Inframolecular protonation process of 6-modified *myo*-inositol 1,4,5-tris(phosphates): substitution effects on the cooperativity between the phosphate groups

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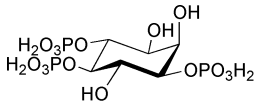
The protonation process of the individual functional groups of four analogues of (±)-*myo*-inositol 1,4,5-tris(phosphate) [Ins(1,4,5)P_3], modified at position 6, were studied by ^{31}P- and ^{1}H-NMR titration experiments in order to gain insight into the reasons for the particular importance of OH6 for the binding of Ins(1,4,5)P_3 to its receptor. The fluorinated derivative (**2**) was studied to evaluate the H-bond donor or acceptor ability of OH6. Compounds **3** and **4** should explain the presence and the configuration of OH6, respectively, whereas the amino analogue (**5**) was considered to delineate the effect of a positively charged group of about the same size as OH6 on the vicinal phosphates P1 and P5. The ^{31}P-NMR curves look alike for compounds **2**–**4** and differ from that of Ins(1,4,5)P_3, as the initial downfield shift of P1 upon protonation is no longer observed. In the ^{1}H-NMR titration curves, the wrongway shift found for H2 of Ins(1,4,5)P_3 is observed for H6 in compounds **3** and **4**. In addition, both phosphorus and proton resonances of compound **5** are influenced by the protonation of the neighbouring NH_2 group. By considering the protonation constants it is shown that the log K_1 values decrease in the order 6-F-Ins(1,4,5)P_3 > 6-deoxy-Ins(1,4,5)P_3 > Ins(1,4,5)P_3 > *epi*-Ins(1,4,5)P_3 > 6-NH_2-Ins(1,4,5)P_3, indicating that the substitution effect is the consequence of the lipophilicity of the substituents, the basicity order following the order of the hydrophobic π constants. Consideration of the microprotonation constants, calculated for all the studied compounds, leads to the conclusion that hydration effects and hydrogen bond donor ability of the equatorial OH6 hydroxyl should mainly account for the differences observed between compounds **2**–**4** and Ins(1,4,5)P_3. The amino derivative **5** remains in a zwitterionic form over almost the entire pH range studied (2.5 < pH < 11.5). Through the presence of an intramolecular hydrogen-bonded ion pair interaction the basicity of all three phosphates is decreased whereas the basicity of the amine increases. The strength of the hydrogen-bonded ion pair appears to be of the same order of magnitude as that of the recently published monoammonium phosphate complexes.

Etude inframoléculaire de la protonation d'analogues du *myo*-inositol 1,4,5-tris(phosphate) modifiés en position 6: effets des substitutions sur la coopérativité entre groupements phosphates. Le processus de protonation individuel des groupements fonctionnels de quatre analogues du (±)-*myo*-inositol 1,4,5-tris(phosphate) (Ins(1,4,5)P_3), modifiés en position 6 a été étudié à partir d'expériences de titrages RMN ^{31}P et ^{1}H afin de mettre en évidence l'importance toute particulière de cette position pour l'expression de l'activité biologique. Ainsi, ont été considérés, le dérivé fluoré (**2**) pour évaluer la capacité d'accepteur ou de donneur de liaison hydrogène de OH6, le dérivé déoxy (**3**) et celui porteur d'un OH axial (**4**) pour rendre compte respectivement de l'importance de la présence et de la configuration du groupement hydroxyle. Enfin, le composé aminé (**5**) a été envisagé pour mettre en évidence l'influence d'un groupement chargé positivement sur les deux phosphates vicinaux P1 et P5.

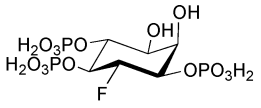
D-*myo*-Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3, **1**] is now well recognised as a major second messenger mediating the intracellular calcium mobilisation that occurs in a wide variety of cellular processes. Ins(1,4,5)P_3, generated in response to activation of cell-surface receptors by numerous ligands, acts through highly stereospecific binding to an endoplasmic reticulum receptor [Ins(1,4,5)P_3R][1–6] In order to elucidate the cellular signal transduction mechanisms, considerable effort has been devoted to the characterisation of the Ins(1,4,5)P_3 receptor and its interaction with the natural ligand. In particular, approaches involving the rational design of Ins(1,4,5)P_3 analogues to map the ligand binding sites of Ins(1,4,5)P_3R have yielded the most crucial structural features for optimal interaction.[7–11] Thus, these studies have revealed the critical importance of the vicinal D-4,5-bisphosphate motif and the enhancing effect of the phosphate group at position 1 (P1). In addition to the phosphates, the three hydroxyl groups play a more or less important role, due to their hydrogen bond acceptor or donor abilities that are involved in both the interaction with the receptor and the setting of the conformation of the ligand in solution. Clearly, OH6 makes a major contribution to the interaction with Ins(1,4,5)P_3R whereas OH2 and OH3 are far less essential. The structure–activity relationship studies undoubtedly afford the structural requirements for agonism at the Ins(1,4,5)P_3R, but these studies only partly account for the subtle mechanisms occurring in the signal transduction processes. In particular, the spatio-temporal characteristics of the Ca^{2+} signalling involve Ins(1,4,5)P_3 and its receptor along with modulators such as Ca^{2+}, pH, ATP, *etc*., which undergo fast local concentration variations in the vicinity of Ins(1,4,5)P_3.[3,7,12–14] Through its coupling domain, Ins(1,4,5)P_3R is the first target for these modulators, but the ligand itself may also, due to its polyfunctional nature, be markedly affected. Thus, it has been shown that Ins(1,4,5)P_3 binding dramatically varies when the pH changes from 7 to 9.[15,16] It is clear also that

within these two pH limits Ins(1,4,5)P_3 undergoes large ionisation state variations. In an attempt to shed new light on the signal transduction mechanism, it is therefore worth examining the protonation process *at each individual* phosphate group and its general influence on the subtle interactions among the functional groups, which ultimately govern the conformation of the molecule and presumably also part of its biological activity. Such an *inframolecular* approach has previously been applied on various inositol phosphates or analogues[17–24] and to Ins(1,4,5)P_3 itself by performing ^{1}H- and ^{31}P-NMR titrations.[23,25]

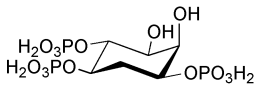
In the present work we report the *inframolecular* behaviour of four analogues of Ins(1,4,5)P_3, modified at position 6, in order to gain insight into the reasons for the particular importance of OH6 for the binding of Ins(1,4,5)P_3 to its receptor. (±)-6-Deoxy-6-fluoro-*myo*-inositol 1,4,5-tris(phosphate) (**2**) was synthesised and studied for evaluating the H-bond donor or acceptor ability of OH6. (±)-6-Deoxy-*myo*-inositol 1,4,5-tris(phosphate) (**3**) and (±)-6-*epi*-inositol 1,4,5-tris(phosphate) (**4**) should explain the presence and the configuration of OH6 respectively. Finally, (±)-6-amino-6-deoxy-*myo*-inositol 1,4,5-tris(phosphate) (**5**) was considered for delineating the effect of a positively charged group of about the same size as OH6 on the vicinal phosphates P1 and P5. The studies were performed in 0.2 M KCl, at 37 °C, near physiological ionic strength and temperature.



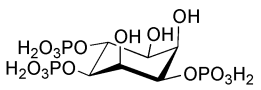
Ins(1,4,5)P_3 (**1**)



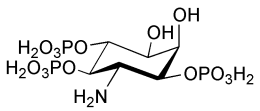
6-F-Ins(1,4,5)P_3 (**2**)



6-deoxy-Ins(1,4,5)P_3 (**3**)



epi-Ins(1,4,5)P_3 (**4**)



6-NH_2-Ins(1,4,5)P_3 (**5**)

Experimental

Materials

(±)-*myo*-inositol 1,4,5-tris(phosphate) (**1**), (±)-6-deoxy-6-fluoro-*myo*-inositol 1,4,5-tris(phosphate) (**2**), (±)-6-deoxy-*myo*-inositol 1,4,5-tris(phosphate) (**3**), (±)-6-*epi*-inositol 1,4,5-tris(phosphate) (**4**) and (±)-6-amino-6-deoxy-*myo*-inositol 1,4,5-tris(phosphate) (**5**) were synthesised as previously described.[26,27]

Potentiometric studies and NMR determinations

Potentiometric and NMR determinations were carried out as previously reported.[18,19,25] The experiments were performed in two steps in which the same initial solution of a compound of about 5×10^{-3} mol dm^{-3} was successively subjected to potentiometric and ^{31}P-NMR or ^{1}H-NMR titrations. The processing of the pH measurements allowed the total concentration of the ligand and the acid as well as the macroscopic protonation constants (by using SUPERQUAD[28,29]) to be determined. The NMR titrations were performed on 0.50 ml of solution in ^2H_2O on a Bruker DPX-300 Fourier transform spectrometer. One-dimensional ^{31}P-NMR spectra were recorded at 121.50 MHz and ^{31}P chemical shifts values were referenced to an external 85% H_3PO_4 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 $\pi/2$ pulse. Typically 1K data points were sampled with a corresponding 0.4 s acquisition time. The spectra had a digital resolution of 1.19 Hz per point. The HypNMR[30] program was used to check the potentiometrically determined protonation constants. ^{1}H-NMR spectra were acquired with water presaturation over a spectral width of 6 ppm using a 3 s relaxation delay and $\pi/2$ 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. Data were zero-filled and a 1 Hz exponential line broadening function was applied prior to Fourier transformation. The temperature in both cases was controlled at 310 ± 0.5 K. The proton and phosphorus resonances of compounds **1**–**5** were assigned by performing proton–proton and phosphorus–proton 2D correlation experiments for a minimum of two suitable pH values thus allowing the titration curves to be unambiguously characterised.

Results and discussion

Macroscopic and microscopic protonation constants

The compounds considered carry three phosphate groups, each group being able to bind only one proton for pH values ranging from 12 to 3. For compound **5**, possessing an additional amino group, a fourth protonation step must be envisaged. The protonation process can be considered macroscopically, leading to macroscopic constants that describe the molecule as a whole. Thus, the macroscopic overall protonation constants β_y quantify the general equilibrium:

$$L^{6-} + yH^+ \overset{\beta_y}{\rightleftharpoons} H_yL^{(6-y)-}.$$

A protonation process occurring stepwise can also be defined by K_y, characterising the equilibrium

$$H_{y-1}L^{(7-y)-} + H^+ \overset{K_y}{\rightleftharpoons} H_yL^{(6-y)-}.$$

These constants can easily be determined from potentiometric and/or ^{31}P-NMR titration curves by treating the pH or chemical shift data with the programs SUPERQUAD[28,29] or HypNMR,[30] respectively.

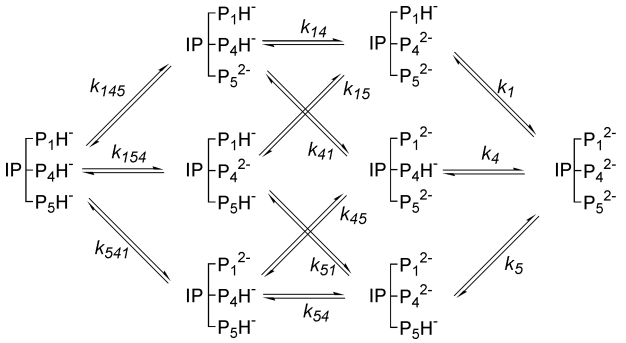
However, if the *inframolecular* protonation processes have to be determined, the microprotonation scheme depicted below and its microprotonation constants have to be resolved. ^{31}P-NMR spectroscopy is a powerful technique, giving easy access to the protonated fraction of each phosphate group,[31–34] provided that the observed chemical shifts for the phosphorus resonances δ_i^{obs} depend mainly on the electronic effects accompanying the variations in the protonation states. Then the protonated fraction $f_{i,p}$ of a phosphate group in position *i* on the inositol ring can be calculated by eqn. (1):

$$f_{i,p} = \frac{\delta_i^{obs} - \delta_{i,d}}{\delta_{i,p} - \delta_{i,d}} \quad (1)$$

where $\delta_{i,p}$ and $\delta_{i,d}$ correspond respectively to the chemical shifts of the protonated and deprotonated fractions of the phosphate in position *i*.

By summing the protonation fraction curves for a given inositol phosphate, the mean number of protons bound per molecule at any pH can be calculated [$\bar{p}$ = f(pH)]. When this curve, as was previously mentioned,[18] is satisfactorily superimposed upon the potentiometric calculated $\bar{p}$ = f(pH) curve, then the individual protonation fractions $f_{i,p}$ are expressed as a function of the macro- and microprotonation constants. For instance, the fraction of protonation of the phosphate at position 1 is defined by the following equation:

$$f_{1,p} = \frac{\beta_3[H^+]^3 + (k_{14}k_1 + k_{15}k_1)[H^+]^2 + k_1[H^+]}{\beta_3[H^+]^3 + \beta_2[H^+]^2 + \beta_1[H^+] + 1} \quad (2)$$



Scheme 1

where k_1, k_{14} and k_{15} refer to the micro-equilibria displayed in Scheme 1. Eqn. (2) is solved by a non-linear regression, introducing the macroprotonation constants generally obtained from the NMR experiments, to give the microprotonation constants.

^{31}P- and ^{1}H-NMR titration curves

The NMR titration curves of the natural ligand **1** are shown in Fig. 1. For compounds **2**–**4** the ^{31}P-NMR curves [Fig. 2(*a*) to 4(*a*)] look similar showing two diphasic curves for phosphates P4 and P5 along with a monophasic one for phosphate P1. Such a pattern corresponds to the superimposition of the titration curves of an inositol monophosphate and an inositol carrying two vicinal phosphates, suggesting that the two phosphate moieties behave independently. Surprisingly, these curves differ markedly from those of Ins(1,4,5)P_3 for which, for instance, P1 undergoes a first downfield shift in the 11 to 7.5 pH range [Fig. 1(*a*)]. The curves of compound **5** (Fig. 5), although keeping the general mono- or biphasic shape, clearly reveal the presence of the additional amino group at position 6 through the initial shift to lower fields experienced by P5 and P1 upon the protonation of the neighbouring NH_2 group. Thus, the chemical shifts of the phosphorus nuclei appear as being mainly influenced by the ionisation state of the phosphate group but also by its functional environment. This will be further ascertained by considering the $\delta_{i,\,p}$ and $\delta_{i,\,d}$ values of compounds **2**–**5** (Table 1).

As stressed in previous work,[23,35] ^{1}H-NMR titration curves bring valuable information on the cooperativity and conformational dynamics of the phosphate groups upon protonation. For purposes of comparison with the compounds studied, the curves of Ins(1,4,5)P_3 are shown on Fig. 1. It can be observed that, as expected, the downfield shift of the protons at the phosphorylated positions parallels the protonation of the corresponding phosphates. Also, the protons bound to non-phosphorylated carbons are affected by the ionisation state of phosphates on neighbouring carbons. Remarkable, however, is the highfield shift of H2, which arises simultaneously with the downfield shift of P1. Both these chemical shift variations occur in the opposite direction from that predicted in the case of protonation processes and have therefore been called "wrongway" shifts.[36–38] The origin of the wrongway shift for compound **1** has been previously discussed and attributed to the formation of a C–H···O hydrogen bond between P1 and H2.[35] At high pH, indeed, in the fully deprotonated form of Ins(1,4,5)P_3, P4 and P5 repel each other, so that P1 is constrained towards H2. In this complex interplay of repulsions and attractions, OH6 has a crucial role in communicating the electrostatic effect between P5 and P1.

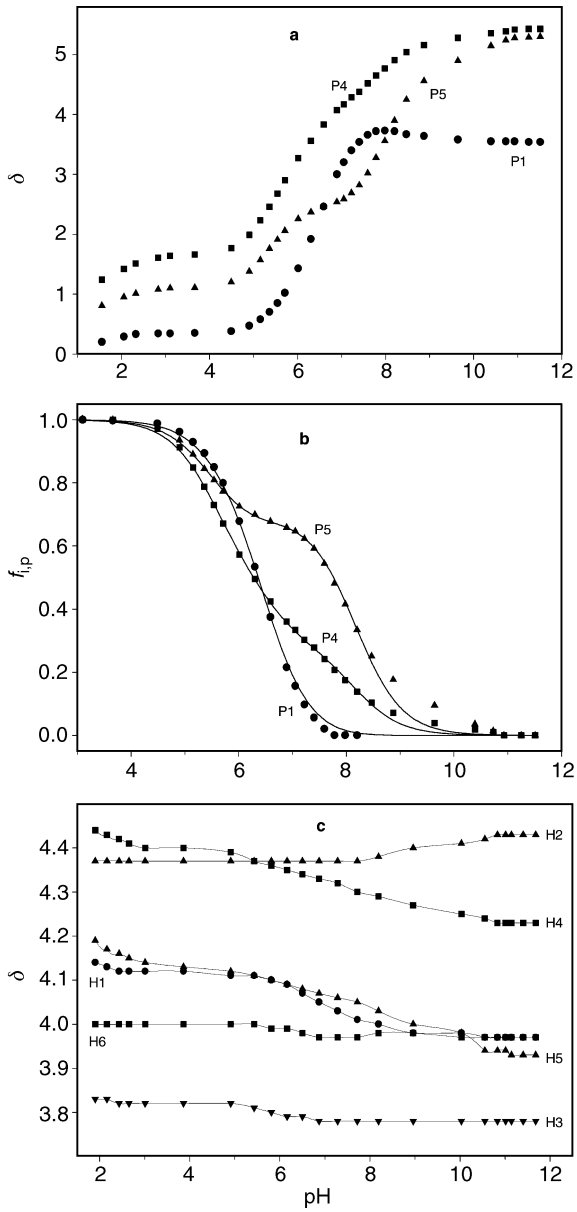


Fig. 1 ^{31}P-NMR and ^{1}H-NMR titrations curves for Ins(1,4,5)P_3 (**1**). (*a*) ^{31}P δ = f(pH) in 0.2 M KCl at 37 °C (100% $^{2}H_2O$); (*b*) the corresponding protonation fraction curves $f_{i,\,p}$; (*c*) ^{1}H δ = f(pH) under the same experimental conditions. The least-squares fit of $f_{i,\,p}$ *vs.* pH is shown as solid lines in (*b*).

The pH dependency of the chemical shifts of the resolved protons in compounds **2**–**5**, are reported in Fig. 2(*c*) to 5(*c*). It can be observed that for all the compounds, the H1, H3, H4,

Table 1 $\delta_{i,\,p}$ and $\delta_{i,\,d}$ values for compounds **1**–**5**

$\delta_{i,\,p\ or\ d}$	Ins(1,4,5)P_3 **1**	6-F-Ins(1,4,5)P_3 **2**	6-deoxy-Ins(1,4,5)P_3 **3**	*epi*-Ins(1,4,5)P_3 **4**	6-NH_2-Ins(1,4,5)P_3 **5**
$\delta_{1,\,p}$	0.34	0.33	0.16	0.40	0.12
$\delta_{4,\,p}$	1.64	1.67	1.36	1.58	1.32
$\delta_{5,\,p}$	1.10	0.70	0.49	0.58	1.38
$\delta_{1,\,d}$	3.73/3.54	4.11	4.12	4.52	4.56/3.90
$\delta_{4,\,d}$	5.43	5.48	5.41	5.85	5.44
$\delta_{5,\,d}$	5.30	3.96	4.01	4.14	4.85/4.21

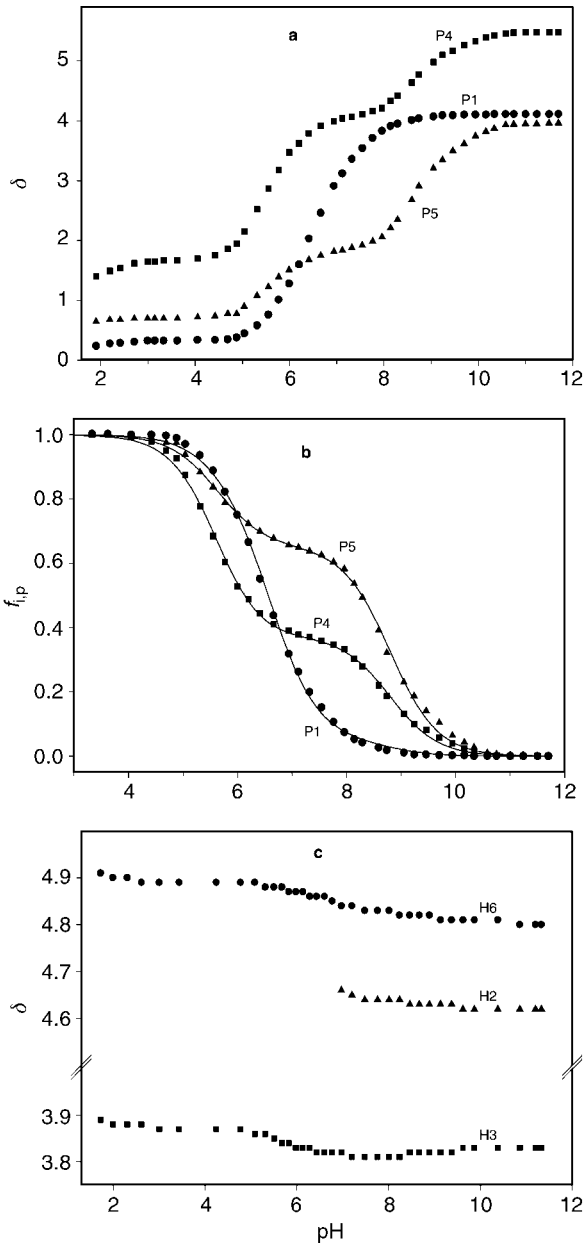


Fig. 2 ^{31}P-NMR and ^{1}H-NMR titrations curves for 6-F-Ins(1,4,5)P_3 (**2**). See caption of Fig. 1 for details.

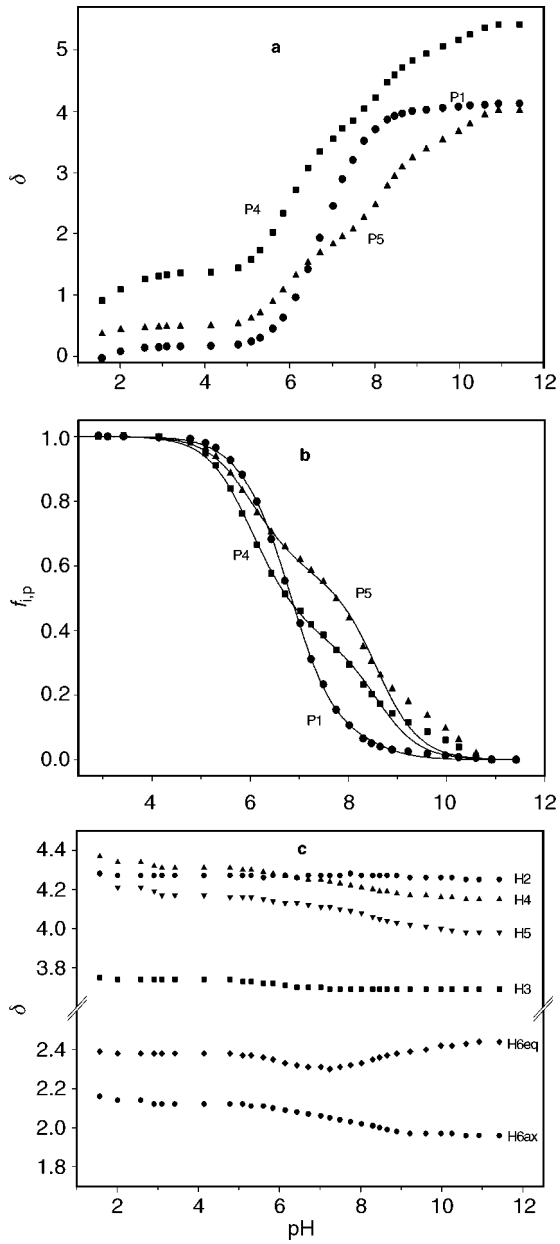


Fig. 3 ^{31}P-NMR and ^{1}H-NMR titrations curves for 6-deoxy-Ins(1,4,5)P_3 (**3**). See caption of Fig. 1 for details.

H5 and H6ax protons behave in an analogous way to the equivalent protons of Ins(1,4,5)P_3, that is they move downfield on going from the highest to the lowest pH values. However, a major difference appears in the behaviour of proton H2. For compounds **2**–**5** the initial H2 wrongway shift observed for **1** no longer occurs and the H2 signal either moves slightly towards lower fields, as expected upon protonation of the neighbouring phosphate (as for **2**, **4**, **5**), or remains at the same position over the entire pH range (as for **3**). In addition, for compounds **3** and **4**, which display an equatorial proton at position 6 (H6eq), there is again, as for H2 of **1**, an initial wrongway shift followed in both cases by a less important shift in the right direction [Fig. 3(*c*) and 4(*c*)]. As already seen previously,[35] the wrongway shift mainly concerns equatorial protons, since it is observed on H6eq but not on H6ax of **3**. Nevertheless, it is not an attribute of equatorial protons since the equatorial H2 of compounds **2**–**5** behaves like the other axial ring protons. Finally, for the amino compound **5**, it is clearly seen that, in addition to the previously mentioned chemical shift variations, all the protons except H2 are more or less sensitive to the protonation of the amino group.

^{31}P- and ^{1}H-NMR chemical shifts: influence of the functional environment and pH variations

The proton and phosphorus chemical shifts at a defined protonation state also contain information about the functional environment. Differences in chemical shifts between **1** and **2**–**5** can be discussed in order to reveal the influence of a hydroxyl configuration inversion or the substitution of an OH group by a fluorine atom, a hydrogen atom or a charged ammonium group. The values in Table 1 show that $\delta_{i,\,p}$ and $\delta_{i,\,d}$ of compounds **2**–**4** are close together, with those of the *epi* derivative (**4**) being slightly higher. If the deoxy compound (**3**) is taken as a reference, it appears that mainly the phosphate P5 experiences the structural variations at position 6. Indeed, $\delta_{5,\,p}$ is displaced to lower fields by about 0.60 ppm in the presence of a vicinal equatorial OH group and by about 0.90 ppm when the 6-position is substituted by NH_3^+. Such an influence might be the result of a local dielectric constant increase induced by the hydrophilicity of both substituents. P1 seems to experience these structural changes to a lesser extent than P5, except in the deprotonated form of Ins(1,4,5)P_3 (**1**), for which the phosphorus chemical shift $\delta_{1,\,d}$ is about 0.75 ppm

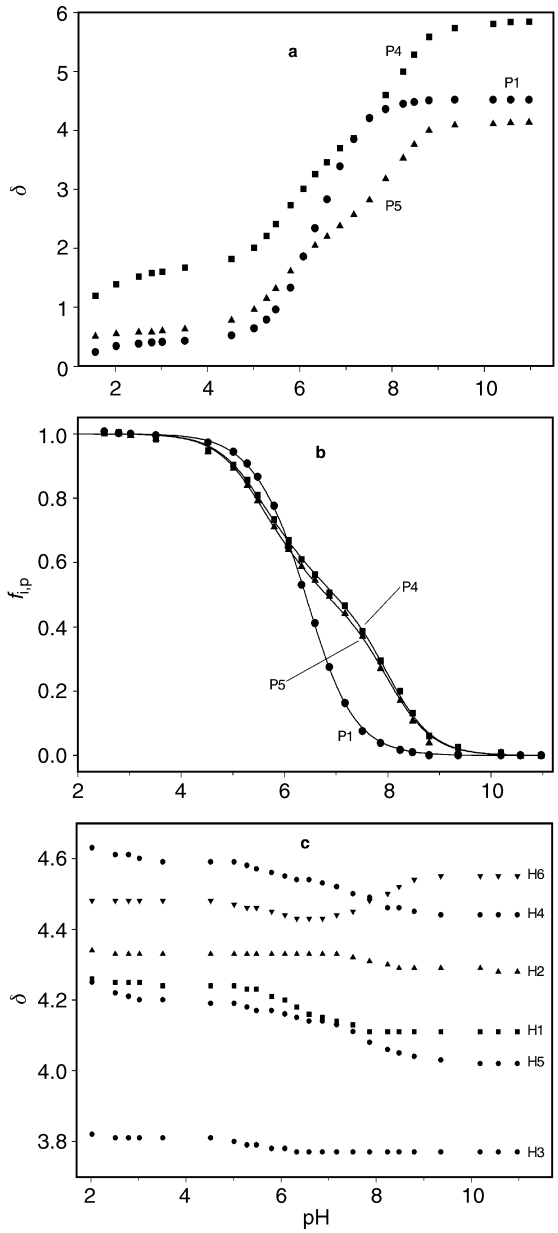


Fig. 4 ^{31}P-NMR and ^{1}H-NMR titrations curves for *epi*-Ins(1,4,5)P_3 (**4**). See caption of Fig. 1 for details.

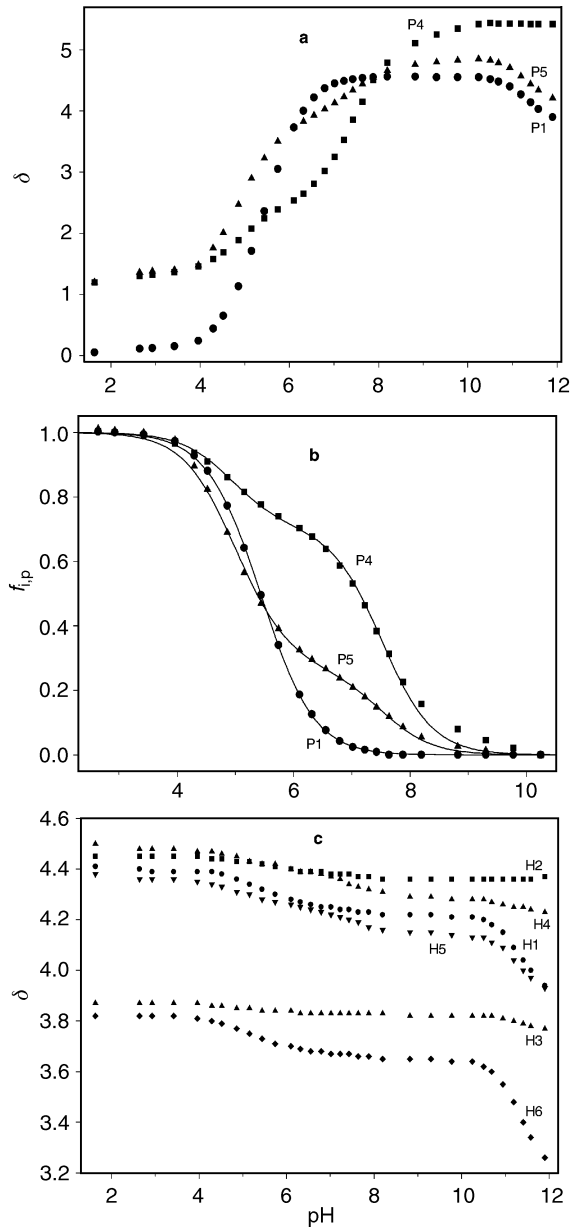


Fig. 5 ^{31}P-NMR and ^{1}H-NMR titrations curves for 6-NH_2-Ins(1,4,5)P_3 (**5**). See caption of Fig. 1 for details.

lower than the equivalent chemical shifts of **2**–**5**. In this case, the low $\delta_{1,d}$ value of **1** is obviously due to the initial P1 wrongway shift. Similarly, the low $\delta_{5,d}$ values of **2**–**5** compared to **1** may also be partly accounted for by a wrongway contribution, even though this contribution is not formally expressed in the general shape of the titration curves. This is strengthened by the observation of the initial highfield shift of the H6 equatorial protons of **3** and **4**, which expresses a P5–H6 interaction such as that between P1 and H2 in **1**.[35]

Inframolecular acid–base properties

Table 2 lists the macroscopic as well as the microscopic protonation constants of the studied compounds. The logarithms of the macroscopic constants referring to the first protonation step (log K_1) decrease in the order 6-F-Ins(1,4,5)P_3 > 6-deoxy-Ins(1,4,5)P3 > Ins(1,4,5)P_3 > *epi*-Ins(1,4,5)P_3 > 6-NH_2-Ins(1,4,5)P_3. This indicates that the position 6 substitution effect on the basicity of the phosphate groups does not occur *via* an electron-inductive effect through σ bonds, but rather is the consequence of the lipophilicity of the substituents. Indeed, the substitution of a hydrogen atom by a fluorine atom leads here to an acid-weakening effect instead of the acid-strengthening effect commonly observed. Thus, the basicity order follows the order of the hydrophobic π constants: F(0.14) > H(0.00) > OH(−0.67) > NH_2(−1.23). Although useful, these results do not provide further information on the manner that the position-6 substitutions influence the acid–base properties of each individual phosphate group. Such information, however, is reavealed from the consideration of the microscopic protonation constants.

For compounds **2**–**4**, it can be seen from Table 2 that the basicity of the individual phosphate groups follows the order P5 ⩾ P4 > P1, whereas for **1**, P5 appears significantly more basic than P4 (log $k_5 = 8.01$ *vs.* log $k_4 = 7.63$). For the three former compounds, the two vicinal phosphates display about the same intrinsic basicity, which, in addition, is larger than that of the isolated phosphate due to the higher negative charge density inherent to their proximity. For Ins(1,4,5)P_3 (**1**) the increased basicity of P5 with respect to P4 may be the result of an extra stabilisation of the proton on P5 through phosphate P1 *via* the equatorial OH6 group. This may be demonstrated by calculating the so-called interactivity parameters previously proposed. For instance, $\Delta \log k_{1-5,4d} = \log k_1 - \log k_{51} = \log k_5 - \log k_{15}$ represents the inter-

Table 2 Logarithms[a] of the macro- and microprotonation constants for **1**–**5**. The potentiometrically determined macroconstants. are shown in italics. log k_i, log $k_{ii'}$ and log $k_{ii'i''}$ represent a general designation for, respectively, the logarithms of the first, second and third stepwise microprotonation constants. $\Delta \log k_{i-i',i''}$ correspond to the interactivity parameters as defined in ref. 22.

Ligand	y	log K_y	i	log k_i	ii'	log $k_{ii'}$	$ii'i''$	log $k_{ii'i''}$	i–i',i''	$\Delta \log k_{i-i',i''}$
Ins(1,4,5)P_3	1	8.17(1)	1	6.44(1)	14	7.77(1)	145	6.0(2)	1–4,5d	−0.14
1		*7.99(2)*	4	7.63(2)	15	7.90(1)	154	5.8(1)	1–5,4d	0.11
	2	6.51(3)	5	8.01(1)	41	6.58(5)	541	6.2(2)	4–5,1d	1.62
		6.56(4)			45	6.39(4)			1–4,5p	0.18
	3	5.48(5)			51	6.33(2)			1–5,4p	0.43
		5.51(5)			54	6.00(5)			4–5,1p	1.95
6-F-Ins(1,4,5)P_3	1	8.80(2)	1	7.51(4)	14	7.45(1)	145	6.2(0.2)	1–4,5d	1.01
2		*8.54(1)*	4	8.37(2)	15	7.65(1)	154	5.9(0.1)	1–5,4d	0.92
	2	6.66(5)	5	8.61(1)	41	6.41(2)	541	6.3(0.2)	4–5,1d	2.25
		6.49(2)			45	6.37(2)			1–4,5p	0.29
	3	5.58(7)			51	6.63(1)			1–5,4p	0.15
		5.31(2)			54	6.11(3)			4–5,1p	1.54
6-deoxy-	1	8.59(2)	1	7.50(1)	14	7.52(2)	145	6.4(2)	1–4,5d	0.62
Ins(1,4,5)P_3		*8.37(2)*	4	8.15(1)	15	7.69(1)	154	6.5(2)	1–5,4d	0.65
3	2	6.92(6)	5	8.33(1)	41	6.89(3)	541	6.6(2)	4–5,1d	1.66
		6.80(5)			45	6.66(2)			1–4,5p	0.27
	3	5.90(9)			51	6.84(3)			1–5,4p	0.29
		5.83(6)			54	6.50(5)			4–5,1p	1.30
epi-Ins(1,4,5)P_3	1	8.02(2)	1	6.58(1)	14	7.53(1)	145	5.8(2)	1–4,5d	0.12
4		*8.07(1)*	4	7.70(2)	15	7.61(1)	154	5.9(2)	1–5,4d	0.12
	2	6.52(5)	5	7.67(1)	41	6.51(4)	541	6.1(2)	4–5,1d	1.45
		6.65(3)			45	6.13(1)			1–4,5p	0.36
	3	5.45(8)			51	6.40(2)			1–5,4p	0.37
		5.72(4)			54	6.34(2)			4–5,1p	1.72
6-NH_2-	0	11.64(5)	1	5.38(9)	14	7.48(4)	145	5.1(2)	1–4,5d	−0.09
Ins(1,4,5)P_3	1	7.52(3)	4	7.37(1)	15	7.0(2)	154	5.5(2)	1–5,4d	−0.14
5		*7.53(7)*	5	6.95(4)	41	5.45(2)	541	5.4(2)	4–5,1d	1.73
	2	5.62(9)			45	5.26(5)			1–4,5p	0.16
		5.82(8)			51	5.56(3)			1–5,4p	0.09
	3	4.8(2)			54	5.6(2)			4–5,1p	1.94
		4.81(9)								

[a] The uncertainties are estimates of the standard deviation as calculated by HypNMR[30] and SUPERQUAD[29] for the macroconstants and by Sigmaplot for the microconstants.

action between P1 and P5, P4 being deprotonated. Also, $\Delta \log k_{1-5,4p} = \log k_{41} - \log k_{541} = \log k_{45} - \log k_{145}$ expresses the same interaction when P4 still carries a proton. It can be observed that for compounds **2**–**4**, $\Delta \log k_{1-5,4d} \approx \Delta \log k_{1-4,5d}$ and $\Delta \log k_{1-5,4p} \approx \Delta \log k_{1-4,5p}$, indicating that P4 and P5 experience the same interaction with P1 which, in addition, can be presumed as poor. On the other hand, for compound **1**, $\Delta \log k_{1-5,4p}$ and $\Delta \log k_{1-5,4d}$ are both greater than, respectively, $\Delta \log k_{1-4,5p}$ and $\Delta \log k_{1-4,5d}$ by about 0.25 log units, revealing a P5–P1 interaction mediated by the equatorial hydroxyl at position 6. Hydration effects should mainly account for the differences observed between compounds **2**–**4** and compound **1**. The deletion of the polar equatorial OH6 of **1** leads to the introduction of a less hydrophilic pocket in between P5 and P1 of compounds **2**–**4**, which locally decreases the effective dielectric constant and thus screens off the latter phosphates. In **4**, although present, the hydroxyl in an axial position may show a different hydration pattern than the equatorial one due to a different fit of the molecule into the three-dimensional hydrogen bonding network of water.[39] In addition to the hydration effects, the hydrogen bond donor ability of the equatorial OH6 hydroxyl, which is lost in compounds **2**–**5** may also participate in the transfer of an electrostatic effect between P1 and P5. Whatever the origin of the phenomenon, the preservation of the P1–P5 cooperativity seems crucial for preserving the biological activity of the molecule.

For the amino derivative **5** the protonation of the amine and the phosphate groups can be considered separately due to the large difference in their respective protonation constants ($>10^4$). As has been recently done for adenophostin A,[40] the microprotonation constants can be derived for the phosphates as for any other trisphosphorylated compounds. Consideration of these provides interesting information about the mutual interactions of neighbouring positively and negatively charged functional groups. Clearly, compound **5** remains under a zwitterionic form over almost the entire pH range studied ($2.5 < \text{pH} < 11.5$). By comparison with the 6-deoxy derivative **3**, it can be seen that the presence of the protonated amino group decreases the basicity of all three phosphates. In contrast, the basicity of the amine greatly increases since, for instance, log $K = 10.62$ for cyclohexylamine at the same temperature.[41] According to the recent publication of Charton and Charton[42] on the mode of transmission of electrical effects, these observations may partly be due to through bond effects but more especially to complex field effects directly propagated through space and involving all the functional groups simultaneously. Indeed, as has been extensively shown over the last few decades[43–53] in anion coordination chemistry, electrostatic and ionic hydrogen bonding interactions provide the bonding forces in complexes between phosphates and protonated amines, leading to base-weakening of the phosphates and base-strengthening of the amines. This, as seen here, remains true for intramolecular phosphate–ammonium interactions. As stated in a recently published work[44] dealing with the thermodynamics of phosphate anion binding by polyammonium receptors, many types of hydrogen bonds may intervene in which both anions and cations can act as acceptors or donors. Among them, the –N–$H^+\cdots{}^{2-}$O–P– hydrogen-bonded ion pair interaction is of primary importance. According to the curves of Fig. 5, this seems to be the predominant interaction between NH_3^+ at position 6 and P1 or P5 in the 7.5 to 10.5 pH range. The chemical shifts of both P1 and P5 phosphates are highfield shifted by about 0.42 ppm when the ion pair dissociates above pH 10.5, presumably indicating similar strengths of interaction between the ammonium

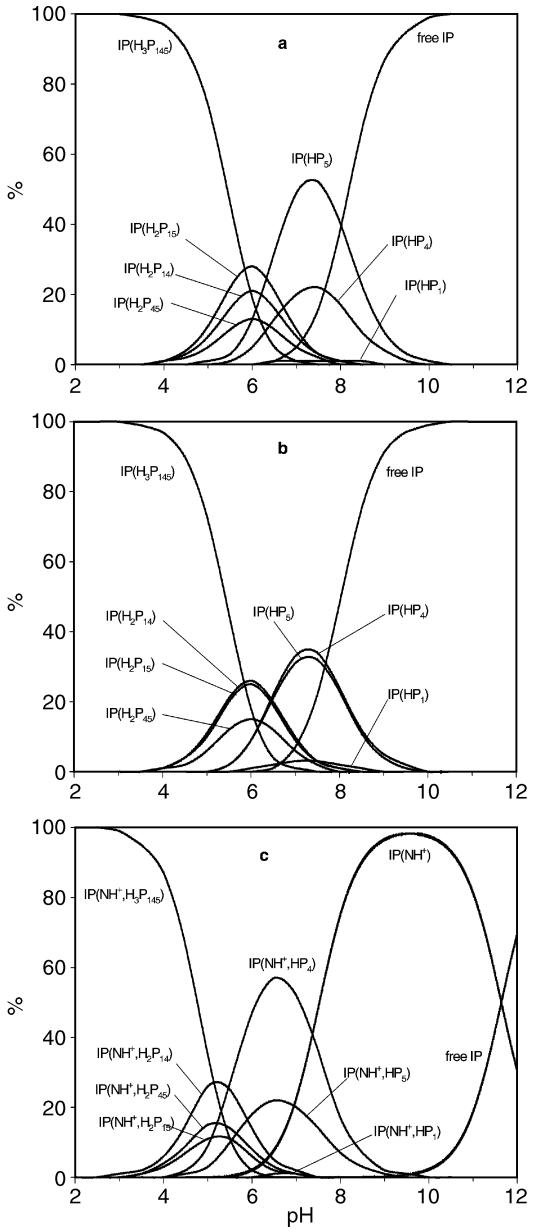


Fig. 6 Relative concentrations of the protonated microspecies of (*a*) Ins(1,4,5)P_3 (**1**), (*b*) *epi*-Ins(1,4,5)P_3 (**4**) and (*c*) 6-NH_2-Ins(1,4,5)P_3(**5**).

group and the deprotonated phosphates P1 and P5. In the same pH region, the chemical shifts of phosphate P4 remain unchanged, which is the sign, as expected, of no direct interaction with the amino group.

The strength of the interaction between the ammonium group and the P1 and P5 phosphates in the 5.5 to 7.5 pH range can be evaluated by calculating the log $k_{s(i)} = \log k_i$(**3**) $- \log k_i$(**5**) values. Provided that the through field effects mentioned above are largely predominant, this value should account for the stability of the –N–$H^+\cdots{}^{2-}$O–P– hydrogen-bonded ion pair. Indeed, it can be considered that log k_i(**5**) depicts the equilibrium –N–$H^+\cdots{}^{2-}$O–P– + $H^+ \rightleftharpoons$ –N–H^+ + $^-$HO–P– whereas log k_i(**3**) corresponds to the equilibrium $^{2-}$O–P– + $H^+ \rightleftharpoons {}^-$HO–P–. Thus, log k_i(**3**) $-$ log k_i(**5**) may be a good approximation of log $k_{s(i)}$, $k_{s(i)}$ standing for the equilibrium constant of –N–H^+ + $^{2-}$O–P– $\rightleftharpoons$ –N–$H^+\cdots{}^{2-}$O–P–. According to the values of Table 2, log $k_{s(1)} = 2.12$ and log $k_{s(5)} = 1.38$, which indicates a much stronger interaction of the ammonium group with P1 than P5. This may be explained considering that the first equivalent of added protons is shared by phosphates P4 and P5. Therefore, P5 experiences an additional –P–$O^{2-}\cdots H^+\cdots{}^{2-}$O–P– interaction with P4, which weakens the –N–$H^+\cdots{}^{2-}$O–P– interaction with regard to P1. It can be noted that the calculated log $k_{s(i)}$, are of the same order of magnitude as the recently published stability constants of monoammonium-phosphate complexes.[44,54] The hydrogen bonded ion pair, as has been demonstrated in ref. 44, is likely to be stabilised by a largely favourable entropic term due to the release of water molecules upon charge neutralisation. Finally, the difference log k_4(**3**) $-$ log k_4(**5**) $= 0.78$ cannot be attributed to an interaction between P4 and the amino group, but rather results from a loss of cooperativity with P5, the latter phosphate being, as has been seen, partially engaged with the neighbouring ammonium group.

Fig. 6 displays the distribution of the various microprotonated species *vs.* pH for compounds **1**, **4** and **5**. These curves allow a direct observation of the protonation state of each phosphate group and illustrate some acid–base properties discussed above. In particular, the IP(HP_5)/IP(HP_4) ratio is >1 for compound **1**, equal to 1 for compound **4** and <1 for compound **5**. This clearly shows the cooperativity between P5 and P1 for the natural ligand **1**, the ideal bisphosphate character of P4 and P5 for the *epi* derivative **4** and the base-weakening effect on P5 of the protonated amino group for compound **5**.

A dramatic loss of binding affinity for the Ins(1,4,5)P_3 receptor of compounds **2**–**5** with regard to the natural ligand is observed, since all the analogues are about two orders of magnitude less active than the parent compound **1**. From the consideration of the ^{31}P- and ^{1}H-NMR titration curves, as well as from the microprotonation constants and the associated interactivity parameters, it can be observed that Ins(1,4,5)P_3 behaves differently from its analogues due to the strategic equatorial hydroxyl at position 6. The importance of this group may arise from the possibility of forming a hydrogen bond with the receptor. However, the reported results clearly show that OH6 in **1** also plays a pivotal relaying role that enables ionisation state information to be transferred from the vicinal P4 and P5 phosphates to the distant P1 phosphate group. The structural and conformational consequences of such a transfer may be of real relevance in the expression of the biological activity.

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