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Purity of Different Preparations of Sodium 3,5-dibromo-4-nitrosobenzenesulphonate and Their Applicability for EPR Spin Trapping

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During the preparation of sodium 3,5-dibromo-4-nitrosobenzenesulphonate (DBNBS) of high purity for electron paramagnetic resonance (EPR) spin-trapping purposes, it was found that the material synthesised as part of the present study differed significantly from some commercially available samples of DBNBS. A thorough chemical characterisation of the contents of the various samples led to the conclusion that the preparations synthesised in the present study, as well as one of four commercially available samples, contained essentially pure DBNBS and had efficient spin-trapping activity. In contrast, the remaining three commercially available samples contained almost exclusively sodium 3,5-dibromo-4-nitrobenzenesulphonate, i.e. a one-oxygen oxidation product of DBNBS, and had little spin-trapping activity. The two compounds were readily separated by reverse-phase high performance liquid chromatography (HPLC). It was further found that the quality of DBNBS preparations may be determined by NMR spectrometry, IR spectrometry, fast atom bombardment-mass spectrometry (FAB-MS) and EPR spectrometry. In particular, UV–Visible spectroscopy may be used to determine A_{308}/A_{280}, which should be greater than 1.8 for a high purity DBNBS preparation.

INTRODUCTION

The compound sodium 3,5-dibromo-4-nitrosobenzenesulphonate (DBNBS) was first reported in 1981, in response to the need of a water-soluble derivative of nitrosobenzene with good spin trapping properties.[1] Since then the mode of action of DBNBS has been investigated in a number of studies. DBNBS is considered to produce stable spin adducts only with carbon-centred radicals and a few heteroatom-centred radicals, such as the sulphite anion radical, $SO_3^{\bullet -}$.[2] However, DBNBS does in fact react very rapidly with the superoxide radical anion, the carbon dioxide radical anion and the hydroxyl radical to form spin adducts which are very unstable with the result that their EPR spectra cannot be seen under normal conditions.[3] An interesting feature of DBNBS is that it reacts with $NO^{\bullet}$ to produce a stable radical, which is not the simple adduct, since no hyperfine splitting due to the ^{14}N-nucleus of the $NO^{\bullet}$ molecule can be seen.[4] Ichimori *et al.*[5] suggested that a diphenyl aminoxyl species was formed, this was later isolated and characterised as the dianion, bis (2,6-dibromo-4-sulphophenyl) nitroxyl by Davies *et al.*[6] We have also shown that the DBNBS-$NO^{\bullet}$ product is stable enough to be suitable for the quantification of $NO^{\bullet}$ liberated from acidified nitrite.[7] A final intriguing property of DBNBS is its ability to react with oxidants to form a radical with a characteristic EPR spectrum, assigned to the DBNBS radical cation,[3] and this has been used to establish the presence of oxidant activity in human uraemic plasma,[8] normal human urine[9] and diseased human synovial tissue.[10] DBNBS has also been used to demonstrate the presence of peroxidatic activity in human lymphoblastic leukaemic cells.[11] Apart from this, in biological systems DBNBS performs the so-called "ene" reaction at a low rate

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with some amino acids to produce a nitroxyl radical even when no radicals are initially present; this artifactual reaction is most pronounced with tryptophan, while proline and cysteine also react to a smaller extent.[12] DBNBS also reacts with olefins to produce artifactual EPR signals.[13]

It is clear from these comments that the use of DBNBS as a spin trap requires considerable care. In line with this there has been some controversy about its use. Most notably, Ozawa and Hanaki[14] observed a stable spin adduct from the reaction between DBNBS and superoxide and assumed it to be the DBNBS-superoxide spin adduct. However, a subsequent study by the same authors showed that the radical was probably the DBNBS spin adduct of the sulphite radical anion, $SO_3^{\bullet -}$.[15] We have found ourselves inadvertently adding to the controversy surrounding DBNBS, because after synthesising a batch of DBNBS for spin trapping purposes we wanted to compare this material to commercially available preparations and, much to our surprise, found that our material differed significantly from some of the commercial preparations. This finding urged us to (1) identify the main compounds present in the investigated mixtures and pure preparations, and (2) determine the purity of the DBNBS in our own preparations and the material from various suppliers. These data presented below illustrate that our own preparations did indeed contain mainly DBNBS, whereas three out of four commercial preparations tested contained mainly sodium 3,5-dibromo-4-nitrobenzenesulphonate (**1**; Fig. 1).

This conclusion is based on extensive spectroscopic and chemical evidence, including FAB-MS, EPR spectrometry, NMR, elemental analysis, HPLC separation, IR spectroscopy, and UV–Vis spectroscopy. We demonstrate that, for all practical purposes, the purity of aqueous DBNBS solutions can be assessed simply by measuring the UV–Vis spectrum of the solution.

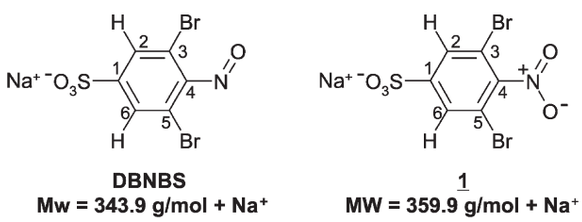

FIGURE 1 The chemical structures of the sodium salts of DBNBS and 3,5-dibromo-4-nitrobenzenesulphonate. 3,5-dibromo-4-nitrobenzenesulphonate is represented by 1. The molecular weight (MW) is given below each structure and the aromatic carbon atoms have been numbered.

EXPERIMENTAL

3,5-Dibromosulphanilic acid, sodium salt (98%), anhydrous sodium acetate (99.995%) and hydrogen peroxide (30%, w/v) were purchased from Sigma-Aldrich Chemical Company (Dorset, England). Acetonitrile (HPLC grade) and glacial acetic acid (99.99 + %) was obtained from Merck Limited (Dorset, UK). DMSO was from Koch-Light Laboratories (Coinbrook, England). DBNBS was obtained commercially from two different suppliers, as four samples, each with a different lot number.

Synthesis of DBNBS

DBNBS was synthesised according to the method of Kaur *et al.*[1] with minor modifications. 3.53 g (10 mmol) 3,5-dibromo-sulphanilic acid, sodium salt and 0.82 g (10 mmol) anhydrous sodium acetate was added to 30 ml glacial acetic acid and 7.9 ml 30% aqueous hydrogen peroxide (78 mmol), and the contents were gently heated until the solution was clear. The solution stood at room temperature for 14 days and was then filtered, except the preparation arising from "synthesis type D" (see below), which was the second crop of crystals after a total reaction time of 28 days at room temperature. In the case of all preparations, the crystals were washed with 5 ml glacial acetic acid. After this, three different washing and crystallisation procedures were used for further purification to produce the preparations A–D (Table I). All samples were then dried for 1 h at 90°C under vacuum, and then overnight at room temperature and atmospheric pressure over P_2O_5. The yields were between 1.1 and 1.2 g (30–33%) and the melting points greater than 300°C. This was similar to the yield (34%) and melting point (>300°C) reported by Kaur *et al.*[1]

DBNBS Characterisation

EPR spectrometry was carried out on a JEOL JES-RE1X spectrometer (JEOL (UK), Welwyn Garden City, England) equipped with an ES-UCX2 cylindrical mode X-band cavity and a JEOL ES-DM1 digital manganese oxide marker. Samples were analysed at room temperature in a WG-LC-11 quartz flat cell (Wilmad Glass, Buena, NJ). The instrument parameters were: Microwave power 4 mW, modulation frequency 100 kHz, modulation width 0.02 mT, sweep width 8 mT, time constant 0.03 s, scan time 120 s, number of data points 8192, receiver gain $1-10 \times 10^3$. The spin trapping efficiency of various DBNBS preparations was calculated from their ability to trap methyl radicals in the following way: A mixture containing 0.34 mg/ml of the preparation,

TABLE I The different procedures used to further purify the batches of DBNBS synthesised (see "Experimental" Section), all of which were found to be spin-trapping-efficient (see "Results" Section). The difference between synthesis types C and D is detailed under "Experimental" Section

Synthesis type (number of independent batches)	Washed with anhydrous diethyl ether	Recrystallised from boiling ethanol	Washed with cold ethanol	Washed with diethyl ether/1,4-dioxane (1:1), then with cold ethanol
A ($n = 4$)	✓	✓	–	–
B ($n = 5$)	✓	–	✓	–
C ($n = 2$)	–	–	✓	✓
D ($n = 2$)	–	–	✓	✓

32 mM DMSO, and 4.8 mM hydrogen peroxide was aspirated into the quartz flat cell (70 μl) and photolysed for 20 s directly on the surface of a 302 nm TM-20 Chromato-Vue Transilluminator (UVP, San Gabriel, CA) set at the high intensity position. The basis of the assay and the main EPR spectrum are described in Fig. 2. The flat cell was inserted into the EPR cavity in a reproducible manner, in order to preserve the cavity Q without the need to retune.[16] Acquisition of the EPR spectrum took place at room temperature and started 120 s after UV irradiation had commenced. The concentration of the spin adduct was quantified from the height from the second positive peak of the third multiplet to the next negative peak. All measurements were done in duplicate, according to a randomised scheme. Control experiments were performed on (1) DBNBS samples alone, (2) DBNBS samples with dimethyl sulphoxide (DMSO) and hydrogen peroxide but without light, and (3) DBNBS samples with light but without DMSO and hydrogen peroxide. No EPR signal was detected in any of the controls. The EPR signal was identified as the methyl radical adduct of DBNBS, on the basis of previous assignments of this radical.[1,13,14]

Further experiments indicated that the assay was approximately linear in spin trap concentration and irradiation time (data not shown). *g*-Values were measured relative to the EPR signals from the manganese marker, assuming that the *g*-value of the point midway between the third and fourth signal of the manganese sextet was 2.00715.

FAB-MS was carried out on a Kratos MS890MS instrument with a Kratos DS90 data system by the Mass Spectrometry Department, Kings College London, UK. The operating conditions were: accelerating voltage, 4 kV; scan speed, 3 s per decade; calibration range, 46–1073 g/mol. The FAB gas was xenon, at 8 kV/2 mA, and the sensitivity was 4.5. The spectrometer was operating in the negative ion mode, using 3-nitrobenzyl alcohol (NOBA) as matrix.

^{13}C and ^{1}H NMR spectra were recorded on a JEOL 8-400 spectrometer using a 5 mm broad band probe. Samples were run in D_2O. IR spectra were recorded on a Perkin-Elmer model 983G instrument coupled to a Perkin-Elmer 3700 data station. Spectra were recorded using diffuse reflectance from a ground mixture of the sample and KBr. UV–Vis spectroscopy was carried out on the different batches of DBNBS. Aqueous solutions containing 0.08 mg/ml DBNBS were placed in 1 cm quartz cells and analysed on a Kontron Uvikon 860 UV double beam spectrophotometer (180–500 nm). Spectra were corrected for the absorbance of the solvent.

Components were separated by reverse-phase HPLC on a Hypersil ODS column (250 mm × 4.6 I.D. × 5 μm). The mobile phase used consisted of acetonitrile and 25 mM sodium acetate/acetic acid buffer (pH 4.0; 20:80, v/v). The flow rate was 1.0 ml/min. UV detection was at 280 nm. Each batch of DBNBS was dissolved in deionised water at a concentration of 0.4 mg/ml, and 20 μl sample was injected. A standard of the starting material (3,5-dibromosulphanilic acid, sodium salt) was also prepared at the same concentration.

Statistical Analysis

The SAS System for Windows, release 6.11 or 6.12 (SAS Institute, Cary, NC, 1996) was used for all statistical calculations. If analysis of variance (ANOVA) was significant at the 5% level, the differences were assessed by Tukey–Kramer's test (more than two groups)[17] or by Student's *t*-test (two groups). Measurements shown in the tables are stated as means and 95% confidence intervals.

RESULTS

Spin Trapping Ability of Different DBNBS Preparations

Thirteen batches of DBNBS were synthesised according to the method described by Kaur *et al.*[1] These batches of DBNBS were sub-divided into four types (A, B, C, D) according to the purification procedure used (Table I). These samples of DBNBS, together with the commercial preparations, were

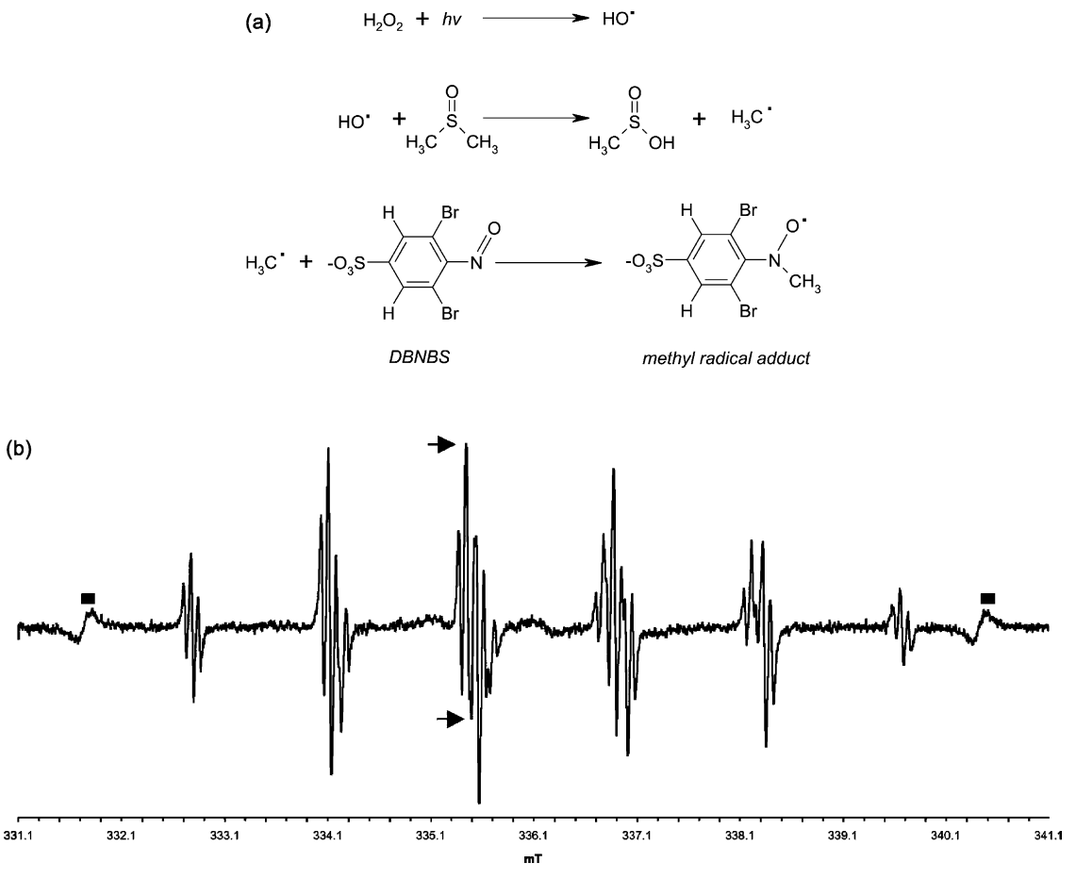

FIGURE 2 Assay used for the determination of spin trapping efficiency. The reactions leading to the production of the methyl radical adduct of DBNBS are shown in (a). See "Experimental" Section for details of the experimental conditions. A representative EPR spectrum of the methyl radical adduct of DBNBS is shown in (b). The DBNBS sample had been synthesised by method C. The EPR signal of the methyl radical adduct of DBNBS shown has $a_N = 1.443$ mT, $a_{CH_3} = 1.351$ mT (3H), $a_{m-H} = 0.074$ mT (2H), and $g = 2.0063$. The peak used for the quantification of the DBNBS spin trapping efficiency is indicated by the two arrows. The signal from the manganese marker is marked with shaded boxes. See "Experimental" Section for the EPR parameters used.

assessed for their efficiency in spin trapping the methyl radical (Fig. 2). All the samples gave the authentic methyl radical adduct of DBNBS (Fig. 2).[18] However, our DBNBS batches and one of the commercial preparations (collectively referred to as "spin-trapping-efficient" samples in the remainder of this paper) differed markedly from the other commercial samples (referred to as the "spin-trapping-inefficient" samples): the spin-trapping-efficient samples had a very large EPR spin trapping efficiency compared to the spin-trapping-inefficient samples, which had almost no spin trapping ability (Table II). In order to ascertain the chemical basis for this difference we analysed the purity and chemical structure of each preparation, using reverse-phase HPLC, FAB-MS, NMR spectrometry, IR spectroscopy and UV–Vis spectroscopy. We compared the results of the group of spin-trapping-efficient samples with the group of spin-trapping-inefficient samples.

Identification and Characterisation of the Main Compounds in the Spin-trapping-efficient and Spin-trapping-inefficient Samples of DBNBS

Fast Atom Bombardment-mass Spectrometry

Figure 3 shows typical FAB-MS spectra of a spin-trapping-efficient and a spin-trapping-inefficient sample, respectively. In Fig. 3(a) a prominent peak is apparent at m/z 344, corresponding to the mass of the 3,5-dibromo-4-nitrosobenzenesulphonate anion (m/z 343.9) in the spin-trapping-efficient samples. Furthermore, the three peaks at m/z 342, 344 and 346 have an intensity ratio of approximately 1:2:1, as expected for a compound containing two bromine atoms (the natural abundance of ^{79}Br and ^{81}Br is 50.54 and 49.46%, respectively). By contrast, the spin-trapping-inefficient sample (Fig. 3(b)) exhibits a very weak peak at m/z 344 but has three new peaks at m/z 358, 360 and 362 (1:2:1), which are barely present in Fig. 3(a).

TABLE II Physico-chemical properties of spin-trapping-efficient and spin-trapping-inefficient preparations of DBNBS—the results of the EPR spectrometric analysis

Sample type	EPR spin trapping efficiency of stored solutions (arb. units)	EPR spin trapping efficiency of freshly made solutions (arb. units)
Spin-trapping-efficient	2440 ± 350	2880 ± 190
Spin-trapping-inefficient	0 ± 750	240 ± 400

DBNBS batches were grouped according to those with high EPR spin trapping efficiency (spin-trapping-efficient samples) and those with little or no spin trapping ability (spin-trapping-inefficient samples). Statistical analysis was performed on the mean values of the duplicate measurements. Stored solutions of DBNBS had been kept for 2 months at room temperature. Number of spin-trapping-efficient samples analysed = 14; number of spin-trapping-inefficient samples analysed = 3. All measurements were performed in duplicate.

NMR and IR Spectroscopy

The results from the ^{13}C-NMR spectroscopy provide two pieces of information. Firstly they show that the major component in the spin-trapping-efficient and -inefficient samples is a different compound, as the chemical shift values are different for the two groups of samples (Table III).

Secondly, the major compound in both the spin-trapping-efficient and -inefficient samples is symmetrically substituted (i.e. identical substituents are attached to C-3 and C-5, and identical substituents are attached to C-2 and C-6), as only four peaks were detected in the ^{13}C-NMR spectra. The peaks were assigned as follows: C-3 and C-5, 110–120 ppm; C-2 and C-6, 130–140 ppm; C-1, 140–147 ppm; and C-4,

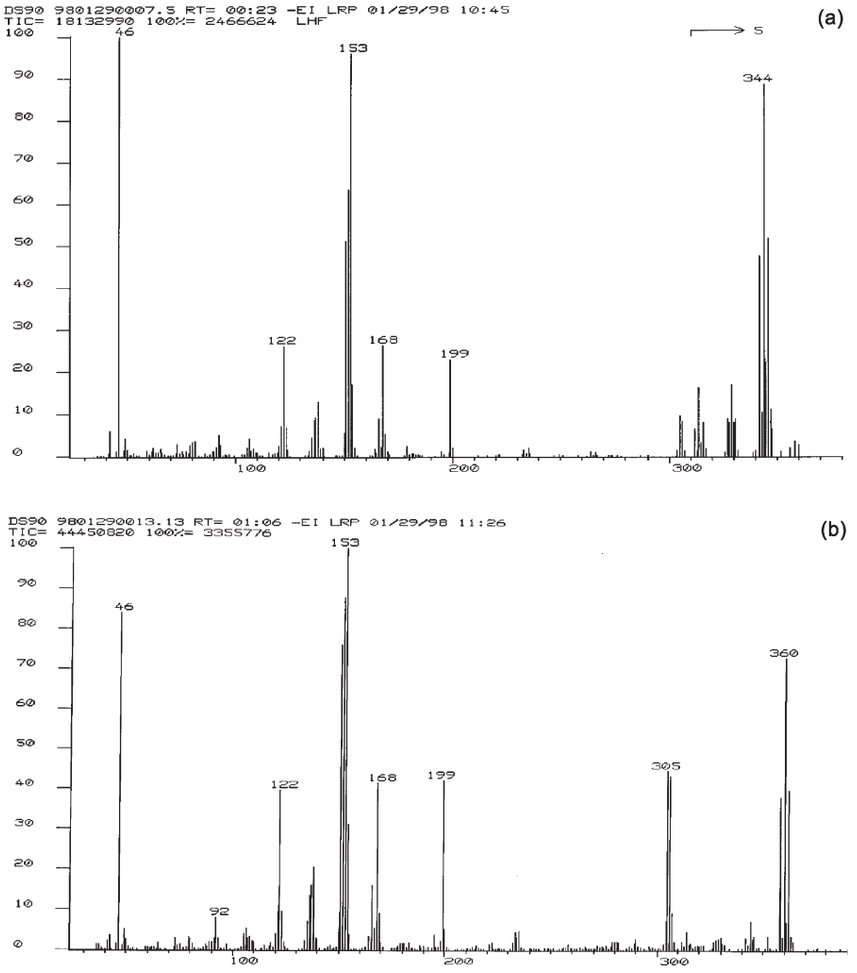

FIGURE 3 Representative FAB-MS spectra of DBNBS samples. The mass spectra shown in (a) and (b) represent the spectrum of a spin-trapping-efficient sample (purification method B) and a spin-trapping-inefficient sample, respectively. The peaks of interest are present at m/z 344 and m/z 360. The peaks at m/z 46, 122, 153, 168, 199 and 306 correspond to the interaction between the NOBA matrix and sodium ions (from NaI). The peaks at m/z 327, 329 and 331 (1:2:1) are probably due to unreacted starting material (3,5-dibromosulphanilate anion, m/z 329.9).

TABLE III Physico-chemical properties of spin-trapping-efficient and spin-trapping-inefficient preparations of DBNBS—results for analysis by ^{13}C NMR spectrometry

	Chemical shift of ^{13}C-NMR peak (ppm)			
Sample type	110–120 ppm	130–140 ppm	140–147 ppm	147–160 ppm
Spin-trapping-efficient	119.5 ± 0.3	131.5 ± 0.3	141.4 ± 0.4	148.0 ± 0.4
Spin-trapping-inefficient	114.0 ± 0.4	130.6 ± 0.4	146.5 ± 0.4	152.5 ± 0.5

Number of spin-trapping-efficient samples analysed = 4; number of spin-trapping-inefficient samples analysed = 3.

147–160 ppm. Figure 1 shows how the different carbon atoms in DBNBS are numbered.

IR spectra obtained from the spin-trapping-efficient and -inefficient samples contained different bands as shown in Table IV, suggesting that the main compounds in the two sample types had different functional groups. The major difference in the IR data was the presence of a strong band at 1281 cm^{-1} in the spin-trapping-efficient samples but not in the inefficient samples. A strong absorption band in the range 1280–1290 cm^{-1} is indicative of an aromatic C-nitroso trans dimer. This suggests that the spin-trapping-efficient samples contain DBNBS in the form of a trans dimer, in agreement with Kaur *et al.*[1] who reported that approximately 98% DBNBS exists in the dimeric state.

HPLC Analysis

HPLC analysis of the spin-trapping-efficient and -inefficient samples confirmed that the main compound in these two groups of samples was different, as two separate main peaks could be identified on the HPLC chromatograms (Table V). The main peak obtained from the spin-trapping-efficient samples had an elution time of about 15 min, whilst the main peak obtained from the spin-trapping-inefficient samples had an elution time of about 18 min. In all samples analysed, a peak was observed at about 4 min that corresponded to the starting material in the synthesis, 3,5-dibromosulphanilic acid. The FAB-MS data demonstrated that the main compound in the spin-trapping-efficient samples was DBNBS. This suggests that the peak eluting at approximately 15 min corresponded to DBNBS, as this was the major peak present in all the HPLC chromatograms of the spin-trapping-efficient samples. The main peak that eluted at approximately 18 min, in the HPLC chromatograms of the spin-trapping-inefficient samples, may therefore be assigned to the compound with an m/z of 360. In one of the spin-trapping-inefficient samples both HPLC peaks (15 and 18 min) could be detected. However, in the other two spin-trapping-inefficient samples tested the peak at 15 min was absent, indicating that these samples did not contain any detectable DBNBS. The mean HPLC peak height of the DBNBS peak at 15 min was significantly higher in the spin-trapping-efficient samples than in the spin-trapping-inefficient samples, showing that the efficient samples contained high levels of DBNBS compared with the inefficient samples.

TABLE IV Physico-chemical properties of spin-trapping-efficient and spin-trapping-inefficient preparations of DBNBS—results of analysis by IR spectrometry

	Position of IR peak (cm^{-1})	
Sample type	1540–1560 cm^{-1}	1280–1290 cm^{-1}
Spin-trapping-efficient	^{m}1552.8 ± 0.4	^{s}1281.5 ± 0.7
Spin-trapping-inefficient	^{s}1544.7 ± 0.9	Absent

Number of spin-trapping-efficient samples analysed = 13; number of spin-trapping-inefficient samples analysed = 3. ^{m}Medium IR absorption band and ^{s}strong IR absorption band.

UV–Vis Spectroscopy

The UV–Vis absorbance scans (200–500 nm) of the spin-trapping-efficient and -inefficient samples were very different, indicating that the main compound in the two groups of samples was different. The spin-trapping-efficient samples exhibited a λ_{max} at 308 nm, whilst in the spin-trapping-inefficient samples this peak was replaced by a much less intense absorbance with a λ_{max} at about 280 nm. The UV–Vis data suggest that the absorbance maximum at 308 nm is characteristic of DBNBS, as this is present in all the spin-trapping-efficient samples, and absent in the majority of spin-trapping-inefficient samples. Table V shows the differences in the mean absorbance at 308 nm in the UV–Vis spectra of the spin-trapping-efficient and -inefficient samples. The spin-trapping-efficient samples had A_{308}/A_{280} ratios of 1.8 or greater. These data demonstrate that UV–Vis spectrometry can be used to determine quickly and easily whether DBNBS has been successfully synthesised.

Effect of Purification

Several experimental parameters were systematically measured for each of the 13 DBNBS batches synthesised. These included the measurement of UV absorbance (λ_{max}), HPLC peak height and EPR spin trapping efficiency in solutions stored for two months at room temperature. Statistically significant

TABLE V Physico-chemical properties of spin-trapping-efficient and spin-trapping-inefficient preparations of DBNBS—results from the analysis of the different batches of DBNBS by UV–Vis spectroscopy and HPLC with UV–Vis detection

Sample type	UV–Vis absorbance at 308 nm	Absorbance at 280 nm of HPLC peak eluting at ~15 min	Retention time of main HPLC peak (min)
Spin-trapping-efficient	0.78 ± 0.10	0.058 ± 0.007	15.24 ± 0.14
Spin-trapping-inefficient	0.36 ± 0.21	0.006 ± 0.015	18.50 ± 0.32

The absorbance at 308 nm was obtained by UV-vis scanning spectrophotometry of an aqueous solution of each entire DBNBS preparation, whilst the absorbance value at 280 nm refers to a different analytical procedure involving UV detection of a single HPLC peak corresponding to DBNBS. For details of the analysis procedures, see the "Experimental" Section. Number of spin-trapping-efficient samples analysed = 14; number of spin-trapping-inefficient samples analysed = 3. All measurements were performed in duplicate.

differences are shown in Table VI. Notably, DBNBS types C and D had a significantly larger height of the main HPLC peak than types A and B. Furthermore, stored DBNBS solutions of type D had a significantly higher spin trapping efficiency than type B, while type C had a significantly higher absorbance at 308 nm than type B. Taken together, these observations suggest that samples C and D had a higher content of DBNBS than samples A and B.

DISCUSSION

Initially the EPR spectrometry results suggested that the spin-trapping-efficient samples contained authentic DBNBS, whereas the spin-trapping-inefficient samples contained a compound that did not react with methyl radicals to form stable spin adducts. Results from mass spectrometry confirmed these findings, by showing that the main component in the spin-trapping-efficient samples had a different molecular ion mass compared to the main component in the spin-trapping-inefficient samples. The molecular ion present at m/z 344 in the spin-trapping-efficient samples has the same molecular ion mass as the anion of DBNBS. It is concluded that the spin-trapping-efficient samples contained authentic DBNBS. One of the spin-trapping-efficient samples (synthesis type C, Table I) was also used successfully in a recent study[6] of the reaction of DBNBS with nitric oxide to produce a stable free radical product.

The main peak of interest in the MS spectra of the spin-trapping-inefficient samples had a mass of m/z 360. Other smaller peaks in this group at m/z 358 and 362 confirm the presence of two bromine atoms, as expected for DBNBS. However, the molecular weight of this ion is 16 atomic mass units higher than that expected for DBNBS, suggesting it corresponds to the DBNBS anion plus an extra oxygen atom. Unless extensive rearrangements have taken place, the number of possible products are limited to two: (a) the nitro derivative (**1**) formed by oxidation of the nitroso group or (b) the phenol derivative (**2**) formed by oxidation of the ring in the 2- or 6-position (Fig. 4).

The unsymmetrical phenol derivative (**2**) could be excluded on the basis of the ^{13}C NMR spectrometry, which demonstrated that both the spin-trapping-efficient and -inefficient samples contained compounds which were symmetrically substituted. However, these data are consistent with the main compound in the spin-trapping-inefficient samples being the nitro derivative, as it is symmetrically substituted in the C2 and C6, and the C3 and C5 positions. Furthermore, the nitro compound was expected to be devoid of spin trapping activity, in agreement with the EPR results. The IR spectroscopy data also confirmed these findings, as the IR absorption indicative of a trans dimer of an aromatic nitroso compound (a strong band at 1280–1290 cm^{-1}) was absent in the spin-trapping-inefficient samples.[19] These IR data confirm that the main compound in the spin-trapping-efficient samples is DBNBS.

The mechanism of the unwanted oxidation of DBNBS to the nitro compound in the spin-trapping-inefficient samples is of interest. The oxidation could take place either in solution or in the solid state of the compound. It is suspected that the commercial,

TABLE VI Statistically significant differences between sample types A–D, as revealed by different analytical procedures: UV–Vis scanning spectrophotometry, HPLC and EPR spectrometry

Sample type	UV absorbance at 308 nm	Absorbance at 280 nm of HPLC peak eluting at ~15 min	EPR spin trapping efficiency (arb. units)
A ($n = 4$)	[a,b]0.75 ± 0.15	[b]0.054 ± 0.008	[a,b]2460 ± 530
B ($n = 5$)	[b]0.64 ± 0.14	[b]0.048 ± 0.007	[b]1850 ± 470
C ($n = 2$)	[a]1.02 ± 0.22	[a]0.077 ± 0.011	[a,b]2870 ± 750
D ($n = 2$)	[a,b]0.94 ± 0.22	[a]0.074 ± 0.011	[a]3300 ± 750
Significance level	$p = 0.0289$	$p = 0.0014$	$p = 0.0201$

The synthesis procedures for the production of samples A–D are specified in Table I. Measurements are stated as 95% confidence intervals. Different superscript letters indicate significantly different measurements ($p < 0.05$). Statistical analysis was carried out as described in the "Experimental" Section. Values labelled "a" are significantly different from values labelled "b". Values labelled "a,b" are not significantly different from values labelled either "a" or "b". EPR spectrometry was performed on solutions of DBNBS that had been stored two months at room temperature.

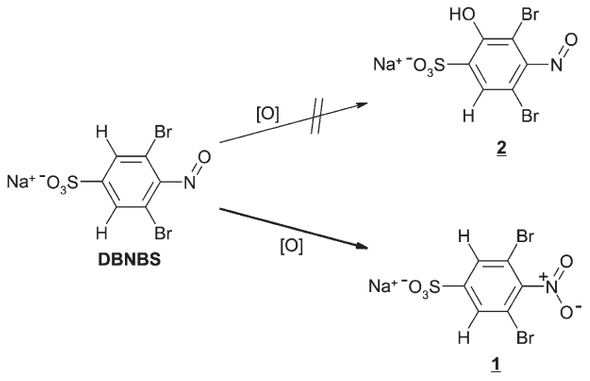

FIGURE 4 Possible chemical structures of the compound formed on addition of a single oxygen atom to DBNBS.

spin-trapping-inefficient samples have not been manufactured strictly according to the procedure of Kaur *et al.*[1] and have been oxidised in solution, for the following two reasons: (1) In general, the oxidation of aromatic amines by peroxides yields a complicated mixture of nitroso and nitro products, and it is therefore only rarely of any synthetic relevance.[20] It is obvious that the procedure of Kaur *et al.* [1] is an exception to this rule, since the pure nitroso compound is isolated. However, the above statement[20] implies that any attempt to accelerate the reaction or change the conditions in any other way may alter the course of the reaction significantly, e.g. towards the formation of the nitro compound. The statement also implies that peroxides or other strong oxidants must be present to effect the oxidation, that is, the oxidation to the nitro compound probably occurs in solution before purification of the product. (2) By contrast, solid DBNBS samples that have been manufactured in strict accordance with the procedure of Kaur *et al.* have been stored at room temperature in the authors' laboratory for more than 10 years without any notable deterioration of spin trapping ability (data not shown). This demonstrates the excellent stability of pure DBNBS in the solid state, so it is unlikely that DBNBS is oxidised in the dry state. It is therefore suggested that manufacturers of DBNBS adhere strictly to the procedure published by Kaur *et al.* without any alterations other than the improved purification procedure described in the present paper.

HPLC and UV spectroscopy provided good methods for assessing the authentic DBNBS content in the spin-trapping-efficient and -inefficient samples. In particular, the distinctive UV spectrum of DBNBS provided a quick and simple way of verifying the presence of authentic DBNBS, using $A_{308}/A_{280} \geq 1.8$. These HPLC and UV spectroscopy data in combination with the EPR spectrometry results demonstrated that the extra washing procedures performed on batches C and D (Table I) improved the purity of the DBNBS synthesised. It is therefore recommended that the DBNBS crystals should be washed with diethyl ether/1,4-dioxane (1:1) and then with cold ethanol in order to obtain a product with optimum spin trapping capability. The first and second crop of DBNBS crystals from the mother liquor are both of high purity.

In summary, the results presented here show that a significant proportion of commercially available DBNBS batches have poor spin trapping properties, as a result of their low content of authentic DBNBS. It is recommended that researchers synthesise their own DBNBS, which is a relatively simple procedure, or that purchasers of DBNBS use one or more of the analysis methods described here to validate the chemical composition of commercial preparations before attempting EPR spin trapping experiments.

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