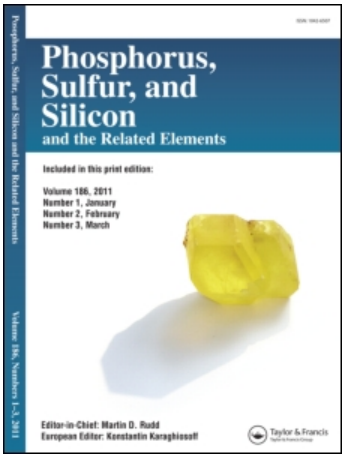

Phosphorus, Sulfur, and Silicon and the Related Elements
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713618290

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To cite this Article Ballereau, Stéphanie , Schlewer, Gilbert and Spiess, Bernard(1997) 'INFRAMOLECULAR PROTONATION STUDIES OF 5-DEOXY-5,5-DIFLUORO-*MYO*-INOSITOL-1,2,6-TRIS(PHOSPHATE)', Phosphorus, Sulfur, and Silicon and the Related Elements, 128: 1, 147 — 151
To link to this Article: DOI: 10.1080/10426509708031570
URL: http://dx.doi.org/10.1080/10426509708031570

Phosphorus, Sulphur, and Silicon, 1997, Vol. 128, pp. 147–151
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INFRAMOLECULAR PROTONATION STUDIES OF 5-DEOXY-5,5-DIFLUORO-*MYO*-INOSITOL-1,2,6-TRIS(PHOSPHATE)

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(Received 28 March 1997; Revised 12 May 1997; In final form 12 May 1997)

The study investigates quantitatively the acid-base properties of 5-deoxy-5,5-difluoro-*myo*-inositol-1,2,6-tris(phosphate) at an inframolecular level by considering the microscopic protonation constants. By comparison with α-trinositol, its inositol parent compound, conclusions are drawn about the influence of an hydroxyl group vicinal to a phosphate group.

Keywords: Inositol-phosphates; protonation; micro-constants; ^{31}P NMR titration

INTRODUCTION

When phosphate anions such as 5-deoxy-5,5-difluoro-*myo*-inositol-1,2,6-tris(phosphate) (2FIns(1,2,6)P_3), are present in biological media, they readily form complexes with the surrounding inorganic or organic cations and interact with the positively charged moieties of the proteins. Such interactions which are largely electrostatic in nature may greatly influence or modulate their mechanisms of action. It has been shown for a long time that the stability of the complexes is governed by the basicity of the phosphate groups.[1,2] However, since the macroprotonation constants only characterise a molecule as a whole, no final conclusion can be drawn on the relative contribution of each individual phosphate group to the complex formation. There is, therefore, a need of an inframolecular picture of the protonation process *i.e.* a picture that describes the

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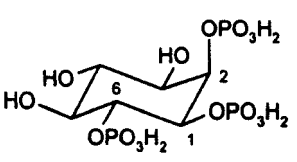
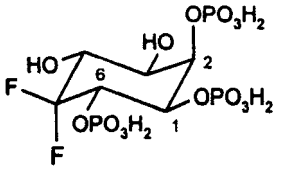


Ins(1,2,6)P_3



2FIns(1,2,6)P_3

ionization state of each phosphate group at any pH. We previously succeeded in resolving the microprotonation scheme of D-*myo*-inositol-1,2,6-tris(phosphate) (Ins(1,2,6)P_3),[3,4] the parent compound of 2FIns(1,2,6)P_3.

The work reported here, applying the same methodology, is part of a comprehensive study aimed at figuring out the main factors that affect the basicity of a phosphate group in the vicinity of several others. The studies were performed by potentiometric and ^{31}P NMR titrations in 0.2 M KCl solution at 37°C, near physiological ionic strength and temperature.

DETERMINATION OF MACROSCOPIC AND MICROSCOPIC CONSTANTS

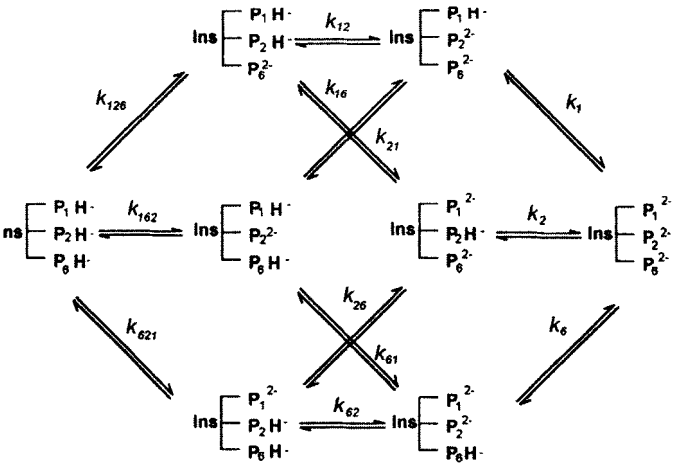
The overall macroscopic protonation constants β_y quantify the general equilibrium:

$$L^{6-} + yH^+ \Leftrightarrow H_yL^{(6-y)-}.$$

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

$$H_{y-1}L^{(7-y)-} + H^+ \Leftrightarrow H_yL^{(6-y)-}$$

However, the description of the ionization state of each individual phosphate group can only be achieved if the microprotonation equilibria have been resolved (Scheme 1). As shown earlier,[3–5] such a resolution becomes possible by performing ^{31}P NMR titrations since the observed chemical shift for any resonance δ_i^{obs} is the weighted average of shifts for the possible protonated and deproton-



SCHEME 1

ated forms. Accordingly $f_{i,p}$, *i.e.* the fraction of protonation of the phosphate in position i can easily be calculated from eq. 1

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

where $\delta_{i,p}$ and $\delta_{i,d}$ are the chemical shifts of the protonated and deprotonated phosphate, respectively.

$f_{i,p}$ can also be expressed as a function of the macro and microprotonation constants and the proton concentration. For instance, the protonation fraction of the phosphate in position 1 ($f_{1,p}$) is defined as:

$$f_{1,p} = \frac{\beta_3[H^+]^3 + (_{12}k_1 + k_{16}k_1)[H^+]^2 + k_1[H^+]}{\beta_3[H^+]^3 + \beta_2[H^+]^2 + \beta_1[H^+] + 1} \tag{2}$$

The desired microconstants are obtained from a non-linear least-squares fit of a given ^{31}P NMR protonation fractions curve, while keeping constant in eq. 2 the values of β_1, β_2 and β_3 previously determined by potentiometry[6] or ^{31}P NMR.[7]

RESULTS AND DISCUSSION

Figure 1 displays the ^{31}P NMR titration curves and the corresponding protonation fractions of 2FIns(1,2,6)P_3. For purpose of comparison the chemical shifts of Ins(1,2,6)P_3 are shown in Figure 1a in solid line. From theses curves, it clearly

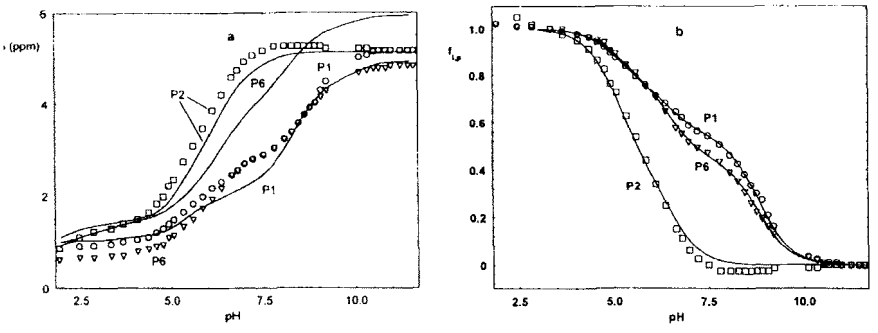


FIGURE 1 Chemical shifts δ from ^{31}P NMR titrations for 2FIns(1,2,6)P_3 (a) and the corresponding protonation fraction curves $f_{i,p}$ (b) as a function of pH in KCl 0.2 M at 37°C. The least-squares fit of $f_{i,p}$ *vs* pH according to type 2 eqs is shown in solid line in b. Potentiometric and ^{31}P NMR determinations were carried out as previously described.[3–5] Phosphorus resonances were assigned by performing 2D correlation experiments at pH 4 and 10.5.

appears that the phosphate P6 is the most affected by introduction of the two fluorine atoms at the 5 position of the inositol ring.

In particular, with regard to Ins(1,2,6)P_3, $\delta_{6,p}$ and $\delta_{6,d}$ of 2FIns(1,2,6)P_3 are highfield shifted by 0.66 and 1.10 ppm respectively whereas the corresponding chemical shifts of the P1 and P2 nuclei remain almost unchanged.

The macroscopic and microscopic protonation constants referring to scheme 1 are reported in Table I. It can be noted that the potentiometric and ^{31}P NMR determined log K_y values are in good agreement. It appears also that log K_1 value is slightly higher for 2FIns(1,2,6)P_3 than for Ins(1,2,6)P_3 (8.44)[4] indicating a gain in the overall basicity of the former. Considering the log k_i values, the increase of basicity can mainly be attributed to the P6 phosphate which has lost a vicinal hydroxyl with regard to its parent compound. Such a behaviour has still been reported for desoxyribonucleotides and ribonucleotides[1,5,8] as well

TABLE I Logarithms of the macro- and microprotonation constants for 2FIns(1,2,6)P_3. 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. The uncertainties are estimates of the standard deviation as calculated by Superquad[6] and Hypnmr[7] for the macroconstants. The calculated interactivity parameters are: $\Delta\log k_{1\text{-}2,6d} = 0.15 \pm 0.02$, $\Delta\log k_{1\text{-}6.2d} = 2.22 \pm 0.16$, $\Delta\log k_{2\text{-}6,1d} = -0.07 \pm 0.10$, $\Delta\log k_{1\text{-}2,6p}$ &equals ;0.76 ± 0.02, $\Delta\log k_{1\text{-}6.2p} = 2.71 \pm 0.06$, $\Delta\log k_{2\text{-}6,1p} = 0.36 \pm 0.08$.

y	*log K*$_y$		*i*	*log k*$_i$	*ii'*	*log k*$_{ii'}$	*ii'i''*	*log k*$_{ii'i''}$
	pot.	*N.M.R.*	*1*	8.53	*12*	6.03	*126*	5.76
			2	6.20	*16*	6.05	*162*	5.59
1	8.79 ± 0.01	8.79 ± 0.02	*6*	8.44	*21*	8.40	*621*	5.63
2	6.48 ± 0.02	6.41 ± 0.04			*26*	8.41		
3	5.07 ± 0.02	5.05 ± 0.07			*61*	6.47		
					62	6.37		

as for Ins(1,4,5)P_3 and its deoxy equivalent. The OH group was presumed to act on the basicity of the phosphate: *i*) through modification of the solvation shell close to the group, *ii*) via hydrogen bonding with the phosphates, *iii*) via electron-inductive effects through σ bonds or through space.

This work clearly shows that the latter reason can be discarded since two fluorine atoms would have a stronger inductive effect than an OH group. Thus, the main explanation for the basicity increase of P6 rests in the inability of the 5 position of 2FIns(1,2,6)P_3 to stabilise the fully deprotonated P6 phosphate by a donating hydrogen bond. In consequence, as is shown by the diphasic shape of the $f_{i,p}$ = f(pH) curves, P1 and P6 tend to behave very similarly and will almost equally share the first equivalent of added protons. On the other hand, the monophasic shape of P2 curve as well as the low value of log k_2 illustrate the independent protonation of the axially orientated P2 phosphate. The above conclusions are strengthened by consideration of the interactivity parameters earlier defined and reported in caption of Table I.

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