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Conformational studies of *myo*-inositol phosphates

Laura G. Barrientos [1], Pushpalatha P.N. Murthy *

Department of Chemistry, Michigan Technological University, Houghton, MI 49931, USA

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Abstract

The discovery of the second messenger role of *myo*-inositol 1,4,5-trisdihydrogenphosphate [Ins(1,4,5)P_3] has triggered tremendous interest in investigating the structure, metabolism, and biological roles of inositol phosphates. Although the conformation of phytic acid [(*myo*-inositol hexakisdihydrogenphosphate), InsP_6] has been the subject of much study, the conformations of lower inositol phosphates such as inositol-pentakis-, tetrakis-, and tris-dihydrogenphosphates have not been investigated. We investigated, by ^{1}H NMR spectroscopy, the conformations of inositol phosphates (InsP_5, InsP_4, InsP_3, InsP_2, and InsP_1) and monitored the influence of pH on conformational preferences. InsP_6 adopts the sterically stable 1ax/5eq (one phosphate in the axial position and five phosphates in the equatorial position) conformation in the pH range 0.5–9.0, and the sterically hindered 5ax/1eq (five phosphates in the axial position and one phosphate in the equatorial position) conformation above pH 9.5. At pH 9.5, both conformations are in dynamic equilibrium. Ins(1,2,3,4,6)P_5 and Ins(1,2,3,5,6)P_5 adopt the 1ax/5eq form in the pH range 1.0–9.0; in the pH range 9.5–13.0, the 1ax/5eq and 5ax/1eq conformations are in dynamic equilibrium. In contrast to InsP_6 and InsP_5, all the lower inositol phosphates (InsP_4 to InsP_1) investigated adopt the 1ax/5eq conformation over the entire pH range, 1.0–13.0. Preference for the 5ax/1eq conformation by InsP_6 and InsP_5 is probably due to decreased electrostatic repulsion between negatively charged vicinal equatorial phosphates in the 1ax/5eq conformation and stabilization of the sterically hindered 5ax/1eq conformation by hydrogen bonding and/or sodium counter-ions bonding between the syn-oriented phosphates. On the basis of conformations adopted by the inositol phosphates (InsP_6 to InsP_1) at different pH, we conclude that the presence of four or five equatorial phosphates on the inositol ring induces a change in the conformation from the sterically unhindered 1ax/5eq structure to the sterically hindered 5ax/1eq conformation, at high pH. This investigation illustrates that the conformational preferences of inositol phosphates

* Corresponding author.

[1] Current address: Chemistry Department, Carnegie Mellon University, Mellon Institute, Box 123, 4400 Fifth Avenue, Pittsburgh, PA 15213, USA.

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at different pH is unique to the particular isomer and does not parallel the behaviour of phytic acid. © 1996 Elsevier Science Ltd.

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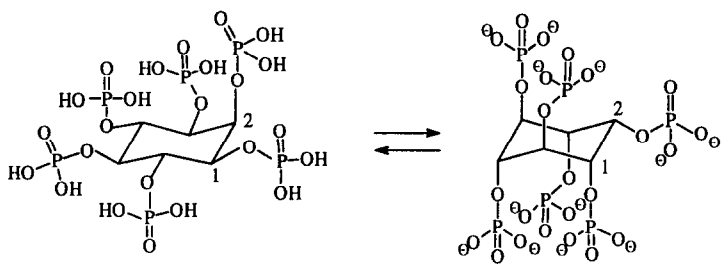
1. Introduction

The discovery that *myo*-inositol 1,4,5-trisdihydrogenphosphate [Ins(1,4,5)P_3] regulates intracellular calcium by binding to specific receptors in endoplasmic reticulum and thereby opening Ca^{+2} channels has resulted in widespread interest in the structure, metabolism, and biological role of inositol phosphates [1–4]. The biological roles of other inositol phosphates such as Ins(1,3,4,5,6)P_5, which modulates the affinity of hemoglobin for oxygen [5], Ins(1,3,4,5)P_4, which regulates cellular calcium at the plasma membrane [1], and InsP_6, which functions as an antioxidant [6] and anticancer agent [7], are being investigated.

The conformation of phytic acid has been a subject of much debate [8–11]. Both X-ray crystallographic and Raman spectroscopy data suggest that, in the solid state, the hydrated dodecasodium salt of phytic acid adopts the conformation that contains five phosphates in axial (ax) positions and one phosphate in equatorial (eq) position (5ax/1eq) (Fig. 1, Structure **2**). The driving force for adopting the sterically hindered 5ax/1eq form is the minimization of electrostatic repulsion between the five contiguous dianionic phosphates in the equatorial positions and stabilization of the axial phosphates by coordination with sodium ions and hydrogen-bonding interactions with water [11]. Understanding the conformation of phytic acid in solution has been more complicated because of the dependence of the conformation on multiple factors of the solvating medium, such as pH, ionic strength, and counter-ions. Costello et al. [10] studied the titration curves of phytic acid with tetrabutylammonium hydroxide and sodium hydroxide and monitored changes in ^{31}P NMR with pH. Taking into consideration both the pK_a values and the changes in ^{31}P NMR they concluded that, in contrast to the solid-state conformation, the solution conformation of phytic acid in the pH range 2–12 is the 1ax/5eq form (Fig. 1, Structure **1**). Emsley and Niazi [8] re-examined the titration



I: 1ax/5eq 2: 5ax/1eq

Fig. 1. Conformations of phytic acid.

curves and ^{31}P NMR of phytic acid under experimental conditions similar to those used by Costello et al. [10] and concluded that phytic acid adopts the 1ax/5eq conformation over the pH range 2–5 and above pH 12, and the 5ax/1eq conformation over the pH range 5–12 and below pH 2. Isbrandt and Oertei [9] investigated the conformation of phytic acid using ^{13}C NMR, ^{31}P NMR, and Raman spectroscopy and concluded that, in aqueous solution, the 1ax/5eq form, called the low-pH form, exists below pH 9.5. Above this pH, the stable conformation is the 5ax/1eq form. The three least acidic protons have pK_a values of 9.2–9.6, and the ring flip to the 5ax/1eq conformation is triggered by the removal of these protons and conversion of all the phosphates to the dianionic form.

The conformations of lower inositol phosphates (InsP_5 to InsP_1) at different pH are unknown. Although previous work has investigated the conformation of InsP_6, no systematic investigation of the conformational preferences of lower inositol phosphates (InsP_5 to InsP_1) has been reported to date. Therefore, conformational preferences of other inositol phosphates are often deduced on the basis of the behaviour of InsP_6. In this paper, we describe a ^{1}H NMR spectroscopic investigation of the conformations of inositol phosphates (InsP_6 to InsP_1) in aqueous solution and monitor the influence of pH on conformational preferences.

2. Results and discussion

NMR investigation was based on the premise that any change in the conformation of the inositol ring would affect the ^{1}H NMR spectroscopic characteristics of inositol phosphates in a diagnostic manner. Change in conformation from the 1ax/5eq to 5ax/1eq form would significantly affect the splitting pattern of the inositol ring protons because of changes in the dihedral angle between vicinal protons. The splitting pattern of the inositol ring protons is due to coupling with two vicinal protons, one on either side, and when phosphorylated, additional coupling with the phosphorous atom. In a molecule with rigid geometry, like the cyclohexane ring of inositol, spin–spin coupling between vicinal protons is dependent on the dihedral angle 'Φ' (Karplus correlation) [12,13]. The coupling constants between two vicinal protons is 2–3 Hz when the relationship is axial–equatorial, (J_{ax-eq}) ($\Phi \sim 60°$), 8–10 Hz when the relationship is axial–axial, (J_{ax-ax}) ($\Phi \sim 180°$), and 2–3 Hz between equatorial–equatorial protons (J_{eq-eq}) ($\Phi \sim 60°$) [12,13]. The value of J_{H-P} in inositol phosphates is about 8–9.5 Hz, similar to J_{ax-ax}, and has been established by the following experiments: (a) Broadband decoupling of phosphorous leads to loss of coupling of about 8–9.5 Hz [14–16]. (b) Comparison of the splitting patterns of ring protons of phosphorylated and nonphosphorylated inositols reveals the loss of coupling of about 8–9.5 Hz due to phosphorous [14–17]. ^{1}H NMR is uniquely capable of providing conformational information because, whereas ^{31}P chemical shifts may be influenced by both pH and conformational changes, and ^{1}H NMR coupling constants are affected only by dihedral angles between vicinal protons.

The conformation of inositol phosphates in aqueous solutions was investigated by ^{1}H NMR spectroscopy at 500 MHz (unless specified). Symmetrical and unsymmetrical

inositol phosphates were analyzed over the pH range 1–13 at intervals of about 1 pH unit.

Conformation of InsP_6.—The ^{1}H NMR spectrum of Ins P_6 was essentially unchanged at pH 0.5–9.0, after which it changed significantly. The ^{1}H NMR spectrum at pH 5.0 is presented in Fig. 2A. The H-2 proton at δ 4.8 is split into a broad doublet due to one large coupling (8.5 Hz) with P and two small couplings (2.5 Hz) with H-1 and H-3 protons, indicating equatorial orientation of the H-2 proton and axial–equatorial relationships with adjacent protons. The magnetically equivalent H-4 and H-6 are split into a quartet (δ 4.35) due to couplings of about 8.5 Hz with vicinal protons (J_{ax-ax}) and phosphorus (J_{H-P}). The assignments are shown in Fig. 2A [17]. Therefore, the splitting pattern is consistent with the 1ax/5eq conformation in which the phosphate at C-2 is oriented in the axial position and the phosphates at C-1, C-3, C-4, C-5, and C-6 are oriented in equatorial positions.

The pH of the solution was increased by the addition of NaOD, and NMR spectra were monitored at pH intervals of 1.0. Fig. 2B is the ^{1}H spectrum of Ins P_6 at pH 9.5. Distinct proton resonances and splitting pattern of either conformation cannot be discerned; instead, broad peaks were observed at room temperature. Loss of distinguishable splitting pattern is caused by exchange broadening of resonances due to the presence of an interconverting mixture of the 1ax/5eq and 5ax/1eq conformers. The rate of interconversion between the 1ax/5eq and 5ax/1eq conformers influences both the chemical shift and the splitting pattern of the ^{1}H NMR spectrum [18–21]. The spectrum (Fig. 2B) indicates that the coalescence point is below room temperature (22 °C). In order to determine the coalescence temperature, the mixture was cooled and temperature-dependent ^{1}H NMR spectra were recorded at 200 MHz; the coalescence temperature was about 6 °C (data not shown). As the temperature was lowered below 6 °C, distinct proton resonances and splitting patterns corresponding to the two conformers became progressively clearer indicating that the rate of interconversion was decreasing. The NMR spectrum at −11 °C (Fig. 2C) shows the presence of the quartet (at around 3.8–4.0 ppm) due to H-4 and H-6 protons of the 1ax/5eq conformer (Fig. 2A) and broadened doublets due to the 5ax/1eq conformation indicating that the two conformers are interconverting at a slow rate relative to the NMR time scale. Raising the pH to 10.0 was accompanied by additional changes in the ^{1}H NMR spectrum of Ins P_6 which then remained unchanged over the pH range 10.0–13.0. Fig. 2D is the ^{1}H spectrum of Ins P_6 at pH 11.0. The splitting pattern of each resonance was observed as broad doublets. The pronounced upfield shift of H-2 is consistent with protons at axial positions. Only one large coupling, J 8.7 Hz, with P is observed for each proton. Broad doublets suggest one large coupling with P and two small couplings ($\sim$ 2–3 Hz) with vicinal protons indicating the presence of the 5ax/1eq conformer. Loss of additional large vicinal couplings suggests flipping of the 1ax/5eq conformation to 5ax/1eq form, thereby changing dihedral angles. The spectrum is consistent with the 5ax/1eq form in which the phosphates at C-1, C-3, C-4, C-5, and C-6 are in the axial position and the phosphate at C-2 is in the equatorial position. DQFCOSY (double quantum filtered correlation spectroscopy) spectrum of phytic acid contains information regarding H–H connectivities (Fig. 3) and provides additional proof of structural assignment. Consistent with the results obtained by previous researchers [9], our results suggest that phytic acid adopts

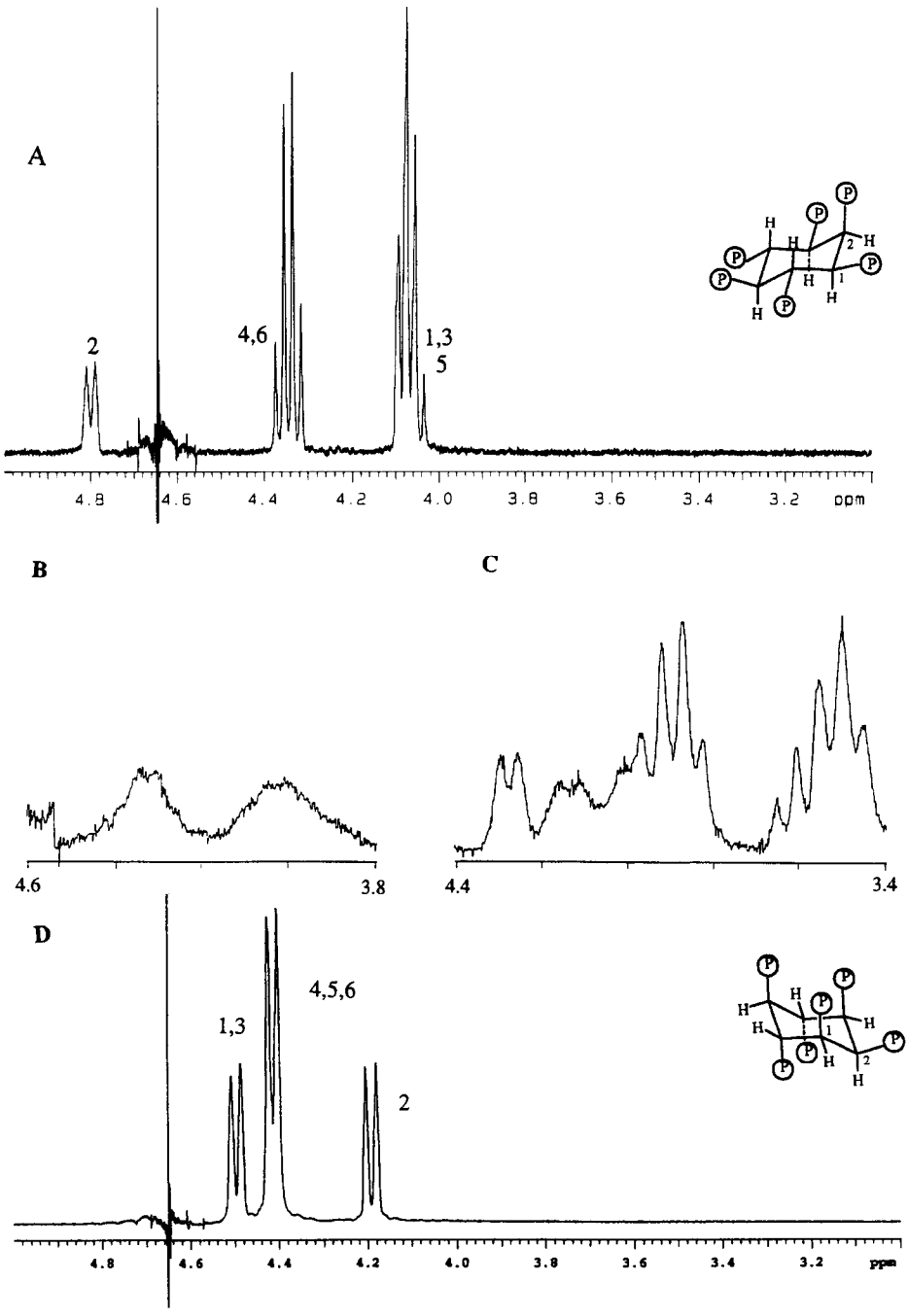


Fig. 2. ^{1}H NMR spectra of InsP_6 at different pH. (A) 5.0; (B and C) 9.5 at room temperature and −11 °C respectively, at 200 MHz; and (D) 11.0.

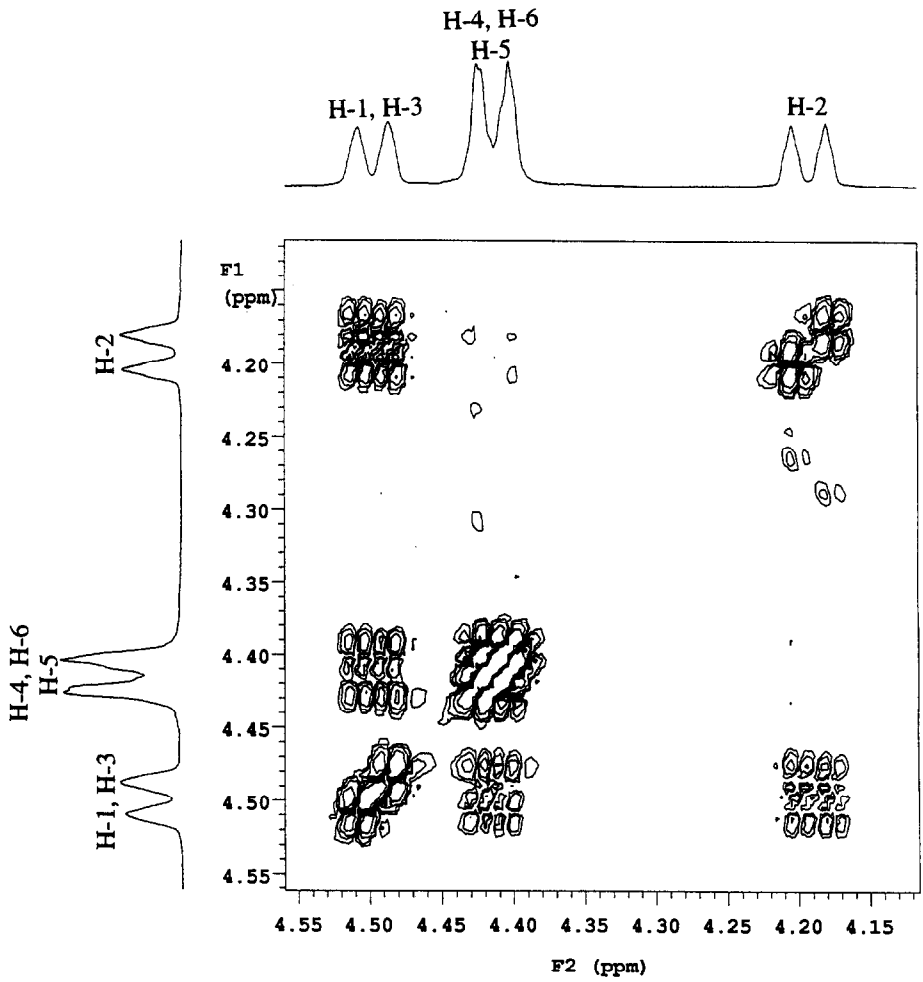


Fig. 3. 2D DQFCOSY of InsP_6 at pH 11.0 with attached ^{1}H spectra. The sweep width in both the F_1 and F_2 dimensions was 456.4 Hz. A total of 128 t_1 increments, each consisting of eight transients with relaxation delay of 4 s between successive transients, were obtained. Shifted Gaussian window was applied in both dimensions. The data matrix was expanded to a 256 × 128 real matrix resulting in digital resolution of 1.8 and 3.6 Hz/point in the F_1 and F_2 dimensions, respectively.

the 1ax/5eq conformation (Fig. 1, Structure **1**) in the pH range 0.5–9.0. In this conformation the five contiguous phosphates are equatorially oriented thereby minimizing steric crowding. In the 5ax/1eq form, unfavorable steric interactions between 1,3,5-triaxial and the 4,6-diaxial phosphates exist (Fig. 1, Structure **2**). According to previous investigations [8,10], six protons have pK_a values in the range of 1–2, three protons in the range 5.0–8.6, and three in the range 9.2–9.6. According to our data, at pH 9.5, close to the pK_a of the three least acidic protons, InsP_6 is present as an interconverting mixture of 1ax/5eq and 5ax/1eq conformers (Structures **1** and **2**, respectively). Removal of all acidic protons from Structure **1** renders the phosphate

groups dianionic (Structure **2**) and thereby results in significant electrostatic repulsion between the equatorial phosphate groups. Above pH 9.5 (10.0–13.0), as the concentration of dianionic phosphates increases, the absence of large coupling constants (J_{ax-ax}) in the NMR spectrum suggests that phytic acid exclusively adopts the 5ax/1eq conformation. The 5ax/1eq conformation adopted at higher pH can reduce the repulsive interactions between the five vicinal equatorial groups in the 1ax/5eq conformer, and, in addition, stabilize the dianionic phosphates by transanular bonding of cations with the 1,3,5-triaxial and the 4,6-diaxial phosphates, and/or hydrogen bonding with solvent [11].

To summarize, consistent with the study of Isbrandt and Oertei [9], InsP_6 adopts the 1ax/5eq conformation in the pH range 0.5–9.0. Around pH 9.5, the 1ax/5eq and 5ax/1eq conformers exist in equilibrium, and in the higher pH range, 10.0–13.0, InsP_6 exclusively adopts the 5ax/1eq conformation.

Conformation of InsP_5.—Symmetrical Ins(1,2,3,4,6)P_5 and unsymmetrical Ins(1,2,3,5,6)P_5 were used to investigate the conformational stability of InsP_5 (Fig. 4

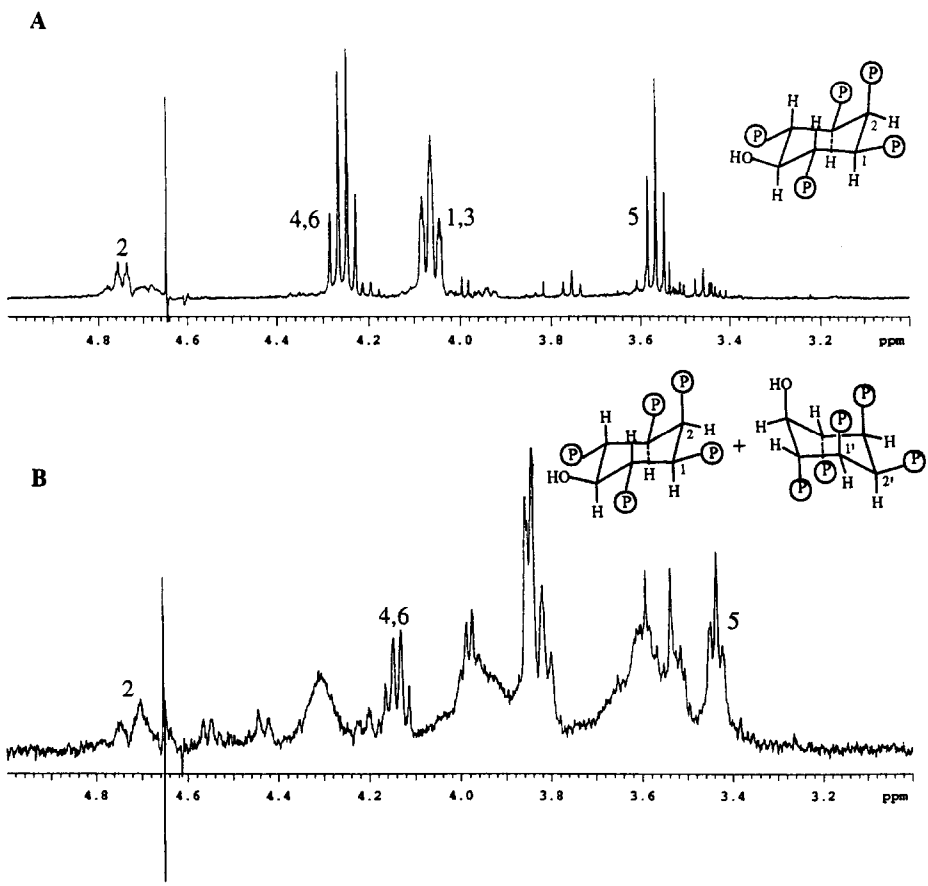


Fig. 4. ^{1}H NMR spectra of Ins(1,2,3,4,6)P_5 at different pH. (A) 5.0 and (B) 11.0.

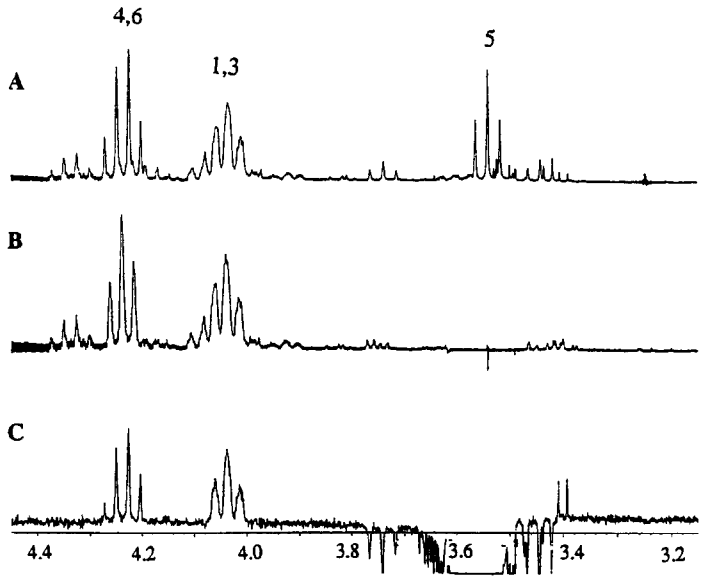


Fig. 5. The 400 MHz spectra of Ins(1,2,3,4,6)P_5 at pH 5 in the δ 3.0–4.5 region. (A) ^{1}H spectrum; (B) Homonuclear (^{1}H) decoupled spectrum obtained by irradiation of H-5; (C) NOE difference spectrum obtained by irradiation of H-5.

and Fig. 6, respectively). The ^{1}H NMR spectrum of Ins(1,2,3,4,6)P_5 was essentially unchanged at pH 1.0–9.0. Fig. 4A illustrates the ^{1}H NMR spectrum at pH 5.0. The chemical shifts and splitting patterns, for example the quartet at δ 4.25 due to large couplings of H-4 and H-6 with vicinal protons (J_{ax-ax}) and with phosphorus (J_{H-P}), are consistent with the 1ax/5eq conformer of Ins(1,2,3,4,6)P_5.

Noticeable changes were observed in the ^{1}H NMR spectrum when the pH was raised to 9.5, and the spectrum then remained unchanged at pH 9.5–13.0. Fig. 4B is the ^{1}H NMR spectrum of Ins(1,2,3,4,6)P_5 at pH 11.0. Exchange broadening of resonances suggests that an interconverting mixture of the 1ax/5eq and 5ax/1eq conformers is present. Even though all the resonances could not be assigned due to exchange broadening, the quartet at δ 4.15 (H-4 and H-6) due to the 1ax/5eq conformer is clearly discernable indicating that, unlike the case of InsP_6, the resonances have not coalesced at room temperature, thereby suggesting that the rate of interconversion is lower than InsP_6.

Additional confirmation of the 1ax/5eq conformation of Ins(1,2,3,4,6)P_5 at pH 5.0 was obtained by an NOE difference experiment. Homonuclear decoupling of H-5 by irradiation at δ 3.55 resulted in the conversion of the quartet at δ 4.21 (H-4 and H-6) to a triplet (Fig. 5B), thereby indicating spin–spin coupling between these resonances and confirming the assignments of H-4, H-5, and H-6. In a NOE difference experiment (Fig. 5C), irradiation of H-5 (δ 3.55) lead to enhancements (less than 1%) at vicinal protons, H-4 and H-6, and, in addition, at H-1 and H-3, thereby indicating the spatial proximity of protons H-1 and H-3 to H-5 as would be expected from 1,3-diaxial interactions of

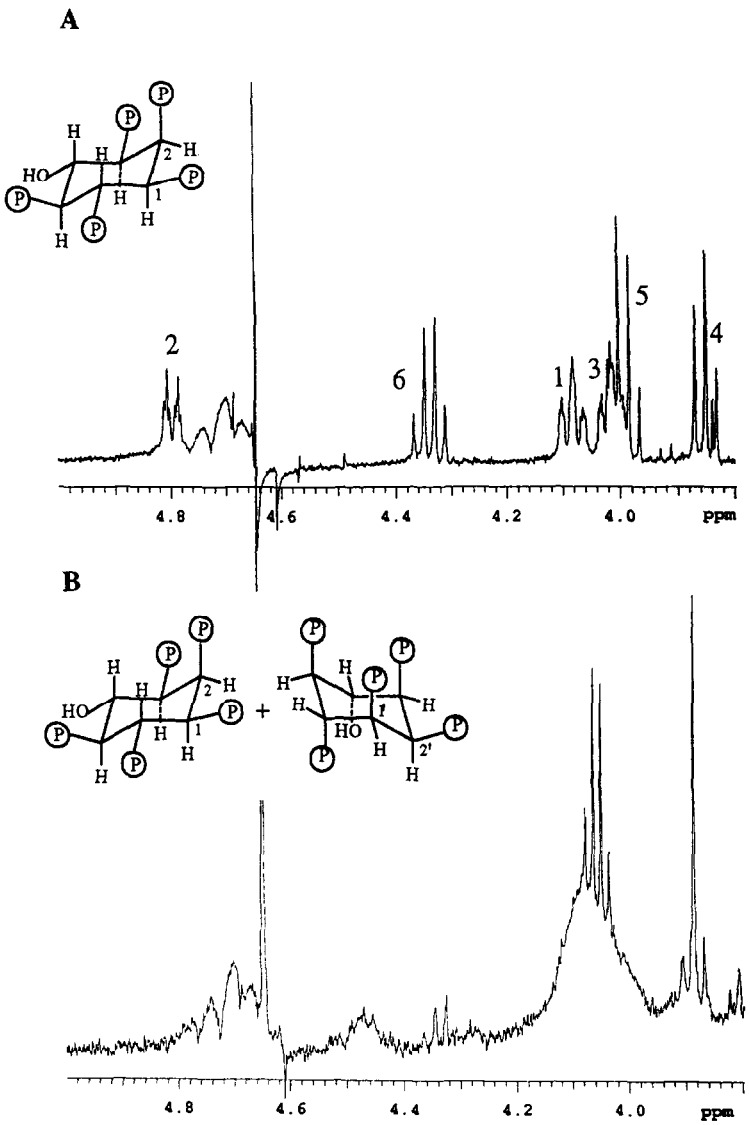


Fig. 6. ^{1}H NMR spectra of Ins(1,2,3,5,6)P_5 at different pH. (A) 5.0 and (B) 11.0.

H-5 with H-1 and H-3 in the 1ax/5eq conformation. In addition, irradiation of H-1 and H-3 protons at δ 4.05 lead to enhancement at H-5 (data not shown).

Ins(1,2,3,5,6)P_5 was also analyzed over the pH range 1.0–13.0. The ^{1}H NMR spectrum of Ins(1,2,3,5,6)P_5 was essentially unchanged between pH 1.0–7.5. The ^{1}H NMR spectrum of Ins(1,2,3,5,6)P_5 at pH 5.0 is presented in Fig. 6A. The assignments are indicated in Fig. 6A, and they are consistent with the structure of the 1ax/5eq conformer of Ins(1,2,3,5,6)P_5. For example, the quartet (J 8.6 Hz) assigned to H-6 is due to diaxial couplings with vicinal protons H-5 and H-1 and with P.

When the pH was raised to above pH 8.0, the ^{1}H NMR spectrum changed significantly and then remained unchanged at pH 8.0–13.0. Fig. 6B, the ^{1}H NMR spectrum at pH 11.0, shows the broadening of resonances due to the presence of an equilibrium mixture of 1ax/5eq and 5ax/1eq conformers. The presence of a well-resolved quartet and triplet overlying the exchange-broadened peaks indicates that, although the coalescence point has not been reached, the mixture is closer to coalescence than Ins(1,2,3,4,6)P_5 under the same conditions.

Ins(1,2,3,4,6)P_5 and Ins(1,2,3,5,6)P_5 both adopt the 1ax/5eq form at pH 1.0–9.0. Above pH 9.5 (10.0–13.0), both InsP_5 molecules are present as an interconverting mixture of the 1ax/5eq and 5ax/1eq conformers. As described above, the dianionic phosphates in the axial positions could be stabilized by sodium counterions and hydrogen bonding. However, in contrast to InsP_6, further increase in pH did not increase the concentration of the 5ax/1eq form probably due to decreased repulsive interactions compared to InsP_6.

The NMR spectra of Ins(1,2,3,4,6)P_5 and Ins(1,2,3,5,6)P_5 contain distinct resonances indicative of the 1ax/5eq conformation over the pH range 9.5–13. At room temperature, Ins(1,2,3,5,6)P_5 is closer to coalescence than Ins(1,2,3,4,6)P_5, indicating that the rate of chair–chair interconversion of Ins(1,2,3,5,6)P_5 is faster than that of Ins(1,2,3,4,6)P_5. This observation suggests that the energy barrier for chair–chair interconversion of Ins(1,2,3,5,6)P_5 is less than that for Ins(1,2,3,4,6)P_5, and, therefore, the proclivity to ring flip is in the reverse order. InsP_6, which has five contiguous, dianionic equatorial phosphates, has a greater tendency to adopt the 5ax/1eq conformation than either of the InsP_5 isomers investigated. The increased proclivity of Ins(1,2,3,5,6)P_5 to ring flip to the 5ax/1eq form is probably due to the presence of three contiguous equatorial phosphates (C-1, C-5, and C-6) compared to two sets of two (C-1, C-6 and C-3, C-4) contiguous equatorial phosphates in Ins(1,2,3,4,6)P_5.

To summarize, Ins(1,2,3,4,6)P_5 exists in the 1ax/5eq form at low pH. At high pH (above 9.5 for Ins(1,2,3,4,6)P_5, and above 8.0 for Ins(1,2,3,5,6)P_5), an equilibrium mixture of the 1ax/5eq and 5ax/1eq forms is present. Unlike InsP_6, which adopted the sterically hindered (5ax/1eq) form over the range pH 10.0–13.0, the exclusive existence of the sterically hindered 5ax/1eq conformation was not observed for either of the InsP_5 isomers investigated.

Conformation of InsP_4.—Unsymmetrical Ins(1,2,3,4)P_4 and Ins(1,2,5,6)P_4 were used to investigate the conformation of InsP_4 molecules).

The splitting patterns in the ^{1}H NMR spectrum of Ins(1,2,3,4)P_4 were essentially unchanged over the entire pH range (1.0–13.0). Fig. 7A is the ^{1}H NMR spectrum at pH 5.0. The assignments are shown in Fig. 7A and they are consistent with the 1ax/5eq conformation. For example, the triplet resonance of H-5 at δ 3.50 is due to large couplings (8.9 Hz) with the axially oriented H-4 and H-6 (J_{ax-ax}).

Comparison of the ^{1}H NMR spectrum at pH 5.0 (Fig. 7A) and at pH 11.0 (Fig. 7B) indicated changes in chemical shifts but not in the splitting pattern. The resonances of protons geminal to a phosphate shifted upfield by about 0–0.10 ppm, and the resonances of protons geminal to a hydroxyl group shifted downfield by about 0.05–0.15 ppm. These changes in the chemical shifts led to overlap of several resonances (H-1 overlapped with H-3, and H-6; H-2 overlapped with the water resonance). The quartet at

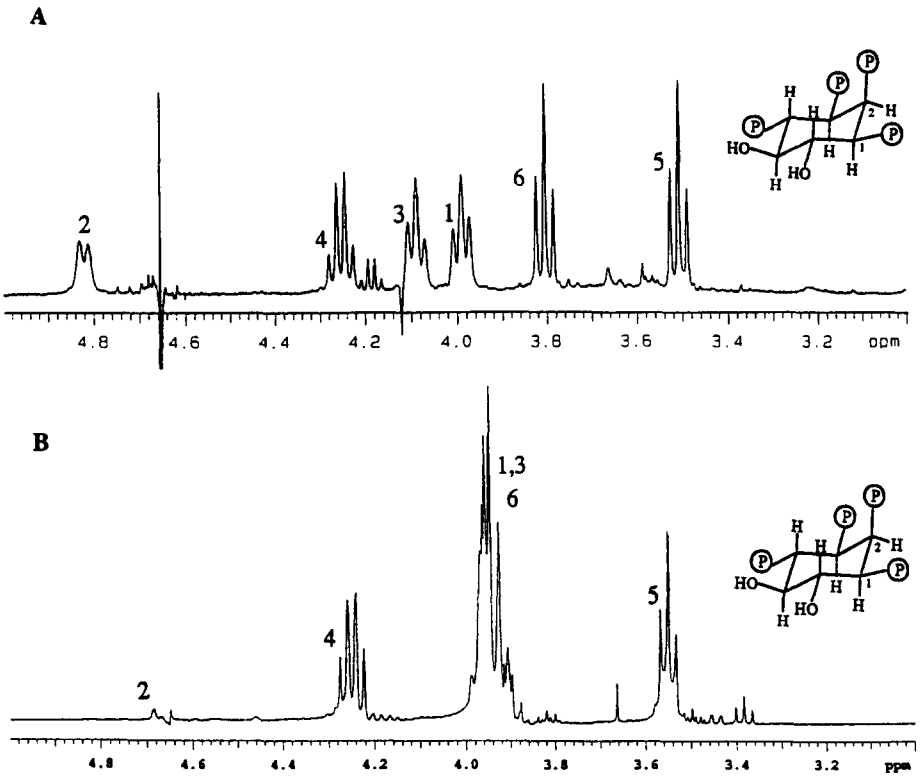


Fig. 7. ^{1}H NMR spectra of Ins(1,2,3,4)P_4 at different pH, (A) 5.0 and (B) 11.0.

δ 4.25 and the triplet at δ 3.55 are consistent with the presence of the 1ax/5eq conformation at high pH.

Similar results were observed for Ins(1,2,5,6)P_4. The splitting pattern in the ^{1}H NMR spectrum was essentially unchanged over the entire pH range (1.0–13.0). The NMR parameters of Ins(1,2,5,6)P_4 at pH 5.0 and 11.0 are listed in Table 1 [22]. Although the H-1 and H-4 resonances overlap, there are identifiable resonances that indicate the presence of the 1ax/5eq conformation. For example, the quartets at δ 4.25 and δ 3.70 (J 8.5 Hz) at pH 11.0 are due to couplings of H-6 and H-5 with axially oriented vicinal protons (J_{ax-ax}), and with phosphorus (J_{H-P}).

To summarize, both Ins(1,2,3,4)P_4 and Ins(1,2,5,6)P_4 exist in the 1ax/5eq form over the pH range 1.0–13.0. No evidence for the 5ax/1eq form was observed.

Conformation of InsP_3.—NMR investigations of inositol phosphates Ins(1,4,5)P_3, Ins(1,2,3)P_3, and Ins(1,2,6)P_3 were conducted over the pH range 1.0–13.0.

The splitting pattern in the ^{1}H NMR spectrum of Ins(1,4,5)P_3 was unchanged over the entire pH range (1.0–13.0) (Table 1). The NMR spectra of Ins(1,4,5)P_3 at pH 5.0 and at pH 11.0 (Table 1) support the presence of the 1ax/5eq conformation at both pH. For example, the doublet of doublets around δ 3.62–3.60 (J 9.5 Hz and 2.9 Hz) is

Table 1
^{1}H NMR chemical shifts of inositol phosphates at pH 5.0 and 11.0 [a]

		H-1	H-2	H-3	H-4	H-5	H-6
Ins(1,2,5,6)P_4	pH 5.0	*4.10td	*4.80dt	3.56dd	3.78t	*3.95q	*4.35q
	pH 11.0	*H [b]	*4.60dt	3.38dd	H [b]	*3.70q	*4.25q
Ins(1,4,5)P_3	pH 5.0	*3.90td	4.16t	3.62dd	*4.20q	*3.95q	3.80t
	pH 11.0	*H [c]	4.22t	3.60dd*	*4.05q	*H [c]	H [c]
Ins(1,2,3)P_3	pH 5.0	*3.92td	*4.80dt	3.92td	3.70t	3.30t	3.70t
	pH 11.0	*3.84td	*4.48dt	*3.84td	3.78t	3.25t	3.78t
Ins(1,2,6)P_3	pH 5.0	*4.05td	*4.80dt	3.50dd	3.65t	3.40t	*4.20q
	pH 11.0	*3.90td	*4.70dt	3.40dd	3.78t	3.45t	*4.18q
Ins(1,2)P_2	pH 5.0	*4.00td	*4.75dt	3.50dd	3.68t	3.25t	3.78t
	pH 11.0	*4.00td	*4.60dt	3.55dd	3.78t	3.35t	3.90t
Ins(2)P_1	pH 5.0	3.52dd	*4.53dt	3.52dd	3.66t	3.25t	3.66t
	pH 11.0	3.45dd	*4.50dt	3.45dd	3.66t	3.25t	3.66t

[a] Data taken from ref. [22]. An * indicates phosphorylated positions. Multiplicity of proton resonances is shown by dd, doublet of doublets; dt, doublet of triplets; q, quarter; t, triplet; td, triplet of doublets.
[b] Broad triplet from δ 3.75 to δ 3.85 (integrates to two protons) due to H-1(td) and H-4 (t).
[c] Multiplet from δ 3.70 to δ 3.80 (integrates to three protons) due to H-1, H-5, and H-6.

characteristic of the axially oriented H-3 alpha to a hydroxyl group. The splitting pattern is due to one large (9.5 Hz) coupling with the axially oriented H-4 and one small (2.9 Hz) coupling with the equatorially oriented H-2. Comparison of spectra at pH 5.0 and pH 11.0 indicate changes in the chemical shifts but not in coupling constants. At high pH, the resonances corresponding to some protons geminal to a phosphate shifted upfield by about 0.1–0.2 ppm, and those corresponding to some protons geminal to a hydroxyl shifted downfield by 0.05 ppm.

Similar results were observed for Ins(1,2,3)P_3. The splitting pattern of the resonances in the ^{1}H NMR spectrum of Ins(1,2,3)P_3 was unaffected by pH changes (Table 1). The NMR characteristics are consistent with the 1ax/5eq conformer. For example, the triplets at δ 3.30–3.25 are due to the H-5 proton geminal to a hydroxyl group. The triplet splitting pattern, J 9 Hz, is due to two vicinal protons, H-4 and H-6, in axial–axial relationships. Similarly, the triplets at δ 3.70–3.78 (J 8.8 Hz) are due to protons H-4 and H-6 in axial–axial relationships with vicinal protons.

The splitting patterns of the ^{1}H NMR spectrum of Ins(1,2,6)P_3 were unaffected by pH. Table 1 lists the NMR characteristics of Ins(1,2,6)P_3 at pH 5.0 and 11.0. Consistent with previous observations for other InsP_3 molecules discussed above, an increase in pH resulted in changes in chemical shifts but not in splitting patterns; at high pH, the resonances corresponding to some protons geminal to phosphates shifted upfield by about 0.05–0.10 ppm, and those corresponding to some protons geminal to hydroxyls shifted down field by 0.10–0.15 ppm. The quartets at δ 4.20–4.18 due to H-6, triplets at δ 3.65–3.78 and δ 3.40–3.45 due to H-4 and H-5, and doublet of doublets at δ 4.05–3.90 due to H-1, are consistent with the 1ax/5eq conformation of Ins(1,2,6)P_3 at both pH 5.0 and 11.0.

In summary, Ins(1,2,3)P_3, Ins(1,4,5)P_3, and Ins(1,2,6)P_3 exist in the 1ax/5eq form over the entire pH range of 1.0–13.0. No evidence for the 5ax/1eq form was observed.

Conformation of InsP_2.—Unsymmetrical Ins(1,2)P_2 was used to investigate the conformation of InsP_2 molecules. The splitting pattern of the resonances in the ^{1}H NMR spectrum of Ins(1,2)P_2 did not change over the pH range (1.0–13.0) (Table 1). Consistent with previous observations, some protons geminal to a phosphate shifted upfield by about 0–0.10 ppm, and some protons geminal to a hydroxyl group shifted downfield by about 0.10 ppm. The splitting patterns and the coupling constants are consistent with the 1ax/5eq conformation of Ins(1,2)P_2. For example, the triplets at δ 3.78–3.90 are due to the coupling of H-6 with vicinal protons in axial–axial relationships (J 9.5 Hz), thereby suggesting the presence of the 1ax/5eq conformation.

Conformation for InsP_1.—Symmetrical Ins(2)P_1 was used to investigate the conformation of InsP_1. The ^{1}H NMR spectrum of Ins(2)P_1 was unaffected by changes in the pH over the range 1.0–13.0 (Table 1). The doublet of triplets (J 8.5 Hz and 2.5 Hz) at δ 4.53–4.50 are characteristic of an equatorially oriented H-2 attached to a phosphorylated carbon. The splitting pattern is due to one large coupling with P (J_{H-P} 8.5 Hz) and two small couplings (J_{ax-eq} 2.5 Hz) with vicinal equatorial protons, H-1 and H-3, thereby suggesting the presence of the 1ax/5eq conformation of Ins(2)P_1.

In summary, ^{1}H NMR analysis clearly indicated the conformational preferences of various inositol phosphates at different pH. In the course of these experiments, the pH of the solutions was changed back and forth between acidic and basic pH values and each time the same pH-dependent spectra were obtained indicating that the pH-dependent spectra are reproducible and that the compounds are stable. InsP_6 favors the 5ax/1eq conformer exclusively at pH > 9 whereas, both Ins(1,2,3,5,6)P_5 and Ins(1,2,3,4,6)P_5 exist as an interconverting mixture of both conformers at high pH. Inositol phosphates with fewer than five phosphates did not provide evidence for favoring the 5ax/1eq form at any pH. The rate of chair–chair interconversion between the 1ax/5eq and 5ax/1eq conformers is as follows: InsP_6 > Ins(1,2,3,5,6)P_5 > Ins(1,2,3,4,6)P_5. This parallels the number of contiguous phosphates in equatorial positions namely, five in InsP_6, three in Ins(1,2,3,5,6)P_5, and two sets of two in Ins(1,2,3,4,6)P_5. However, it is noteworthy that Ins(1,2,5,6)P_4, which also has three contiguous equatorial phosphates, does not show any preference for the 5ax/1eq form and neither does Ins(1,2,3,4)P_4, which has one set of two contiguous equatorial phosphates.

On the basis of conformations adopted by *myo*-inositol phosphates (InsP_6 to InsP_1) at different pH, we conclude that the presence of four or more equatorial phosphates on the inositol ring induces a change in the conformation from the sterically unhindered 1ax/5eq conformation to the sterically hindered 5ax/1eq conformation, at high pH. The electrostatic repulsion due to four or more equatorially oriented dianionic phosphates disfavours the 1ax/5eq conformation. These results suggest that phosphorylated derivatives of *scyllo*-inositol, in which all the phosphates are in the equatorial orientation (6eq), would exhibit a greater preference for the sterically hindered 6ax orientation at basic pH than the corresponding *myo*-isomers. When the phosphates are syn-axially oriented, they have the ability to form strong coordination bonds with sodium counterions and thereby minimize electrostatic repulsions. These data clearly illustrate that the conformational preferences of phytic acid at different pH cannot be used as a guide to predict the conformations of other inositol phosphates.

3. Experimental

Crude wheat-bran phytase was purchased from Sigma Chemical Company, St. Louis, MO. Alkaline phytase was extracted from lily pollen (*Lilium longiflorum* L. Cv Nellie White, 1991 harvest) as previously described [17]. Ins(1,4,5)P_3 was purchased from Sigma Chemical Company, St. Louis, MO. Ins(1,2,3,5,6)P_5, Ins(1,2,5,6)P_4, Ins(1,2,6)P_3, Ins(1,2)P_2, and Ins(2)P_1 were produced by wheat-bran phytase-catalyzed hydrolysis of phytic acid as described below. Ins(1,2,3,4,6)P_5, Ins(1,2,3,4)P_4, and Ins(1,2,3)P_3 were produced by alkaline phytase-catalyzed hydrolysis of phytic acid as described below.

Inositol phosphates produced by wheat-bran phytase.—Crude wheat bran phytase (22 mg solid containing 2.3 mg protein as estimated by the Bio-Rad assay) was dissolved in water (10 mL). The solution was first filtered through a 0.22 μ filter to remove undissolved solids and then through a 30,000 MW cut-off membrane filter (Millipore) to remove low molecular weight impurities, and lyophilized. Phytic acid (20 μmol, 20 mg of sodium salt) was dissolved in H_2O (0.7 mL), and the pH was adjusted to 5.0 with acetic acid. Phytase (4.4 mg of protein) was added to the solution of phytic acid, and the temperature was maintained at 25 °C. To monitor the reaction, aliquots of the reaction mixture were separated by HVE at different time periods. Inositol phosphates were separated by HVE and extracted from the paper as previously described [17]. The mixture of inositol phosphates was then dried and dissolved in D_2O for NMR analysis.

Inositol phosphates produced by alkaline phytase.—Typically, to isolate 10 mg of Ins(1,2,3)P_3, a solution (6 mL) containing 6 mg of alkaline-phytase containing protein of specific activity (2.0 ± 0.3) μmolP_i/h/mg protein [17] was mixed with an aq solution (6 mL) containing phytic acid (4 mM), Tris-HCl buffer (0.2 M, pH 8.0), CaCl (2 mM), and sodium fluoride (20 mM). After 48 h at 37 °C, only InsP_3 was observed. To isolate Ins(1,2,3,4)P_4 (200 mg), a more concentrated solution of phytic acid (14 mM) was mixed with 35 mL of the same enzyme preparation. Only Ins(1,2,3,4)P_4 was observed after 4 days at 37 °C. Ins(1,2,3,4,6)P_5 were obtained only as a mixture of InsP_6 or Ins(1,2,3,4)P_4. The separation and purification was performed by HVE as previously described [17].

Nuclear magnetic resonance spectroscopy.—Spectra were recorded on a 500 MHz Varian VXR-500, 400 MHz Varian Unity Inova-400, and a 200 MHz Varian XL-200 spectrometers. Inositol phosphates (0.5–2 mg) were first dissolved in deionized water (0.7 mL), the pH adjusted to 1.0 with HCl, lyophilized, and redissolved in D_2O (0.7 mL). ^{1}H NMR spectra were recorded at different pH over the range 1–13. The pH was increased by adding aliquots of NaOH (10 N) in D_2O. For final pH adjustments perdeuterated acetic acid was used when necessary. The pH of solutions in NMR tubes (5 mm o.d.) were measured with a combination pH electrode (Wilmad Glass Company). The pH meter was calibrated using the following standards: oxalic acid (0.1 M, pH 1.30); standard pHydrion buffers (pH 4.0, 7.0, and 10.0; Metrepak). The pH values reported in this paper are readings of the glass electrode uncorrected for deuterium effects. All spectra were recorded at 25 °C. One-dimensional ^{1}H NMR spectra were obtained at 499.84 MHz. ^{1}H chemical shifts were referenced to the residual proton absorption of the solvent, D_2O (δ 4.67). The acquisition conditions were as follows:

spectral windows 6738 Hz; pulse width, 50–90° tipping angle. For 1D spectra typically, 8–64 scans with recycle delays of 6 s. between acquisitions were collected. The residual H_2O resonance was suppressed by a 2s selective presaturation pulse.

Variable-temperature NMR experiments were performed on a Varian XL-200 spectrometer. The temperature was calibrated by monitoring peak separations of MeOH at different temperatures [23].

DQFCOSY was obtained using a 5 mm $^{1}H/^{19}F$ probe. The pulse sequence was that of Piantini et al. [24]. Experimental details and processing parameters are given in the figure legend.

Homonuclear decoupling experiments and cycle NOE difference experiments (CYCLENOE) [25] were performed on a Varian Unity Inova 400 spectrometer.

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