* = 9.5 Hz, 1H, H-3), 4.28 (dd, *J* = 9.5, 9.5 Hz, 1H, H-1), 4.31 (ddd, *J* = 9.5, 9.5, 9.5 Hz, 1H, H-4), 4.44 (ddd, *J* = 9.5, 9.5, 9.5 Hz, 1H, H-5), 4.57 (ddd, *J* = 9.5, 9.5, 9.5 Hz, 1H, H-6).; ^{13}C NMR (125 MHz, D_2O, TSP = 0.00) δ 72.33 (C-3), 72.69 (br t, C2), 77.68 (br d, C-1), 79.45 (br d, C-6), 80.42 (br d, C-4), 80.57 (br d, C-5). ^{31}P NMR (202 MHz, D_2O, external reference: triphenyl phosphate = –17.3): δ −0.25, 0.36 (2P), 0.65.

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Conflicts of interest

There are no conflicts to declare.

Supplemental data

Supplemental data to this article can be found online at..

Notes and references

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32 To determine the stereochemistry of the product, ketone **7** was reduced by $NaBH_4$ under same condition as described. The resulting alcohol was identical with the precursor of **7**, 1,3,4,5,6-pentabenzyl *myo*-inositol.

- *Myo*-inositol 1,4,5,6-tetrakisphosphate was isolated from chicken eggshell.
- The racemic inositol tetrakisphosphate and its deuterated analog were synthesized.
- ACC as a calcium source for chick embryo was prepared.
- The origin of inositol phosphates in eggshell were discussed.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: