Communication

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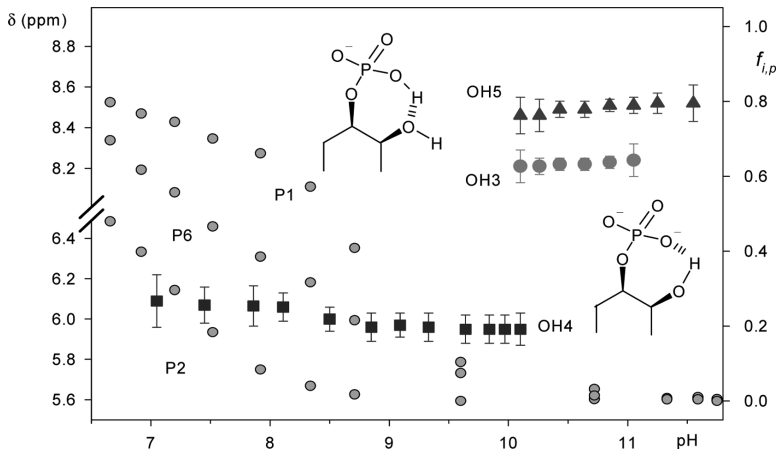

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^{1}H NMR Titrations of Hydroxy Protons in Aqueous Solution as a Method of Investigation of Intramolecular Hydrogen-Bonding in Phosphorylated Compounds: Examples of *myo*-Inositol 2-Phosphate and *myo*-Inositol 1,2,6-Tris(phosphates)

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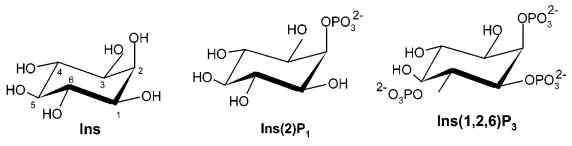
O−H···O hydrogen bonds (HB) have been extensively studied due to the key role they play in the structure and properties of water, proteins, DNA, and carbohydrates. However, since a relatively nonpolar environment is generally required for the physical observation of HBs, few studies describe hydrogen bonding in aqueous solution. NMR spectroscopy is in this instance a very powerful technique[1−3] which, in particular, can be applied in strictly controlled experimental conditions to the detection of the hydroxy protons. Thus, the chemical shifts of the OH protons, the measurement of their vicinal coupling constants (^3J(H,OH)), exchange rates with water, and temperature dependence of the chemical shifts ($\Delta\delta/\Delta t$) provide important information about HBs.[4] Such techniques were recently nicely applied by Sandström et al.[5−8] to carbohydrates in conformational analysis and in an attempt to detect HB interaction.[5−10] However, for hydroxy compounds being at the same time polyacid bases, most of the above-mentioned techniques can hardly be used due to the acid−base character of these molecules and the multiple charges they carry. It is indeed well-known that the detection of OH signals in water by ^{1}H NMR spectroscopy is hampered by an acid-catalyzed fast exchange of OH protons with H_2O. Therefore, for compounds displaying acid−base properties, the hydroxy proton signals are expected to be broad and the couplings difficult to observe. In addition, temperature dependence cannot accurately be determined since these signals can only be recorded in a narrow temperature range. Finally, exchange rates are very sensitive to pH and catalysis by small traces of impurities. To the best of our knowledge, no data have been reported on hydroxy protons in aqueous solution of compounds carrying one or more basic groups. In this study, we present ^{1}H NMR hydroxy proton titration experiments for *myo*-inositol 2-phosphate (Ins(2)P_1) and *myo*-inositol 1,2,6-tris(phosphates) (Ins(1,2,6)P_3) and wish to demonstrate that by following OH signals versus pH, evidence can be brought for HB interaction between the hydroxy and phosphate groups.



The studied molecules belong to the *myo*-inositol phosphates family which includes compounds that have received intensive study due to their fundamental cellular role in signal transduction. The molecules chosen in this work are also, more generally, models for compounds carrying simultaneously OH and phosphates groups, such as RNAs, nucleotides, phosphorylated sugars, for which the knowledge of intramolecular HB interaction is crucial in the understanding of their biological function.

To compare the pH dependency of the hydroxy proton signals for related neutral and charged compounds, the titration of *myo*-inositol (Ins) was first performed. Due to the symmetry of the molecule, four titration curves (see Supporting Information) can be observed ranging from pH 4.5 to 9.7.

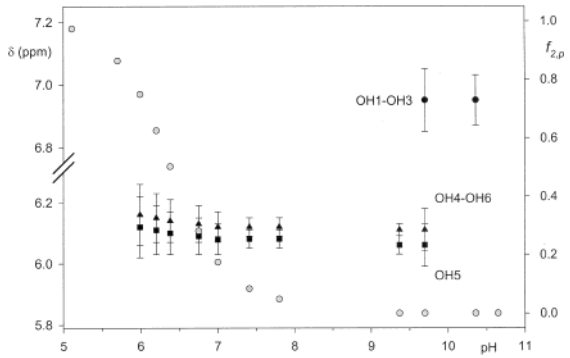


Figure 1. δ vs pH of the hydroxy protons for *myo*-inositol 2-phosphate in 80% H_2O−20% $(CD_3)_2CO$ at −15 °C. The bars correspond to twice the half-height width of the signals. The curve with open circles represents the phosphate protonation curve.

The chemical shifts of the hydroxy protons are, as expected, pH independent and follow the general rules observed for neutral carbohydrates.[2] In particular, the resonance of the axial OH2 proton is shifted upfield with regard to the equatorial OH protons, and among the latter, the OH1−OH3 protons are the less deshielded due to the axial configuration of their neighboring OH2 group. The ^3J(H,OH) vicinal coupling constants, which can only be measured for the three middle resonances, are close to 5.5 Hz, representing rotational averaging of the OH groups. As was previously demonstrated,[3] the broadening of the resonances at the acidic and basic extremities of the curves is caused by the following equilibria:

$$\text{Ins-OH} + \text{H}_3\text{O}^+ \rightleftarrows \text{Ins-OH}_2^+ + \text{H}_2\text{O} \qquad (1)$$

$$\text{Ins-OH} + \text{OH}^- \rightleftarrows \text{Ins-O}^- + \text{H}_2\text{O} \qquad (2)$$

Figure 1 displays the OH titration curves for Ins(2)P_1, which differs from the former compound by an axial phosphate group at position 2 of the *myo*-inositol ring. On the same figure is shown the protonation fraction of phosphate P2 ($f_{2,p}$) vs pH, calculated as previously indicated[11] from the chemical shift of the phosphorus resonances recorded concurrently with those of the OH resonances.

From these curves it appears that the δ values of the OH groups two (OH4−OH6) or three (OH5) centers removed from the axial phosphate are close to those of Ins or carbohydrate OHs, although appearing only at pH 6. These resonances also move slightly upfield when pH increases, by experiencing the deprotonation of P2. Interestingly, the OH protons vicinal to phosphate P2 (OH1−OH3) appear as broad signals at pH 9.7 and 10.4, deshielded by ca. 1.1 ppm with regard to Ins.

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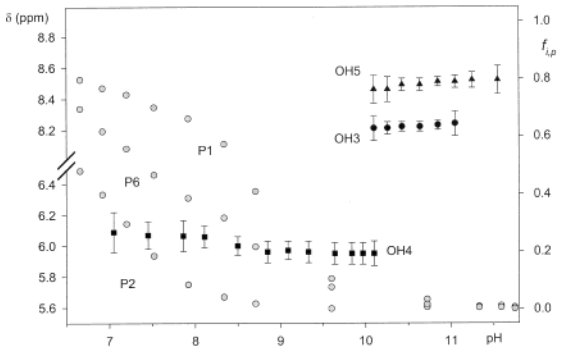
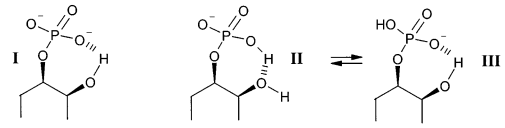


Figure 2. δ vs pH of the hydroxy protons for *myo*-inositol 1,2,6-tris-(phosphates) in 80% H_2O−20% $(CD_3)_2CO$ at −15 °C. The curves with open circles represent the phosphate protonation curves.

Figure 2 shows the OH proton titration curves and protonation fraction curves for Ins(1,2,6)P_3. Again, the OH4 proton, only surrounded by two equatorial hydroxy groups, keeps a chemical shift close to that in Ins, appears at pH 7 until pH 10.2, and remains slightly sensitive to the deprotonation of the phosphate groups. Remarkably, OH3 and OH5 hydroxy protons are only present when their neighboring phosphate group (P2 and P6, respectively) are fully deprotonated. On the other hand, both signals persist until pH 11 for OH3 or 11.5 for OH5, i.e., much higher pHs than in the case of neutral species. In addition, from Ins to Ins(1,2,6)P_3, the δ values of these protons are shifted ca. 2.4 ppm downfield. Taking into account all these observations, the following types of HBs can be proposed:



Indeed, it is generally assumed that a marked downfield shift of hydroxy protons can be attributed to HB formation. Although stereoelectronic effects due to the proximity of a charged group may operate through space and affect the chemical shifts,[10] it is likely that δ values reaching 8.5 ppm, values thus far never observed in aqueous solution for hydroxy protons of nonaromatic compounds, are the result of C−OH···$^{2-}$$O_3$P−O type I hydrogen bonding. McAllister et al.[12] recently concluded that, within a family of similar substrates, a proton shift of one unit downfield implies an approximately 1.5 kcal/mol stronger HB. If this remains valid for our system, the replacement of a OH group by a phosphate group, strengthened the HB by ca. 3.6 kcal/mol. The hypothesis of a type I HB is reinforced by the 2 pH unit shift, from Ins to Ins(1,2,6)P_3, of the limit where the OH proton is still observable. Clearly, setting such a bond competes with OH^-, displaces equilibrium (2) to the left, leads to the basicity increase of the hydroxy group and slows down the exchange rate of its proton with the aqueous medium. In addition, since OH5 is both downfield-shifted with regard to OH3 and can be observed at higher pHs, it can be concluded that *trans* diequatorial C−OH···$^{2-}$$O_3$P−O HBs are more stable than *cis* ones. Type II C−HO···$^{-}$$HO_3$P−O and type III C−OH···$^{-}$$HO_3$P−O HBs are, on the contrary, expected to occur as long as the hydroxy neighboring phosphate remains protonated. The consequence of type II HB on the OH signal is analogous to that of H_3O^+ described in eq 1. It is likely that by setting such a bond, the basicity of the oxygen decreases, weakening the O−H bond and leading to a faster exchange of the hydroxy proton with water. Type III HB should contribute to stabilize the OH proton. However, inspection of the phosphate protonation curves along with the hydroxy proton signal shows that type III HBs are not strong enough to counterbalance the acid-catalyzed fast proton exchange.

Examination of the pH dependence of the hydroxy protons of inositol phosphates bring clues on the intramolecular HBs which, in complex and subtle rearrangements, govern their biological properties. In particular, such bonds oppose the electrostatic repulsion of deprotonated phosphates, allowing the retention of the biologically active 1-axial/5-equatorial (1-ax/5-eq) conformation of the *myo*-inositol ring. For instance, D-*myo*-inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4), the well-known second messenger,[13] keeps the 1-ax/5-eq conformation[14] over the whole pH range; whereas its 6-deoxy analogue shows a pronounced distorted chair conformation at high pH. By moving in Ins(1,3,4,5)P_4 phosphate P3 on the neighboring C6 carbon, the very biologically relevant *myo*-inositol 3,4,5,6-tetrakisphosphate (Ins(3,4,5,6)P_4) is obtained. For the latter, the only two *cis*- and *trans*-diequatorial type I HBs are not energetically sufficient to compensate the mutual repulsion of the phosphate groups leading at high pH (pH 10) to the flipping of the *myo*-inositol ring.[15]

This study is a starting point for the investigation of the HBs between phosphate and hydroxy groups. It must be further proved that, as for other systems,[12] the strength of such HBs is in linear relationship with the associated proton NMR chemical shift. On the other hand, by using the inframolecular approach that we recently proposed,[11,16,17] we are able to determine the intrinsic basicity of a phosphate group in polyphosphorylated compounds. Thus, since the strength of HBs is dependent on the pK_a's of the hydrogen bond donor and hydrogen bond acceptor, valuable information on the long-standing question[18] of the basicity of the hydroxy groups should be obtained.

Acknowledgment. We thank Persorp Pharma for providing the Ins(1,2,6)P_3.

Supporting Information Available: ^{1}H NMR spectra for Ins, and the corresponding titration curves; materials and NMR methods (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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