

D-6-Deoxy-*myo*-inositol 1,3,4,5-tetrakisphosphate, a mimic of D-*myo*-inositol 1,3,4,5-tetrakisphosphate: biological activity and pH-dependent conformational properties

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Abstract

D-6-Deoxy-*myo*-inositol 1,3,4,5-tetrakisphosphate [D-6-deoxy-Ins(1,3,4,5)P₄] **3** is a novel deoxygenated analogue of D-*myo*-inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P₄] **2**, a central and enigmatic molecule in the polyphosphoinositide pathway of cellular signalling. D-6-Deoxy-Ins(1,3,4,5)P₄ is a moderate inhibitor of Ins(1,4,5)P₃ 5-phosphatase [1.8 μM] compared to Ins(1,3,4,5)P₄ [0.15 μM] and similar to that of L-Ins(1,3,4,5)P₄ [1.8 μM]. In displacement of [³H] Ins(1,4,5)P₃ from the rat cerebellar Ins(1,4,5)P₃ receptor, while slightly weaker [IC₅₀ = 800 nM] than that of D-Ins(1,3,4,5)P₄ [IC₅₀ = 220 nM], **3** is less markedly different and again similar to that of L-Ins(1,3,4,5)P₄ [IC₅₀ = 660 nM]. **3** is an activator of I_{CRAC} when inward currents are measured in RBL-2H3-M1 cells using patch-clamp electrophysiological techniques with a facilitation curve different to that of Ins(1,3,4,5)P₄. Physicochemical properties were studied by potentiometric ³¹P and ¹H NMR titrations and were similar to those of Ins(1,3,4,5)P₄ apart from the observation of a biphasic titration curve for the P1 phosphate group. A novel vicinal phosphate charge-induced conformational change of the inositol ring above pH 10 was observed for D-6-deoxy-Ins(1,3,4,5)P₄ that would normally be hindered because of the central stabilising role played by the 6-OH group in Ins(1,3,4,5)P₄. We conclude that the 6-OH group in Ins(1,3,4,5)P₄ is crucial for its physicochemical behaviour and biological properties of this key inositol phosphate.

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D-*myo*-Inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] **1** (Fig. 1), produced in response to stimulation of a wide variety of cellular membrane receptors, exhibits well-known second messenger properties [1] mediating a large number of calcium-regulated signal transduction events [2]. Ins(1,4,5)P₃ releases Ca²⁺ from intracellular stores and activates store-operated Ca²⁺ influx through plasma membrane channels collectively known as store-

operated channels (SOCs) [3]. The best characterised SOCs are Ca²⁺ release activated Ca²⁺ channels (CRAC) which pass the inward Ca²⁺ current, I_{CRAC}. After this Ca²⁺ release the signal must be removed and this is accomplished by one or more metabolic pathways: Ins(1,4,5)P₃ 5-phosphatase [yielding Ins(1,4)P₂] and Ins(1,4,5)P₃ 3-kinase which generates D-*myo*-inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P₄, **2**, Fig. 1]. While the role of Ins(1,4,5)P₃ and its calcium mobilising ability is well defined in many tissues, exact reasons for the rapid production of Ins(1,3,4,5)P₄ following receptor

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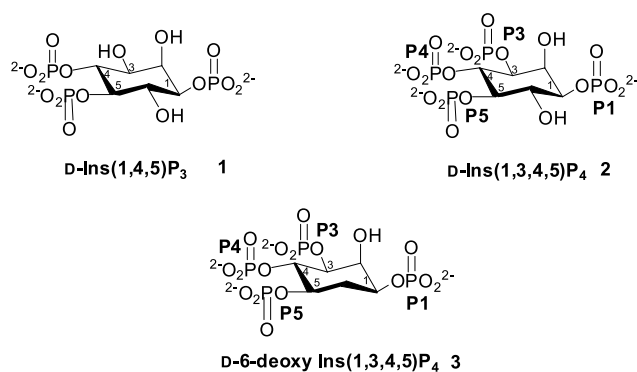


Fig. 1. Structures of Ins(1,4,5)P₃ 1, Ins(1,3,4,5)P₄ 2, and 6-deoxy-Ins(1,3,4,5)P₄ 3.

stimulation are still less well defined. Essentially, there are three broad possibilities: (1) the phosphorylation of Ins(1,4,5)P₃ to Ins(1,3,4,5)P₄ is considered to be an off-switch terminating the release of calcium by Ins(1,4,5)P₃; (2) Ins(1,3,4,5)P₄ is a metabolic intermediate with no other function; and (3) Ins(1,3,4,5)P₄ has a second messenger function itself, initiated through binding to its own specific intracellular receptor.

A role mediating Ca²⁺ entry through plasma membrane channels has been proposed. An Ins(1,3,4,5)P₄-sensitive Ca²⁺-permeable channel has been characterised from endothelial cells [4] and Ins(1,3,4,5)P₄ binding proteins have been identified from pig and rat cerebellum [5] and porcine platelets [6]. The latter example has been characterised and identified as a putative Ins(1,3,4,5)P₄ receptor designated as GAP^{IP4BP} [7]. GAP^{IP4BP} is a Ras GTPase-activating protein that in vitro is regulated by Ins(1,3,4,5)P₄ and contains C-terminal Ins(1,3,4,5)P₄-binding pleckstrin homology (PH) domains [8]. The interaction of Ins(1,3,4,5)P₄ with the PH domain of Bruton's tyrosine kinase (Btk) may be involved in B-cell activation and development [9]; mutations in the Btk PH domain causing human X-linked agammaglobulinaemia (XLA) and murine X-linked immunodeficiency (Xid) are associated with dramatically reduced Ins(1,3,4,5)P₄-binding activity [10]. We reported an X-ray crystal structure of synthetic Ins(1,3,4,5)P₄ [11] bound to the PH domain of Btk [12], that has been used to define a model describing the Ins(1,3,4,5)P₄-binding site within the PH domain of GAP^{IP4BP} [13].

Recently, we suggested a role for Ins(1,3,4,5)P₄ in facilitating store-operated Ca²⁺ influx in cells by inhibition of Ins(1,4,5)P₃ 5-phosphatase [14]. It is known that Ins(1,3,4,5)P₄ is hydrolysed by the same 5-phosphatase that hydrolyses Ins(1,4,5)P₃, but the enzyme has a 10-fold higher affinity and 100-fold lower *V*_{max} for Ins(1,3,4,5)P₄ than it does for Ins(1,4,5)P₃ [15], implying that Ins(1,3,4,5)P₄ can protect Ins(1,4,5)P₃ against hydrolysis and therefore increase its overall effectiveness.

Ins(1,3,4,5)P₄ sensitises Ins(1,4,5)P₃-mediated activation of the store-operated Ca²⁺ current *I*_{CRAC} through inhibition of type I Ins(1,4,5)P₃ 5-phosphatase. Consequently, after cell stimulation has ceased Ins(1,3,4,5)P₄ will have a longer half-life than Ins(1,4,5)P₃ and will protect any new Ins(1,4,5)P₃ that is formed by subsequent stimulation. This inhibition of metabolism results in increased levels of available Ins(1,4,5)P₃. The antagonistic action of Ins(1,3,4,5)P₄ was also revealed with higher concentrations of Ins(1,3,4,5)P₄ acting to inhibit Ins(1,4,5)P₃ receptors. Through these regulatory effects on Ins(1,4,5)P₃ metabolism, Ins(1,3,4,5)P₄ is suggested to behave as a potent bi-modal regulator of cellular sensitivity to Ins(1,4,5)P₃ that provides both facilitatory and inhibitory feedback on Ca²⁺ influx.

In endothelial cells [4] and neurons [16,17] Ins(1,3,4,5)P₄ has been shown to activate Ca²⁺ channels in the plasma membrane. At high concentrations Ins(1,3,4,5)P₄ can also interact with Ins(1,4,5)P₃ receptors on the endoplasmic membrane with differing effects. In some cell types it has been shown to inhibit the actions of Ins(1,4,5)P₃ [18], whereas in others it apparently mimics its action by causing the mobilisation of Ca²⁺ [19]. This apparent contradictory nature of Ins(1,3,4,5)P₄ could be accounted for through the presence of at least three isoforms of the Ins(1,4,5)P₃ receptor in mammals with each possibly responding differently to Ins(1,3,4,5)P₄. To date, three types of Ins(1,4,5)P₃ receptors have been cloned [20] and are expressed in varying abundance in different tissues [21]. Recently, first three-dimensional structures of the type I Ins(1,4,5)P₃ receptor have been reported [22a,22b] and also an X-ray crystal structure of the ligand-binding domain in complex with Ins(1,4,5)P₃ [22c].

In order to further investigate the biological function of Ins(1,3,4,5)P₄ at several potential biological targets the synthesis of structural analogues of this molecule is of continuing interest. We recently reported the synthesis of 6-deoxy-Ins(1,3,4,5)P₄ [23] in racemic and chiral form, as well as the synthesis of D-2-deoxy-Ins(1,3,4,5)P₄ from D-glucose [24]. We now report the protonation constants and an overview of the protonation process of the individual phosphate groups for D-6-deoxy-Ins(1,3,4,5)P₄ (3, Fig. 1) and the results of the interaction of D-6-deoxy-Ins(1,3,4,5)P₄ with Ins(1,4,5)P₃ 5-phosphatase and Ins(1,4,5)P₃ receptors. The results are compared to the biological and acid–base behaviour of Ins(1,3,4,5)P₄ to aid in the understanding of the enigmatic role of Ins(1,3,4,5)P₄.

Experimental

Materials. Full synthetic details for the preparation of D-Ins(1,3,4,5)P₄ [11] and D-6-deoxy-Ins(1,3,4,5)P₄ [23] have previously been reported. All materials used for titrations and biological evaluations

were spectroscopically homogeneous and were used as their tri-ethylammonium salts.

5-Phosphatase inhibition and Ins(1,4,5)P₃ receptor-binding assays [14]. Assays of Ins(1,4,5)P₃ 5-phosphatase activity were performed at pH 7.2 using purified recombinant enzyme expressed in *Escherichia coli*. Ins(1,4,5)P₃ receptor-binding assays were performed using microsomal fractions of rat cerebellum prepared as described. The assay mixture (0.5 ml) contained 50 mM Hepes/NaOH buffer, pH 7.2, 1 mM EDTA, 6 nM [³H]Ins(1,4,5)P₃, and ~30 μg microsomal fraction, in the presence of various concentrations of compounds of interest. Incubation was performed on ice for 10 min, followed by the separation of bound radioactivity from free form by centrifugation (15,000 rpm for 10 min). Non-specific binding (150–200 dpm) was determined in the presence of 10 μM Ins(1,4,5)P₃ and was subtracted from that in its absence to determine the specific binding (4000–5000 dpm).

Electrophysiology. For patch-clamp experiments, RBL-2H3-M1 cells grown on glass coverslips were transferred to a recording chamber and kept in a standard modified Ringer's solution of the following composition (in mM): NaCl 145, KCl 2.8, CsCl 10, CaCl₂ 10, MgCl₂ 2, glucose 10, and Hepes–NaOH 10, pH 7.2. The standard intracellular pipette-filling solution contained (in mM): Cs-glutamate 145, NaCl 8, MgCl₂ 1, ATP 0.5, and GTP 0.3, pH 7.2, adjusted with CsOH. The internal solution was supplemented with a mixture of 10 mM Cs-BAPTA and 4.3–5.3 mM CaCl₂ to buffer [Ca²⁺]_i to resting levels of 100–150 nM and avoid spontaneous activation of I_{CRAC}. Patch-clamp experiments were performed in the tight-seal whole-cell configuration at 21–25 °C. High-resolution current recordings were acquired by a computer-based patch-clamp amplifier system (EPC-9, HEKA, Lambrecht, Germany). Patch pipettes had resistances between 2 and 4 MΩ after filling with the standard intracellular solution. Immediately following establishment of the whole-cell configuration, voltage ramps of 50 ms duration spanning the voltage range of –100 to +100 mV were delivered from a holding potential of 0 mV at a rate of 0.5 Hz over a period of 300–800 s. All voltages were corrected for a liquid junction potential of 10 mV between external and internal solutions. Currents were filtered at 2.3 kHz and digitised at 100 μs intervals. Capacitive currents and series resistance were determined and corrected before each voltage ramp using the automatic capacitance compensation of the EPC-9. For analysis, the very first ramps before activation of I_{CRAC} (usually 1–3) were digitally filtered at 2 kHz, pooled, and used for leak-subtraction of all subsequent current records. The low-resolution temporal development of inward currents was extracted from the leak-corrected individual ramp current records by measuring the current amplitude at –80 mV.

Potentiometric studies and NMR determinations. Potentiometric and NMR determinations were carried out as previously reported [25,26]. The experiments were performed in two steps in which 0.50 cm³ of the same initial solution of 6-deoxy-Ins(1,3,5,6)P₄, 3.0 × 10^{–3} mol dm^{–3} in ²H₂O was successively subjected to potentiometric and NMR titrations. It should be noted that the glass electrode was calibrated in a concentration scale and the measurements were done in ²H₂O, so that pH here means the co-logarithm of the concentration of ²H⁺. The processing of the pH measurements allowed the total concentration of the ligand and the acid, as well as the macroscopic protonation constants, to be determined. One-dimensional ³¹P NMR spectra were recorded at 121.50 MHz on a Bruker DPX-300 Fourier transform spectrometer. ³¹P chemical shift values were referenced to an external 85% H₃PO₄ signal at 0.00 ppm with downfield shifts represented by positive values. Spectra were acquired over a spectral width of 10 ppm using a 0.1 s relaxation delay and a π/2 pulse. Typically 1K data points were sampled with a corresponding 0.4 s acquisition time. Data were zero-filled and a 1 Hz exponential line broadening function was applied prior to Fourier transformation. The HypNMR program was used to check the potentiometrically determined protonation constants. The ¹H NMR titration was performed on the same equipment as before operating at 300.13 MHz. Spectra were acquired

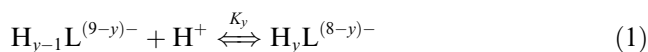
with water presaturation over a spectral width of 6 ppm using a 3 s relaxation delay and a π/2 pulse. 4K data points were sampled with a corresponding 1.14 s acquisition time. The spectra had a digital resolution of 0.44 Hz per point. The temperature was controlled at 310 ± 0.5 K. The proton and phosphorus resonances were assigned by performing proton–proton and phosphorus–proton 2D thus allowing the titration curves to be characterised.

Results and discussion

Macroscopic and microscopic protonation constants

Determination of structural and thermodynamic parameters at an intramolecular level (i.e., at each functional group) is one of the key issues in the comprehension of molecular interactions. In addition to structure–activity studies, NMR and potentiometric investigations may also contribute to delineate the electronic and conformational demands for optimal binding in the receptor cleft. This is especially true if the parameters that are considered are related to the individual functional groups of the molecules. We have previously determined the acid–base properties of Ins(1,3,4,5)P₄ at an intramolecular level [27]. It is therefore interesting to compare such properties with those of the 6-deoxy analogue. Using both ³¹P and ¹H NMR titration curve analysis provides valuable information on the conformational dependence of the inositol ring with respect to the ionisation state of the individual phosphate groups and in particular the dynamics of the complex intramolecular interactions which govern the conformation of the phosphate groups and of the inositol ring [28–35]. Thus, this physicochemical part first describes the protonation pattern of the 6-deoxy-Ins(1,3,4,5)P₄ and then analyses the conformational consequences of the phosphate protonation process.

In the 12–2 pH range, 6-deoxy-Ins(1,3,4,5)P₄ is able to bind four protons which are involved in the following macroscopic stepwise protonation equilibria:



characterised by the related K_y constants, where y takes the values 1–4. It can be noted that $\log K_y$ correspond to the usual $\text{p}K_y$ values.

Derivation of the microprotonation schemes is straightforward for difunctional ligands, becomes more complicated for trifunctional ligands such as inositol-trisphosphates [25], but appears as a very intricate task for tetrafunctional molecules, since the number of microspecies to consider is, respectively, 4, 8, and 16. Fortunately, ³¹P NMR spectroscopy is well suited to monitor the ionisation state of individual phosphates, the chemical shift variations of the phosphorus nuclei with pH being mainly the result of the protonation state variations of the phosphate groups. Thus, even for tetrafunctional ligands, the relative protonation degree of

each phosphate group can be calculated at a given pH from the protonation fraction curves. These protonation fractions can be easily obtained according to the following equation:

$$f_{i,p} = \frac{\delta_i^{\text{obs}} - \delta_{i,d}}{\delta_{i,p} - \delta_{i,d}}, \quad (2)$$

where δ_i^{obs} , $\delta_{i,p}$, and $\delta_{i,d}$ correspond, respectively, to the observed chemical shift and the chemical shifts of protonated and deprotonated fractions of the phosphates in position i .

The ^{31}P NMR titration curves of 6-deoxy-Ins(1,3,4,5) P_4 from which the protonation fraction curves are calculated are displayed in Fig. 2. The general shape of these curves differs only slightly from those of Ins(1,3,4,5) P_4 (curves not shown). As for the latter, P3 and P5 behave similarly and P4 shows the usual features of an equatorial phosphate centrally located between two other equatorial phosphates. Only the P1 curves look different in the 8–12 pH range: the curve for Ins(1,3,4,5) P_4 is monophasic with a slight deshielding upon protonation at the beginning of the curve, whereas that of 6-deoxy-Ins(1,3,4,5) P_4 is biphasic.

Differences also appear on the macroprotonation constants of both ligands. These constants determined by NMR titration [36] in the same experimental conditions (0.2 M KCl in 100% $^2\text{H}_2\text{O}$ at 37 °C) are listed in Table 1. It can be observed that there is no unique tendency in the variation of the acid–base character of both compounds. If $\log K_1$ and $\log K_2$ are higher for Ins(1,3,4,5) P_4 than for its 6-deoxy analogue, the inverse occurs for $\log K_3$ and $\log K_4$. This is in line with the observations that can be made on the protonation fractions ($f_{i,p}$) vs pH curves (Fig. 3).

On these curves it can be seen that the P3, P5, and especially P1 curves for 6-deoxy-Ins(1,3,4,5) P_4 are shifted to higher pH with respect to those of Ins(1,3,4,5) P_4 , thereby indicating a more basic character

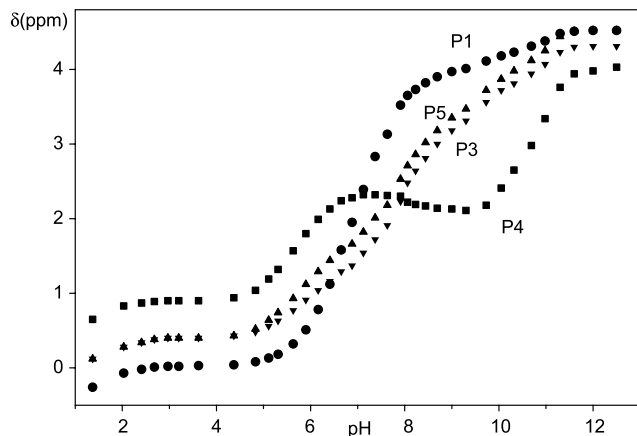


Fig. 2. Chemical shifts (δ) from ^{31}P NMR titrations for 6-deoxy-Ins(1,3,4,5) P_4 as a function of pH in KCl 0.2 M at 37 °C ($^2\text{H}_2\text{O}$).

Table 1
Logarithms of the stepwise macroprotonation constants ($\log K_y$) for 6-deoxy-Ins(1,3,4,5) P_4 and Ins(1,3,4,5) P_4

| y , ligand | $\log K_y$, 6-deoxy-Ins(1,3,4,5) P_4 | $\log K_y$, Ins(1,3,4,5) P_4 |
|--------------|--|--|
| 1 | 10.44 (0.04) | 10.79 (0.03) |
| 2 | 7.08 (0.02) | 8.00 (0.02) |
| 3 | 6.87 (0.03) | 6.56 (0.03) |
| 4 | 5.68 (0.02) | 5.33 (0.02) |

The uncertainties are estimates of the standard deviation as calculated by HypNMR [36]. It can be noted that $\log K_y$ also correspond to the classical $\text{p}K_a$ values which refer to a proton dissociation process.

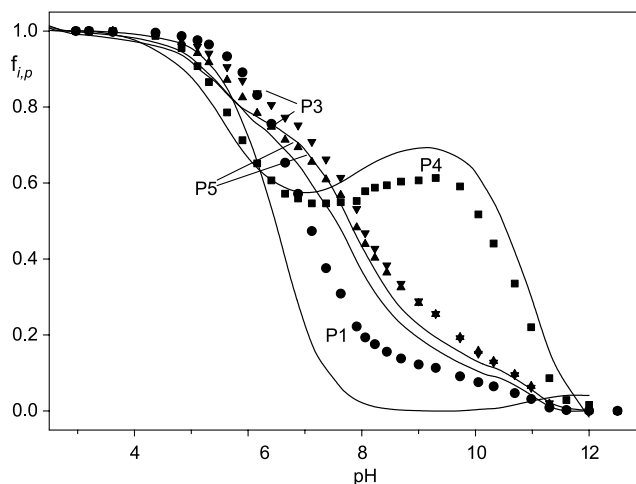


Fig. 3. Protonation fraction curves $f_{i,p}$ as a function of pH in KCl 0.2 M at 37 °C ($^2\text{H}_2\text{O}$) for 6-deoxy-Ins(1,3,4,5) P_4 (points) and Ins(1,3,4,5) P_4 (solid line).

for these phosphates of 6-deoxy-Ins(1,3,4,5) P_4 . However, the shift is inverted for P4 showing that P4 is more basic for Ins(1,3,4,5) P_4 .

To shed light on these observations, ^1H NMR titration experiments of both compounds were performed. The curves for Ins(1,3,4,5) P_4 (Fig. 4A) are fully in line with those previously obtained for Ins(1,4,5) P_3 and related compounds [33]. Upon protonation of a neighbouring phosphate, the ^1H resonances generally move downfield due to the decreased electron density occurring at this phosphate. The opposite trend earlier called “wrongway shift” [37,38] has recently been attributed to the establishment of a C–H···O hydrogen bond between a phosphate oxygen constrained to closely approach a neighbouring equatorial hydrogen atom [33]. This effect is particularly obvious for H2, undergoing a highfield shift of 0.17 ppm between pH 10.8 and 7.0, which primarily results from the protonation of P4 but also P3 and P5 phosphates. Partial neutralisation of their negative charges releases the strong constraints due to both P3–P4 and P4–P5 repulsions. A similar behaviour, although much less marked, is shown for the axial H6 which slightly experiences the P4–P5 repulsion. From Fig. 4B, it can be seen that the ^1H NMR titration curves

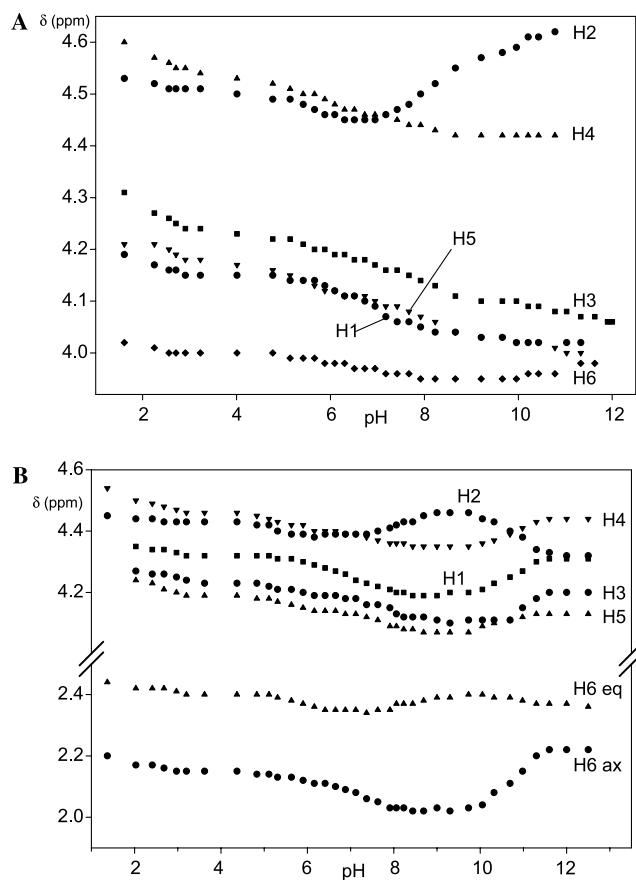


Fig. 4. Chemical shifts δ from ^1H NMR titrations for Ins(1,3,4,5) P_4 (A) and 6-deoxy-Ins(1,3,4,5) P_4 (B) as a function of pH in 0.2 M KCl at 37 °C ($^2\text{H}_2\text{O}$).

for 6-deoxy-Ins(1,3,4,5) P_4 appear much more complicated. From pH 12.3 to 10 the curves of all the protons take exactly the opposite direction than those of Ins(1,3,4,5) P_4 , then below pH 10 they behave again as expected. This is, according to previous observations [34], clearly the sign of a conformational change in the inositol ring. A closer look at its $^3J_{\text{H,H}}$ coupling constants shows that at pH 12.3 $^3J_{4,5} \approx ^3J_{3,4} \approx 6.6$ Hz, whereas the remaining $^3J_{\text{H,H}}$ axial–axial coupling constants are nearly 9.2 Hz, both values accounting for dihedral angles which correspond to a pronounced distorted chair conformation (Fig. 5). In between pH 11 and pH 10, $^3J_{4,5}$ and $^3J_{3,4}$ suddenly reach 9.2 Hz, i.e., coupling constants expected for a classical chair inositol

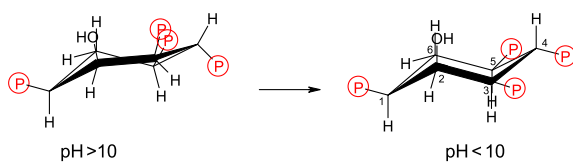


Fig. 5. Inositol ring distortion occurring at pH 10 for 6-deoxy-Ins(1,3,4,5) P_4 .

ring. The transition from a distorted to an undistorted conformation accompanies the protonation of the P4 phosphate which again relieves the strong repulsions between the three vicinal phosphate groups. Even though the phosphates are exactly at the same position for Ins(1,3,4,5) P_4 as for its 6-deoxy analogue, such a distortion does not take place due to strong hydrogen bonds established between OH6 and the P1 and P5 phosphates. Nevertheless, for 6-deoxy-Ins(1,3,4,5) P_4 , the electrostatic repulsions are not sufficient to provoke the flipping of the inositol ring as recently described for the Ins(1,4,5,6) P_4 carrying four vicinal phosphate groups [34]. Inspection of a simple molecular model shows that, due to the ring distortion for 6-deoxy-Ins(1,3,4,5) P_4 in comparison to Ins(1,3,4,5) P_4 , the interactions between the vicinal phosphates are weakened and correlatively the P1–P3, P1–P4, and P1–P5 interactions are strengthened. As a consequence, P4 is more basic for Ins(1,3,4,5) P_4 than for its 6-deoxy analogue. However, the higher basicity of P1 for the latter may rather result from the deletion of the polar equatorial OH6 producing a less hydrophilic pocket in between P1 and P5 which decreases the effective dielectric constant and therefore increases the basicity of the phosphates [35].

The differences observed in the acid–base behaviour of both the studied compounds, although notable, should not solely account for any potential differences in their biological activity. As for Ins(1,4,5) P_3 , OH6 seems to play a central role by engaging in strong hydrogen bonds with its neighbouring phosphates. By doing so it participates in the transfer of an electrostatic effect between P1 and P5 [21], but also prevents the inositol ring distortion that would result from the repulsion of vicinal phosphate groups.

Ins(1,4,5) P_3 5-phosphatase inhibition and Ins(1,4,5) P_3 receptor binding

Ins(1,4,5) P_3 releases Ca^{2+} from stores and activates store-operated Ca^{2+} influx through plasma membrane channels collectively known as store-operated channels (SOCs). The best characterised SOCs are Ca^{2+} release activated Ca^{2+} channels (CRAC) which pass the inward Ca^{2+} current, I_{CRAC} . The relationship between Ins(1,4,5) P_3 and I_{CRAC} activation has been reported to be non-linear, exhibiting a steep dose–response curve [39,40]. This non-linearity is thought to be due to Ins(1,4,5) P_3 metabolism, because the non-metabolisable analogue Ins(2,4,5) P_3 has been shown to cause a more graded I_{CRAC} activation. Recent evidence suggests that a functionally distinct store is involved in activating I_{CRAC} and that depletion of this store requires fairly high Ins(1,4,5) P_3 levels [39,41]. It seems likely that the lower sensitivity is due to significant Ins(1,4,5) P_3 metabolism which effectively lowers Ins(1,4,5) P_3 available to gate the

Ins(1,4,5)P₃ receptors in these stores. Ins(1,4,5)P₃ 5-phosphatase has been implicated as a major contributing factor to the non-linearity of I_{CRAC} activation. We recently reported the Ins(1,4,5)P₃-mediated activation of I_{CRAC} in living cells by Ins(1,3,4,5)P₄ and other pharmacological 5-phosphatase inhibitors [14]. Based on structural considerations 6-deoxy-Ins(1,3,4,5)P₄ was also believed to be inhibitory to Ins(1,4,5)P₃ 5-phosphatase—6-deoxy-Ins(1,4,5)P₃ was shown to be a moderately potent inhibitor of erythrocyte 5-phosphatase and we reported that Ins(1,3,4,5)P₄ (IC_{50} 0.15 μ M) is a more potent 5-phosphatase inhibitor than previously described synthetic inhibitors *L-chiro*-Ins(2,3,6)P₃ (IC_{50} 0.23 μ M) and *L-chiro*-Ins(1,4,6)P₃ (IC_{50} 0.30 μ M) [14]. We found that 6-deoxy-Ins(1,3,4,5)P₄ inhibits 5-phosphatase at least one order of magnitude less potently than Ins(1,3,4,5)P₄ [Fig. 6A, Ins(1,3,4,5)P₄ IC_{50} 0.15 μ M, 6-deoxy-Ins(1,3,4,5)P₄ IC_{50} 1.8 μ M]. This value is the same as that for *L*-Ins(1,3,4,5)P₄ that we previously reported [14]. Looked at in terms of binding orientation in the active site of 5-phosphatase we can assume it to be most likely that 6-deoxy-Ins(1,3,4,5)P₄ (Fig. 7C) binds in the same way as Ins(1,3,4,5)P₄ (Fig. 7A) and that *L*-Ins(1,3,4,5)P₄ binds probably in the relative orientation shown in Fig. 7B with the polyphosphate spatial configuration maintained, but the axial 2-position and equatorial 6-position hydroxyls binding in the sites normally occupied by the equatorial

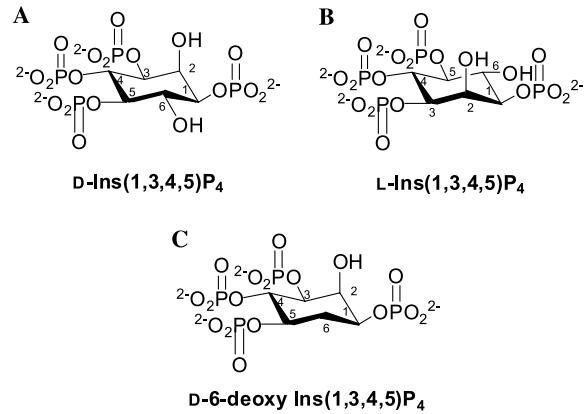


Fig. 7. Proposed relative ligand-binding orientations for Ins(1,4,5)P₃ receptor and 5-phosphatase interactions: (A) Ins(1,3,4,5)P₄; (B) *L*-Ins(1,3,4,5)P₄ is shown in a binding orientation in which it can mimic its enantiomer Ins(1,3,4,5)P₄; and (C) *D*-6-deoxy-Ins(1,3,4,5)P₄ is shown in a binding orientation in which it can mimic Ins(1,3,4,5)P₄ and *L*-Ins(1,3,4,5)P₄. Note the relative differences at C-2 and C-6.

6-OH of (A), the CH₂ of (B), and the axial 2-OH of (A) and of (B), respectively.

As 6-deoxy-Ins(1,3,4,5)P₄ behaves as a 5-phosphatase inhibitor we then looked at the potential interaction of this molecule with Ins(1,4,5)P₃ receptors (Fig. 6B). The Ins(1,4,5)P₃ receptor binding assays show that 6-deoxy-Ins(1,3,4,5)P₄ inhibits Ins(1,4,5)P₃ binding to the Ins(1,4,5)P₃-receptor only slightly less effectively

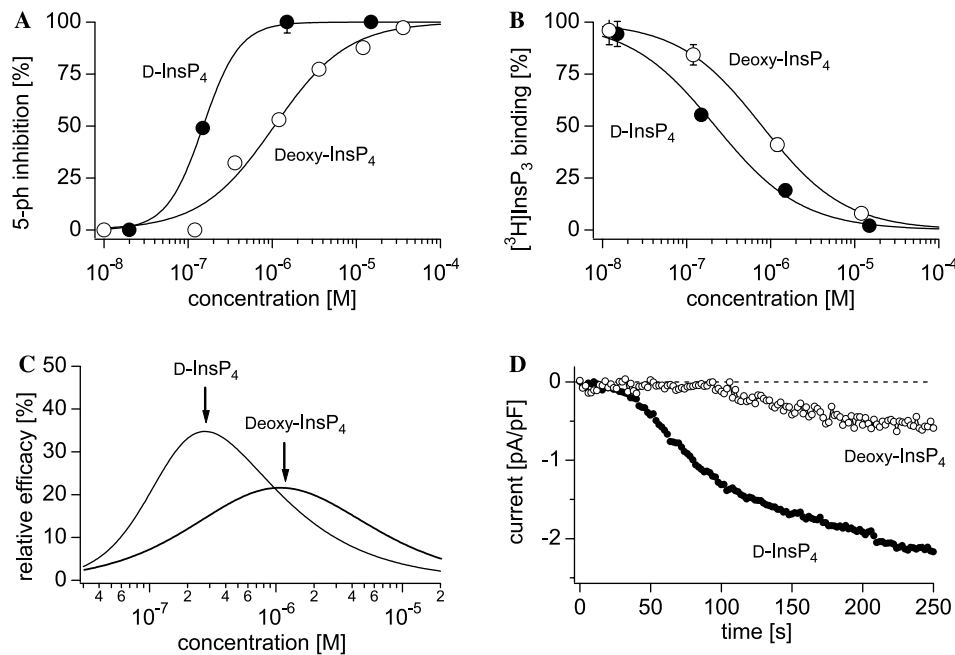


Fig. 6. Ins(1,3,4,5)P₄ and 6-deoxy-Ins(1,3,4,5)P₄ inhibit 5-phosphatase and Ins(1,4,5)P₃ receptor binding. (A) Inhibition profile of Type I 5-phosphatase activity by ligands (see Experimental). Data points are averages \pm SEM ($n = 3$). (B) Inhibition profile of [³H]Ins(1,4,5)P₃ binding to InsP₃ receptors for ligands (see Experimental). Data points are averages \pm SEM ($n = 3$). (C) Facilitation profiles of the two ligands. Net facilitation curves reflect the product of inhibition profiles of 5-phosphatase activity and Ins(1,4,5)P₃ binding of InsP₃ receptors from (A) and (B). (D) Average inward currents carried by I_{CRAC} at -80 mV. Cells were co-perfused with either Ins(1,3,4,5)P₄ or 6-deoxy-Ins(1,3,4,5)P₄ and sub-threshold levels of Ins(1,4,5)P₃ (1 μ M). Traces are averages of experiments in which *D*-Ins(1,3,4,5)P₄ ($n = 14$) and *D*-6-deoxy-Ins(1,3,4,5)P₄ ($n = 8$) were used at 20 μ M.

[$IC_{50} = 800$ nM] than $\text{Ins}(1,3,4,5)\text{P}_4$ [$IC_{50} = 220$ nM]. $\text{Ins}(1,4,5)\text{P}_3$ -receptor binding and 5-phosphatase inhibition would have opposing effects on $\text{Ins}(1,4,5)\text{P}_3$ -mediated activation of I_{CRAC} , with the first contributing a positive and the second, a negative effect. The resulting net facilitation can be calculated as the product of these two factors. This analysis is illustrated in Fig. 6C for both $\text{Ins}(1,3,4,5)\text{P}_4$ and 6-deoxy- $\text{Ins}(1,3,4,5)\text{P}_4$, where the product of the superimposed 5-phosphatase inhibition and $\text{Ins}(1,4,5)\text{P}_3$ -receptor binding curves yields in each case a bell-shaped net facilitation curve. The amplitude of the latter (Y -axis) roughly reflects efficacy, whereas potency is reflected by the location of the curve on the X -axis. Compared to $\text{Ins}(1,3,4,5)\text{P}_4$, 6-deoxy- $\text{Ins}(1,3,4,5)\text{P}_4$ is, as predicted, an effective facilitator of I_{CRAC} , but less potent than the natural inositol phosphate. Qualitatively, these predictions are borne out by patch-clamp experiments which clearly show that $\text{Ins}(1,3,4,5)\text{P}_4$ is a more effective facilitator of I_{CRAC} than 6-deoxy- $\text{Ins}(1,3,4,5)\text{P}_4$ (Fig. 6D). 6-Deoxy- $\text{Ins}(1,3,4,5)\text{P}_4$ is, however, a slightly more effective I_{CRAC} facilitator than the $\text{Ins}(1,3,4,5)\text{P}_4$ enantiomer L - $\text{Ins}(1,3,4,5)\text{P}_4$ [14], which had an IC_{50} for [^3H] $\text{Ins}(1,4,5)\text{P}_3$ displacement of 660 nM.

If we assume that 6-deoxy- $\text{Ins}(1,3,4,5)\text{P}_4$ and L - $\text{Ins}(1,3,4,5)\text{P}_4$ most likely also bind to the $\text{Ins}(1,4,5)\text{P}_3$ receptor in the relative modes as shown (Fig. 7) then, not surprisingly, placing the axial 2-OH group of L - $\text{Ins}(1,3,4,5)\text{P}_4$ in the position normally occupied by the equatorial 6-OH group of D - $\text{Ins}(1,3,4,5)\text{P}_4$ [or $\text{Ins}(1,4,5)\text{P}_3$] is presumably in SAR terms equivalent to deoxygenation of D - $\text{Ins}(1,3,4,5)\text{P}_4$ at the 6-position. Since the relative IC_{50} values for L - $\text{Ins}(1,3,4,5)\text{P}_4$ and D -6-deoxy- $\text{Ins}(1,3,4,5)\text{P}_4$ are very similar we can assume that the equatorial 6-OH group of L - $\text{Ins}(1,3,4,5)\text{P}_4$, binding at a site normally occupied by the axial 2-OH group of D - $\text{Ins}(1,3,4,5)\text{P}_4$ [or $\text{Ins}(1,4,5)\text{P}_3$] does not have a significant effect on binding. This observation is perfectly in line with the established SAR for $\text{Ins}(1,4,5)\text{P}_3$ binding to its receptor where the 2-position modifications found in *scyllo*- $\text{Ins}(1,4,5)\text{P}_3$ [equatorial 2-OH] and 2-deoxy- $\text{Ins}(1,4,5)\text{P}_3$ [no 2-OH group] have a minimal effect on potency [1b].

Structural considerations indicated that 6-deoxy- $\text{Ins}(1,3,4,5)\text{P}_4$ should most likely have an inhibitory effect on $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase and this has been demonstrated with 6-deoxy- $\text{Ins}(1,3,4,5)\text{P}_4$ behaving as a relatively potent 5-phosphatase inhibitor. Through this inhibition 6-deoxy- $\text{Ins}(1,3,4,5)\text{P}_4$ like $\text{Ins}(1,3,4,5)\text{P}_4$ facilitates Ca^{2+} influx by sensitising $\text{Ins}(1,4,5)\text{P}_3$ -mediated activation of I_{CRAC} . This action is shared by other 5-phosphatase inhibitors and therefore appears to be a general mechanism involved in regulating $\text{Ins}(1,4,5)\text{P}_3$ -mediated Ca^{2+} signalling [14]. However, 6-deoxy- $\text{Ins}(1,3,4,5)\text{P}_4$ has a more varying and reduced potency with respect to the natural ligand $\text{Ins}(1,3,4,5)\text{P}_4$ in all

biological indicators examined. This can be rationalised to some extent through consideration of the inframolecular data presented here.

It is particularly revealing here that phosphates 1, 3, and 5 of 6-deoxy- $\text{Ins}(1,3,4,5)\text{P}_4$ are more basic than the corresponding groups of the natural polyphosphate and that the inverse is true for the 4-phosphate. This alteration in the physicochemical status of 6-deoxy- $\text{Ins}(1,3,4,5)\text{P}_4$ with respect to $\text{Ins}(1,3,4,5)\text{P}_4$ could explain the observed differences in the biological activity of these structurally similar inositol phosphates. Thus, changes in charge distribution could account for the reduced potency of 6-deoxy- $\text{Ins}(1,3,4,5)\text{P}_4$ —any interaction between these phosphate groups and an $\text{Ins}(1,3,4,5)\text{P}_4$ receptor that are key to activity will undoubtedly be affected by reduced/increased basicity of the phosphate moieties. Furthermore, it has been shown that OH-6 plays a central role in the conformational preference of the inositol ring. The OH-6 motif participates in the electrostatic effect between phosphates 1 and 5, as well as preventing inositol ring distortion. Consequently, removal of this stabilising effect could have important consequences for biological activity. Indeed, we show here that deletion of OH-6 has dramatic effects upon the relative basicity of the individual phosphate groups—a consequence that has been shown to influence the conformation of 6-deoxy- $\text{Ins}(1,3,4,5)\text{P}_4$. The ^1H NMR titration studies revealed that at relatively high pH a distorted chair conformation is formed in which the three vicinal phosphates are strongly repulsed by each other. Upon protonation of phosphate 4, as the pH is lowered, the inositol ring of 6-deoxy- $\text{Ins}(1,3,4,5)\text{P}_4$ returns to an undistorted conformation. This transition from distorted to undistorted conformations does not occur in $\text{Ins}(1,3,4,5)\text{P}_4$ where such conformational changes are hindered by the formation of a hydrogen bond between phosphate 5 and OH 6, presumably making the inositol ring more rigid. Due to the resultant ring distortion that occurs in 6-deoxy- $\text{Ins}(1,3,4,5)\text{P}_4$ interactions between vicinal phosphates are weakened and P1–P3, P1–P4, and P1–P5 interactions are strengthened. The biological consequences of such pH-dependent conformational changes have been implicated in the binding mechanisms and regulation processes of $\text{Ins}(1,4,5,6)\text{P}_4$ and $\text{Ins}(3,4,5,6)\text{P}_4$ [34].

The protonation constants of inositol polyphosphates containing several vicinal phosphate groups are known to vary by several orders of magnitude and it is known that the conformational equilibrium of such phosphates is governed by the ionisation state of the phosphate groups concerned. This, in itself, strongly depends upon the immediate ionic environment in which the molecule in question finds itself. Consequently, the observed difference in the relative potencies of $\text{Ins}(1,3,4,5)\text{P}_4$ and its 6-deoxy congener could potentially be attributed to such

a phenomenon occurring in the microenvironment of the ligand–receptor interaction.

Thus, in summary, we have demonstrated that D-6-deoxy-Ins(1,3,4,5)P₄ is a moderate inhibitor of Ins(1,4,5)P₃ 5-phosphatase compared to natural Ins(1,3,4,5)P₄, thus illustrating a role for the 6-hydroxyl group in the binding of Ins(1,3,4,5)P₄ to this enzyme. 6-Deoxy-Ins(1,3,4,5)P₄, however, inhibits the binding of [³H]Ins(1,4,5)P₃ to its receptor only slightly less effectively than Ins(1,3,4,5)P₄. The resulting bell-shaped facilitation curve for 6-deoxy-Ins(1,3,4,5)P₄ reveals a compound of overall lower efficacy and potency and this molecule is an effective facilitator of *I*_{CRAC}, but less effective than Ins(1,3,4,5)P₄. The pH-dependent protonation characteristics of 6-deoxy-Ins(1,3,4,5)P₄ were determined and found to be broadly similar to those of Ins(1,3,4,5)P₄, apart from the data for P1. ¹H NMR titrations uncovered evidence for a novel conformational charge of the inositol ring above pH 10 for 6-deoxy-Ins(1,3,4,5)P₄, something that is normally prevented in Ins(1,3,4,5)P₄ because of the stabilising effect of the 6-OH group.

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