N-tert-butylmethanimine N-oxide is an efficient spin-trapping probe for EPR analysis of glutathione thiol radical

Melanie J. Scott, Timothy R. Billiar & Detcho A. Stoyanovsky

The electron spin resonance (EPR) spin-trapping technique allows detection of radical species with nanosecond half-lives. This technique is based on the high rates of addition of radicals to nitrones or nitroso compounds (spin traps; STs). The paramagnetic nitroxides (spin-adducts) formed as a result of reactions between STs and radical species are relatively stable compounds whose EPR spectra represent “structural fingerprints” of the parent radical species. Herein we report a novel protocol for the synthesis of N-tert-butylmethanimine N-oxide (EBN), which is the simplest nitrone containing an α-H and a tertiary α′-C atom. We present EPR spin-trapping proof that: (i) EBN is an efficient probe for the analysis of glutathione thiol radical (GS•); (ii) β-cyclodextrins increase the kinetic stability of the spin-adduct EBN/•SG; and (iii) in aqueous solutions, EBN does not react with superoxide anion radical (O2•−) to form EBN/•OOH to any significant extent. The data presented complement previous studies within the context of synthetic accessibility to EBN and efficient spin-trapping analysis of GS•.
Results and Discussion

Synthesis of EBN. EBN, the simplest nitrore containing an α-H and a tertiary α'-C atom, has been extensively used as a reagent for cycloaddition reactions. In early spin-trapping studies with nitroso compounds, Challant et al. noted that EBN can be used as an alternative ST for detection of carbon-centered radicals. However, EPR spin-trapping data obtained with this nitrore have not been reported thus far.

Coupling either of 2-methyl-2-nitroso-propane with diazomethane (CAUTION, highly toxic compound) or of aqueous formaldehyde with N-tert-butyldimethylamine (BHA) affords EBN in good to excellent yields. Following the latter protocol, we attained vacuum distillation of EBN, but failed to obtain a nitrore fraction free of trace amounts of nitroxides, which ultimately interfere with EPR spin-trapping experiments. Purification of the nitrone by activated charcoal or by column chromatography also proved difficult as the end products exhibited comparable polarity. Hence, we optimized the synthetic protocol via assessment of that was free of trace amounts of nitroxides, which ultimately interfere with EPR spin-trapping experiments.

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Spin-trapping of GS* by EBN. The metabolism of redox-sensitive xenobiotics often proceeds with generation of free radicals, which, in turn, react with thiols to form thyl radicals. As glutathione is the most abundant cellular thiol, its oxidation by free radicals to GS* is a preponderant reaction, and the formation of GS* is viewed as a toxicological event as this radical species abstracts H atoms from cellular molecules, reacts with sulphydryls to form disulphides, and adds to double bonds.

The detection of GS* in biological matrices is difficult because its half-life is in the nano-to micro-second scale. Research in the 1980s demonstrated that GS* reacts with 5,5-dimethyl-1-pyrroline N-oxide (DMPO) to form DMPO-SG (Fig. 2), which exhibits a specific four-line EPR spectrum. While this protocol proved instrumental in the elucidation of fundamental redox reactions of GSH, its application is limited by the low stability of DMPO-SG (t1/2 ≃ 50 s). Recent analyses of the kinetics of formation and decay of a number of GS*-derived spin-adducts have identified 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) and trans-Mito-DEPMPO as STs that form kinetically more stable spin-adducts with GS- than DMPO (Fig. 1). To extend the structure-activity relationship study of the spin-trapping analysis of GS*, we have carried out experiments with EBN, which is a common structural motif of a number of widely-used STs (Fig. 2; common bonds in red).

The data presented in Fig. 3A show the spin-trapping of GS* with EBN. We generated GS* via photolytic homolysis of the S-N bond of S-nitrosoglutathione (GSNO). At ambient luminescence (<400 lux), the reaction system consisting of GSNO and EBN did not exhibit any EPR activity (Fig. 3A1, black trace). Irradiation of the solution with visible light (515 nm cutoff filter; 1 × 10^5 Lux) led to the appearance of a seven-line EPR spectrum with hyperfine splitting constants (in mT) of a_π = 0.729 and a_S = 1.607, which was assigned to EBN-SG (Fig. 3A1, red tracings). In Fig. 3A2 is presented a computer simulation of the EPR spectrum of EBN-SG. When GS* was spin-trapped by 50 mM EBN in the presence of 100 mM and 200 mM DMPO, the magnitude of the EPR spectrum of EBN-SG decreased by 33% and by 66%, respectively, indicating that K_{E[EBN]} ≃ 1.5 k_{E[DMPO]} (data not shown; comparison of the rate constants were made as reported in ref. 33). The dismutation of EBN-SG by cyclodextrins (CDs). The dismutation of α-H nitroxides to nitrones and hydroxyamines is a major reaction pathway leading to the decay of spin-adducts. The reaction proceeds via formation of a nitroxide-dimer wherein a single electron transfer from nitrogen to oxygen yields the ion pair...
“hydroxylamine anion/o xo ammonium cation”; tautomerization of the o xo ammonium cation, with concomitant release of a proton, leads to the formation of a new nitrone (Fig. 4; reviewed in ref. 34).

One strategy to increase the kinetic stability of spin-adducts containing α-H atoms is to impede their dimerization via inclusion into CDs. Cyclodextrins are cyclic polymers consisting of 6, 7 or 8 glucopyranoside units (α-CD, β-CD or γ-CD) which have the shape of toroids (7.9 Å), with the larger and smaller openings exposed to the solvent. They have hydrophilic interfaces and hydrophobic cavities with diameters of 4.7, 6, and 7.5 Å (α-, β- and γ-CD, respectively). CDs have a propensity for forming non-covalent inclusion complexes with a variety of hydrophobic organic molecules33,36, including nitroxides37–40. The spin-trapping of superoxide anion radical (O_2^•−) with a series of STs in the presence of β-Me-CD has been shown to proceed with inclusion of the corresponding spin-adducts in the cavity of the cyclodextrin, thus increasing their half-lives41–46.

The EPR spectra presented in Fig. 5 were obtained with generation of EBN/SG in the presence of α-, β-, β-Me-, and γ-CD. In all experiments, EBN and CDs were used at 15 mM concentration (saturated solution of β-CD, 17 mM). As compared to the spectrum of EBN/SG, new EPR-active species were formed in the presence of β- and β-Me-, but not in the presence of α- and γ-CD. We observed the same spectral changes upon addition of CDs to pre-formed EBN/SG (data not shown). Although marked spectral changes were detected in the hyperfine splitting constants of EBN/SG upon its inclusion in CDs, resolved signals from the free and bound nitroxide were not obtained, and hence we did not assess the constants of the corresponding inclusion complexes. In the presence of β-CD, the EPR signal of EBN/SG increased linearly with increases in the concentration of GSNO (0.005–0.2 mM). Since α- and γ-CD differ from β- and β-Me-CD only in the sizes of their cavities, spectral changes due to surface adsorption of the nitroxide on the outside of the cavities and/or spin-trapping of secondary radicals generated by reactions between GS^* and/or EBN/SG with CDs can be excluded. Furthermore, since β-CD has a hydrophobic channel with a diameter of 6 Å, inclusion of the whole EBN/SG radical can be ruled out as well. The decrease in T_{1/2} in the presence of β-CDs indicates that the nitroxide function was compartmentalized in a more hydrophobic milieu, suggesting that the tert-butyl side of the spin-adduct was included in the CDs, whereas the hydrophilic, glutathionyl part of the molecule remained exposed to the bulk water.

In Fig. 6A, we show comparative kinetics of the decays of EBN/SG, DMPO/SG, and DEPMPO/SG. The apparent T_{1/2} values of 50 and 120 seconds for DMPO/SG and DEPMPO/SG were in good agreement with previous studies29,30,32, and the decay of EBN/SG and DEPMPO/SG exhibited similar kinetic profiles. Next, we assessed the kinetics of decay of EBN/SG in the presence of CDs (Fig. 6B); in the presence of β-CD, the apparent T_{1/2} of EBN/SG increased from 120 s to 450 s, and the spectrum of the spin-adduct was readily detectable 90 min after interruption of the photolytic generation of GS^*. Although EBN/SG formed inclusion complexes with both β- and β-Me-CD, the stability of the spin-adduct in the presence of β-Me-CD was lower (T_{1/2} = 190 s). This suggests that either the rate of release of EBN/SG from its complex with β-Me-CD was higher than from β-CD, presumably due to decreased hydrogen bonding between methylated OH groups and the nitroxide, or to the methyl groups obstructing its inclusion in the cyclodextrin.

Spin-trapping of O_2^•− and hydroxyl radical (HO^•) by EBN. A considerable research effort has been directed toward the identification of STs that can be used for analysis of O_2^•− and HO^•, which are radical species of considerable importance as reaction intermediates in various biological, radiolytic, and photochemical processes. In 1974, Harbour et al. reported that, in aqueous solutions, O_2^•− can be spin-trapped by DMPO37. Although this method has been extensively used, it has considerable analytical limitations; the reaction of DMPO with O_2^•− is rather slow (k_{DMPO/O_2} = 10 M^{−1}s^{−1}), DMPO/•OH is an unstable spin adduct (T_{1/2} = 50 s), and the analysis is affected by trace amounts of transition metal ions and pH variations30,48. In 1995, Frejaville et al. found that DEPMPO, an α-phosphorus-containing analogue of DMPO, reacts with O_2^•− to form DEPMPO/•OOH, which is 15 times more stable than DMPO/•OOH43. Rosen et al. observed that increases in the...
bulkiness of alkyl substituents in third position of the pyrroline ring of DMPO leads to complete inhibition of the spin-trapping of O$_2$•$^-$, as indicated by the formation of the corresponding nitroxides, while Allouch et al.

Figure 3. Spin-trapping of photolytically generated thyl radicals by EBN. Reactions were carried out at 25 °C in 0.1 M phosphate buffer (pH 7.4) containing EBN (50 mM) and S-nitrosothiol (5 mM). A1 - EPR spectra of GSNO and EBN prior to (light off; black trace) and after illumination (light on; red traces); 2 - computer simulation of the EPR spectrum of EBN/SG; 3 - EPR-monitored decay of EBN/SG (light off). Consecutive spectra were recorded with time intervals of 30 s (1) and 150 s (3). Arrows indicate the directions of the spectral changes. B - EPR spectra were recorded after illumination of S-nitrosothiols and EBN for 5 min; 1 - S-nitroso cysteine and EBN; 2 S-nitroso N-acetyl-D-penicillamine and EBN; S-nitroso 2-methyl-2-propanethiol and EBN. EPR spectra were recorded at an Amplification of 100.

Figure 4. Nitroxides containing α-H atoms dismutate to nitrones and hydroxylamines.
found that introduction of electron-withdrawing groups in Cα position of acyclic STs inhibits the spin-trapping of O2− facultatively.

In a theoretical analysis of cyclic STs, Villamena et al. reported that the electronic density on the nitronyl C atom noticeably changes with introduction of substituents in Cα−δ and thus affects the reactions of nitrones with O-centered radicals. However, these studies have not been extended to a structure-activity relationship that allows prediction of the spin-trapping affinity of nitrones for specific radical species.

Our attempts to obtain the spin-adduct of EBN with O2− in aqueous solutions proved unsuccessful. In phosphate buffer (0.1 M; pH 7.4) containing catalase (300 U/mL) and EBN (50–200 mM), we did not observe the formation of EPR-active species upon addition of up to 0.4 mM KO2 (Fig. 7A1; stock solution of KO2 was prepared in DMSO containing an equimolar amount of 18-Crown 6). Similarly, we did not observe formation of EBN/• −OH when O2− was enzymatically generated by the system xanthine oxidase (XO; 50 mU/mL; Fig. 7A2). Substitution of EBN with DEPMPO in the HX/XO system resulted in the appearance of the typical EPR spectrum of DEPMPO/• −OH (Fig. 7A3), thus indicating that O2− was generated in the reaction solution. Superoxide dismutase (SOD; 30 U/mL) fully inhibited the formation of DEPMPO/• −OH (Fig. 7A4). In contrast to O2−, the generation of HO• in a Fenton-like system containing EBN led to the appearance of a well resolved nine-line EPR spectrum, which we assigned to EBN/• +OH (Fig. 7B2; in mT, aH = 0.613 and an = 1.125; H2O2 + Fe2+ → HO• + Fe3+ + HO−; EBN + HO• → EBN/• +OH). No EPR activity was observed if any one of the reagents was omitted from the reaction system (Fig. 7B1). Introduction of DMSO in the reaction system led to the formation of EBN/• +CH3, which exhibited a distinct EPR spectrum (Fig. 7B3; CH3S(O)CH3 + HO• → CH3• + CH3S(O)OH; EBN + CH3• → EBN/• +CH3). The identity of the latter nitroxide was confirmed by EPR analysis of authentic EBN/• +CH3, which was synthesized by methylation of EBN with CH3MgI (Fig. 7B4; in mT, aH = 1.199 and an = 1.854).

**Figure 5. Effects of CDs on the EPR spectrum of EBN/• SG.** EPR spectra (Amplification, 500) were recorded after photolytic homolysis of GSNO (5 mM) for 3 min in the presence of EBN (15 mM) and CDs (15 mM). All other incubation conditions are the same as indicated in the legend to Fig. 2.
We further carried out experiments to assess the reaction of EBN with $O_2^{-•}$ in an aprotic solvent. Addition of a solution of 18-Crown 6/KO$_2$ (final concentration, 0.4 mM) in anhydrous DMSO to DMSO containing EBN (20 mM) and H$_2$O (0.05 mM; Fig. 8A1) led to the appearance of a nine-line EPR spectrum, reflecting the formation of EBN/$•$OOH (Fig. 8A2; in mT, a$_H$ = 0.483 and a$_N$ = 0.975). The magnitude of the EPR signal decreased by 15% for 6 min, indicating that EBN/$•$OOH was unstable under these reaction conditions. An identical EPR spectrum was observed when a solution of EBN in DMSO was treated with H$_2$O$_2$ (20 mM) and Et$_3$N (100 mM), thus supporting the assignment of the EPR spectrum to EBN/$•$OOH; in the latter reaction system, a nucleophilic addition of HOO$^{-}$ to the nitronyl C atom yielded EBN/OOH hydroxylamine, which autooxidized to EBN/$•$OOH nitroxide (data not shown). Dilution of a DMSO solution of EBN/$•$OOH with phosphate buffer (pH 7.4) resulted in a decrease of the EPR signal by 15% for 6 min, indicating the instability of the EPR spectrum.

Figure 6. Effects of CDs on the decay of EBN/$•$SG. Photolytic homolysis of GSNO to GS$^•$ was carried out for 3 minutes in the presence of DMPO, DEPMPO and EBN. Thereafter, the light was switched off and decreases in the EPR spectra of spin-adducts were recorded over time in the absence (A) and the presence (B) of CDs (15 mM). Nitrones were used at concentrations of 50 mM (A) and 15 mM (B). The spin concentration of nitroxides was determined by double integration of the EPR signals using 4-hydroxyl-1-TEMPO as a standard. All other reaction conditions are the same as indicated in the legend to Fig. 2. The data in panels B are presented as mean values of three independent experiments ± the standard error.

Figure 7. Spin-trapping of $O_2^{-•}$, HO$^•$ and CH$_3$• by EBN. Reactions were carried out in 0.1 M phosphate buffer (pH 7.4) containing EDTA (0.05 mM). A1 - EBN (50 mM), catalase (300 U/ml) and KO$_2$ (0.2 mM); A2 - EBN, catalase, HX (0.5 mM) and XO (50 mU/mL); A3 - DEPMPO (20 mM), catalase, HX and XO; A4 - DEPMPO, catalase, HX, XO and SOD (30 mU/mL). B1 - H$_2$O$_2$ (0.05 mM) and EBN (20 mM); B2 - plus Fe(NH$_4$)$_2$SO$_4$ (0.01 mM); B3 - DMSO (0.2 M), EBN and H$_2$O$_2$ plus Fe(NH$_4$)$_2$SO$_4$; B4 - authentic EBN/$•$CH$_3$. EPR spectra were recorded at an Amplification of 1000.
in the disappearance of the EPR spectrum of EBN/*OOH (Fig. 8A3) in less than 30 seconds, which is the approximate time required for sample preparation and data acquisition. However, we did observe the presence of trace amounts of a relatively stable nitroxide, less than 5% of the expected concentration of EBN/*OOH, with hyperfine coupling constants suggesting the formation of EBN/*OH (Fig. 8A3 and 4-Amplification 100 and 4000, respectively).

In spin-trapping experiments, nitroxides are analyzed by EPR under steady-state conditions, where their rates of formation and decomposition define the analytical sensitivity of the corresponding protocol. While the data presented in Fig. 8A indicate that EBN/*OOH is an unstable compound in aqueous solutions, we were interested to assess the rate of the reaction of EBN and O$_2$ with O$_2$ in phosphate buffer (pH 7.4). To this end, we carried out a competitive kinetics study of the reaction of DEPMPO with O$_2$ in the presence of EBN, where the source of O$_2$ was 18-Crown 6/KO$_2$. Addition of O$_2$ (final concentration, 0.1 mM) to a solution of DEPMPO (10 mM) in 0.1 M phosphate buffer (Fig. 8B; Inset, trace 1) resulted in the formation of DEPMPO/*OOH (Fig. 8B; Inset, trace 2). The magnitude of the EPR signal of DEPMPO/*OOH decreased linearly with increasing concentrations of EBN, with 50% inhibition of the formation of DEPMPO/*OOH at ~100 mM EBN. These data indicate that the rate constant of the reaction of EBN with O$_2$ is one order of magnitude lower than that of DEPMPO (k$^{\text{Superoxide}}_{\text{DEPMPO}} = 15 \text{M}^{-1}\text{s}^{-1}$). Hence, in contrast to DMPO and its analogues, EBN is a suitable ST for analysis of O$_2$ formed in organic but not in aqueous solutions, where both the rate constant of its reaction with O$_2$ and the stability of EBN/*OOH are relatively low.

**Spin-trapping of enzymatically-generated GS with EBN.** The low reactivity of EBN with O$_2$ in aqueous solutions suggests that this nitroxide can be used for spin-trapping analysis of secondary, O$_2$-derived radicals in biological systems. This possibility is illustrated by the data presented in Fig. 9A. Generation of O$_2$ by HX/XO in the presence of EBN, GSH, and catalase resulted in the formation of EBN/*SG; in neutral aqueous solutions, O$_2$ oxidized GSH to GS with a rate constant of 10$^3$ M$^{-1}$s$^{-1}$. Addition of SOD to the reaction system fully inhibited the formation of EBN/*SG (data not shown). In this system, the spin-trapping selectivity of EBN was reminiscent to the affinity of 2-H-imidazole-1-oxide for thiyl radicals but not for O$_2$.

To further validate EBN as a spin-trapping probe for GS, we assessed the formation of GS in a reaction system consisting of myeloperoxidase from human leucocytes (MPx; 0.2 units/mL), phenol (0.01 mM), GSH (1 mM), and H$_2$O$_2$ (0.1 mM). In this system, phenol undergoes oxidation to phenoxyl radical, which is reduced back to phenol by GSH with concomitant generation of GS; in turn, GSH reacts with the latter to form a disulfide anion radical which transfers an electron to O$_2$, yielding GSSG and O$_2$. In cells, this reaction sequence can occur without apparent consumption of phenol and GSH, whose concentration is maintained via reduction of GSSG by glutathione reductase (Fig. 10).

In the absence of EBN, the complete reaction system did not exhibit any EPR activity (Fig. 9B1), indicating that the concentration of radical species was below the detection limit of the EPR spectrometer. Addition of EBN led to the appearance of the EPR spectrum of EBN/*SG, whose intensity increased until a steady-state
Figure 9. Spin-trapping of enzymatically-generated $\text{O}_2^-$•. Reactions were carried out at 25°C in 0.1 M phosphate buffer (pH 7.4). A- EBN (15 mM), β-CD (15 mM), GSH (2 mM), HX (0.5 mM), XO (50 mU/mL), and catalase (300 U/mL). B1- Phenol (0.01 mM), MPx (0.2 units/mL), $\text{H}_2\text{O}_2$ (0.1 mM), and GSH (1 mM); B2- Phenol, MPx, $\text{H}_2\text{O}_2$, GSH and EBN (20 mM). Consecutive spectra were recorded with time interval of 120 s. Arrows indicate the directions of the spectral changes. EPR spectra were recorded at an Amplification of 1000.

Figure 10. Phenol phenoxy radical oxidizes GSH to GS•.
Concentration of EBN/SG has been reached. Elimination of H₂O₂ by addition of catalase to the reaction system led to disappearance of the EPR signal of EBN/SG with a kinetic profile that was identical to that presented in Fig. 6A (data not shown).

Conclusions
The data presented herein complement previous studies within the context of synthetic accessibility to EBN and efficient spin-trapping analysis of GS. From all nitrones tested thus far, trans-Mito-DEPMPO, DEPMPO and EBN form the most stable spin adducts with GS (t₁/₂ trans-Mito-DEPMPO/SG = 730 sec; t₁/₂ DEPMPO/SG = 120 sec; and t₁/₂ EBN/SG = 120 sec). As compared to EBN, however, the synthesis of EPR grade DMPO analogues requires higher experimental effort. The relatively high rate of addition of GS* to EBN, the kinetic stability of EBN/SG, and the well-resolved EPR spectrum of EBN/SG define this nitrone as an efficient molecular probe for GS*.

Materials and Methods
Reagents.
All reagents used were purchased from Sigma-Aldrich Co. (St. Louis, MO). N-tert-butylhydroxylamine was synthesized as described in ref. 54. The solutions used in EPR-STs experiments were prepared in deionized and Chelex 100-treated water and in potassium phosphate buffer (pH 7.4). S-nitroso thiolis were prepared by treatment of thiols with ethyl nitrite as reported in ref. 55. Methylation of EBN with CH₃MgI was carried out as reported in ref. 56.

Synthesis of N-tert-butylmethanimine oxide (EBN). Under an atmosphere of helium, a suspension of BHA hydrochloride (0.65 g; 5.2 mmol), paraformaldehyde (0.46 g; 15.3 mmol), anhydrous Na₂SO₄ (3.5 g; 24.6 mmol) and NaHCO₃ (2.5 g; 30 mmol) in 30 mL CH₂Cl₂ was stirred at 25 °C for 5 hours. Progression of the reaction was monitored with a SPD M10Avp Shimadzu diode array detector. Upon completion of the reaction, the suspension was filtered and the filtrate rotor-evaporated (30 °C; 250 torr), affording EBN as a colorless liquid (yield, 100%).

Visible (white) light was provided by a Sylvania lamp type DWY (625 W) equipped with a spherical reflector. The light source was positioned 30 cm from the front of the EPR cavity, where Telfon tubing containing a solution of GSNO, EBN and DTPA (0.1 mM) in 0.1 M phosphate buffer (pH 7.4) was placed. The aperture of the cavity was equipped with a 515 nm cut-off filter (colored glass filter OG515; Melles Griot BV; Aalsbergen, Nederland). Light intensity was measured with a Datalogging Light Meter (Model 850008; SPER Scientific, LTD; Scottsdale, AZ).

EPR Spectroscopy. EPR spectra were recorded at room temperature using a JEOL-RE1X spectrometer (Kyoto, Japan). Spectrometer settings were: field center 335.094 mT, microwave power 10 mW, sweep time 30–120 s, time constant 0.1 s, and modulation width 0.2 mT. EPR spectra simulations were performed with a JEOL computer program.

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Author Contributions
M.J.S. and D.A.S. planned the research work. D.A.S. synthesized EBN and performed EPR experiments. All the authors discussed the results and commented on the manuscript. D.A.S. wrote the manuscript.

Additional Information
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