Synthesis and use as spin-trap of 5-methyl-5-phosphono-1-pyrroline *N*-oxide (DHPMPO). pH Dependence of the EPR parameters of the spin adducts

Jean-Louis Clément,*[a] Stéphane Barbati,[a] Claudine Fréjaville,[a] Antal Rockenbauer[b] and Paul Tordo[a]

[a] *Laboratoire Structure et Réactivité des Espèces Paramagnétiques UMR 6517, CNRS-Universités d'Aix-Marseille I et III, Av. Esc. Normandie Niemen, 13397Marseille, Cédex 20, France. E-mail: tordo@srepir1.univ-mrs.fr; Tel: 33 491 632 851*

[b] *Chemical Research Center, Institute for Chemistry, H-1525 Budapest, PO Box 17, Hungary*

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5-Methyl-5-phosphono-1-pyrroline *N*-oxide (DHPMPO), the acid analogue of DEPMPO, is synthesized by hydrolysis of the diethoxyphosphoryl moiety of DEPMPO. A variety of carbon- and oxygen-centred radicals are trapped with DHPMPO and the corresponding EPR spectra are analysed. We investigate the influence of pH on the coupling-constant-values of DHPMPO-CH_3 which behaves like a pH-sensitive probe. The two pK_a-values (1.31 and 7.58) are obtained from the simultaneous calculation of ten EPR spectra recorded in the pH range 0.5–9, using new two-dimensional simulation software.

Introduction

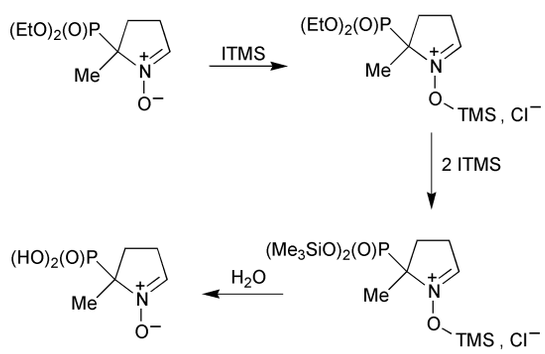
During oxidative stress serious cellular damage can result from the uncontrolled production of oxygen-centred radicals.[1] Spin-trapping combined with electron paramagnetic resonance (EPR) is a powerful tool for the detection and characterization of these radicals.[2] For a long time 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) has been the most popular spin-trap used to investigate the role of oxygen free radicals in biological milieux. However, the use of DMPO is limited by several drawbacks[3] and progressively a more convenient nitrone, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO), has been used instead of DMPO.[4] In our continuing effort to design new nitrones for biological spin-trapping, we prepared and explored the spin-trap properties of 5-methyl-5-phosphono-1-pyrroline *N*-oxide (DHPMPO) which is the acid analogue of DEPMPO. Our results are presented below.

Results and discussion

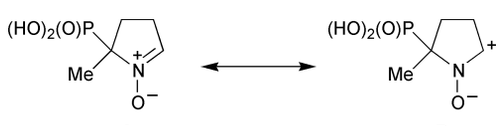
Synthesis

DEPMPO (1 eq.) was stirred for 4.5 h at room temperature in methylene dichloride with iodotrimethylsilane (ITMS) (3 eq.).[5] After removal of the solvent the reaction mixture was dissolved in acetone and addition of water led to a precipitate of DHPMPO (76% yield). The formation of DHPMPO can be accounted for as shown in Scheme 1.

^{1}H NMR monitoring of the reaction showed that the nitronyl function is silylated first. When hydrolysis was performed after addition of one equivalent of ITMS, the starting DEPMPO was recovered. The reaction rate was slower with bromotrimethylsilane and 20 h at 30 °C were necessary to complete the reaction. In DMSO-d_6 the chemical shifts of the DEPMPO and DHPMPO nitronyl carbon are very close, δ_C 133.6 and 134.9, respectively (δ_C 128.8 for DMPO). For DHPMPO in D_2O, a significant influence of pH on this chemical shift was observed (δ_C 150.0 at pH 1.5, 143.8 at pH 7.0). This trend can be explained by the influence of the pH on the electron-withdrawing effect of the phosphono group. When the pH increases, the ionization of the phosphono group increases and its electron-withdrawing effect decreases. Then, the relative amount of the mesomeric structure **B** (Scheme 2) decreases and as observed the nitronyl carbon is more shielded.



Scheme 1



Scheme 2

Spin-trapping studies

Hydroxyl radical. The hydroxyl radical spin adduct (Fig. 1) was obtained by incubating DHPMPO (0.1 M) with H_2O_2 (2 mM) and $FeSO_4$ (2 mM) in 0.1 M phosphate buffer at pH 7.0. We obtained an EPR signal simulated as a superimposition of spectra corresponding to two diastereoisomers (Table 1). This signal was inhibited by the presence of catalase (600 U mL^{-1}) in the incubation mixture and the same EPR signal was observed when a 0.1 M phosphate buffer solution of DHPMPO and H_2O_2 (1%) was photolysed. Significant changes of the

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Table 1 Calculated EPR hyperfine splittings for DHPMPO spin adducts[a]

Spin adduct	a_N/mT	a_H/mT	a_P/mT		
DHPMPO-OH	1.45	1.34	4.07	48.3%	
	1.47	1.51	4.24	51.7%	
DHPMPO-OOH[b]					
trans[c]	1.27	4.32	1.0; 0.1; (0.05 × 3; 0.04 × 2; 0.06)[d]	74%	65%
	1.2	4.49	1.3; 0.1; (0.05 × 3; 0.04 × 2; 0.06)[d]	26%	
cis	1.26	3.39	0.79; 0.06 × 3	35.0%	
DHPMPO-CH_3					
SAH_2	1.52[e] (1.52)[f]	2.18 (2.19)	4.41 (4.41)		
SAH^-	1.56 (1.56)	2.19 (2.19)	4.16 (41.6)		
SA^{--}	1.60 (1.6)	2.20 (2.2)	3.80 (3.83)		
DHPMPO-CH_3					
(pH 1.5)	1.54	2.18	4.25		
(pH 7.0)	1.56	2.19	4.11		
(pH 9.0)	1.59	2.20	3.87		
DHPMPO-CO_2^-	1.49	1.79	4.37		
DHPMPO-CH_2OH	1.51	2.10	4.19		
DHPMPO-O-Bu^t[b]	1.32	1.03	4.19		

[a] In phosphate buffer at pH 7.0 unless otherwise mentioned. [b] In pyridine. [c] $K_{exchange} = 2.4 \times 10^6\ s^{-1}$. [d] The variation of these couplings with chemical exchange was not considered. [e] Calculated from two-dimensional simulation. [f] From the plot of a_P *versus* pH.

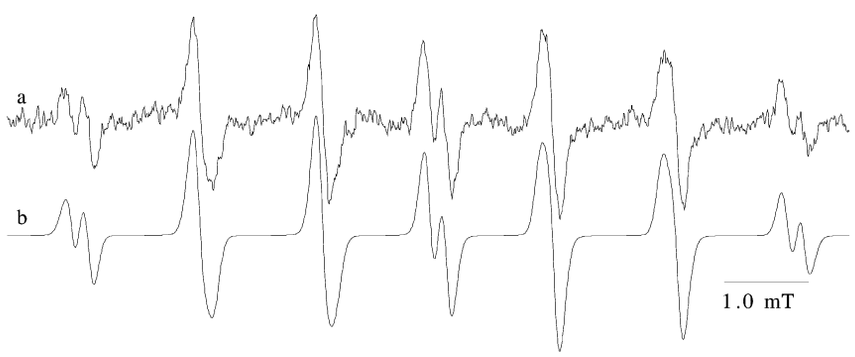


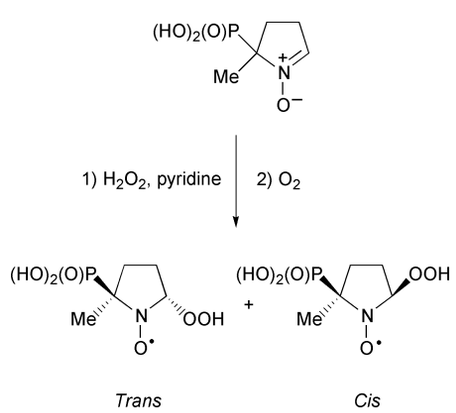
Fig. 1 (a) EPR spectrum of DHPMPO-OH spin adduct formed in the reaction of H_2O_2–Fe^{2+} in the presence of DHPMPO, (b) calculated spectrum. Concentrations: DHPMPO: 0.1 M, H_2O_2: 2.0 mM, Fe^{2+}: 2.0 mM. Settings: modulation amplitude 0.05 mT, time constant 0.128 s, sweep time 120 s, gain 5×10^4, microwave power 10 mW.

diastereoisomer ratio, the coupling-constant-values, and the linewidth were observed in the pH range 1–9 (data not shown). However, a satisfactory analysis of these changes has not yet been obtained. The nucleophilic addition of water to DHPMPO was not observed even in the presence of a high concentration of Fe^{3+} (40 mM). This result is certainly the consequence of the high affinity of the phosphono group for Fe^{3+}, which probably inhibits the activation of the nitronyl function.[6]

Superoxide radical. Either the hypoxanthine/xanthine oxidase system or a light–riboflavine–diethylenetriaminepentaacetic acid (DTPA) combination was used to generate the superoxide radical in the presence of DHPMPO at different pH-values. In all our attempts only the DHPMPO-OH spin adduct was observed. No signal was observed in the presence of superoxide dismutase (SOD) (85 U mL^{-1}), thus suggesting that the DHPMPO-OH spin adduct resulted from a rapid decomposition of the superoxide adduct.

Nucleophilic addition of H_2O_2 (from 0.16 up to 1.3 M) to DHPMPO (0.1 M) in pyridine yielded a strong EPR signal (Fig. 2), which was assigned to DHPMPO-OOH. As has been observed for the DEPMPO-OOH adduct in organic solvents,[7] the EPR signal of DHPMPO-OOH arises from the superimposition of the spectra of the *trans* and *cis* diastereoisomers (Scheme 3, Table 1).

Furthermore, the spectrum of the major diastereoisomer (*trans*) showed a characteristic linewidth alternation. The DHPMPO-OOH adduct in pyridine was very persistent even in the presence of a large concentration of H_2O_2 (1.3 M). When the pyridine solution of DHPMPO-OOH was diluted with phosphate buffer (pH 6, 7 or 9) the EPR signal faded at once.



Scheme 3

Other radicals. *tert*-Butoxyl radical (*t*-BuO$^\bullet$) was produced by UV photolysis of di-*tert*-butyl peroxide in pyridine. In the presence of DHPMPO we observed the spectrum of a single diastereoisomer, most likely corresponding to a *trans* geometry of the phosphono and *tert*-butoxy groups. Anomeric interaction between the aminoxyl group and the C–OBu^t bond forces the bond into a pseudoaxial geometry and as a result the $a_H(\beta)$-value is low (Table 1).[8]

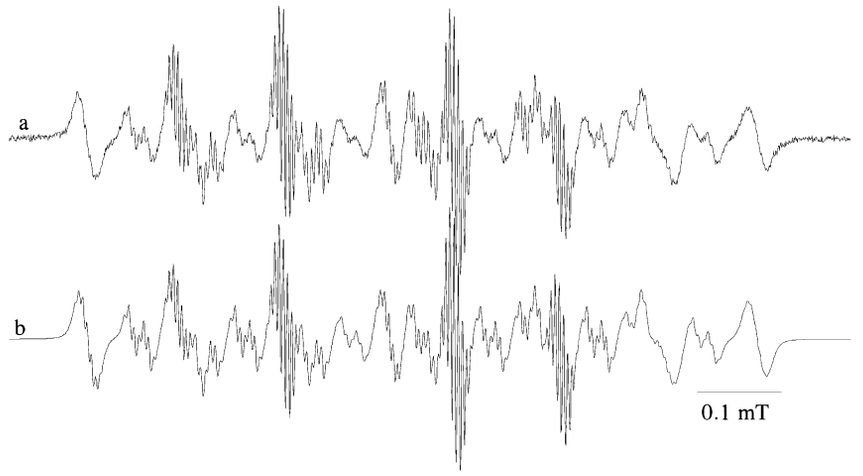


Fig. 2 (a) EPR spectrum of DHPMPO-OOH spin adduct formed in the addition reaction of H_2O_2 on DHPMPO in pyridine and subsequent oxidation with O_2 and degassing with N_2, (b) calculated spectrum. Concentrations: DHPMPO: 0.1 M, H_2O_2: 3 M. Settings: modulation amplitude 0.03 mT, time constant 0.128 s, sweep time 480 s, gain 1.25×10^4, microwave power 5 mW.

A Fenton reaction carried out in the presence of either sodium formate or methanol and DHPMPO was used to generate, at pH 7.0, the $^{\bullet}CO_2^-$ and $^{\bullet}CH_2OH$ spin adducts, respectively. The trapping of $^{\bullet}CO_2^-$ and $^{\bullet}CH_2OH$ generated only one diastereoisomer, exhibiting a_N and a_H coupling constants (Table 1) close to those observed for DEPMPO analogues. However, for both spin adducts, the phosphorus coupling is significantly lower, in agreement with a lower electron-withdrawing effect of the partially ionized phosphono group.

Methyl radical. The trapping of methyl radical was investigated over the pH range 0.5–9. Phosphate buffer solutions of DHPMPO (0.1 M) were adjusted to the desired pH, then DMSO (10% v/v) and H_2O_2 (1% v/v) were added. Samples were degassed by bubbling nitrogen and placed in a flat quartz cell which was illuminated within the cavity of the EPR spectrometer. Typical spectra of DHPMPO-CH_3 recorded at different pH-values are shown in Fig. 3. While the line position in the EPR spectra recorded at different pH-values showed a significant variation, the linewidth remained practically the same, indicating a fast exchange between the preferred conformers of the three species SAH_2, SAH^- and SA^{--} (Scheme 4).

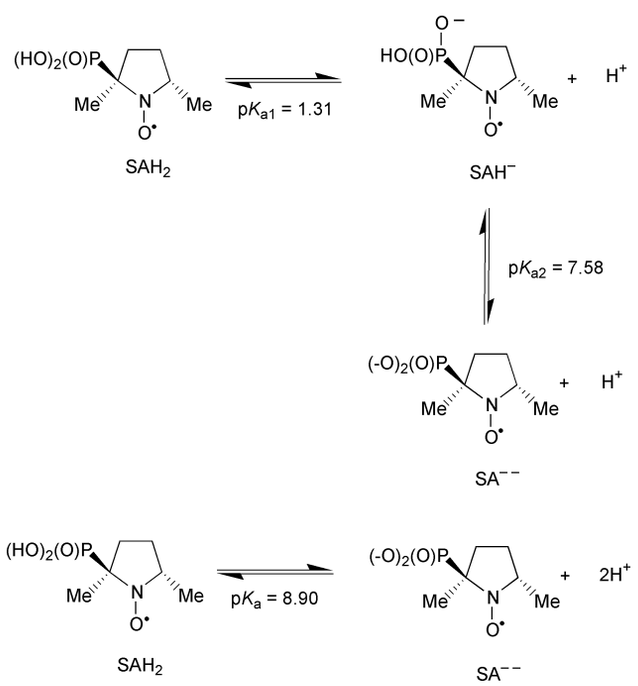
The pH-dependent EPR parameters, namely the *g*-factor and the coupling constants, can be described as weighted means of individual values of respective species [eqn. (1)].

$$\langle a_P \rangle = p(SAH_2)\, a_P(SAH_2) + p(SAH^-)\, a_P(SAH^-) + p(SA^{--})\, a_P(SA^{--}) \quad (1)$$

The 10 EPR spectra recorded at different pH-values between 0.5 and 9 were simultaneously fitted by an original two-dimensional simulation procedure (Fig. 3).[9] The experimental spectra were calculated assuming the existence of only *trans* diastereoisomers (Scheme 4) and using 15 adjustable parameters: the *g*-value, three coupling constants for each species, the linewidth and pK_{a1}- and pK_a-values. The pH-dependent populations [$p(SAH_2)$, $p(SAH^-)$ and $p(SA^{--})$] were computed from the mass-balance equations [eqn. (2)].

$$[SAH^-] = K_{a1}[SAH_2][H^+]^{-1};\ [SA^{--}] = K_a\,[SAH_2][H^+]^{-2} \quad (2)$$

The calculated p*K*-values are indicated in Scheme 4 and the coupling-constant-values are listed in Table 1. The pK_{a1}- and pK_{a2}-values can also be determined from the plot of a_P (obtained from each individually calculated EPR spectrum) *versus* pH (Fig. 4). The values (1.4 and 7.5, respectively) were in very good agreement with those calculated using the two-dimensional simulation. Furthermore, assuming that for the pH ranges $0.5 \le pH \le 3$ and $6 \le pH \le 9$ only two species may be considered (SAH_2/SAH^- and SAH^-/SA^{--}, respectively) the EPR coupling constants for each species can be calculated and were found to match very well (Table 1) the values obtained from the two-dimensional simulation.



Scheme 4

The p*K*-values (1.31 and 7.58) agree very well with those observed for alkyl phosphonic acids.[10] The a_H coupling is almost the same for the three species, indicating that deprotonation does not induce significant changes on the geometry of the preferred conformers. Therefore the significant change of the phosphorus coupling must be attributed to the decrease of the electron-withdrawing effect of the phosphono group on successive deprotonation. As the electron-withdrawing effect decreases the P_{3S} character of the β-C–P bond decreases and for the same geometry the β-phosphorus coupling decreases.[11]

Conclusions

DHPMPO can be easily obtained from DEPMPO *via* formation of the bistrimethylsilyl ester which is then hydrolysed. DHPMPO is a good trap for both hydroxyl radical and carbon-centred radicals. However, the DHPMPO-superoxide adduct

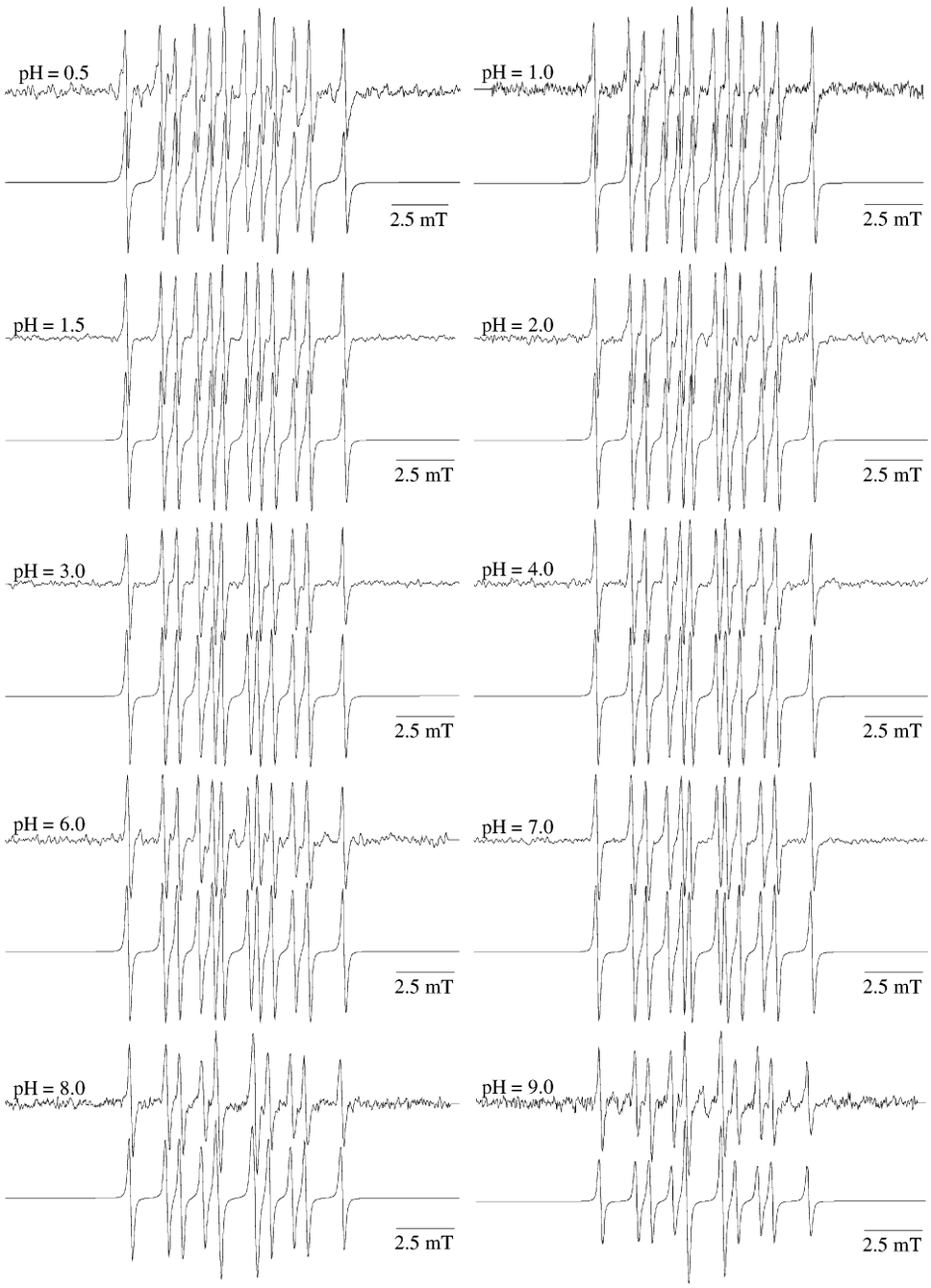


Fig. 3 Experimental and calculated EPR spectra of DHPMPO-CH_3 spin adduct formed in reaction of H_2O_2/*hv* in the presence of DMSO and DHPMPO at various pH-values. Concentrations: DHPMPO: 0.1 M, H_2O_2: 1% v/v, DMSO 10% v/v. Settings: modulation amplitude 0.05 mT, time constant 0.128 s, sweep time 120 s, gain 5×10^4, microwave power 10 mW.

was observed only in pyridine; in the presence of water it rapidly decomposes to the DHPMPO-OH adduct.

The phosphorus coupling constant of the DHPMPO spin adducts is very sensitive to pH, and the p*K*-values for the DHPMPO-CH_3 spin adduct were obtained from the plot of a_P *versus* pH. These p*K*-values, as well as the coupling constants for the diprotonated, monoprotonated and unprotonated forms, were easily obtained from the simultaneous calculation of a series of EPR spectra recorded at different pH-values. This two-dimensional simulation software appears to be a very powerful tool to analyse the influence of pH on pH-sensitive EPR probes.

Experimental

Chemicals

All chemicals and organic solvents are commercially available and used as supplied. CH_2Cl_2 and CH_3COCH_3 were purchased at the highest grade and stored before used on molecular sieves (3 Å). DEPMPO was synthesized as described previously.[12] The mp of DHPMPO was measured on a Büchi 535 apparatus and is uncorrected.

NMR and EPR

^{31}P Spectra were recorded at 40.53 MHz (Bruker AC 100 instrument) with 85% H_3PO_4 as external reference. ^1H and ^{13}C spectra were recorded on a Bruker AMX 400 at 400 MHz and 100 MHz, respectively.

EPR spectra were recorded on a Varian E 109 spectrometer. All experiments were performed in phosphate buffer (0.1 M) previously stirred for 6 h in the presence of Chelex 100 provided by Sigma Chemical Company. Spectrometer settings and reagent concentrations are given in the relevant figures.

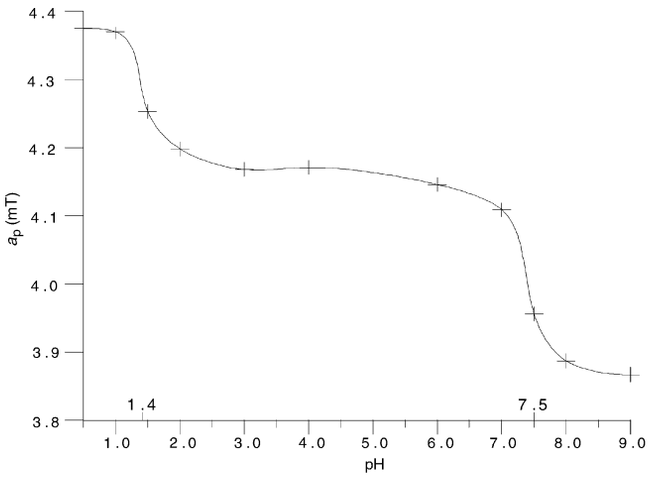


Fig. 4 a_P *versus* pH for DHPMPO-CH_3.

Synthesis of 5-methyl-5-phosphono-1-pyrroline *N*-oxide (DHPMPO)

DEPMPO (0.537 g, 2.30 mmol) was dissolved in dry CH_2Cl_2 (20 mL) under inert atmosphere, and ITMS (1.37 g, 6.85 mmol) was added with a syringe. The mixture was stirred during 4.5 h and then the solvent was removed under reduced pressure. The residue was dissolved in acetone (15 mL) and a precipitate of DHPMPO was observed after addition of a few drops of water. The solid was filtered off, washed with dry acetone, and dried under vacuum to give a slightly yellow powder (0.308 mg, 76%) (Found: C, 33.10; H, 5.90; N, 7.65. Calc. for $C_5H_{10}NO_4P$: C, 33.50; H, 5.63; N, 7.82%); mp 121 °C; ^{31}P NMR (D_2O; pH 1.5) δ_P 21.6; ^{13}C NMR (D_2O; pH 1.5) δ_C 20.8, 27.6, 30.7, 75.9 (d, J = 145.0 Hz), 150.0; ^{13}C NMR (D_2O–NaOD, pH 7.0) δ_C 21.9, 26.7, 30.7, 75.9 (d, J = 138.0 Hz), 143.8 (d, J = 7.0 Hz); ^{13}C NMR (DMSO d_6) δ_C 20.8, 25.3, 30.0, 73.8 (d, J = 145.0 Hz), 134.9; ^{1}H NMR (D_2O; pH = 1.5) δ 1.54 (3H, d, J = 13.5 Hz), 2.1 (1H, dt, J = 8.3 and 13.4 Hz), 2.7 (1H, m), 2.75 (2H, m), 7.45 (m, 1H).

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