Exploratory study of diazoketones as spin traps for nitric oxide†

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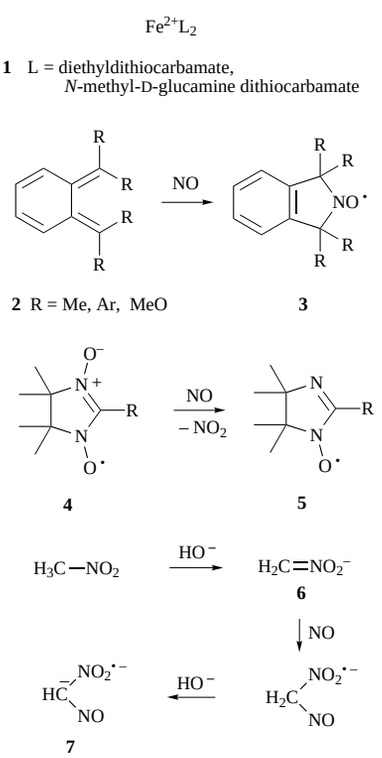
2-Diazocycloheptanone traps nitric oxide to produce a mixture of radicals consisting mainly of the (*Z*)-iminoxyl radical together with minor amounts of the (*E*)-iminoxyl radical, a dialkyl aminoxyl and an acyl aminoxyl. Best EPR spectra have been obtained from solutions in *tert*-butylbenzene which give signals lasting for several hours in the dark. Nitric oxide in aqueous solution could be detected by means of the EPR spectrum obtained on extraction with a *tert*-butylbenzene solution of the spin trap. Nitric oxide release by isoamyl nitrite has been disclosed with the same spin trap although, under photolytic conditions, the spectrum is dominated by the dialkyl aminoxyl. The main stable product from the reaction of nitric oxide with 2-diazocycloheptanone has been found to be cyclohexanecarboxylic acid. Satisfactory EPR spectra were not obtained from nitric oxide using 5-diazouracil as spin trap in aqueous solution.

Introduction

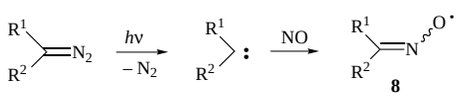
Continuing discoveries of the ubiquity and significance of nitric oxide (NO) in a wide range of biological roles has powered a search for direct and effective methods of detecting and quantifying this molecule. Because of their high sensitivity, wide applicability and potential for zonal mapping of NO, EPR spectroscopic methods based on several families of spin traps have been examined, for example, iron nitrosyl complexes (**1**)[1,2] and *o*-quinodimethane derivatives **2** (NOCTs), which yield persistent cyclic aminoxyl radicals **3**, (Scheme 1).[3–5] Several



Scheme 1

groups[6,7] have shown that NO quantitatively converts nitronyl aminoxyl radicals **4** to imino aminoxyl radicals **5**. A bonus of this method is that the nitrite released can be quantified by conventional Griess analysis. An unusual method[8] employed anion **6**, obtained by deprotonation of nitromethane at high pH. Nucleophilic attack of **6** on NO gave a radical anion which was deprotonated to give an EPR active radical dianion **7**. All the spin traps so far devised have been non-ideal and have suffered from one or more disadvantages, such as lack of specificity, the need for photolysis, complex spectra, short-lived spectra or bio-incompatibility. There is an unmistakable need for an improved EPR method and the research described in this paper was undertaken as part of a search for more effective spin traps for NO.

It was reported some time ago that NO could be trapped in solution by carbenes, photochemically generated from diazo-compounds, to afford long-lived iminoxyl radicals **8** [reaction (1)], which could easily be detected by EPR spectroscopy.[9–11]



(1)

Significantly, it was observed that certain diazo-compounds, including some diazoketones and aryldiazoalkanes, reacted directly with NO, in the absence of irradiation, and thus without the intermediacy of carbenes, to produce similar EPR active iminoxyl radicals.[11] A range of efficient synthetic routes to diazoketones is available[12] which makes a variety of functionalisation and structural types accessible. It was plainly worth testing compounds of this type as spin traps for NO and we report here our results with two dissimilar diazoketones.

Results and discussion

For analytical purposes it is desirable that the EPR spectra of the NO spin adducts have few, narrow lines, that they be long-lived and that they form without the need for photolysis. Previous reports[10,11] suggested that 2-diazocycloalkanones might fulfil these criteria so we made tests with 2-diazocycloheptanone **9**. Diazo spin trap **9** (DAST **9**) was prepared from cycloheptanone, *via* a Claisen condensation to introduce a formyl group, followed by treatment of the 2-(hydroxymethylene)cycloheptanone with *p*-toluenesulfonyl azide, according to the literature procedure.[13] It was safely purified by distillation *in vacuo* but, although it remained analytically pure for long periods in the dark under nitrogen, best results were obtained with freshly distilled material.

Control experiments with **9** alone in hydrocarbon solvents showed no signals with or without photolysis [Fig. 1(*a*) and (*b*)].

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Table 1 9.4 GHz EPR spectra of radicals observed on reaction of 2-diazocycloheptanone (**9**) with NO at 25 °C in *tert*-butylbenzene

Radical					
No.	Structure	Type	*g*-factor	*a*(N)/G	*a*(other)/G[a]
1	**11b**	(*Z*)-Iminoxyl	2.0055	26.5	1.65 (1H), 0.90 (2H), 0.49 (1H), 0.44 (1H), 0.23 (1H)
2	**11a**	(*E*)-Iminoxyl	2.0055	33.5	2.10 (1H), 1.54 (1H), 0.58 (2H)
3[b]		Di-*tert*-alkyl aminoxyl	2.0060	13.6	
4		acyl aminoxyl	2.0068	7.7	

[a] Hfs checked by computer simulation. [b] May be a superposition of several aminoxyl radicals.

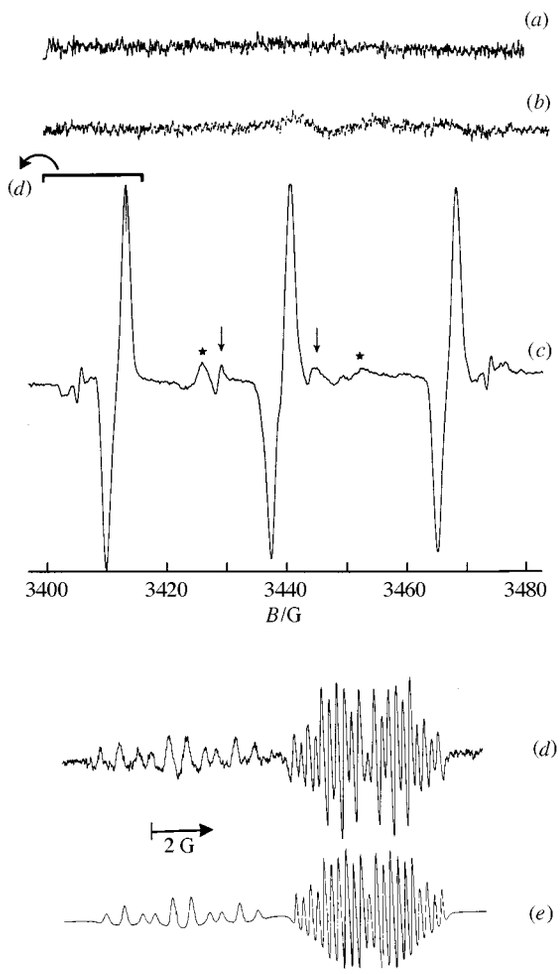


Fig. 1 9.4 GHz EPR spectra obtained with 2-diazocycloheptanone (**9**) in *tert*-butylbenzene solution at 25 °C. (*a*) DAST **9** alone in the dark; (*b*) DAST **9** alone on photolysis; (*c*) DAST **9** on addition of NO in the dark; (*d*) scale expansion of low field region (bracketed) at −20 °C under high resolution conditions with second derivative presentation; (*e*) computer simulation of (*d*) with hfs from Table 1. The three main lines on spectrum (*c*) correspond to the (*Z*)-iminoxyl radical **11b**, the multiplets outside this correspond to the (*E*)-iminoxyl radical **11a**. The stars on spectrum (*c*) indicate the outer lines of the dialkyl aminoxyl (no. 3); the vertical arrows indicate the outer lines of the acyl aminoxyl (no. 4).

When pure NO was bubbled into a solution of **9** (0.27 mol dm^{-3}), or when a saturated hydrocarbon solution of NO was added by syringe, a strong spectrum developed in minutes [Fig. 1(*c*)]. The spectrum of the major species present (no. 1) consisted of a 1 : 1 : 1 triplet [*a*(N) = 26.5 G], each component having $\Delta H_{pp} \sim 3$ G,‡ but this was accompanied by three minor radicals all showing a triplet due to coupling of each unpaired electron with a single nitrogen nucleus. EPR parameters for all four species are recorded in Table 1. The magnitudes of the *a*(N) values, their *g*-factors, and comparison with previous

‡ 10 G = 1 mT.

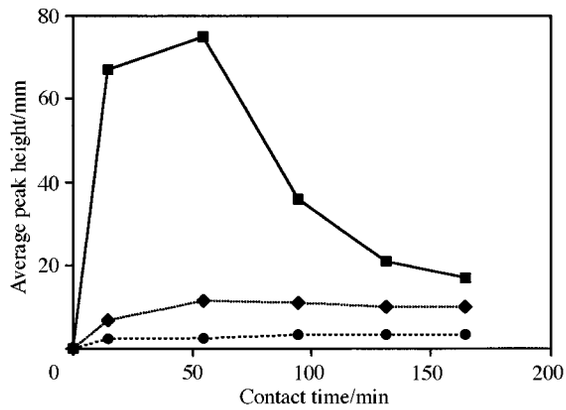
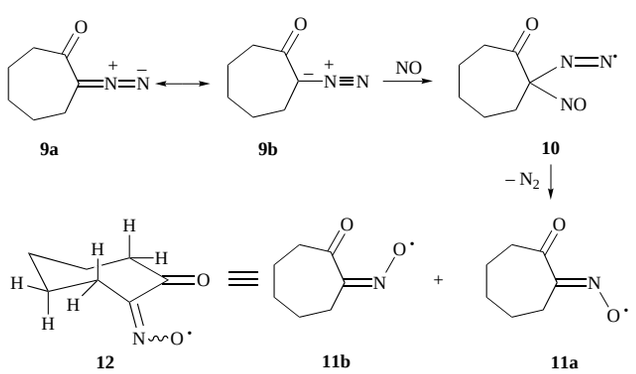


Fig. 2 Evolution of the signal intensities from a solution of DAST **9** as a function of contact time with NO. (■) (*Z*)-Iminoxyl **11b** (no. 1); (◆) dialkyl aminoxyl (no. 3); (●) acyl aminoxyl (no. 4). Signal intensities expressed as the average of the heights from the baseline (mm) of the outer two lines from each radical.

work [11,14] firmly establishes that the major radical (no. 1) and the outermost radical (no. 2) are the (*Z*)- and (*E*)-iminoxyls **11b** and **11a** (Scheme 2), respectively. Better resolution than that



Scheme 2

reported previously was achieved [Fig. 1(*d*)] and a satisfactory simulation [Fig. 1(*e*)] was obtained with the hyperfine splittings (hfs) recorded in Table 1. It is likely that the lowest energy conformation of **11b**,**a** has the chair structure **12** with the C=O and C=N bonds coplanar. By analogy with related cyclic iminoxyl radicals [14] the two largest ^{1}H hfs of both conformers will originate from the non-equivalent hydrogens at C(3); it is not possible to assign the rest of the hfs with certainty. The *g*-factors and hfs of radicals nos. 3 and 4 in Table 1 show that they are di-*tert*-alkyl aminoxyl and an acylalkyl aminoxyl, respectively. No additional hfs were resolved under high resolution for either no. 3 or no. 4. The *a*(N) value for no. 3 is on the low side for a di-*tert*-alkyl aminoxyl radical and this suggests that one (possibly both) of the *tert*-alkyl groups contains an electron-withdrawing substituent adjacent to the tertiary carbon atom.

The relative intensities of **11b** and the two aminoxyls, as a function of contact time with NO, are shown in Fig. 2. All four radicals initially grew in intensity with time, but after *ca.* 60 min decay of the iminoxyl radical signals took place more rapidly

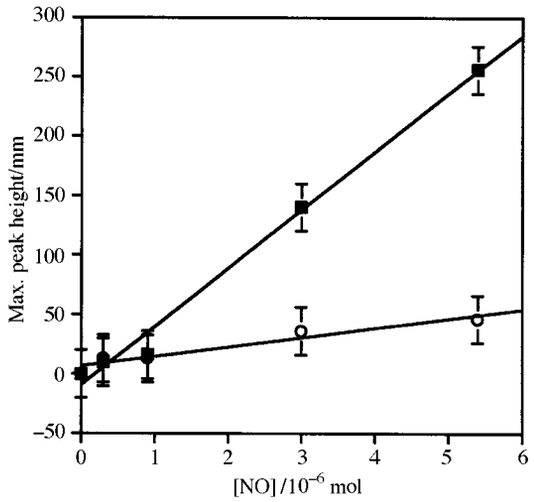


Fig. 3 Plot of EPR signal intensity as a function of the amount of NO (mol) added. (■) Iminoxyl, no. 1; (○) dialkyl aminoxyl, no. 3.

than that of radicals 3 and 4, so that the latter eventually dominated the spectrum. Provided the sample was kept in the dark, at or below room temperature, the major iminoxyl was still detectable 12 h later. Photolysis of the solution led to a short-lived increase in the iminoxyl signals followed by a more rapid degradation and dominance of radicals 3 and 4. Experiments with various concentrations of DAST **9** (0.019–0.27 mol dm^{-3}) showed somewhat erratic results. The concentration of NO in *tert*-butylbenzene will be[15] *ca.* 1.2×10^{-2} mol dm^{-3}; it was found that provided freshly distilled **9** was used, and light and oxygen were excluded, the most intense signals were obtained with [**9**] > 0.05 mol dm^{-3}.

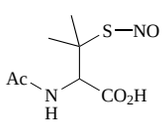
The sensitivity of the method to NO concentration was evaluated by means of experiments in each of which an aliquot of DAST **9** in *tert*-butylbenzene (600 μl of 0.27 mol dm^{-3}) was mixed with a measured volume of a saturated solution of NO (25, 75, 250, 450 μl). In each experiment the development of the signals was monitored at room temperature by EPR over a period of *ca.* 90 min. The development of the signals was somewhat erratic; maximum signal intensity was usually attained in 10–20 min, but up to 60 min could be needed. The maximum peak-to-peak signal heights, *h* (average of outer lines), for the iminoxyl [no. 1, *h*(1)] and dialkyl aminoxyl [no. 3, *h*(3)] are plotted in Fig. 3 as a function of the quantity of NO added. The latter was calculated assuming NO saturation (1.2×10^{-2} mol dm^{-3}).[15]

The iminoxyl signals showed a satisfactorily linear response for NO amounts $>5 \times 10^{-7}$ mol and linear regression gave: [NO]/mol = 0.020*h*(1) + 0.21 ($r = 0.995$). The dialkyl aminoxyl signals also increased with the quantity of NO used although the increase was much smaller. The correlation coefficient of the linear regression line for *h*(3) was only 0.963 suggesting the relationship may be non-linear. It is evident that use of DAST **9** enables amounts of NO to be detected in hydrocarbon solution at 0.5 μmol and above, *i.e.* concentrations of >*ca.* 5×10^{-4} mol dm^{-3}. However, quantification is problematic because of variations in signal intensity with time and inconsistency in the distribution of the signal between iminoxyl and aminoxyl species.

The use of DAST **9** in other solvents was also investigated. When saturated solutions of NO in CH_3CN were added to **9** (0.033 mol dm^{-3}) in CH_3CN the EPR spectra were *ca.* two orders of magnitude weaker than in *tert*-butylbenzene and showed the acyl aminoxyl and the dialkyl aminoxyl, but none of the iminoxyl radical. DAST **9** was very sparingly soluble in water and no spectra were observed on addition of NO. However, when a solution of **9** (0.033 mol dm^{-3}) in *tert*-butylbenzene was shaken with a saturated aqueous solution of NO good signals due to **11** were obtained. However, under these conditions, the signal reached its maximum *ca.* 10 min after mixing and decayed to zero by *ca.* 60 min.

To test the selectivity of **9** as a spin trap, a solution (0.2 cm^3 of 0.2 mol dm^{-3}) was mixed with a saturated solution of NO_2 in *tert*-butylbenzene (0.6 cm^3). A strong EPR spectrum, dominated by the dialkyl aminoxyl, with only minor iminoxyl [*h*(1)/*h*(3) ~0.09] was obtained immediately. The spectrum decayed over a period of several hours in which the signals due to both these two radicals weakened. Similar results were obtained for a range of concentrations of **9** and NO_2. Thus, both NO and NO_2 interact with **9** to produce strong spectra, but NO gives mainly iminoxyl (nos. 1 and 2), whereas NO_2 gives dialkyl aminoxyl (no. 3), particularly during the first hour after mixing.

A potential application of DASTs would be in the detection and estimation of NO emitted from nitroso-releasing drugs such as nitrosothiols. We first investigated *S*-nitroso-*N*-acetyl penicillamine (SNAP, **13**) which is known to release NO in aqueous solution particularly in the presence of copper(I) salts.[16] However, **13** was not sufficiently soluble in *tert*-butylbenzene and no spectra were observed. Not surprisingly, in view of the weak signals obtained with pure NO, no spectra were observed for SNAP with DAST **9** in CH_3CN solution either. Isoamyl nitrite **14** is a known source of NO, particularly on photolysis. *tert*-Butylbenzene solutions containing DAST **9**



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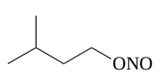

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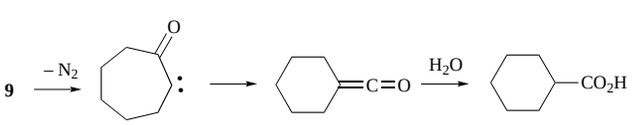
with **14** gave strong EPR spectra on photolysis showing mainly the dialkyl aminoxyl radical (no. 3), minor acyl aminoxyl (no. 4) and traces of iminoxyl **11**. At first sight this seems more consistent with NO_2 release by **14**. However, as reported above, photolysis leads to rapid quenching of iminoxyl signals. It is probable therefore that the photolysis needed to generate NO from **14** simultaneously quenched the iminoxyl EPR signals. Maximum aminoxyl intensities were only a little less than observed for solutions containing 10^{-6} mol NO, but quantification of NO release from **14** was not attempted because complete conversion of the iminoxyl signal to aminoxyl cannot be guaranteed. Similar solutions in CH_3CN also yielded EPR spectra of the dialkyl aminoxyl but, in keeping with all experiments in this solvent, the signals were very weak.

A possible mechanism for iminoxyl radical formation in the dark, similar to that suggested by Chapman and Heckert,[9] is outlined in Scheme 2. Initial nucleophilic attack by the stabilised carbanion **9b** on NO affords a short-lived diazenyl radical **10** which will rapidly lose N_2 with production of the pair of iminoxyl radicals **11a**,**b**. In addition, photolysis of **9**, even by stray long wavelength light, will generate the corresponding carbene **15** which will rapidly trap NO, as shown in reaction (1), to produce more of the same pair of iminoxyl radicals. The di-*tert*-alkyl aminoxyl and acyl aminoxyl cannot be identified with certainty, but several plausible possibilities exist.

Thermal or photochemical decomposition of DAST **9** will generate carbene **15** (Scheme 3). In addition to its intermolecular reaction with NO, **15** is expected to rapidly undergo

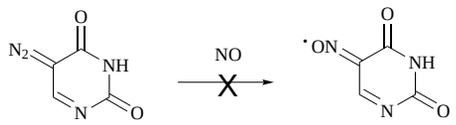


15 **16** **17**

Scheme 3

a Wolf rearrangement leading to ketene **16** which will easily hydrolyse to cyclohexanecarboxylic acid **17**. A GC–MS analysis of the products from reaction of DAST **9** with NO indicated that **17** was the major product with only traces of other unidentified components being observed.

A diazoketone with significant water solubility was needed to test the method under conditions appropriate for *in vivo* NO detection. Accordingly, 5-diazouracil (**18**) was prepared and an



18

aliquot of an aqueous solution (0.028 mol dm^{-3}) was mixed with an equal volume of an aqueous solution of NO. However, no EPR spectra were observed in the dark, or on photolysis at room temperature. Similarly, solutions of **18** in CH_3CN gave no EPR signals on mixing with NO. When this solution was photolysed, a weak ill-defined spectrum containing >11 lines could be observed at low temperatures (*ca.* −40 °C). The spectrum could not be analysed unambiguously because of broad lines and imperfect resolution but it appeared to contain one radical with a(N) = 14.8 G together with at least one additional species. Obviously, a di-*tert*-alkyl aminoxyl may have formed but, because of the weakness of the spectra, and absence of iminoxyl radicals, DAST **18** is unsuitable as a spin trap for NO in water.

Although strong EPR spectra could be obtained from reaction of diazoketones with NO, the partitioning of the spectra between iminoxyl and aminoxyl species severely limits the usefulness of the process, particularly for quantification. The failure of DAST **18** to trap NO is currently not understood and further work with water soluble analogues is needed.

Experimental

^{1}H NMR spectra were obtained at 300 MHz on a Bruker AM 300 spectrometer for $CDCl_3$ solutions containing $SiMe_4$ as internal standard. GC–MS analyses were carried out with a Finnigan Incos 50 quadrupole mass spectrometer coupled to a Hewlett Packard HP5890 capillary gas chromatograph fitted with a 25 m HP 17 column (50% phenyl methyl silicone). *p*-Toluenesulfonyl azide was prepared as described in the literature.[17] 2-Diazocycloheptanone was made by the method of Regitz *et al.*[13] stored at 4 °C and freshly distilled on a vacuum line prior to use; δ_H 2.4–2.6 (4H, m), 1.6–1.8 (6H, m). 5-Diazouracil was prepared by the method of Thurber and Townsend,[18] mp 195 °C (lit.,[20] 195–197). Pure nitric oxide[19] was made by dropwise addition of a solution of sodium nitrite (3.45 g, 0.05 mol in 25 cm^3 H_2O) to an L-ascorbic acid solution (3.52 g, 0.02 mol in 20 cm^3 H_2O) with stirring. Both solutions, together with that containing the substrates, were degassed by bubbling nitrogen for *ca.* 40 min prior to the start of NO generation. The NO gas was passed through a bubbler of conc. H_2SO_4 and the outlets from the apparatus were protected from air seepage with bubblers containing paraffin oil. EPR spectra were obtained with a Bruker ER 200D spectrometer operating at 9.4 GHz with 100 kHz modulation. Samples were prepared in 4 mm od quartz tubes or, for solutions in H_2O or CH_3CN, in capillary tubes (1 mm id) and degassed by bubbling nitrogen for 15 min. Photolyses were carried out in the EPR resonant cavity with unfiltered light from a 500 W super pressure Hg lamp focused onto the sample tubes by a series of quartz lenses. Spectra were simulated with a program originally written by Heinzer.[20]

Reaction of DAST 9 with NO

A solution of DAST **9** (usually 0.5 cm^3 of a 0.033 mol dm^{-3} solution in *tert*-butylbenzene) was placed in the EPR tube which was sealed with a rubber septum. After degassing with nitrogen, either NO gas was bubbled directly through, or an aliquot of a saturated solution NO in *tert*-butylbenzene was added with a syringe. The tube was then transferred to the EPR spectrometer. After *ca.* 12 h reaction the mixture was analysed by GC–MS which showed one major product (cyclohexanecarboxylic acid **17**) together with several minor unidentified components. A library search on the largest of the minor components indicated that it was an ester of **17**, but which ester could not be established with certainty. Reactions in other solvents, with NO_2 and with DAST **18** were carried out using analogous procedures.

Acknowledgements

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