Indirect detection of hydroxyl radicals using spin trapping and gas chromatography–mass spectrometry

Pritesh Mistry, Nigar Najim, Ashley Purdie and Ian Podmore*

**Biomedical Sciences Research Institute, School of Environmental and Life Sciences, University of Salford, Salford M5 4WT, UK*

In this study, hydroxyl radicals have been converted into methyl radicals by reaction with DMSO which, following capture by the spin-trap α-[4-pyridyl N-oxide]-*N*-*t*-butyl nitrone (POBN, 1), produced a novel dimethyl product (POBN-Me_2, 3) identified by GC–MS. Identification was confirmed when DMSO was replaced by a deuterated analogue (d_6-DMSO).

Keywords: Spin trapping, gas chromatography–mass spectrometry, α-[4-pyridyl *N*-oxide]-*N*-*t*-butyl nitrone, free radicals, Fenton reaction

The hydroxyl free radical (•OH) has the ability to react with all cellular biomolecules, including DNA, lipids and protein. It has been implicated in the pathogenesis of a number of disease states and, therefore, accurate measurement, both *in vitro* and *in vivo*, is vital to improve our understanding of mechanism and in evaluating the potential benefits of antioxidants, such as vitamins and phenolic compounds.[1] The principal method for detecting and measuring unstable free radicals is the electron paramagnetic resonance (EPR) spin trapping technique, in which radicals react with a nitrone to form a relatively more stable nitroxide radical (see Fig. 1). Unfortunately, the nitroxide formed from the addition of the hydroxyl radical still lacks stability, making measurements by EPR *in vitro* and, particularly, *in vivo* rather limited. Mass spectrometry (MS) coupled to a suitable chromatographic system (*e.g.* gas chromatography (GC)) offers an important alternative approach to measuring free radical spin adducts as it is possible to detect both paramagnetic and non-paramagnetic species.[2-6] Detection of •OH by spin trapping may be further improved by the use of dimethyl sulfoxide,[4] which reacts with •OH at near diffusion-controlled rates to generate, amongst other products, the methyl radical (reaction (2), Scheme 1), which in turn may then be trapped by a nitrone, *e.g.*, α-[4-pyridyl *N*-oxide]-*N*-*t*-butyl nitrone [POBN, (**1**)] (Fig. 1).

In our study, hydroxyl radicals were produced chemically using the "Fenton" reaction (reaction (1), Scheme 1). The reaction was performed at room temperature in aqueous 50 mM potassium phosphate buffer (pH 7.4). The reaction

$$Fe^{2+} + H_2O_2 \rightarrow {}^{-}OH + Fe^{3+} + {}^{\bullet}OH \quad (1)$$

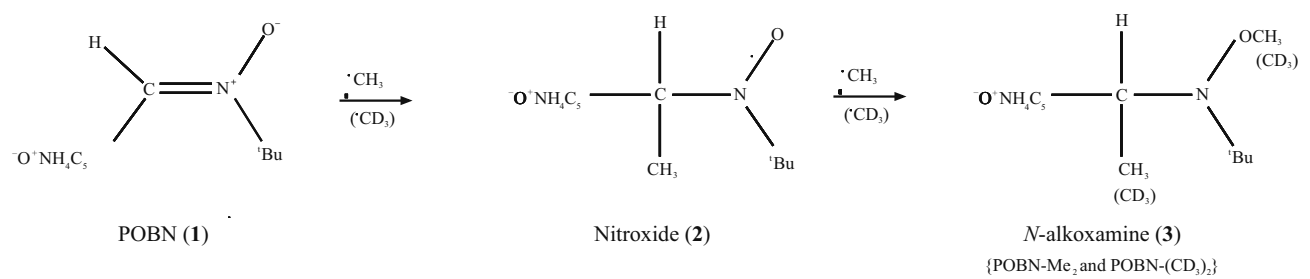
$$^{\bullet}OH + (CH_3)_2SO \rightarrow CH_3SOOH + {}^{\bullet}CH_3 \quad (2)$$

$$^{\bullet}OH + (CD_3)_2SO \rightarrow CH_3SOOH + {}^{\bullet}CD_3 \quad (3)$$

Scheme 1

mixture (10 ml) contained 10 mM DMSO, 1 mM EDTA, 1 mM ascorbate, 10 mM H_2O_2, 5 mM Fe^{2+}, and 5 mM POBN. The reaction was initiated by the addition of Fe^{2+} and left for 5 minutes. Ascorbate was added to the reaction to reduce Fe^{3+} back to Fe^{2+}. 1 ml of reaction mixture was then extracted with chloroform (1 ml) and 1 μl of the chloroform layer injected onto a GC column.

Figure 2 shows the GC chromatogram obtained following injection of the chloroform extract. A broad peak (12.5–16.0 minutes) is observed and has been identified as unreacted POBN. In addition, a major peak is seen at 14.9 minutes which is not found in a corresponding chromatogram when the reaction is carried out in the absence of Fe^{2+} (data not shown). The electron ionisation (EI, 70eV) mass spectrum (Figure 3b) shows peaks characteristic of a novel POBN dimethyl adduct (POBN-Me_2, **3**, Fig. 1) with a weak molecular ion for this compound at *m/z* 224 – an addition of 30 *m/z* to POBN. Peaks at *m/z* 208 $(M-16)^+$ and 193 $(M-31)^+$ correspond to loss of oxygen from the pyridyl-*N*-oxide group and a further loss of methyl (probably from the *t*-butyl group), respectively. The identity of **3** was confirmed when the reaction was carried using d_6-DMSO in place of DMSO. The resulting $^{\bullet}CD_3$ radicals (reaction (3), Scheme 1) were also found to add to POBN giving a dialkyl adduct (POBN–$(CD_3)_2$, Figs 1 and 3a). The molecular ion can now be seen at *m/z* 230 – an addition of 36 *m/z* to POBN. In Fig. 3a there are peaks at *m/z* 214, 199 and 158, all of which are 6 *m/z* higher than the corresponding peaks for POBN–Me_2 (Fig. 3b), confirming the replacement of six hydrogen atoms with six deuterium atoms. The use of deuterated DMSO assists in the identification of mass spectrometric fragments, *e.g.* a peak at *m/z* 122 (Fig. 3b) is shifted to *m/z* 125 (Fig. 3a). Clearly, a fragment containing one of the original methyl groups that added to POBN has been lost – probably a *t*-$BuNOCH_3$ (*t*-$BuNOCD_3$) radical. A further loss of oxygen (from the pyridyl-*N*-oxide), either prior to or after formation of this fragment, gives rise to the peak at *m/z* 106 (Fig. 3b) or *m/z* 109 (Fig. 3a). Ions at



POBN (**1**) Nitroxide (**2**) *N*-alkoxamine (**3**)
{POBN-Me_2 and POBN-$(CD_3)_2$}

Fig. 1 The addition of a methyl (or $^{\bullet}CD_3$) radical to the spin trap POBN (**1**) to give the paramagnetic nitroxide (**2**). Further addition of a methyl (or $^{\bullet}CD_3$) radical yields the novel *N*-alkoxamine (**3**) (POBN-Me_2 or POBN-$(CD_3)_2$).

* Correspondent. E-mail: i.podmore@salford.ac.uk.

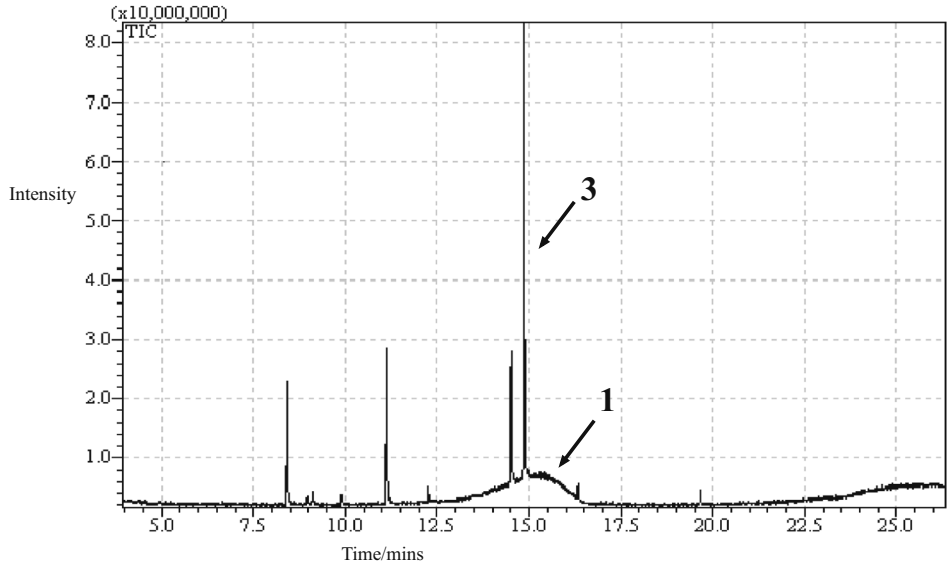


Fig. 2 Gas chromatogram showing a major peak at 14.9 minutes corresponding to a novel dimethyl adduct POBN-Me_2 (3). GC conditions were as follows: GC column stationary phase was essentially non-polar consisting of 95% polydimethylsiloxane with 5% phenyl content (0.25 μm thickness), the column was 30 m in length and 0.25 mm internal diameter. GC-MS analyses were carried out on a Varian CP3800 gas chromatograph interfaced to a 1200 triple quadrupole mass spectrometer. Helium was used as a carrier gas at a flow rate of 1 ml min^{-1}. The GC injector port and transfer line temperature were set at 250°C. Column oven temperature was set at 120°C for 2 minutes and then increased at a rate of 20°C min^{-1} up to 300°C, where it remained for 3 minutes.

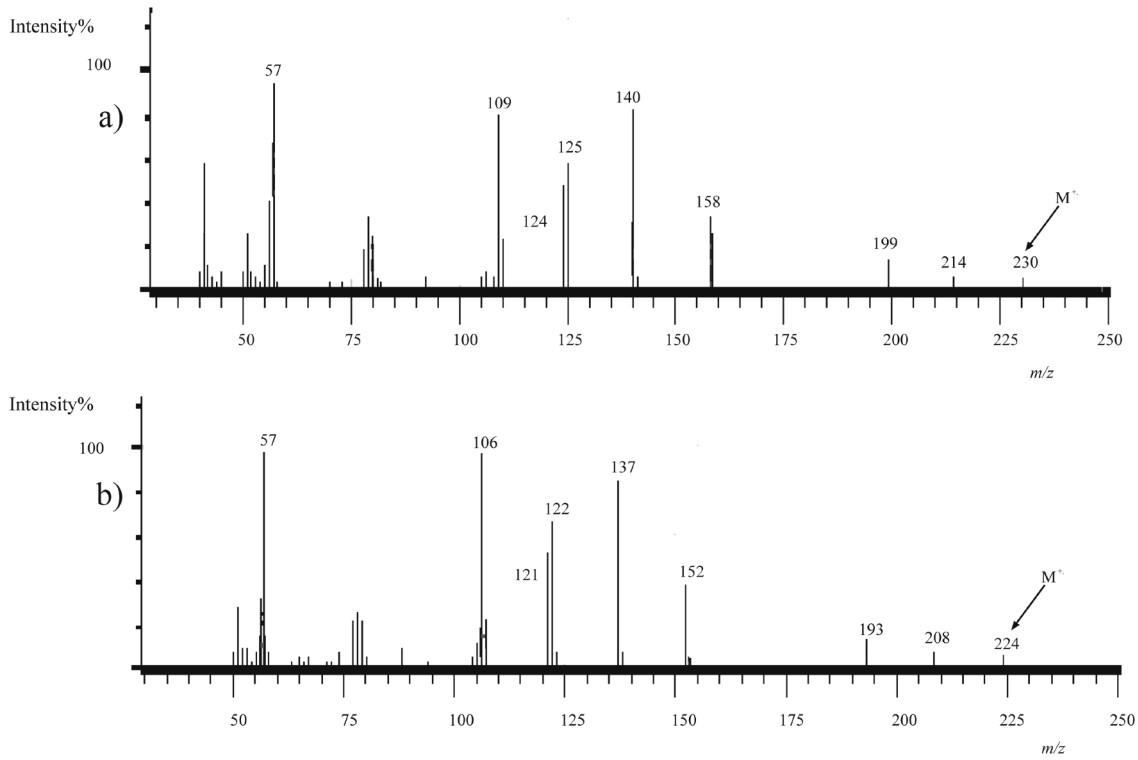


Fig. 3 Electron ionisation (EI) mass spectra corresponding to **3a** POBN-$(CD_3)_2$ and **3b** POBN-Me_2.

m/z 137/140 must also derive from loss of a fragment containing one of the added methyl/CD_3 groups – indeed these may have been formed from direct loss of CH_3 (CD_3) from the fragments at *m/z* 152 (158). The peak at *m/z* 57 is found in both mass spectra and may be attributed to the *tert*-butyl cation ($^+CMe_3$).

To the best of our knowledge, the 1,3-addition of methyl ($^\bullet CD_3$) radicals to POBN has not been reported before. However, Janzen et *al.*[7] and Iwamura and Inamoto[8] have previously observed similar adducts to POBN when trapping radicals from the thermolysis of azo compounds. Also, a recent study using HPLC–EPR/MS identified many of the species produced in a "Fenton" reaction containing DMSO.[4] The authors did not, however, report the detection of POBN-Me_2. Therefore, in conclusion, we have identified a novel product of methyl radical addition to the spin trap α-[4-pyridyl *N*-oxide]-*N*-*t*-butyl nitrone using GC–MS which, since cells are able to tolerate small amounts of DMSO, may prove a useful biomarker of hydroxyl radical production, particularly *in vitro*. This will be the subject of further investigation.

Acknowledgements

The authors wish to thank the University of Salford (Research Investment Fund) for financial support.

Received 3 April 2008; accepted 30 May 2008
Paper 08/5200 doi: 10.3184/030823408X333427

References

1 B. Halliwell and M. Whiteman, *Br. J. Pharmacol.*, 2004, **142**, 231.
2 H. Iwahashi, C.E. Parker, R.P. Mason and K.B. Tomer, *Analyt. Chem.*, 1992, **64**, 2244.
3 I.D. Podmore, *J. Chem. Res. (S)*, 2002, 574.
4 S.Y. Qian, M.B. Kadiiska, Q. Guo and R.P. Mason, *Free Radi. Biol. Med.*, 2005, **38**, 125.
5 H. Suezawa, K. Abe, M. Hirota and T. Ishii, *Chem. Lett.*, 1981, 1049.
6 H. Sang, E.G. Janzen, C.M. DuBose, E.J. Geels and J.L. Poyer, *J. Chem. Soc., Perkin Trans. 2*, 1996, 1985.
7 E.G. Janzen, P.H. Krygsman, D.A. Lindsay and D.L. Haire, *J. Am. Chem. Soc.*, 1990, **112**, 8279.
8 M. Iwamura and N. Inamoto, *Bull. Chem. Soc. Jpn*, 1970, **43**, 856.