Measurement of the ^{31}P–^{31}P Geminal Coupling Constant in Pyrophosphate using the Scalar Relaxation between ^{17}O and ^{31}P Nuclei

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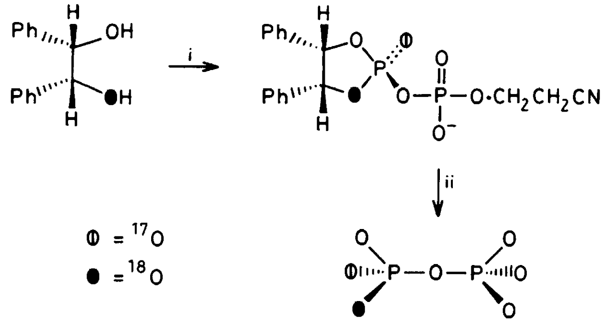
Although the scalar relaxation between the quadrupolar nucleus ^{17}O and a phosphorus atom to which it is bonded can cause extreme broadening of the ^{31}P resonance, it does not decouple the ^{31}P nucleus even in a tightly coupled system; consequently the ^{31}P–^{31}P geminal coupling constant in pyrophosphate can easily be measured at low magnetic field.

During our studies with chiral [^{16}O,^{17}O,^{18}O]phosphate esters and anhydrides we had occasion to make P-1-(*S*)-[^{16}O,^{17}O,^{18}O]pyrophosphate.[1] The synthetic route adopted is outlined in Scheme 1 and follows the general method we have developed for the synthesis of chiral [^{16}O,^{17}O,^{18}O]phosphate esters and anhydrides.[2]

The ^{18}O site was enriched to 99 atom % ^{18}O, but the ^{17}O site contained 1.8 atom % ^{16}O, 44.0 atom % ^{17}O, and 54.2 atom % ^{18}O. The sample of P-1-(*S*)-[^{16}O,^{17}O,^{18}O]pyrophosphate contained therefore a large amount of P-1-[$^{18}O_2$]pyrophosphate and a small amount of P-1-[^{18}O]pyrophosphate. The ^{31}P n.m.r. spectrum of the sample is shown in Figure 1.



Scheme 1. *Reagents:* i, $P^{17}OCl_3$, pyridine, followed by $\mathrm{NC{\cdot}CH_2{\cdot}CH_2{\cdot}O{\cdot}P\overset{-}{O}_3H(C_8H_{17})_3\overset{+}{N}H}$; ii, Na–liq. NH_3.

^{18}O causes an upfield shift on a ^{31}P resonance when directly bonded to phosphorus, and the calculated chemical shift difference between the two phosphorus atoms in P-1-[$^{18}O_2$]pyrophosphate is *ca.* 0.04 p.p.m.[3] It has been demonstrated recently that when all four oxygen atoms around one phosphorus atom in pyrophosphate are replaced by ^{18}O the chemical shift difference is large enough to observe a typical AB spin–spin coupling pattern, albeit at very high magnetic field (202.5 MHz for ^{31}P), from which the ^{31}P–^{31}P geminal coupling constant of 21.1 Hz was obtained.[4] In Figure 1(a) the outer lines (1 and 5) of the AB system of P-1-[$^{18}O_2$]pyrophosphate can just be discerned (at 81.01 MHz) but the much more intense inner lines are not resolved (peak 3). In the expanded presentation in Figure 1(b) the weak outer lines (1 and 5) can be seen more easily. These lines are not spinning side bands since the sample was spinning at 37 Hz. Thus, the main peak (3) at −9.00 p.p.m. is assigned to the unresolved central lines of the AB spin–spin system in P-1-[$^{18}O_2$]pyrophosphate {and the trace of P-1-[^{18}O]pyrophosphate}, and the very small signals (1 and 5) on each side, are assigned to the outer lines of the AB system from which the ^{31}P–^{31}P geminal coupling constant of 21.1 Hz is obtained in agreement with the value obtained by Marschner *et al.*[4]

The doublet centred at −8.98 p.p.m. (2 and 4) is assigned to P-1-(*S*)-[^{16}O,^{17}O,^{18}O]pyrophosphate. The ^{31}P n.m.r. spectrum has been measured at three spectrometer frequencies [36.43, 81.01 (Figure 1), and 162 MHz] and in each case a doublet with lines of equal intensity separated by 21.1 Hz was observed, confirming that the doublet is due to spin–spin

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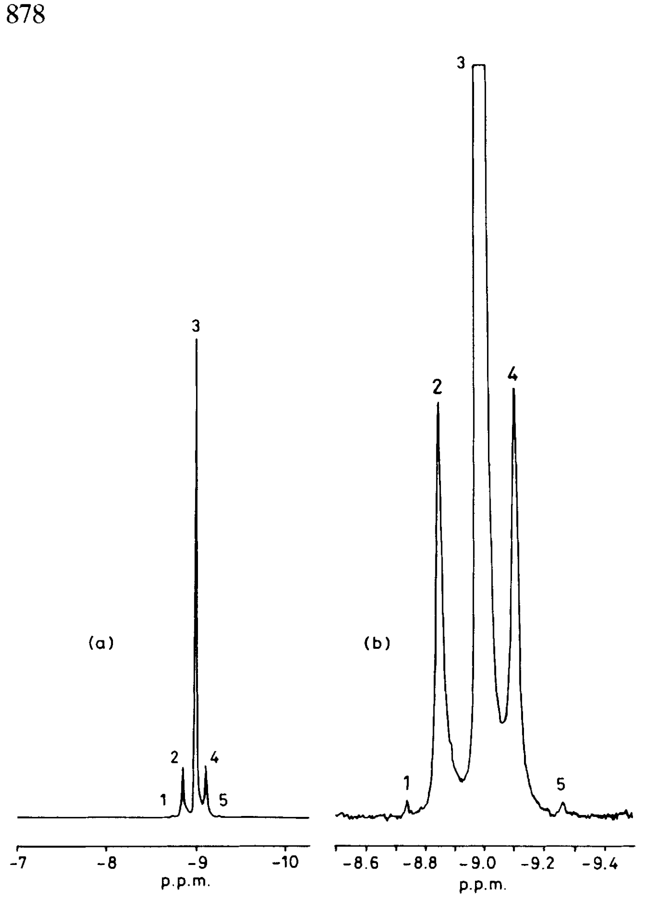
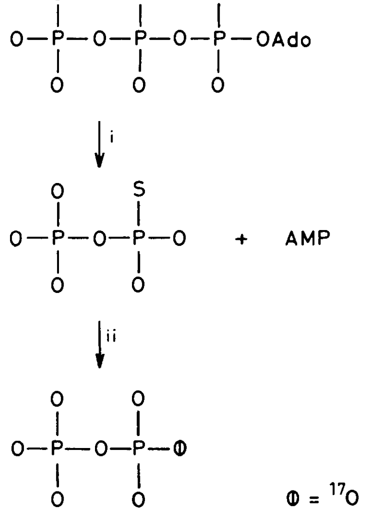


Figure 1. The ^{31}P n.m.r. spectrum (at 81.01 MHz) of (a) P-1-(*S*)-[^{16}O,^{17}O,^{18}O]pyrophosphate and P-1-[^{18}O$_2$]pyrophosphate and (b) the same spectrum expanded. The central line (3) at −9.00 p.p.m. and the two very weak outer lines (1 and 5) are assigned to P-1-[^{18}O$_2$]-pyrophosphate and the doublet (2 and 4) centred at −8.98 p.p.m. (*J* 21.1 Hz) is assigned to the PO$_3$ residue in P-1-(*S*)-[^{16}O,^{17}O,^{18}O]pyrophosphate.



Scheme 2. *Reagents:* i, snake venom phosphodiesterase; ii, *N*-bromosuccinimide, [^{17}O]water–dioxane.

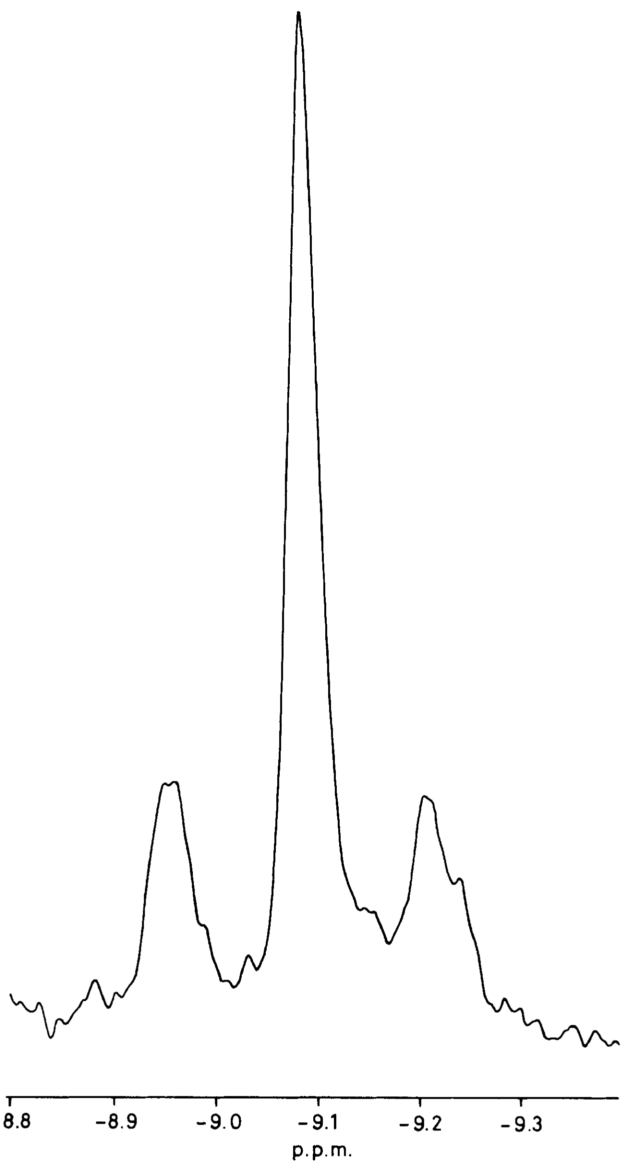


Figure 2. The ^{31}P n.m.r. spectrum (at 81.01 MHz) of [^{17}O]pyrophosphate containing unlabelled pyrophosphate and [^{18}O]pyrophosphate. The central line at −9.1 p.p.m. is assigned to unlabelled and [^{18}O]pyrophosphate and the doublet centred at a similar chemical shift (*J* 21 Hz) is assigned to the PO$_3$ residue of [^{17}O]pyrophosphate.

coupling; by contrast the centre of the AB system (line 3) moved progressively away from the centre of gravity of the doublet (lines 2 and 4) at higher spectrometer frequency, since this is due to a chemical shift difference caused by the isotope shift. Comparison of the area under the limbs of the doublet with that under the AB system assigned to P-1-[^{18}O$_2$]pyrophosphate is in accord with the isotopic composition of the [^{17}O]water used in the synthesis.

It is well established that when ^{17}O is directly bonded to phosphorus the ^{31}P resonance is generally extremely broad.[5] Moreover, it is known from previous studies with ^{17}O incorporated into ATP that this broadening does not decouple the ^{31}P nucleus to which it is attached from its neighbouring ^{31}P nucleus.[6] However, it was not obvious that in a tightly coupled system {the chemical shifts of the ^{31}P nuclei in P-1-(*S*)-[^{16}O,^{17}O,^{18}O]pyrophosphate are unlikely to differ by more than 0.03 p.p.m.} the coupling would be observed and the lines be of equal intensity.

In order to confirm this observation monothiopyrophosphate was obtained by hydrolysing adenosine 5′-O-[2-(*R*)-thiotriphosphate] with snake venom phosphodiesterase (Scheme 2).[7] The monothiopyrophosphate was then converted into [^{17}O]pyrophosphate by treatment with *N*-bromosuccinimide in dioxane–[^{17}O]water.[8] Similar treatment using unlabelled water gave pyrophosphate which appeared as a singlet at −9.1 p.p.m. in the ^{31}P n.m.r. spectrum. The [^{17}O]pyrophosphate sample was expected to contain unlabelled pyrophosphate and [^{18}O]pyrophosphate since the isotopic composition of the [^{17}O]water used was 13.8 atom % ^{16}O, 50.7 atom % ^{17}O, and 35.5 atom % ^{18}O. The ^{31}P n.m.r. spectrum (Figure 2) shows an unresolved singlet assigned to unlabelled pyrophosphate and [^{18}O]pyrophosphate (an AB system is not now observed) and a doublet (*J* 21 Hz) centred at essentially the same chemical shift. The doublet is assigned to the phosphorus atom not bonded to ^{17}O in [^{17}O]pyrophosphate and the ratio of the area of the doublet to that of the singlet at −9.1 p.p.m. is in accord with that expected from the known isotopic composition of the [^{17}O]water used, thus confirming the earlier observation.

Scalar relaxation of the 'second kind' occurs when a dipolar nucleus A ($I = \frac{1}{2}$) is coupled to a quadrupolar nucleus X ($I > \frac{1}{2}$), but the observed effect depends on the quadrupolar relaxation time (T_q) of X and the scalar coupling constant between A and X.[9] For ^{17}O ($I = \frac{5}{2}$) the quadrupolar relaxation time for small molecules is of the order of milliseconds,[10] and $^1J(^{31}P^{17}O)$ is 104 Hz for non-bridging oxygens in pyrophosphate (measured by ^{17}O n.m.r. spectroscopy).[11] The condition $1/T_q = ca.\ 2\pi J$ applies therefore and the scalar relaxation of the dipolar nucleus A will be given by equations (1) and (2), where T_1sc. and T_2sc. are the longitudinal and transverse scalar relaxation times of A, and ω_A and ω_X are the Larmor precession frequencies of A and X respectively.

$$\frac{1}{T_1\mathrm{sc.}} = \frac{8\pi^2 J^2 I(I+1)}{3} \cdot \left[\frac{T_q}{1+(\omega_A-\omega_X)^2 T_q^2}\right] \quad (1)$$

$$\frac{1}{T_2\mathrm{sc.}} = \frac{4\pi^2 J^2 I(I+1)}{3} \cdot \left[T_q + \frac{T_q}{1+(\omega_A-\omega_X)^2 T_q^2}\right] \quad (2)$$

Since $(\omega_A-\omega_X)^2 T_q^2 \gg 1$ for ^{31}P and ^{17}O, scalar relaxation will dominate T_2 of the phosphorus nucleus to which ^{17}O is coupled but will leave T_1 essentially unaffected. Moreover, since $1/T_q$ = ca. $2\pi J$, the ^{31}P nucleus bonded to ^{17}O will be split into 6 lines (^{17}O has $I = \frac{5}{2}$) which are not observed owing to broadening caused by scalar relaxation. Nevertheless, the ^{31}P–^{17}O coupling makes the chemically equivalent ^{31}P nuclei magnetically non-equivalent, which in the absence of the isotope shift would be an AA′X system where X is ^{17}O. In fact the chemical shift difference expected between the ^{31}P nuclei due to the isotope shift is not resolved at this spectrometer frequency {note that the inner lines of the AB system of P-1-[$^{18}O_2$]pyrophosphate were not resolved} and hence the ^{31}P resonance not bonded to ^{17}O appears as a doublet with lines of equal intensity.

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