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Characterisation of free radical spin adducts of the cyclic β-phosphorylated nitrone DEPMPO using tandem mass spectrometry

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Abstract

The application of the spin trapping technique coupled with mass spectrometry for the detection of free radicals using the nitrone diethyl-(2-methyl-1-oxido-3,4-dihydro-2*H*-pyrrol-2-yl)phosphonate (DEPMPO) is described. In a first step, the fragmentation pathway of this β-phosphorylated nitrone was studied by MS/MS. Then, DEPMPO was used to trap the free radicals •OH, $^{\bullet}CH_2OH$ and $^{\bullet}CH_3$, and structure elucidation of the adducts obtained was performed by tandem mass spectrometry. The fragmentation pathway of the two carbon-centred radical adducts mainly proceeds via the loss of the diethoxy(oxido)phosphoranyl radical. In contrast, the nitroxide formed after •OH trapping exclusively dissociated via the release of the radical initially trapped. This last decomposition pathway was unexpected in terms of radical stability but could be accounted for when considering the •OH elimination could be favoured by an intramolecular hydrogen transfer, leading to the formation of a stable oxazirane intermediate. This spin trapping/mass spectrometry technique using DEPMPO provides a powerful tool for the identification of short-lived free radicals in complex mixtures, without preliminary chromatographic separation.

Keywords: Free radicals; Spin trapping; Phosphorylated nitrone; Electrospray ionisation; Tandem mass spectrometry

1. Introduction

Since the EPR/spin trapping technique was first introduced [1–3], studies in this field have proliferated with numerous applications in the detection of free radicals formed in solution chemistry as well as in biology or medicine. To date, more than 5500 publications on this topic are quoted in CAPLUS. In this technique, a transient free radical typically reacts with a double bond of a diamagnetic compound (the spin trap) to form a longer-lived radical (the spin adduct), capable of being detected by conventional electron paramagnetic resonance (EPR) spectroscopy (Scheme 1) [4–8]. Nitrones are frequently used as spin trapping agents, notably in biological media, the spin adduct being in this case a nitroxide. Ideally, the splitting pattern of the spin adduct EPR spectrum should provide crucial information about the structure of the radical trapped. A typical EPR spectrum of an aldonitrone-radical adduct shows six lines, due to hyperfine couplings of the unpaired electron with the nitrogen and the β-hydrogen nuclei. The adducts obtained with β-phosphorylated aldonitrones, such as diethyl-(2-methyl-1-oxido-3,4-dihydro-2*H*-pyrrol-2-yl)phosphonate (DEPMPO, **1**), present 12 lines, because of a strong extra coupling with the phosphorus nucleus, very sensitive to the nature of the radical trapped, which facilitates the adduct identification [9]. Unfortunately, even with these nitrones, a given set of EPR parameters may not always clearly characterise a particular spin adduct, and the EPR-spin trapping technique essentially allows to determine the general type of radical trapped. For instance, oxygen- and carbon-centred radicals could be clearly distinguished using DEPMPO [9]. Using this ^{31}P-labelled spin trap, the EPR spectral patterns of the adduct could also differ for radicals centred on a primary or on a sterically hindered tertiary carbon [10]. But carbon-centred radicals with similar structures gave DEPMPO-spin adducts with identical EPR signals [11,12]. Note also that the EPR parameters of a given spin adduct may vary significantly

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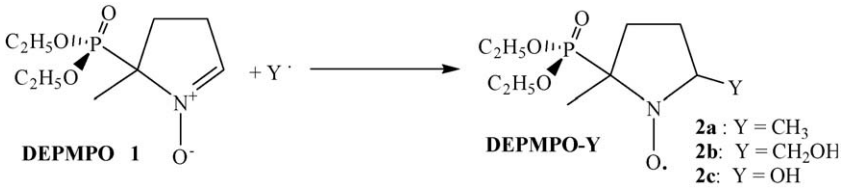


Scheme 1. Formation of the spin adducts DEPMPO-Y **2** by trapping a free radical Y• using DEPMPO **1**.

with the nitroxide environment, for instance with the solvent polarity. This means that it is impossible to know the detailed radical structure by using the sole EPR spectroscopy. To overcome this problem, other techniques that provide additional information about the structure of the adduct should be used. It has been shown that mass spectrometry (MS) could be a suitable tool to detect and identify spin adducts, and this technique was more particularly applied to the characterisation of radical adducts of the nitrones benzylidene(*tert*-butyl)azane oxide (PBN) [13,14], 4-{[*tert*-butyl(oxido)imino]-methyl}pyridine 1-oxide (POBN) [15–18] and 2,2-dimethyl-3,4-dihydropyrrole *N*-oxide (DMPO) [19–24]. Thus, several research groups have used high performance liquid chromatography (HPLC) to isolate radical spin adducts of these nitrones which were further detected by MS and identified by tandem mass spectrometry (MS/MS) [13–20]. However, collection of all analytes from HPLC for MS structure elucidation is laborious and time-consuming. Even when an on-line HPLC–MS system is used, it can lead to decomposition of the initially formed spin adduct via different reactions that could occur during elution. In recent works, Domingues and coworkers described the MS detection and the MS/MS structure determination of free radical spin adducts in complex mixtures, without preliminary HPLC separation [21–24]. Note however that in their studies only the nitrone DMPO was used to trap various free radicals. In addition, hydroxylamines or nitrones obtained after oxido-reductive processes of the spin adducts have been frequently detected rather than the initially formed nitroxides themselves [13–24]. To our knowledge, spin trapping/MS experiments have never been performed using β-phosphorylated nitrones. On the other hand, it has been shown that these compounds are generally superior to their non-phosphorylated analogues when it comes to spin trapping efficiency [8,9,25–28]. This prompted us to examine the possibility of identifying various DEPMPO-spin adducts by MS using electrospray ionisation (ESI) and to characterise them by MS/MS. Our very first results obtained in this field are presented herein.

2. Experimental

2.1. Materials

The nitrone DEPMPO was synthesised, purified and identified in our laboratory according to procedures described previously [9,29]. All the chemicals were purchased from Sigma–Aldrich (St. Louis, MO). Solvents were of the highest grade of purity commercially available and used as received.

2.2. Spin trapping

Hydroxyl radical was produced in water by a standard Fenton system (0.2% H_2O_2, 2 mmol L^{-1} ethylenediamine tetracetic acid, and 1 mmol L^{-1} $FeSO_4$). The free radicals $^{\bullet}CH_2OH$ and $^{\bullet}CH_3$ were generated by performing a Fenton reaction in water in the presence of methanol (10%) or dimethylsulfoxide (DMSO, 10%), respectively. All spin trapping experiments were conducted in tridistilled water in the presence of 20 mmol L^{-1} nitrone. For each experiment, 100 μL of reaction medium were prepared. The systems were allowed to react for 1–2 min, and the medium was rapidly extracted with either diethylether or CH_2Cl_2 (ca. 300 μL). An aliquot (ca. 50 μL) of the organic phase was analysed by EPR spectroscopy in order to verify the presence of the nitroxide spin adduct. The rest of the organic phase was evaporated under reduced pressure to remove the solvent and dissolved in acidified methanol for MS analysis. EPR assays were carried out at room temperature in capillary tubes by using a computer-controlled Bruker EMX spectrometer operating at X-band with 100 kHz modulation frequency. The instrument settings were as follows: non-saturating microwave power, 20 mW; modulation amplitude, 1 G; receiver gain, 5×10^5; time constant, 1.28 ms; scan time, 120 s; scan width, 120 G. Computer simulation of the EPR spectra were achieved using the program elaborated by Duling [30].

2.3. Mass spectrometry

All experiments were performed with a Sciex API III Plus triple quadrupole system with a pneumatically assisted electrospray interface (Sciex, Thornhill, Canada). The interface temperature was 54 °C throughout all experiments. Ultrahigh-purity (UHP, 99.999%) nitrogen was used as the curtain gas in the API source at a flow rate of 0.6 L/min, and zero-grade air was the nebulizing gas, at a flow rate of 0.8 L/min. Positive mode electrospray ionization was performed at 5 kV and the orifice voltage was set at 50 V. Resolution for both quadrupole was set at 0.7 amu fwhh. Mass calibration was performed on poly(propylene glycol) solution. Tandem mass spectrometry (MS/MS) measurements were based on collision-induced dissociations (collision energy: 20 eV, laboratory frame), using UHP argon as the target gas, at a collision gas target of 90×10^{15} molecules/cm^2. The API III Hyperspec workstation and API software version 2.6 were used on a Power Macintosh 8100/80 for instrument control, data acquisition and data processing. A syringe pump (Harvard Apparatus, South Natick, MA) was used for sample direct introduction at a 5 μL/min flow rate.

3. Results and discussion

3.1. Fragmentation of the nitrone DEPMPO 1

Since no MS study of β-phosphorylated nitrones have ever been published, the fragmentation pathways of DEPMPO was first studied by tandem mass spectrometry, with the aim of making the structure elucidation of its spin adducts easier. The positive ion ES-mass spectrum of DEPMPO showed a major peak at *m/z* 236 as well as a secondary signal at *m/z* 258 that could be attributed to the protonated molecule $[M+H]^+$ and to the sodium adducts $[M+Na]^+$, respectively. The MS/MS spectrum of *m/z* 236 shown in Fig. 1 exhibits abundant fragment ions at *m/z* 80, 81, 82, 98, 144 and 162. On the basis of this spectrum analysis, a partial fragmentation pathway of *m/z* 236 could be proposed (Scheme 2).

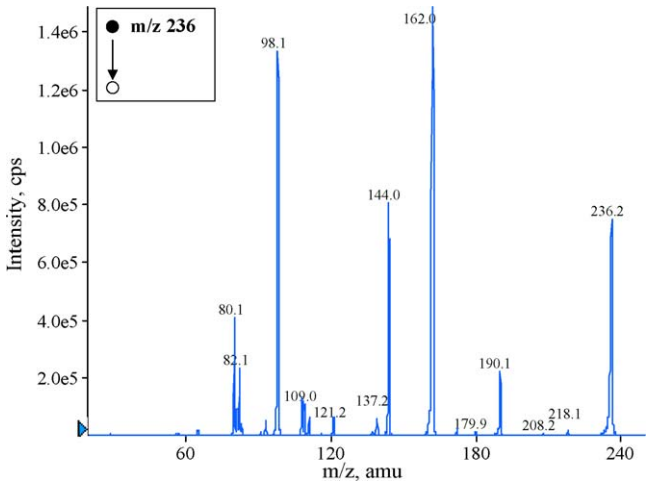
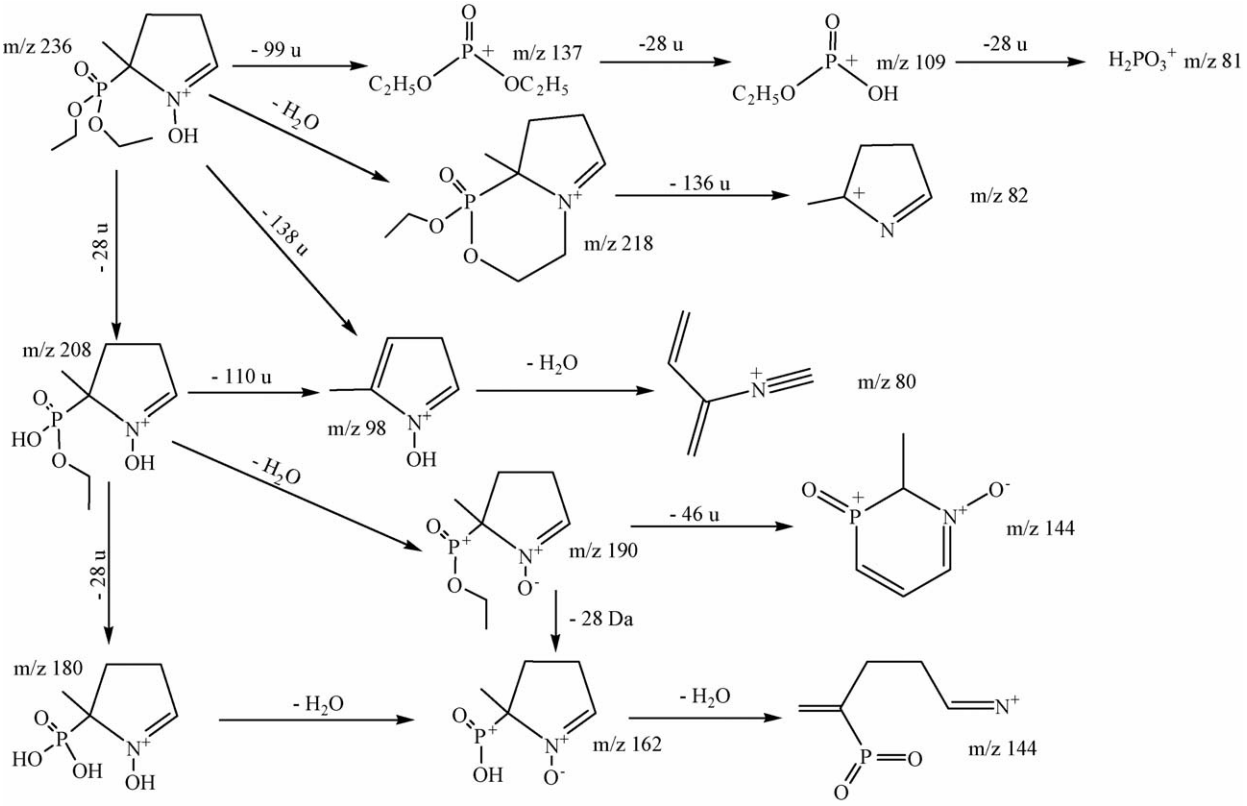


Fig. 1. ESI–MS/MS spectrum of the protonated DEPMPO **1**.

3.2. Detection and identification of DEPMPO-spin adducts

Throughout this text, the nitroxide spin adduct **2** formed by trapping a radical Y by DEPMPO will be denoted DEPMPO-Y. All the spin adducts were first formed in water as described in the experimental section. The media were then rapidly extracted and the organic layer was analysed by EPR spectroscopy. The nitroxides **2a** and **b** thus formed showed an EPR spectrum consisting of a triplet of doublets, due to hyperfine couplings of the unpaired electron with the nitrogen and the β-hydrogen nuclei, splitted by a large phosphorus coupling. In the case of **2c**, hyperfine couplings with the nitrogen and the β-hydrogen nuclei are very close, which leads to an EPR spectrum exhibiting eight lines only instead of 12. Computer simulation of the EPR spectra of the nitroxides **2a–c** yielded the values listed in Table 1 for the hyperfine coupling constants a_N, a_H and a_P. In each case studied, this preliminary analysis allowed to confirm the presence of a paramagnetic nitroxide spin adduct in the organic phase. The samples were evaporated under reduced pressure to remove the solvent and dissolved in acidified methanol for MS analysis.

This approach was first applied to the DEPMPO–CH_3 nitroxide. The positive mode ES mass spectrum of the mixture predominantly showed the presence of the DEPMPO sodium adduct



Scheme 2. Fragmentation patterns of the protonated DEPMPO **1**.

Table 1
Hyperfine coupling constants of spin adducts **2a–c** determined by computer simulation of the EPR spectra recorded in organic solvent

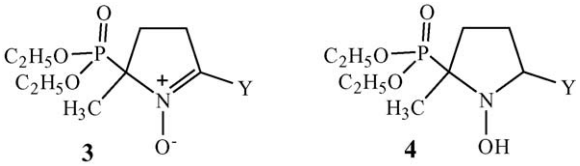
Nitroxides	a_N (G)	a_H (G)	a_P (G)	Solvent
2a: DEPMPO–CH_3	14.1	46.9	20.3	CH_2Cl_2
2b: DEPMPO–CH_2OH	14.3	49.1	20.1	CH_2Cl_2
2c: DEPMPO–OH	11.4	50.3	11.9	Et_2O

at *m/z* 258. The mass spectrum also showed three peaks of much lower abundance at *m/z* 272, 273 and 274. The peak observed at *m/z* 273 could correspond to the sodium adduct of the nitroxide **2a** obtained after trapping the methyl radical by DEPMPO, i.e., [DEPMPO–CH_3 + Na^+], while the two others could respectively be assigned, on the basis of their *m/z* value, to the sodium adducts of the nitrone **3a** and the hydroxylamine **4a**, formed by oxidation, reduction, and/or dismutation of **2a** (Scheme 3).

In order to confirm this hypothesis, attempts to elucidate the structure of these three ions were performed by MS/MS (Fig. 2). The presence of a peak at *m/z* 23 in the fragmentation spectrum of *m/z* 273 (Fig. 2b) confirmed the nature of the cation adducted to the molecule. By far, the most abundant fragment ion was observed at *m/z* 136. It would likely arise from the loss of the diethoxy(oxido)phosphoranyl radical, i.e., $^{\bullet}P(O)(OEt)_2$. The high abundance of this fragment ion indicates a very fast decomposition reaction, which is consistent with a radical-initiated rupture that would yield an even *m/z* value ion. This result would thus imply that the precursor ion at *m/z* 273 was a radical cation, thereby confirming that the molecule was originally a radical, as observed by EPR spectroscopy. The formation of other ions observed in the MS/MS spectrum could be rationalised from DEPMPO–CH_3 sodium adduct dissociation. However, their very low abundance indicates that the precursor ion decomposition proceeds almost exclusively via the loss of the fairly stable radical $^{\bullet}P(O)(OEt)_2$.

In contrast with *m/z* 273, the MS/MS spectrum resulting from *m/z* 272 dissociation mainly shows very low abundance peaks (Fig. 2a). Comparison of fragment ions obtained from *m/z* 272 and 273 precursor ions clearly indicates that the peak at *m/z* 273 arises from an ion with a different structure and cannot be considered only as a ^{13}C contribution in *m/z* 272 isotopic pattern. Fragmentation of *m/z* 272 could be explained from the structure of the nitrone **3a** sodium adduct.

The ion at *m/z* 274 exhibits a different decomposition spectrum from *m/z* 272 and 273 (Fig. 2c). The collision-induced



3a : Y = CH_3 **4a** : Y = CH_3
3b: Y = CH_2OH **4b**: Y = CH_2OH
3c: Y = OH **4c**: Y = OH

Scheme 3. Formulae of nitrones **3** and hydroxylamines **4** formed from nitroxides **2**.

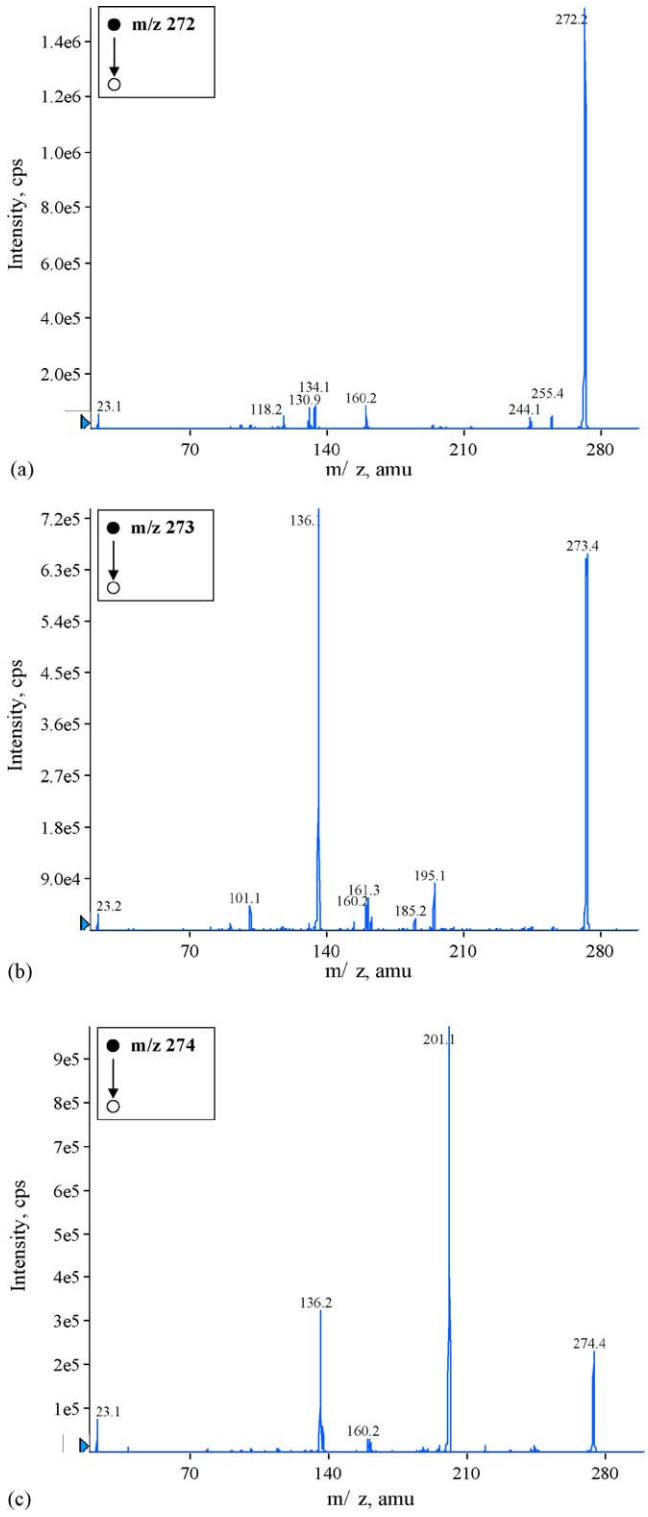
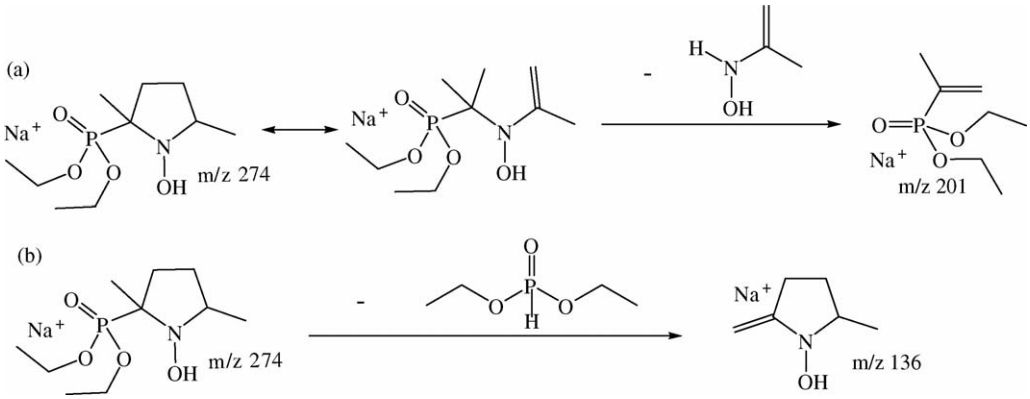


Fig. 2. ESI–MS/MS spectra of the sodium adducts of (a) nitrone **3a**, (b) nitroxide **2a** and (c) hydroxylamine **4a**.

dissociation of this sodium adduct (a fragment ion is detected at *m/z* 23) mainly gives rise to the formation of two fragment ions. The ion at *m/z* 201 would arise from the reaction depicted in Scheme 4a which implies the presence of a methyl group in β position towards a hydroxylamine. The ion at *m/z* 136 would result from the loss of a diethyl phospho-



Scheme 4. Fragmentation patterns of the sodiated **4a**.

nate molecule (Scheme 4b). Note that although the structure of *m/z* 272 and 273 should theoretically enable the fragmentation illustrated in Scheme 4b, this 138 u neutral loss is observed with much lower abundance from *m/z* 272 whereas it is not detected from *m/z* 273. In the latter case, this neutral loss could fairly be assumed to be completely hindered by the much faster diethoxy(oxido)phosphoranyl radical elimination.

Following an analogous procedure as for DEPMPO–CH_3, the spin adduct DEPMPO–CH_2OH **2b** was formed in water and the medium was analysed in positive mode electrospray mass spectrometry after extraction. The MS spectrum mainly revealed the presence of sodiated DEPMPO at *m/z* 258. As in the previous case, a low abundance peak was detected at the expected *m/z* 289 value for the nitroxide **2b** sodium adduct, together with small peaks at *m/z* 288 and 290, which were assumed to be sodium adducts of **3b** and **4b**, respectively. The *m/z* 289 ion has been submitted to fragmentation and the resulting MS/MS spectrum shows a dominant peak at *m/z* 152 (Fig. 3). This favoured 137 u loss from the precursor ion lead to conclude the *m/z* 289 ion is a radical cation able to lose $^{\bullet}P(O)(OEt)_2$ radical upon fragmentation. Another fragment ion is noticeable at *m/z* 258 as it would account for the loss of the hydroxymethyl radical (31 u) from the precursor ion, yielding the sodiated nitrone **1**. The low abundance of this peak in the MS/MS spectrum indicates this decomposition is less favoured than the *m/z* 289 → *m/z* 152 transition. Interestingly, DEPMPO–CH_3 sodium adduct was not shown to undergo such an alternative radical loss (i.e., loss of $^{\bullet}CH_3$) in the time-frame of the MS/MS experiment. This difference will be discussed later. Using notably the fragmentation pathway proposed for DEPMPO **1** (see Scheme 2), the other minor peaks in the MS/MS spectrum could be identified and confirmed the structure of the expected nitroxide **2b**. Finally, fragment ions observed in the MS/MS spectra of *m/z* 288 and 290 could be explained when considering these ions as the sodium adducts of **3b** and **4b**, respectively (data not shown). This last result again shows, as in the case of DEPMPO–CH_3, that the DEPMPO–CH_2OH spin adduct easily undergoes oxidation, reduction and/or dismutation reactions.

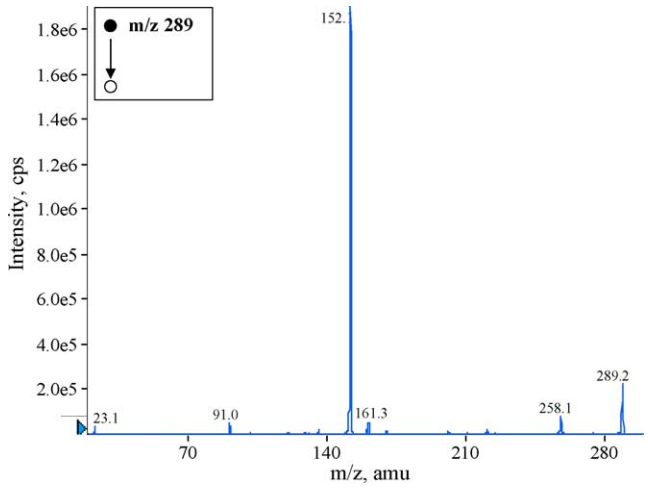
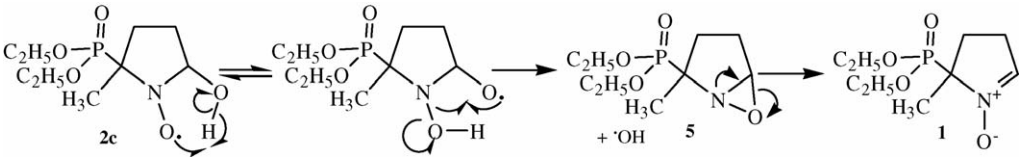


Fig. 3. ESI–MS/MS spectrum of the nitroxide **2b** sodium adduct.

MS analysis of the extracted mixture obtained after generating $^{\bullet}OH$ in the presence of DEPMPO showed, beside the predominant peak of [DEPMPO + Na^+] at *m/z* 258, a peak of low abundance at *m/z* 253 that could correspond to the expected protonated nitroxide, [DEPMPO–OH + H^+]. Note that, in contrast to the two previous cases, a protonated molecule was formed rather than a sodium adduct. In addition, the peaks observed at *m/z* 252 and 254 are very small, which would mean that the hydroxyl radical spin adduct would be essentially detected as the nitroxide **2c** rather than as oxidised and reduced forms **3c** and **4c**, respectively. Two major peaks were observed in the MS/MS spectrum of *m/z* 253 (Fig. 4). The peak at *m/z* 236 would illustrate a favoured loss of the hydroxyl radical, leading to the protonated nitrone **1**, which would further decompose to yield *m/z* 162, as described in Scheme 2. No peak was observed at *m/z* 116, indicating the loss of the diethoxy(oxido)phosphoranyl radical from the protonated DEPMPO–OH did not occur in the time-frame of the experiment.

The competing losses of $^{\bullet}P(O)(OEt)_2$ and $^{\bullet}Y$ during collision-induced dissociations of the three ionised spin adducts studied could not strictly be related to radical relative stabilities, as should be expected according to radical cation fragmentation rules [31]. When comparing the radicals in term of heat of formation in the gas phase, the following scale could be established: $^{\bullet}CH_3$ (145.7 kJ/mol) [32] < $^{\bullet}OH$ (39.0 kJ/mol) [32] < $^{\bullet}CH_2OH$ (−9 kJ/mol) [33] < $^{\bullet}P(O)(OEt)_2$. Interestingly, no referenced data could be found about the diethoxy(oxido)phosphoranyl radical. However, a low performance calculation (CS MOPAC,



Scheme 5. Proposed mechanism for •OH elimination as observed from the protonated nitroxide **2c**.

AM1) was performed and although the obtained absolute values are very approximate (ΔH_f° (•CH_3) = 130.6 kJ/mol, ΔH_f° (•OH) = 3.8 kJ/mol, ΔH_f° (•CH_2OH) = −111.0 kJ/mol, ΔH_f° (•$P(O)(OEt)_2$) = −684.5 kJ/mol), the relative stability scale could be validated. With the aim of better understanding the particular behaviour of **2c**, for which the loss of the hydroxyl radical is much more favoured than the loss of the diethoxy(oxido)phosphoranyl radical, we have looked more closely at the data available concerning the behaviour of this nitroxide in solution. In a recent study [34], Villamena et al. have shown that the nitroxide DEPMPO–OH decayed in aqueous media mainly by a first order process with a rate constant slightly lower than $10^{-4}\,s^{-1}$. During this reaction, •OH could be released, while the formation of nitroxides different than **2c** never occurred [8,9,12,34]. This more particularly demonstrates that the leaving of the •$P(O)(OEt)_2$ is not involved in DEPMPO–OH decay in water, otherwise this radical would have been trapped by DEPMPO and the resulting spin adduct would have been detected by EPR spectroscopy. On the other hand, the nitroxides **2a** and **b** shows half-life times higher than 6 h in neutral aqueous media and their decay mechanism proceeds almost exclusively by second order dismutation [8,12]. A likely hypothesis that could explain the unimolecular decomposition of DEPMPO–OH is given in Scheme 5. The formation of the stable oxazirane intermediate **5** [29] would thus explain •OH release. A similar mechanism might be envisaged in the gas phase to account for the faster release of •OH as compared to the loss of •$P(O)(OEt)_2$, in spite of the respective stabilities of the two radicals. In this case, the first step, i.e., hydrogen transfer, could be favoured by the existence of intra-molecular H-bonding [34]. All these results clearly show that **2c** behaves differently than the carbon-centred radical adducts **2a** and **b** not only in the gas phase as ionic species undergoing collision-induced dissociations but also in liquid phase as studied by EPR spectroscopy.

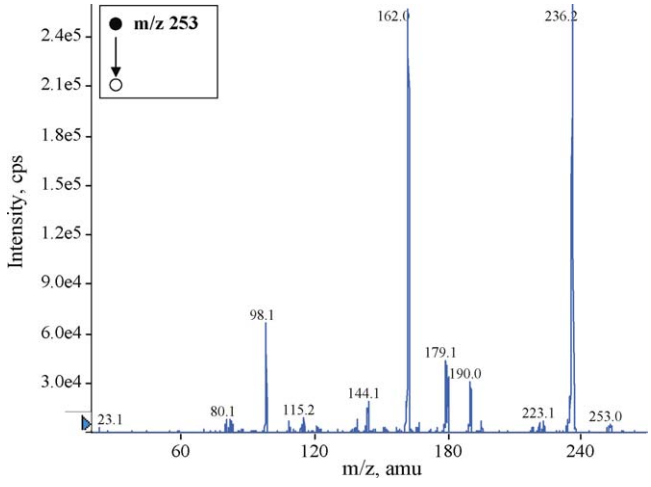


Fig. 4. ESI–MS/MS spectrum of the protonated nitroxide **2c**.

4. Conclusion

The experiments above demonstrate the feasibility of using tandem mass spectrometry for direct identification of nitroxide spin adducts from complex mixtures, without preliminary chromatographic separation. In this field, the spin trap DEPMPO shows major advantages over non-phosphorylated nitrones. Contrarily to nitrones commonly employed in spin-trapping/mass spectrometry approach, the use of DEPMPO allows the nitroxide form of the spin adducts to be systematically detected in mass spectrometry. This is particularly valuable as collision-induced dissociations of the electrosprayed ion were shown to proceed via a rapid radical loss which is diagnostic of the radical initially trapped. Carbon-centred radical spin adducts of DEPMPO show a unique fragmentation pathway via the loss of the diethoxy(oxido)phosphoranyl radical •$P(O)(OEt)_2$. This would permit unambiguous identification in case of an addend with a complex structure. In the case of the nitroxide formed after trapping •OH by DEPMPO, the fragmentation pathway mainly proceeds via the release of the hydroxyl radical, which was tentatively explained by a favourable intramolecular hydrogen transfer. In summary, this spin trapping/mass spectrometry technique using DEPMPO provides a powerful tool, complementary to the EPR detection, for the identification of short-lived free radicals in complex mixtures that could find interesting applications both in radical chemistry and biology.

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