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Analysis of *myo*-inositol phosphates by 2D ^{1}H-n.m.r. spectroscopy

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ABSTRACT

2D ^{1}H-N.m.r. spectroscopy applied to phosphorylated *myo*-inositols can indicate the number and the sites of phosphorylation, and up to three isomers can be completely analyzed simultaneously. The resonance of the sole equatorial proton (H-2) is shifted furthest downfield (to ~4.7 p.p.m.) when C-2 is phosphorylated; when C-5 is unphosphorylated, the H-5 resonance has the lowest chemical shift (~3.3 p.p.m.). The number of sites of phosphorylation can be determined from the sum of the chemical shifts for the resonances in a given *myo*-inositol phosphate and, based on the data presented, an algorithm can be constructed that will identify *any myo*-inositol phosphate on the basis of the chemical shifts.

INTRODUCTION

myo-Inositol hexakisphosphate (phytic acid or IP_6) is a common storage form for phosphorus in plants and is abundant in grain and seed[1]. Little is known about its metabolic pathways, but its stepwise dephosphorylation by phosphatases[2] yields *myo*-inositol mono- (IP_1), bis- (IP_2), tris- (IP_3), tetrakis- (IP_4), and pentakis-phosphate (IP_5). Recently, interest in IP_3 has increased due to the discovery that two of its isomers may act as second messengers in mammalian cells[3].

There have been numerous studies in which ^{13}C- (refs. 4–6), ^{31}P- (refs. 2, 6–9), and ^{1}H- (refs. 6, 9, 10) n.m.r. techniques have been used to determine the number of phosphate groups bound to the inositol ring and to identify the conformation of various inositol phosphates. We have used the 2D-n.m.r. technique to assign the signals to specific protons in a series of inositol phosphates. The number of phosphate groups and their positions can be determined from the multiplet structure of these signals.

A major advantage of 2D-n.m.r. techniques is the simplicity with which the signals can be assigned in small cyclic molecules[11], through cross-peaks in correlation spectroscopy (COSY) experiments.

EXPERIMENTAL

Sample preparation. — The synthesis and purification of the *myo*-inositol phosphates in Table I was performed at Perstorp Pharma (Perstorp, Sweden) and will be

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described elsewhere. The samples for n.m.r. analysis contained one, or mainly one, unknown *myo*-inositol phosphate.

Each sample was dissolved in D_2O (1.0 mL), the pH (not corrected for isotopic effects) was adjusted to 5.5 with μL amounts of NaOD or DCl, and the solution was lyophilized. Each sample was then redissolved in D_2O (0.5 mL), to give a solution in the mM range that was transferred to a 5-mm standard n.m.r. tube.

N.m.r. spectroscopy. — All ^{1}H-n.m.r. experiments were performed on a Nicolet 360-WB spectrometer at 361.79 MHz. Chemical shifts were referenced to the signal of residual HOD at 25° or 33°.

1D ^{1}H-n.m.r. spectra were recorded with a selective inversion sequence, 180°–t_1–90°–t_2, in order to suppress the residual HOD signal. The t_1 delay was therefore adjusted to allow the HOD resonance to relax to $M_z = 0$ after the inversion pulse. Typically, a spectral width of $\pm$2000 Hz was used to collect 8192 data points with up to 2048 scans.

2D ^{1}H Correlation (COSY) experiments were acquired with quadrature detection in both dimensions, using the standard pulse sequence and 16-step phase cycling in order to suppress quad images as well as axial peaks. Routinely, 64–128 scans were accumulated in 512 blocks of 1024 datapoints with a spectral width of $\pm$600 Hz. The t_1 increment was set equal to the dwell time in f2, 833 μs, which made the digital resolution identical in both dimensions.

Resolution enhancement of the 2D spectra was achieved by double exponential multiplication, and the spectra were symmetrized around the diagonal[12].

RESULTS

The first stage of the analysis of a sample of phosphorylated *myo*-inositol involved assignment of the ^{1}H signals, after which the positions phosphorylated were identified. The chemical shifts of the ^{1}H resonances for all of the *myo*-inositol isomers are given in Table I.

Assignments of ^{1}H signals. — The only equatorial proton in *myo*-inositol is H-2, and its signal has a characteristic fine structure caused by two small (~2 Hz) *gauche* couplings which result in a narrow triplet when C-2 is unphosphorylated and two triplets separated by 10 Hz ($^3J_{P,H}$) when it is phosphorylated. Therefore, H-2 is the natural starting position for the assignment of the ^{1}H-n.m.r. spectra. In addition, the signal with the greatest downfield shift belongs to H-2 when C-2 is phosphorylated; when C-2 is unphosphorylated, the H-2 resonance still has one of the highest chemical shifts.

For some compounds, the signal for H-2 was not seen in the 1D ^{1}H-n.m.r. spectrum recorded initially at 25°. However, when the spectrum was obtained at 33°, only the HOD resonance was shifted to lower frequency and the signal for H-2 could then be seen. 2D-n.m.r. spectra were mostly recorded at 25°.

As shown in Fig. 1 where OX is either OH or phosphate, each proton gives cross-peaks to the neighbouring two protons. Since *myo*-inositol is a symmetrical

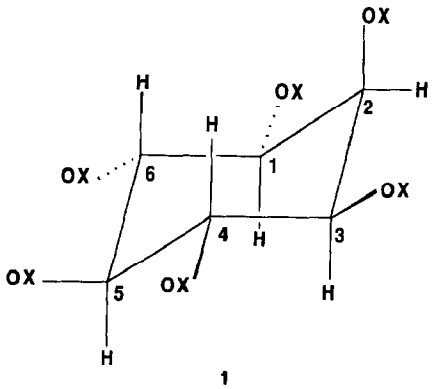
TABLE I

Chemical shifts (relative to that of HOD at 25°, 4.81 p.p.m.) of the ^1H resonances for *myo*-inositol phosphates

Inositol	*Phosphorylated Position*	*Position*					
		H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*
IP_0		3.56	4.09	3.56	3.66	3.32	3.66
IP_1	1	3.97	4.28	3.58	3.72	3.34	3.79
	2	3.58	4.58	3.58	3.72	3.31	3.72
IP_2	1,2	4.02	4.81[b]	3.58	3.73	3.37	3.82
	1,4	4.03	4.43	3.77	4.25	3.56	3.88
	1,6	4.11	4.26	3.63	3.75	3.57	4.33
	2,4	3.60	4.59	3.74	4.23	3.48	3.77
	4,5	3.65	4.11	3.74	4.31	4.02	3.84
IP_3	1,2,3	4.15	4.97	4.15	3.82	3.43	3.82
	1,2,4[a]	4.04	4.81[b]	3.77	4.27	3.57	3.90
	1,2,5[a]	4.12	4.81[b]	3.69	3.90	3.97	3.97
	1,2,6	4.17	4.81[b]	3.63	3.83	3.55	4.34
	1,3,4[a]	4.05	4.45	4.14	4.35	3.59	3.87
	1,4,5	4.05	4.30	3.75	4.31	4.05	3.94
	1,4,6	4.14	4.28	3.79	4.28	3.67	4.40
	1,5,6	4.14	4.24	3.69	3.84	4.05	4.44
	2,4,6	3.77	4.64	3.77	4.30	3.66	4.30
IP_4	1,2,4,5	4.00	4.67	3.67	4.24	3.90	3.83
	1,2,5,6	4.12	4.70	3.63	3.83	4.01	4.40
IP_5	1,2,4,5,6	4.20	4.73	3.77	4.36	4.20	4.46
IP_6		4.26	4.99	4.26	4.52	4.24	4.52

[a] The sample contained more than one isomer. [b] The signal was hidden under the signal for HOD.

molecule, either of the two cross-peaks from H-2 can be related to H-3. However, since the positions phosphorylated should have as low numbers as possible, the assignments might have to be "reversed" once the positions of the phosphate groups are identified. By the same procedure, H-4 was assigned *via* the cross-peak between H-3 and H-4, and so on.



1

Identification of the positions of phosphorylation. — Upon phosphorylation, the resonance of the proton at the position phosphorylated is shifted downfield. A similar but smaller effect occurs if the surrounding carbons are phosphorylated.

Due to the large $^3J_{H,P}$ value (10–12 Hz), each ^1H signal will contain information on whether the corresponding position is phosphorylated or not. In addition, the spin coupling between two axial protons is also 10–12 Hz, whereas that between equatorial and axial protons is ~2 Hz. This latter coupling, however, could seldom be resolved in our spectra. The multiplicities of the signals for H-1/6 in *myo*-inositol are listed in Table II. The effect of the coupling between equatorial H-2 and axial H-1 and/or H-3 on the multiplicity is omitted.

In Fig. 1, the complete assignment of a *myo*-inositol phosphate is traced out. The signals for H-4,5 are quartets and hence (Table II) their positions are phosphorylated; accordingly, all of the other positions are unphosphorylated. Thus, the compound is 4,5-IP_2.

Fig. 2 shows the 2D spectrum of a sample that contained two isomeric *myo*-inositol phosphates in the ratio 2:1. Only one H-2 signal could be found (s, 4.25 p.p.m.).

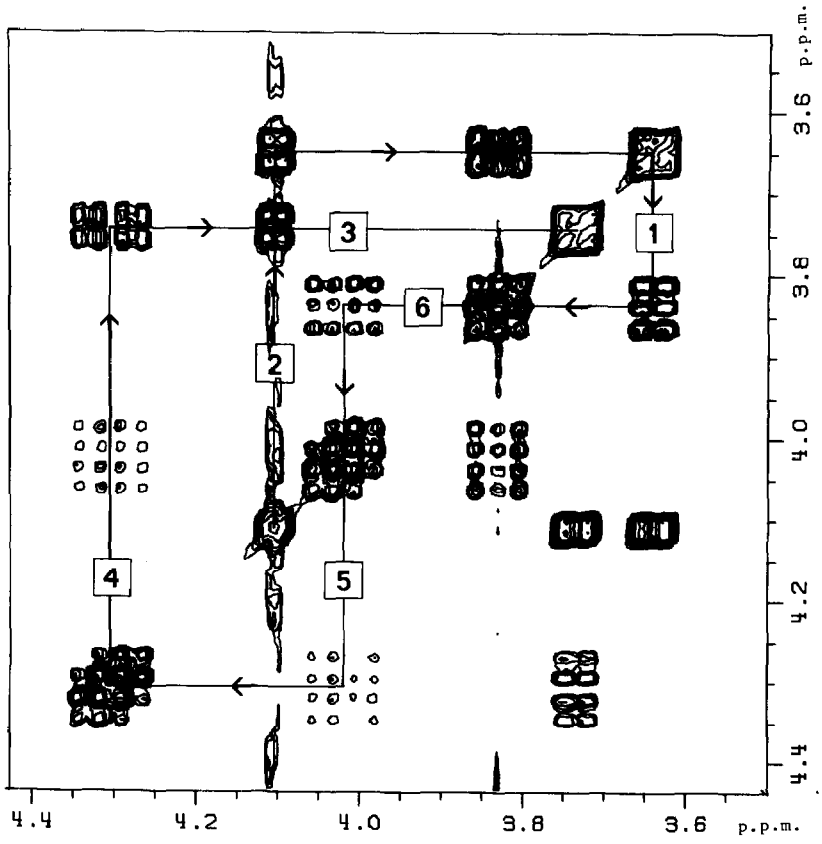


Fig. 1. 2D-N.m.r. spectrum of 4,5-IP_2.

TABLE II

Multiplicity[a] of the ^1H signals

Position	*Phosphorylated*	*Not phosphorylated*
1	t	d
2	d	s
3	t	d
4	q	t
5	q	t
6	q	t

[a] Key: s, singlet; d, doublet; t, triplet; q, quartet.

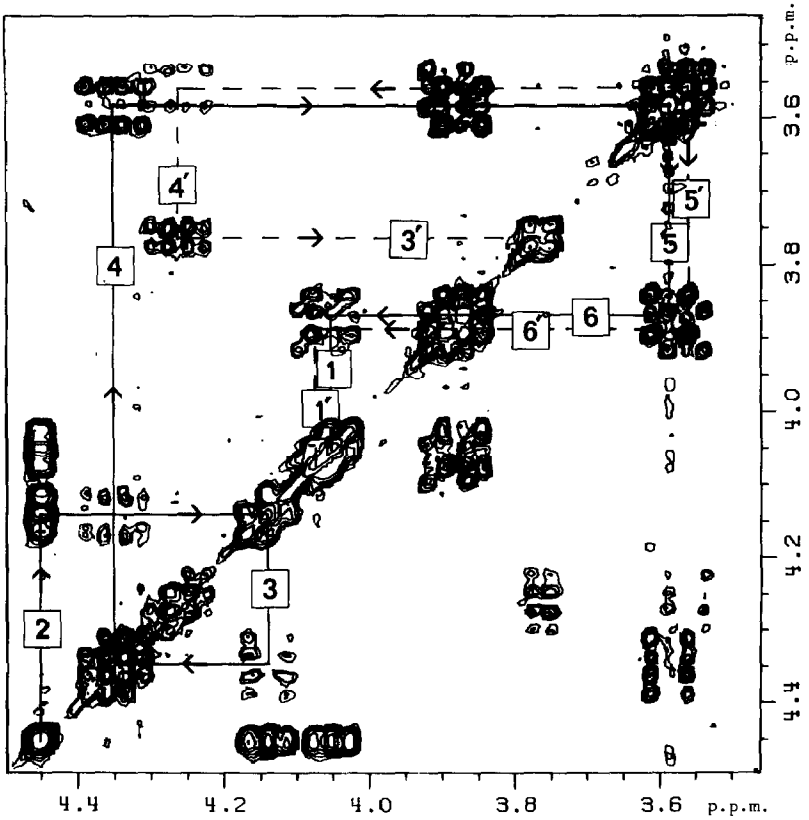


Fig. 2 2D-N.m.r. spectrum of a 2:1 mixture of 1,3,4-IP_3 and 1,2,4-IP_3.

The missing H-2 signal is close to the signal for residual HOD, and was suppressed. However, when the 1D spectrum was recorded at 33°, this H-2 signal appeared as a doublet downfield of the HOD signal. Working from the shift of the H-2 resonance, the assignments of the other signals can be obtained, as shown in Fig. 2. Furthermore, the signals for H-1,3 (2 t) and H-6 (q) indicate that these positions are phosphorylated, and readjustment of the numbering gives the structure 1,3,4-IP_3.

The other isomer was identified by starting from the signal for H-5′ (t, lowest shift in the spectrum). The signals for H-5,5′ (2 t) partially overlap in the 1D spectrum. However, in the 2D spectrum, the two signals could be identified easily since their cross-peaks are partially resolved. From the H-5′ signal, the H-4′ and H-6′ signals can be assigned, leading to the assignments of H-3′ and H-1′. The last two signals showed only one cross-peak each, since the H-2′ signal is suppressed by the water irradiation as described above. The multiplicity of the signals (Table II) for H-4′ (q), H-1′ (t), and H-2′ (d) indicates the compound to be 1,2,4-IP_3.

DISCUSSION

Cerdan *et al.*[9] proposed the following tentative algorithm to describe the effect on the chemical shift for the resonance of any axial proton in *myo*-inositol phosphate upon phosphorylation of any position except C-2,

$$\Delta\delta = n_1\alpha + n_2\beta + n_3\gamma,$$

where $\Delta\delta$ is the difference in chemical shift of a particular ^1H resonance compared with that of the corresponding resonance for *myo*-inositol, n_1 specifies if the position in question is phosphorylated ($=1$) or not ($=0$), and n_2 and n_3 give the number of positions phosphorylated that are adjacent and twice-removed positions, and α–γ are empirical constants found from least squares fitting of the equations to the experimental data. Based on five substances, these constants were found as follows: $\alpha = 0.44 \pm 0.02$, $\beta = 0.18 \pm 0.01$, and $\gamma = 0.02$[9]. Based on the 8 *myo*-inositol phosphates in Table I that were not 2-phosphorylated, the constants are $\alpha = 0.48 \pm 0.02$, $\beta = 0.15 \pm 0.01$, and $\gamma = 0.05 \pm 0.01$. Using all 20 compounds in Table I, including those that are 2-phosphorylated, a similar set of constants is obtained, namely, $\alpha = 0.49 \pm 0.01$, $\beta = 0.10 \pm 0.01$, and $\gamma = 0.05 \pm 0.01$, suggesting that the shift of a ^1H resonance from *any myo*-inositol phosphate can be calculated. The correlation between the calculated and the experimental chemical shifts are subject to some uncertainties for *both* sets of data. For example, if the salt content in various samples differed significantly, this would affect the ^1H-n.m.r. chemical shifts. However, there is a clear correlation between the sum of the chemical shifts and the number of phosphorylated positions. The result of a least squares fit shows that an increase of 0.75 ± 0.05 p.p.m. in the sum of the chemical shift is to be expected for each additional phosphate group. This accords with the value of 0.81 ± 0.03 p.p.m. ($0.49 + 2 \times 0.11 + 2 \times 0.05$) derived from the position specific algorithm presented above. The algorithm was tested as follows. For IP_2, IP_3, and IP_4, the theoretical chemical shifts of all (9, 11, and 9, respectively) possible isomers were calculated. For a given sample, the 6 experimental shifts were used to construct the 720 (6!) possible combinations of shifts. These combinations were fitted to the 9 or 11 combinations of theoretically calculated shifts, by calculating the least square sum of the pairwise difference in shift. The algorithm is self consistent, in that it correctly identifies all 5 IP_2's, 9 IP_3's, and 2 IP_4's for which experimental data are available. For two IP_2's and one IP_4 with *near* degeneracy of the chemical shifts, the fitting resulted in pairwise interchange

of two fitted shifts as compared to the experimental observation. This situation, however, did not prevent the fitting procedure from arriving at the correct assignments of isomers. Thus, the equation may be useful for the identification of an unknown *myo*-inositol phosphate from the chemical shift data only.

In order to aid in confirming assignments, it is possible to consider qualitative information regarding the shift of the resonance of a specific proton. In the spectrum for phytic acid, where all six positions are phosphorylated, as well as for *myo*-inositol itself, the chemical shifts of the resonances decrease in the order H-2 > H-4, H-6 > H-3, and H-1 > H-5. The same trend is found also for the other *myo*-inositol phosphates, although the exact relative position depends on how many of the neighbouring positions are phosphorylated.

It is clear from the spectra that the highest chemical shift found in a spectrum is for the H-2 resonance, when C-2 is phosphorylated. If C-2 is not phosphorylated, then the resonance from H-2 still has a high chemical shift, but not necessarily the highest. In this situation, the resonances of both H-4 and H-6 can be shifted to a higher frequency than that of H-2 if *their* positions are phosphorylated. The exact positions of all three signals depend on how many of their neighbours are phosphorylated.

Furthermore, if C-5 is not phosphorylated, the H-5 resonance invariably has the lowest chemical shift; if C-5 is phosphorylated, it still has one of the lowest, in competition with the signals from H-1 and H-3. Again, the exact position depends on the extent to which the neighbouring positions are phosphorylated.

It is possible, with good accuracy, to identify *any myo*-inositol phosphate only from its 1D ^{1}H.n.m.r. chemical shift data. For confirmation, it is of help to include multiplet structure and qualitative trends of the chemical shifts. If several isomers of similar concentration are present in the same sample, 2D ^{1}H-n.m.r. analysis may be needed in order to assign the resonances to the appropriate isomer.

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