Detection of ethanal-derived free radicals by spin-trapping and headspace thermal desorption gas chromatography-mass spectrometry (TD-GC-MS)

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Detection and identification of ethanal-derived spin-trapped free radicals using headspace thermal desorption gas chromatography-mass spectrometry (TD-GC-MS)

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ABSTRACT

In this study, we demonstrate a novel approach to the detection and identification of the products of spin-trapped free radicals. Hydroxyl free radicals were generated by Fenton-based chemistry in the presence of ethanal and the spin-trapping agent *N*-*tert*-butyl-α-phenylnitrone (PBN). The resulting volatile compounds present in the reaction vial headspace were collected using thermal desorption (TD) and analysed by gas chromatography-mass spectrometry (GC-MS). Eleven compounds were detected in the headspace, and their identification was aided by using either a fluorinated or deuterated analogue of PBN as an alternative spin trap and/or deuterated ethanal (CD_3CHO) as the secondary source of free radicals. The electron-ionisation (EI) mass spectra clearly demonstrate the "capture" of methyl radicals; two of the compounds detected were identified as containing one methyl group derived from ethanal, and four were shown to contain two methyl groups. This study demonstrates that sampling the reaction headspace using TD-GC-MS is a viable method for analysing products of free radical trapping, and potentially may be applied to a wide range of free radical systems.

INTRODUCTION

Many free radicals are highly reactive and require the technique of spin-trapping for their detection, which typically uses a nitrone or nitroso compound as the trapping agent and often results in the formation of a nitroxide (Marchand *et al.* 2017). The main technique for observing these nitroxides is Electron Paramagnetic Resonance (EPR) spectroscopy, however, the use of mass spectrometry-based techniques, coupled to a suitable form of chromatography, provides an alternative way of identifying the products of spin-trapped radicals and thus the radical itself (for examples, see: Qian *et al.* 2005; Janzen *et al.* 1990; Parker, Iwahashi & Tomer 1991; Zhang, Wang & Guo 2006; Mistry *et al.* 2008).

Thermal desorption (TD) is a well-known technique for the sampling of volatile organic compounds (VOCs) (Forbes, Staymates & Sisco 2017; Huilian *et al.* 2017). The TD unit includes a trap (sorbent tube) used to collect the VOCs, which is then heated for the release of adsorbed compounds followed by analysis typically using GC-MS. Sampling in the vial headspace with this solvent free approach can be done in minutes or even in seconds and provides separation of the volatile species from the non-volatile solvent and its ionic/polar components (Callan, Walsh & Dowding 1993). TD offers therefore, an excellent opportunity to detect and identify the volatile products of spin-trapped free radicals.

Hydroxyl free radicals (·OH) may be generated chemically by the well-known Fenton reaction. They have been shown to react rapidly with the aldehyde ethanal to produce acetyl radicals, with a rate constant (k) for the reaction of 3.6×10^{9} M^{-1} s^{-1}, and methyl radicals, the latter by decarbonylation of the acetyl radical (**scheme 1**) (Nakao *et al.* 2000). In this reaction, however, methyl radicals may also be generated by a non-Fenton pathway, with direct nucleophilic

addition of a peroxy anion ($^{-}$OOH) to ethanal and subsequent reduction by Fe^{2+} (**scheme 2**; Nakao, Ouchi & Augusto 1999). Previous EPR studies have demonstrated that all three radicals (methyl, acetyl, and hydroxyl) may be detected when using different spin traps (Nakao, Ouchi & Augusto 1999; Nakao *et al.* 2000). In addition, a study by Jenkins *et al.* (1997) using UV-irradiation of ethanal solutions also identified the presence of acetoxyl ($CH_3CO_2^{\bullet}$) radicals, along with those of acetyl radicals.

In the current study, a Fenton-based system in the presence of ethanal has been used to generate free radicals, which have then been trapped by either N-*tert*-butyl-α-phenylnitrone (PBN) or a derivative (figure 1). The resulting products have been collected from the headspace of the reaction vial by thermal desorption and analysed by gas chromatography-mass spectrometry (TD-GC-MS). The main purpose of the study is to develop a quick and simple method of identifying free radicals by detecting the volatile products of free radical spin-trapping.

MATERIALS AND METHODS

Ethylene diaminetetraacetic acid (EDTA), L-ascorbic acid, di-potassium hydrogen phosphate (K_2HPO_4), and N-*tert*-butyl-α-phenylnitrone (PBN) were all purchased from Sigma-Aldrich (Suffolk, UK). Ethanal-d_3 was obtained from CDN Isotopes (Dunmow, UK). Ammonium ferrous sulfate hexahydrate $\{Fe(NH_4)_2(SO_4)_2.6H_2O\}$ was purchased from Fluka Biochemika (Loughborough, UK). Ethanal and hydrogen peroxide (30% w/v) were obtained from Alfa Aesar (Lancashire, UK). Derivatives of PBN were synthesised in our laboratory using the method of Hinton & Janzen (1992) and further purified by sublimation.

Generation and trapping of ethanal derived radicals

A standard method reported previously (Mistry *et al.* 2008; Podmore, Cunliffe & Heshmati 2013) was used for the generation and trapping of free radicals. PBN was utilised as the main spin trapping agent and replaced by one of its derivatives (figure 1) for confirmation of the identity of the products. The Fenton-based reaction (total volume approximately 10 cm^3, containing: 1 cm^3 hydrogen peroxide {3% w/v}, 1 cm^3 ascorbic acid {0.1 moles L^{-1}}, 1 cm^3 EDTA {0.01 moles L^{-1}}, 5 cm^3 potassium phosphate buffer {0.1 moles L^{-1}} 1 cm^3 PBN or derivative {0.1 moles L^{-1}}, 1 cm^3 Fe^{2+} salt {0.01 moles L^{-1}} and 0.132 cm^3 ethanal (or d_3-ethanal) {0.24 moles L^{-1}}) was carried out at pH 7.4 in a 25 cm^3 beaker. The reaction was initiated by adding the Fe^{2+} salt as the final component and the mixture left for 5 minutes. The total reaction mixture was then transferred to a 40 cm^3 vial which was set aside for 3 minutes to allow headspace saturation.

Headspace analysis

An Easy-VOC pump (Markes International, Pontyclun-UK) was used to extract the volatile organic compounds (VOCs), including the volatile products of spin-trapping, from the headspace. SVI™ sorbent tubes (Perkin Elmer, Llantrisant, UK) were used for sampling and were conditioned at 350 °C before the analysis. The collected headspace volume was set at 50 cm^3 and the pump released after 30 seconds. A Turbomatrix 300 thermal desorber (Perkin Elmer, UK) was used to extract the adsorbed VOCs from the sorbent tube. The sorbent tube was purged for 5 minutes using oxygen-free nitrogen gas (BOC, UK). Primary desorption was conducted at 330 °C for 5 minutes using helium as a carrier gas. Separation of the extracted VOCs was carried out using a Clarus 5800 gas chromatograph (Perkin Elmer, UK) equipped with a Restek capillary column with mainly polydimethylsiloxane as the stationary phase (Rtx-5; non-polar). The capillary column was 30 meters in length with a diameter of 0.25 mm and

the film coating thickness of the stationary phase was 0.25 µm. The initial column temperature was set at 100 °C for 5 minutes and was programmed to increase at 5 °C/minute to 150 °C, and finally to increase to 300 °C at 40 °C/minute where it was held for 2 minutes. The mass spectrometer used to detect the eluted compounds consisted of an electron-ionisation (EI) source and single quadrupole mass analyser (Clarus 800MS from Perkin Elmer, UK). Total ion chromatograms (TICs) were obtained by recording EI mass spectra from a scan range of 45-500 m/z.

RESULTS

Figure 2 shows the TIC obtained from TD-GC-MS headspace analysis of the Fenton-based reaction mixture containing ethanal as a secondary source of free radicals and PBN as the spin-trapping agent. The following compounds have been assigned to the peaks in the chromatogram, as follows: peak 1 - either N-*tert*-butyl-N-methyl-hydroxylamine (**1a**) or a methyl adduct of *tert*-butylhydroaminoxyl (**1b**); peak 2 - paraldehyde (**2**); peak 3 - either di-*tert*-butyl hydroxylamine (**3a**) or a *tert*-butyl radical adduct of *tert*-butylhydroaminoxyl (**3b**); peak 4 - phenyl methanimine (**4**); peak 5 - benzaldehyde (**5**); peak 6 - N-methoxy-1-phenylethanimine (**6**); peak 7 - methoxy-(1-phenylethylidene)-amine (**7**); peak 8 - the hydroxylamine of a PBN-methyl-adduct (HO-PBN-CH_3) (**8**); peak 9 - a dimethyl adduct of PBN (PBN-Me_2) **(9**); peak 10 - 2,3-diphenylbutane isomers (**10**). For each compound structure see figure 3. The compounds have been assigned by interpretation of the electron-ionisation (EI) mass spectra when using either PBN or one of its derivatives as the trapping agent and using ethanal or d_3-ethanal (CD_3CHO) as the secondary source of free radicals (e.g. figure 4 shows the EI-mass spectrum of peak 6 from the chromatogram in figure 2). The peak observed at 1.53 minutes is detected in the vial headspace of a control sample (data not shown), which

contains no Fenton reactants. It may be attributed to dimethylsiloxane, of which the source may either be the GC column stationary phase or the thermal desorption 'trapping' material .

Table 1 shows the m/z values of key ions, including the molecular ion ($M^{+\cdot}$), found in the EI-mass spectrum of each compound observed in the chromatogram (figure 2). The peak labelled 1 in figure 2 corresponds to either N-*tert*-butyl-N-methyl-hydroxylamine (^{t}Bu-NMeOH) (**1a**) or a methyl radical adduct of *tert*-butylhydroaminoxyl (MeONH^{t}Bu) **(1b**). The peak at m/z 57 confirms the presence of a tert-butyl group. The base peak (m/z 88) is generated in the ion source of the mass spectrometer by loss of a methyl radical from the *tert*-butyl group of the molecular ion (m/z 103), which is confirmed by experiments when replacing ethanal with d_3-ethanal (CD_3CHO) as a secondary free radical source. It is possible that both **1a** and **1b** are derived from a breakdown product of PBN. Previous studies have suggested that the hydrolysis of PBN gives rise to the formation of 2-methyl-2-nitrosopropane (MNP) and benzaldehyde (Atamna, Paler-Martinez & Ames 2000; Turnbull et *al*. 2001). Also, hydroxyl radical addition to the carbon of the C=N bond of PBN is known to form an unstable nitroxide, which may then dissociate to benzaldehyde and the *tert*-butyl-hydroaminoxyl (TBHA) radical (**scheme 3;** Kotake & Janzen, 1991; Jerzykiewics et *al*. 2011). Since MNP is a known spin trap for methyl radicals (Rosenthal, Mossoba & Riesz, 1981) it may ultimately lead to the formation of **1a**. As a nitroxide, TBHA has the potential to trap carbon-centred radicals such as $\cdot CH_3$ (Wright & English 2003) and thus lead to the formation of **1b**. It is not clear from the EI mass spectrum which compound, **1a** or **1b**, is responsible for peak 1, and neither MNP nor TBHA are observed in the chromatogram, however, when ethanal is replaced in the reaction mixture by the deuterated analogue (CD_3CHO), the *m/z* value of the molecular ion increases by 3 units to 106, thus confirming the presence of an ethanal-derived methyl group in the structure (table 1). It is possible, therefore, that a methyl radical (formed in the Fenton-based mixture from the reaction

between ·OH and ethanal; **scheme 1**) adds to either MNP or TBHA (**scheme 4**); if to the former, then the resulting nitroxide may be reduced in the presence of ascorbate to form **1a** (**scheme 4a**).

Peak 2 in figure 2 (retention time 1.8 minutes) corresponds to paraldehyde (compound **2**). The EI mass spectrum shows a molecular ion at m/z 132, which changes to m/z 141 when ethanal is replaced in the reaction mixture by CD_3CHO (table 1). This compound is oberved in the absence from the reaction mixture of either Fe^{2+}, hydrogen peroxide or the spin trap (data not shown) and is, therefore, not derived from Fenton-based chemistry. It is formed by self-aldol condensation of ethanal molecules (Georgieff, 1966; Hill, Miessner & Öhlmann,1989).

Peak 3 in the chromatogram mostly likely corresponds to either di-*tert*-butylhydroxylamine (**3a**) or a *tert*-butyl adduct of TBHA (^{t}BuONH^{t}Bu; **3b**). The peak in the EI-mass spectrum at m/z 57 confirms the presence of a *tert*-butyl group. The molecular ion and base peak have m/z values of 145 and 74, respectively. **3a** may possibly be formed by the trapping of a *tert*-butyl radical by MNP (as mentioned earlier, a potential breakdown product of PBN) to produce the di-*tert*-butylnitroxide radical (not observed in the chromatogram shown in figure 2) which subsequently reduces in the presence of ascorbate to di-*tert* butylhydroxylamine. Formation of the di-*tert*-butylnitroxide radical has been observed in previous studies, where the source of the *tert*-butyl radical has been attributed to MNP itself (Turnbull et *al*. 2001; Jerzykiewics et *al*. 2011). However, in this study, replacing ethanal in the reaction mixture with CD_3CHO causes a shift in the m/z value of the molecular ion to 154, indicating the incorporation of 9 ethanal-derived deuterium atoms (possibly as a deuteron-*tert*-butyl radical) into compound **3** (table 1). It is also possible that peak 3 may instead correspond to **3b**, which could be generated by the

trapping of a *tert*-butyl (or d_9-*tert*-butyl radical) radical by TBHA. From the EI-mass spectra, it is not possible to determine which of the compounds, **3a** or **3b**, is responsible for peak 3.

Peaks 4 and 5 in figure 2 at 2.33 and 2.61 minutes, respectively, correspond to phenyl methanimine (**4**) and benzaldehyde (**5**). The EI mass spectrum of **4** gives a moleular ion at m/z 105, which changes to m/z 111 and m/z 123, respectively, when PBN is replaced by either d_6-PBN and F-PBN in the reaction mixture (table 1). These changes may be accounted for by the differences in the masses of the spin traps and thus demonstrate that **4** does not contain any ethanal-derived methyl hydrogen atoms. Benzaldehyde is most likely formed from the addition of a hydroxyl radical to the carbon of the C=N bond in PBN and subsequent breaking of the single bond between the carbon and nitrogen (**scheme 3**). This is supported by the corresponding EI-mass spectra when PBN is replaced by either F-PBN or d6-PBN in the reaction **mixture (see table 1).**

The EI mass spectrum of peak 6 shown in figure 4 corresponds to N-methoxy-1-phenylethanamine (**6**). No peak is observed at m/z 57 confirming the absence of a *tert*-butyl group. The molecular ion ($M^{+\cdot}$) has an m/z value of 151. The fragment at *m/z* 136 is formed in the ion source of the mass spectrometer by the loss of a methyl radical from the molecular ion. Dissociation of the bond between the carbon and nitrogen in $M^{+\cdot}$ gives the base peak at *m/z* 105. Further evidence confirming the identity of **6** is obtained when either using a different PBN derivative as the spin-trap or replacing ethanal with d_3-ethanal in the reaction mixture. When PBN is replaced by F-PBN or by d_6-PBN, the molecular ion m/z values increase by 18 and 6 units, respectively. When ethanal is replaced by CD_3CHO, then all molecular ions

increase by 6 m/z units (table 1) indicating the incorporation of two deuteron-methyl groups into the structure.

Peak 7 in figure 2 corresponds to methoxy-(1-phenylethylidene)amine (**7**). The absence of the peak at m/z 57 suggests that there is no *tert*-butyl group in the structure, and the base peak at m/z 77 (shifting to m/z 82 when d_6-PBN, rather than PBN, is used as the spin trap) demonstrates the presence of a phenyl group derived from PBN. The molecular ion has an m/z value of 149. Loss of the methoxy group from molecular ion in the ion source of the mass spectrometer gives a peak at *m/z* 118. Once again, further evidence confirming the identity of the compound is obtained when either using a different PBN derivative as the spin-trap or replacing ethanal with d_3-ethanal in the reaction mixture. When PBN is replaced by F-PBN or by d_6-PBN, the molecular ion m/z values increase by 18 and 5 units, respectively; the latter increase demonstrates that the deuterium atom on the carbon of C=N has been lost on forming **7**. When ethanal is replaced by CD_3CHO, then all molecular ions increase by 6 m/z units (table 1) indicating the incorporation of two deuteron-methyl groups into the structure. Compound **7** is most likely generated from compound **6** by loss of the two hydrogen atoms, one attached to the carbon and the other to the nitrogen of C=N; this process may either occur in the Fenton-based reaction mixture or possibly during the desorption process.

Peak 8 (figure 2) is most likely a hydroxylamine formed from the mono-methyl radical adduct to PBN (**8**; HO-PBN-CH_3). The peak at m/z 57 demonstrates the presence of a *tert*-butyl group. The molecular ion for the compound can be seen at *m/z* 193. The fragment at *m/z* 178 is formed in the ion source of the mass spectrometer by the loss of a methyl radical from the *tert*-butyl group of the molecular ion, whilst the loss of 2-methyl-1-propene (from $M^{+\cdot}$) gives a peak at

m/z 137. The base peak at *m/z* 91 is a tropylium ion formed by the rearrangement of a benzyl cation. Replacing PBN with either d_6-PBN or F-PBN in the reaction mixture results in an increase in the m/z values of both the molecular ion and base peak by 6 and 18 units, respectively (table 1). Using d_3-ethanal as the secondary source of free radicals in the reaction mixture increases the m/z values of all the molecular ions by 3 units, clearly demonstrating the incorporation of a single deuteron-methyl group into the structure. It is assumed that compound 8 is formed following addition of a methyl radical to the carbon of the C=N site in PBN to form the nitroxide, with subsequent reduction by ascorbate to the hydroxylamine (**scheme 5**). However, nitroxides may also be generated by so-called inverted spin-trapping, which involves oxidation of the spin-trap followed by nucleophilic attack, or the "Forrester-Hepburn mechanism" involving nucleophilic attack of the spin trap to generate the hydroxylamine followed by reduction (Leinisch *et al.* 2011). There is no evidence, either in this study or from previous studies, to support a route other than genuine spin-trapping, but the possibility cannot be discounted. The nitroxide (mono-methyl spin adduct) itself is not observed in the chromatogram in figure 2 but has been seen in many previous studies by EPR spectroscopy (for example: Jerzykiewicz *et al*. 2011; Jenkins *et al*. 1997).

The most intense peak in the chromatogram shown in figure 2 corresponds to a di-methyl-adduct of PBN (PBN-Me_2) (**9**). In a previous study, this compound was synthesised using a Grignard reagent and analysed using GC with EI mass spectrometry (Janzen *et al*. 1985). The EI-mass spectrum observed for compound **9** in the present study contains exactly the same spectral pattern as that shown by Janzen et *al*. (1985): the molecular ion peak is at *m/z* 207; the fragment at *m/z* 192 is formed in the ion source of the mass spectrometer by the loss of a methyl radical from the *tert*-butyl group of the molecular ion; the loss of 2-methyl-1-propene from the molecular ion gives the peak at *m/z* 151; and, in addition, the breaking of the bond between the

carbon and nitrogen in the molecular ion generates the base peak at *m/z* 105. Further evidence confirming the identity of **9** is obtained in this study by either using a different PBN derivative as the spin-trap or by replacing ethanal with d_3-ethanal in the reaction mixture. When PBN is replaced by F-PBN or by d_6-PBN, the molecular ion m/z values increase by 18 and 6 units, respectively (table 1). When ethanal is replaced by CD_3CHO, all the molecular ion m/z values increase by a further 6 units (table 1) indicating the incorporation of two deuteron-methyl groups into the structure.

The two peaks observed at 15.96 & 16.25 minutes in the chromatogram in figure 2 correspond to isomers of 2,3-diphenylbutane (**10**). There is a weak molecular ion at m/z 210. Breaking of a C-C bond in the molecular ion, which occurs in the ion source of the mass spectrometer, gives the base peak at *m/z* 105. When PBN is replaced by either F-PBN or d_6-PBN, the molecular ion m/z values increase, respectively, by 36 and 12 units and the base peak values increase by 18 and 6 units (table 1). This confirms the presence of two phenyl-CH groups in the structure of compound **10**. Furthermore, replacing ethanal with the deuterium analogue, gives rise to an increase in the m/z values of all the molecular ions of 6 units (table 1). This clearly indicates the incorporation of 6 deuterium atoms and thus two deuteron-methyl groups into the structure. The mechanism for the formation of **10** is not clear. The structure suggests the co-addition of two phenyl-C·(H)CH_3 radicals, which are possibly derived from the nitroxide intermediate (shown in scheme **5**) or the hydroxylamine (**8**). It is also not clear whether this takes place in the reaction mixture or during the thermal desorption process.

DISCUSSION

As expected, the results demonstrate that hydroxyl and methyl radicals are formed during the Fenton reaction in the presence of ethanal. Products of the reaction of ·OH with PBN include benzaldehyde (**5**) which has been identified directly in the headspace. Although not observed directly, MNP (and/or TBHA) may also be formed by this reaction (**scheme 3**), as suggested by the presence of compounds **1a/1b** and **3a/3b**. Previous studies using EPR spectroscopy that have followed the fate of the nitroxide radical formed when ·OH has attached to the carbon of the C=N in PBN, **have suggested that benzaldehyde**, MNP and di-*tert*-butyl nitroxide are formed as products (Kotake & Janzen, 1991; Atamna *et al.* 2000; Turnbull *et al.* 2001). Reinke *et al.* (2000) also demonstrated that the hydroxyl radical may add at different positions to the phenyl ring of PBN to give hydroxylated PBN isomers. In the current study however, these isomers were not observed and it is likely that, as with PBN itself, they are present in the reaction mixture but insufficiently volatile to enter the vial headspace, and thus not detected by TD-GC-MS.

The current study demonstrates the trapping of methyl radicals, either directly by PBN or indirectly by the dissociation products of PBN. Products of the direct addition of $\cdot CH_3$ to PBN include compounds **8** and **9**. Also, **6** and **7** are likely to be products of methyl radical addition to PBN, although it is not clear at what stage in the process PBN dissociates (prior to or post trapping of the radical). Interestingly, the nitroxide resulting from a single methyl radical adding to the carbon of the C=N of PBN (scheme **5**), which is detected by EPR spectroscopy, is not observed here. This is in accord with our previous observations using POBN as the spin trap and DMSO as the source of methyl radicals (Mistry *et al*. 2008; Podmore *et al*. 2013). There are several possible explanations for this, including: lack of volatility of the nitroxide; the nitroxide being reduced in the presence of ascorbate to form **8**; or, addition of a second methyl radical to the nitroxide oxygen to form the dimethyl adduct (**9**). Boyd & Boyd (1994)

carried out a computational study which demonstrated that the formation of di-adducts of PBN was energetically favoured over simply the formation of monoadducts. Their calculations showed that initial radical addition to the carbon site of the C=N bond is most favoured, followed by secondary addition to the nitroxide oxygen. Compounds **6**, **7** and **9** are all dimethyl adducts but as mentioned previously, it is not clear whether **6** and **7** are formed from the decomposition of **9**. It is also not clear whether such formation has occurred in the reaction mixture or subsequent sampling and analysis by TD-GC-MS. Compounds **1a/1b**, **8** and **10** also provide further evidence for the trapping of methyl radicals.

The compound responsible for peak 3 (figure 2) cannot be unequivocally identified from its EI-mass spectrum, nonetheless, the presence of a *tert*-butyl group in the structure is evident. Experiments using both CD_3CHO and/or d_6-PBN (or F-PBN) as alternatives to CH_3CHO and PBN, respectively, in the reaction mixture confirm the incorporation of a *tert*-butyl group and identify ethanal as its source; in all cases where CD_3CHO was used, an increase of 9 m/z units was observed in the mass spectrum (table 1). The mechanism for this process is not yet known and is the subject of further investigation.

In this work, Fenton-based chemistry has been used to generate ethanal-derived free radicals which may be trapped by PBN and their volatile products extracted from the headspace using the solvent-free extraction approach of thermal desorption (TD). GC-MS, along with the use of PBN derivatives as alternative spin traps and a stable isotope-labelled compound as a secondary source of free radicals, has allowed identification of the extracted compounds. This novel approach to free radical detection potentially offers the opportunity to develop biomarkers or "fingerprints" of free radical production in many chemical or biological systems.

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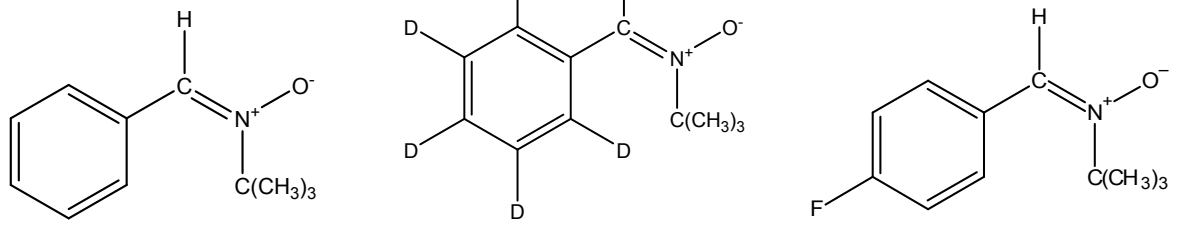
Table 1: Molecular ion ($M^{+\cdot}$) m/z values for compounds detected by headspace TD-GC-MS analysis of the Fenton-based mixture containing spin-trap and aldehyde (see materials and methods for experimental details).

Compound number	Spin-trap and aldehyde included with the Fenton system	Compound name or formula	Molecular ion ($M^{+\cdot}$) m/z value	Significant ions m/z values[a]
1a or 1b	PBN + CH_3CHO	^{t}Bu-NMeOH or MeONH^{t}Bu	103	88, 57
	PBN + CD_3CHO	t-Bu-NCD_3OH or D_3CONH^{t}Bu	106	91, 57
	d_6-PBN + CH_3CHO	^{t}Bu-NMeOH or MeONH^{t}Bu	103	88, 57
	d_6-PBN + CD_3CHO	t-Bu-NCD_3OH or D_3CONH^{t}Bu	106	91, 57
	F-PBN + CH_3CHO	^{t}Bu-NMeOH or MeONH^{t}Bu	103	88, 57
2	PBN + CH_3CHO	Paraldehyde	132	131, 117, 45
	PBN + CD_3CHO	d_9-Paraldehyde	141	140, 123, 48
3a or 3b	PBN + CH_3CHO	di-*tert*-butylhydroxylamine or ^{t}BuONH^{t}Bu	145	130, 74, 57
	PBN + CD_3CHO	di-*tert*-butyl (d_9) hydroxylamine or $(CD_3)_3$ONH^{t}Bu	154	139, 80, 57
	d_6-PBN + CH_3CHO	di-*tert*-butylhydroxylamine or ^{t}BuONH^{t}Bu)	145	130, 74, 57
	d_6-PBN + CD_3CHO	di-*tert*-butyl (d_9) hydroxylamine or $(CD_3)_3$ONH^{t}Bu	154	139, 80, 57
	F-PBN + CH_3CHO	di-*tert*-butylhydroxylamine or ^{t}BuONH^{t}Bu	145	130, 74, 57
4	PBN + CH_3CHO	Phenyl methanimine	105	104, 78
	PBN + CD_3CHO	Phenyl methanimine	105	104, 78
	d_6-PBN + CH_3CHO	d_6- Phenyl methanimine	111	110, 84
	F-PBN + CH_3CHO	F-Phenyl methanimine	123	122, 96
5	PBN+ CH_3CHO	Benzaldehyde	106	105, 77
	d_6-PBN+ CH_3CHO	d_6-Benzaldehyde	112	111, 83
	F-PBN+ CH_3CHO	F-Benzaldehyde	124	123, 95
6	PBN + CH_3CHO	(PBN-56)-Me_2	151	136, 119, 105
	PBN + CD_3CHO	(PBN-56)-$(CD_3)_2$	157	139, 122, 108
	d_6PBN + CH_3CHO	(d_6PBN-56)-Me_2	157	142, 125, 111
	d_6PBN + CD_3CHO	(d_6PBN-56)-$(CD_3)_2$	163	145, 128, 114
	FPBN + CH_3CHO	(F-PBN-56)-Me_2	169	154, 137, 123
7	PBN + CH_3CHO	(PBN-58)-Me_2	149	134, 118, 77
	PBN + CD_3CHO	(PBN-58)-$(CD_3)_2$	155	137, 122, 77
	d_6PBN + CH_3CHO	(d_6PBN-59)-Me_2	154	139, 123, 82
	d_6PBN + CD_3CHO	(d_6PBN-59)-$(CD_3)_2$	160	142, 126, 82
	F-PBN + CH_3CHO	(F-PBN-58)-Me_2	167	152, 136, 95
8	PBN + CH_3CHO	CH_3-PBN-OH	193	178, 137,91, 57
	PBN + CD_3CHO	CD_3-PBN-OH	196	181, 140, 91, 57
	d_6PBN + CH_3CHO	CH_3-d_6PBN-OH	199	184, 143, 97, 57
	d_6PBN + CD_3CHO	CD_3-d_6PBN-OH	202	187, 152, 97, 57
	FPBN + CH_3CHO	CH_3-FPBN-OH	211	196, 164, 109
9	PBN + CH_3CHO	PBN-Me_2	207	192, 151, 136, 105, 57
	PBN + CD_3CHO	PBN-$(CD_3)_2$	213	198, 157, 139, 108, 57
	d_6PBN + CH_3CHO	d_6PBN-Me_2	213	198, 157, 142, 111, 57
	d_6PBN + CD_3CHO	d_6PBN-$(CD_3)_2$	219	204, 163, 145, 114, 57
	FPBN + CH_3CHO	F-PBN-Me_2	225	210, 169, 154, 123, 57
10	PBN + CH_3CHO	MeCHPh-CHPhMe	210	105,77
	PBN + CD_3CHO	CD_3CHPh-CHPhCD_3	216	108, 77
	d_6PBN + CH_3CHO	MeCDd_5Ph-CDd_5PhMe	222	111, 82
	d_6PBN + CD_3CHO	CD_3CD(d_5Ph)-CD(d_5Ph)CD_3	228	114, 82
	FPBN + CH_3CHO	MeCH(FPh)-CH(FPh)Me	246	123, 95

a Base peak values are shown underlined.



N-*tert*-butyl-α-phenylnitrone **(PBN)** **d_6 -** N-*tert*-butyl-α-phenylnitrone **(d_6PBN)** 4-fluoro-- N-*tert*-butyl-α-phenylnitrone **(FPBN)**

Figure 1: The structures of PBN derivatives used as free radical spin-traps in this study.

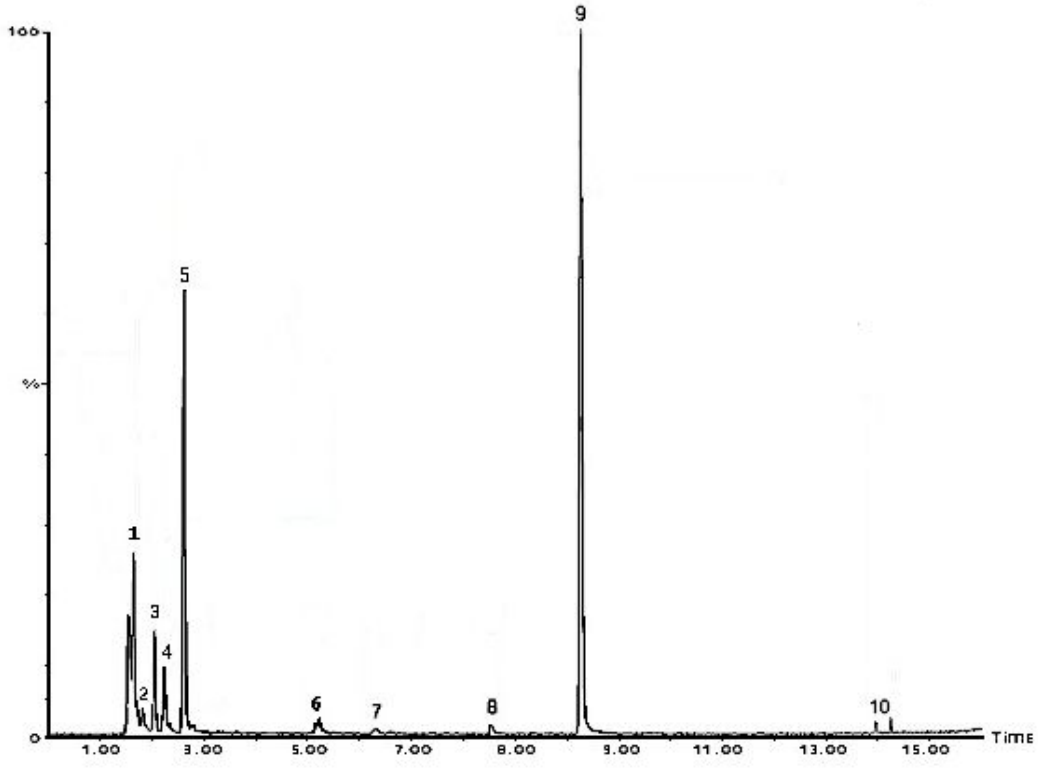


Figure 2: Total ion chromatogram (TIC) obtained from the headspace TD-GC-MS analysis of the Fenton-based reaction mixture containing ethanal and PBN (see materials and methods for further details).

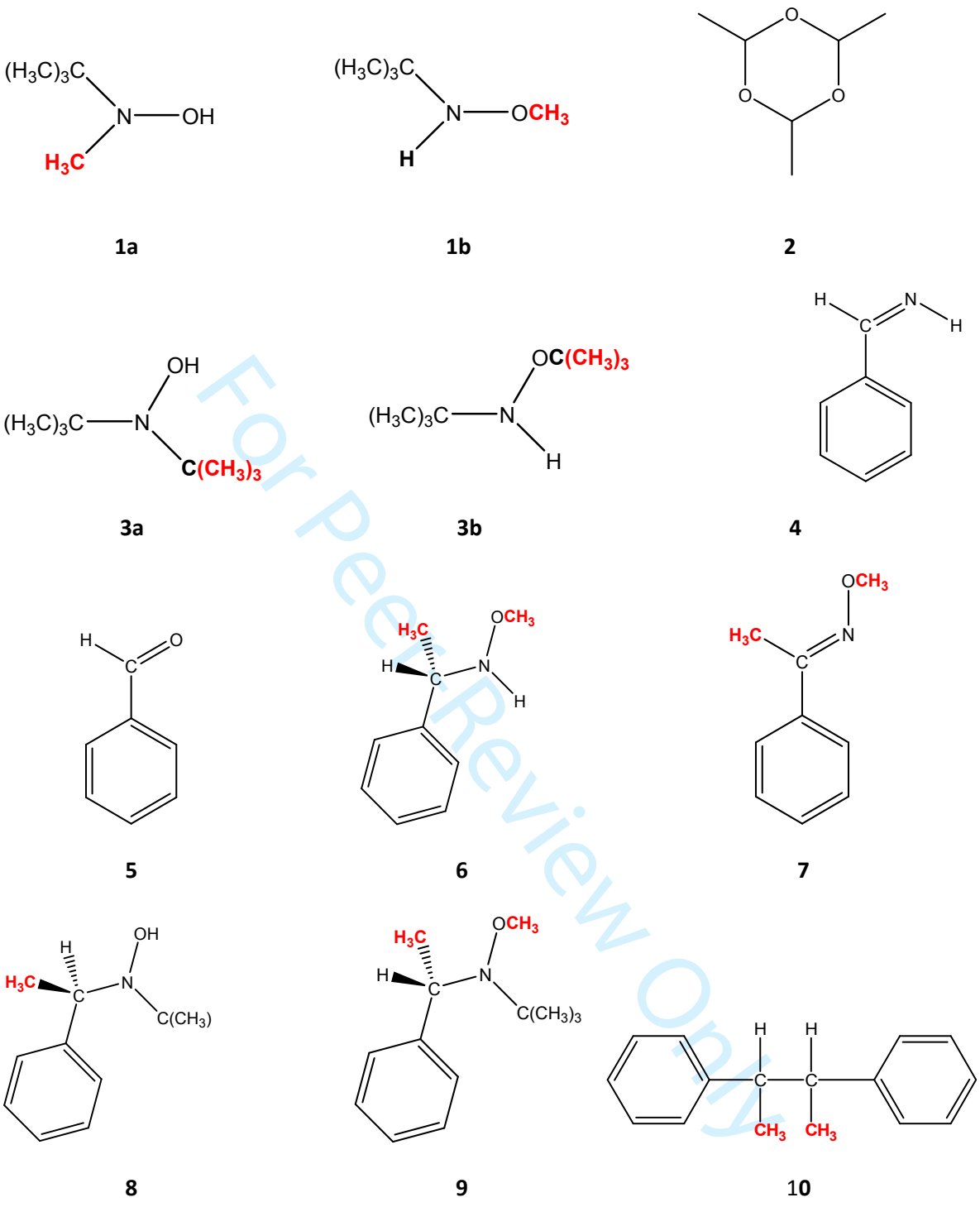


Figure 3: The structures of compounds identified in the vial headspace by TD-GC-MS analysis of a Fenton-based reaction mixture containing PBN and ethanal. The methyl groups in red are derived from ethanal. For compounds 1 and 3, there are two possible structures (labelled a or b).

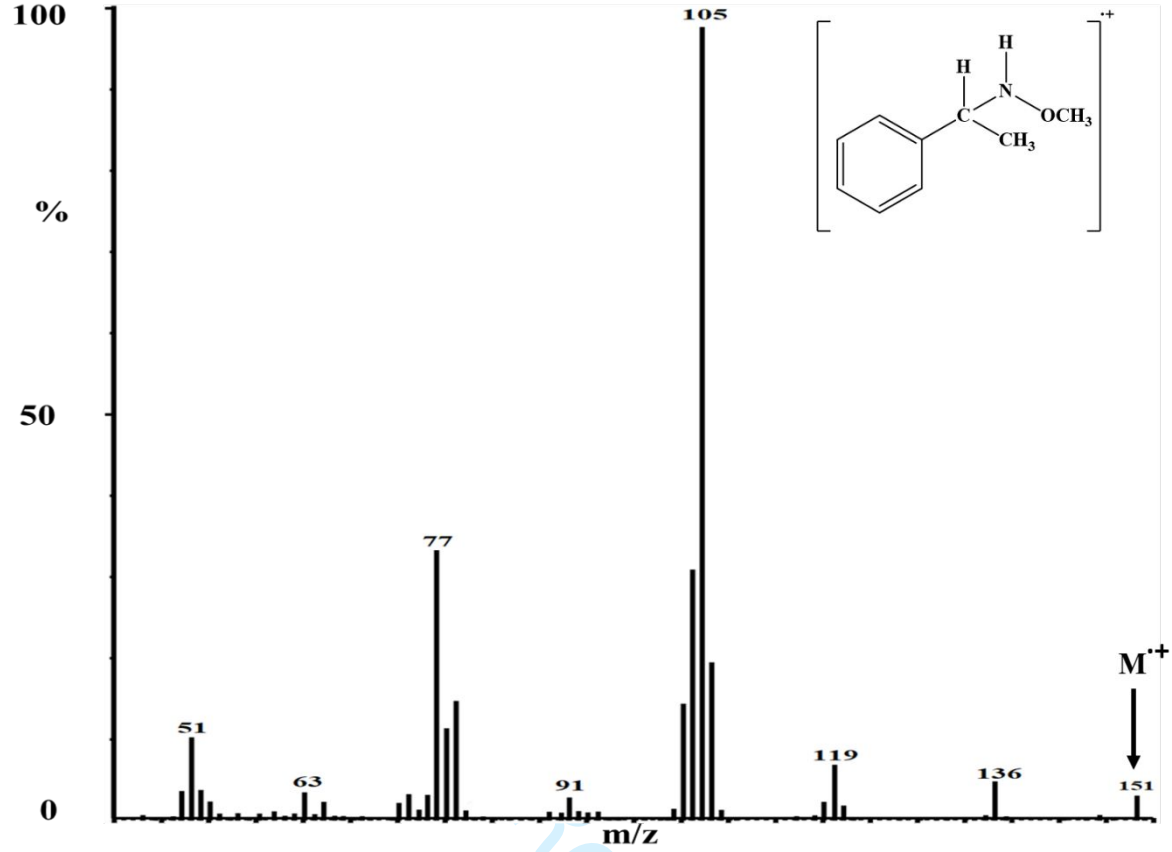
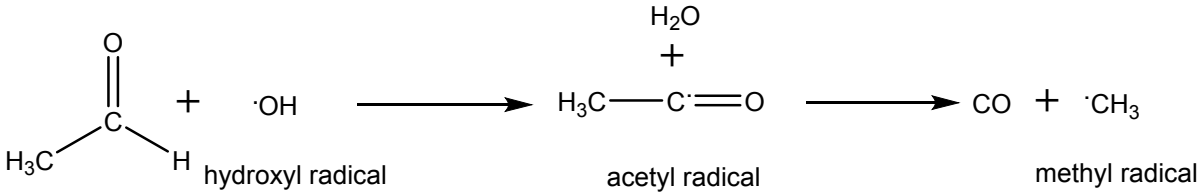
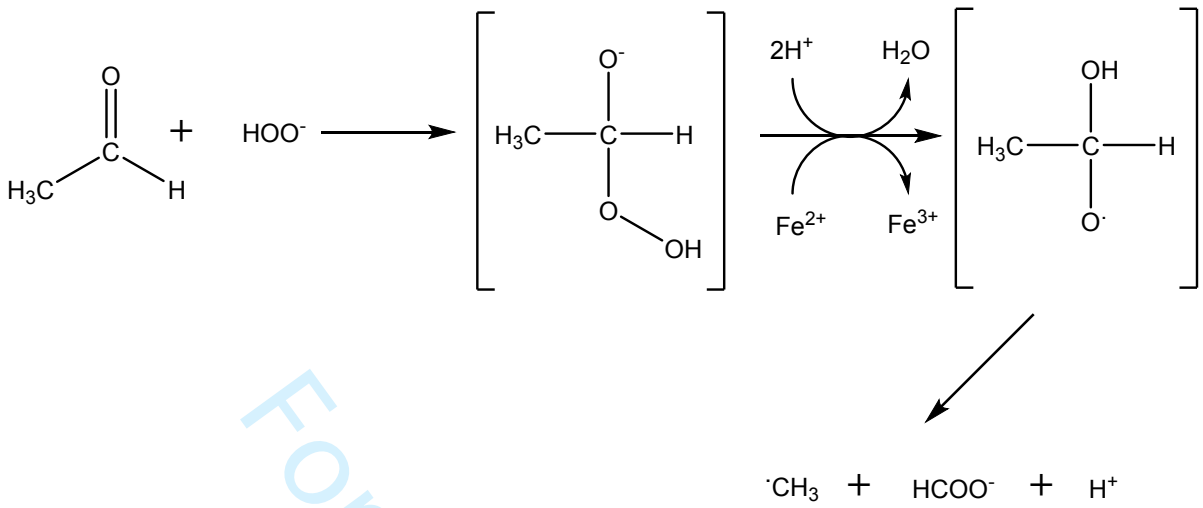


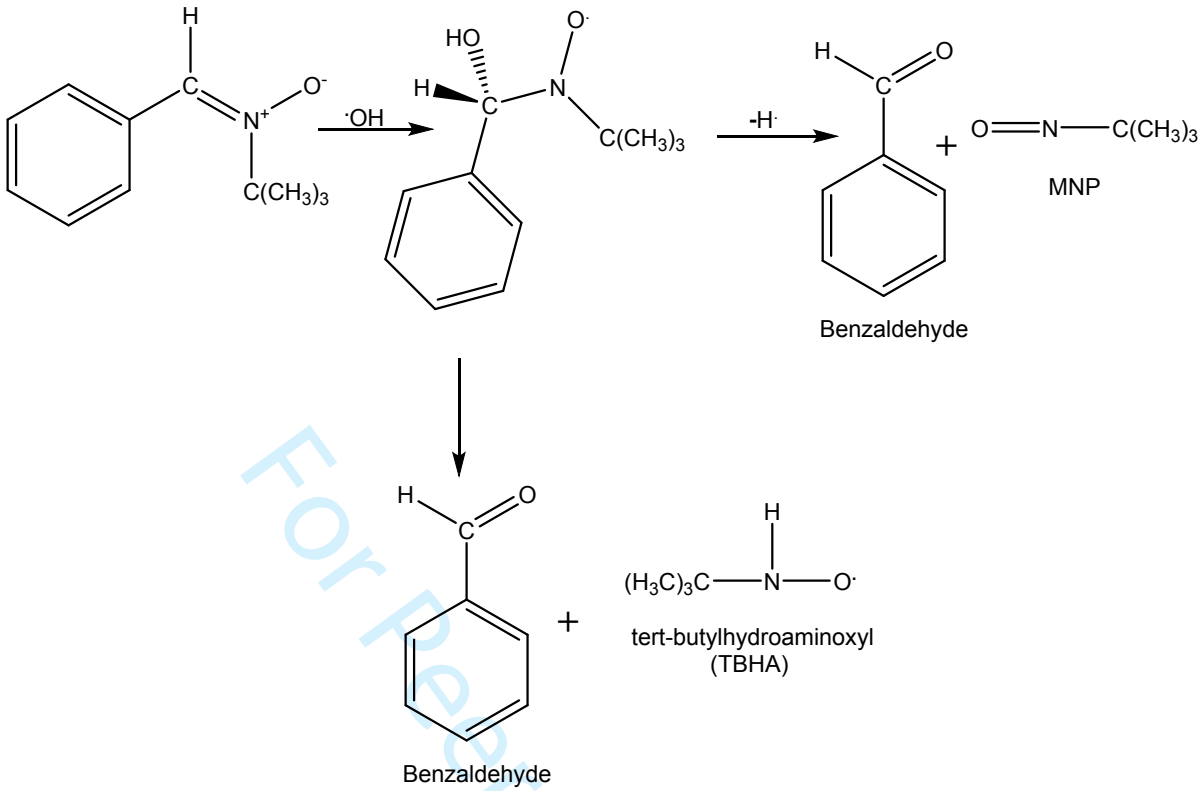
Figure 4: EI mass spectrum of peak 6 (retention time 5.22 minutes) corresponding to N-methoxy-1-phenylethanamine {(PBN-56)-Me_2}. The molecular ion ($M^{+\bullet}$) for the compound can be seen at *m/z* 151 (structure shown in top right-hand corner) and the base peak at m/z 105.



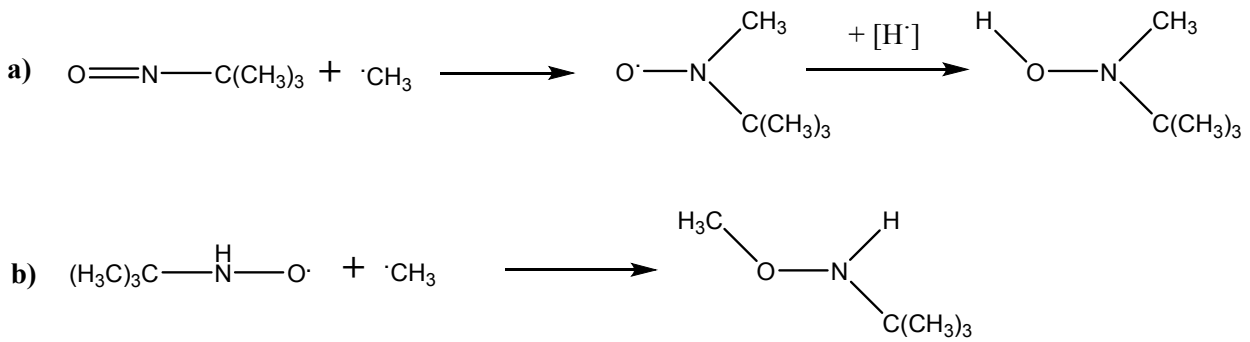
Scheme 1: Hydroxyl radical reaction with ethanal (Nakao *et al.* 2000).



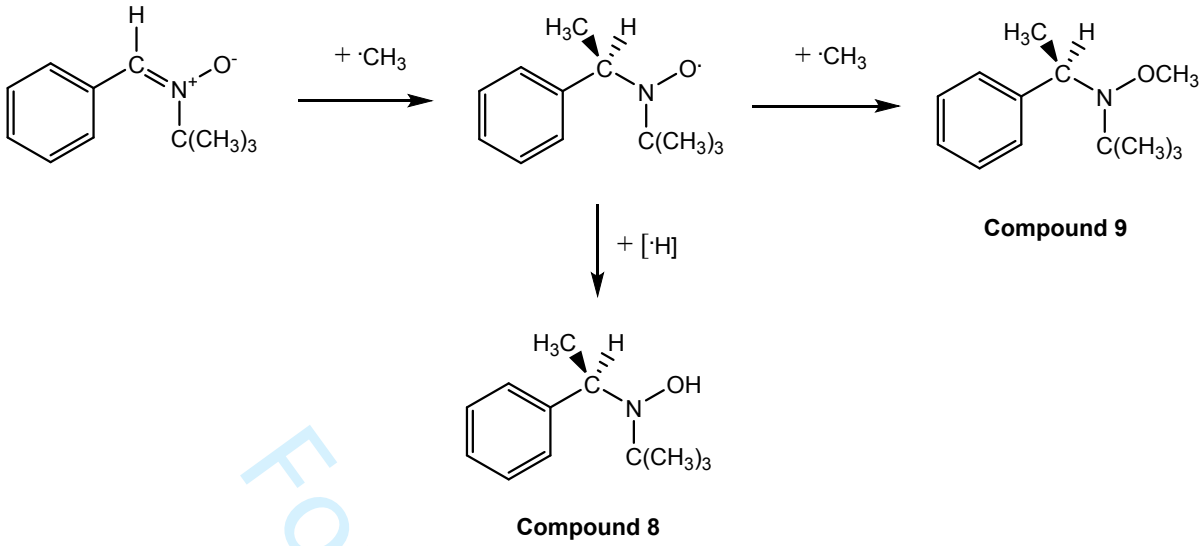
Scheme 2: Nucleophilic addition of the hydroperoxyl anion to ethanal (Nakao, Ouchi & Augusto 1999).



Scheme 3: Possible mechanisms for the formation of benzaldehyde, 2-methyl-2-nitrosopropane (MNP), and *tert*-butylhydroaminoxyl (TBHA) (Kotake & Janzen, 1991; Jerzykiewics et *al*. 2011).



Scheme 4: Addition of a methyl radical to a) MNP (and subsequent reduction to the hydroxylamine) or b) *tert*-butylhydroaminoxyl (TBHA).



Scheme 5: Suggested mechanism for the formation of compounds 8 and 9.