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The Use of a Nitroxide Probe in DMSO to Capture Free Radicals in Particulate Pollution

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A profluorescent nitroxide was used to evaluate the oxidative potential of pollution derived from a compression ignition engine fuelled with biodiesel. The reaction products responsible for the observed fluorescence increase when a DMSO solution of nitroxide was exposed to biodiesel exhaust were

Introduction

Pollution-derived particulate matter (PM) is widely recognized as impacting significantly on human health,^[1,2] particularly in the areas of respiratory and cardiovascular disease.^[3–7] The underlying toxicological mechanisms by which particles induce adverse health effects are complex; however, central to these mechanisms is the ability of inhaled PM to induce cellular oxidative stress and promote inflammatory responses within tissues.^[8] PM contains and/ or is able to generate free radicals and related reactive oxygen species (ROS), which result in oxidative stress at the sites of deposition, thereby triggering a cascade of events associated with inflammation and, at higher concentrations, cell death.

The direct detection of free radicals and ROS in PM is limited by their short half-life, low concentration, and high reactivity. Recently, we pioneered a new approach that uses an extremely sensitive profluorescent nitroxide,^[9] 9-(1,1,3,3tetramethyliosindoline-2-oxyl-5-ethynyl)-10-(phenylethynyl)anthracene (1),^[10] to assess the oxidative potential of all types of particulate pollution. The advantages of this

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determined by using HPLC/MS. The main fluorescent species was identified as a methanesulfonamide adduct arising from the reaction of the nitroxide with DMSO-derived sulfoxyl radicals.

method are that it facilitates the direct measurement of particulate-derived free radicals and related ROS during sampling (without relying on post-exposure derivatization) and allows relatively unstable redox products (such as hydroxylamine **2**) to be detected. We have used profluorescent nitroxide **1** to detect free radicals and related ROS derived from PM in cigarette smoke,^[11] biomass combustion in a pellet boiler and logwood stove,^[12] and a compression ignition engine using ethanol fumigation technology^[13] or diesel alternative fuels.^[14,15] This probe displays only weak fluorescence due to the presence of the nitroxide radical, but exhibits a steady increase in fluorescence emission upon exposure to various sources of PM.



To date, however, the specific chemical species responsible for the observed fluorescence increase upon exposure of 1 to PM has not been identified. Herein we describe the development of a high-performance liquid chromatography/ mass spectrometry (HPLC/MS) method and its use to identify the fluorescent products that result upon exposure of profluorescent nitroxide 1 to PM derived from a compression ignition engine using biodiesel. We can now report that the reaction of 1 in DMSO with biodiesel exhaust gives predominately methanesulfonamide 3, rather than the expected methoxyamine 4 or hydroxylamine 2.^[16] Further-

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more, we have discovered a hitherto unrecognized fragmentation of DMSO in the presence of biodiesel exhaust.

Results and Discussion

To validate the use of HPLC/MS as a method for the detection of particulate-derived adducts of 1, we first obtained reverse-phase (RP) HPLC retention times and high-resolution mass spectrometry (HRMS) data for nitroxide 1, methoxyamine 4, and acetoxyamine 5 (Table 1, Entries 1–3). We also formed hydroxylamine 2 (a potential fluorescent reaction product) by treating 1 with hydrazine hydrate in DMSO. The formation of 2 was confirmed by HPLC ($t_R = 6.3 \text{ min}$) and HRMS ($m/z = 492.2313 \text{ [M + H]}^+$; Table 1, Entry 4). Methoxyamine 4 ($t_R = 10.6 \text{ min}$) was also produced upon reaction of 1 with hydrazine hydrate in DMSO (see Supporting Information).

Our previous sampling of PM from various combustion sources was undertaken by bubbling generated aerosol into an impinger^[17] containing a solution of **1** in DMSO to avoid solvent loss and subsequent variations in fluorescence measurements. However, the majority of PM sampling in this field is undertaken by collecting particles on a filter (for practicality and efficiency reasons) and often involves subsequent extraction by sonication into a solvent.^[18] Thus, we next investigated the effect of sonication on a solution of **1** in DMSO by using our HPLC/MS method.

Interestingly, we observed two peaks in the RP HPLC chromatogram after sonication of a 10-mM solution of **1** in DMSO for 60 min (Table 1, Entry 5). The first component at 4.2 min was assigned by electrospray ionization mass spectrometry (ESI-MS) to secondary amine **6**, but the second component at 5.5 min did not give an identifiable ion by ESI-MS. Its retention time was similar to that of nitroxide **1** (at 5.2 min); however, it was substantially more fluorescent in the HPLC trace with fluorescence detection ($\lambda_{ex} = 430$ nm, $\lambda_{em} = 485$ nm; see Supporting Information).

To identify this new product, sonication of 1 was performed on a preparatory scale (20 mg). Methanesulfonamide 3 and methanesulfinamide 7, which both gave retentions at 5.5 min following analysis by RP HPLC, were isolated in yields of 28 and 21%, respectively. The structures of these products were confirmed by NMR spectroscopy (see Supporting Information) and mass spectrometry (Table 1, Entry 5). Further evidence for the structure of 3 was provided by the observed strong S=O asymmetric and symmetric stretching frequencies at 1316 and 1142 cm⁻¹ in the infrared (IR) spectrum. One plausible mechanism for the formation of methanesulfonamide 3, secondary amine 6, and methanesulfinamide 7 upon sonication of 1 in DMSO is shown in Scheme 1. Sonication is known to cause



Scheme 1. Proposed mechanism for the formation of **3**, **6**, and **7** from the sonication of **1** in DMSO.

Table 1. Identification of adducts of nitroxide 1 by using HPLC/MS.

Entry	Sample	HPLC retention (min) ^[a]	ESI-MS obtained	Assignment
1	1	5.2	491.2252	$1 (m/z = 491.2249 [M + H]^+)$
2	4	10.6	506.2476	4 $(m/z = 506.2484 [M + H]^+)$
3	5	6.2	534.2430	5 $(m/z = 534.2433 [M + H]^+)$
4	$1 + NH_2NH_2 H_2O$	6.3	492.2313	2 $(m/z = 492.2327 [M + H]^+)$
5	1 + sonication ^[b]	4.2	476.2375	6 $(m/z = 476.2378 [M + H]^+)$
		5.5	553.2074 ^[f]	$3 (m/z = 553.2076 [M]^+)$
		5.5	475.2302 ^[g]	$7 (m/z = 475.2300 [M + H^+ - S(O)CH_3])$
6	$1 + H_2O_2$	4.2	_	6 ^[h]
	2 2	5.5	_	$3^{[h]}$ and $7^{[h]}$
7	$1 + AAPH^{[c]}$	14.7	_	10
8	$1 + AAPH^{[d]}$	5.4	_	4 ^[h]
		6.4	_	$3^{[h]}$ and $7^{[h]}$
9	1 + PM (biodiesel) ^[e]	5.2	554.2 ^[i]	$3 (m/z = 554.2 [M + H]^+)$

[a] At 430 nm using a C18 column in 60% THF/40% water at 1 mL/min. [b] Sonication of a 10-mM solution of 1 in DMSO at 460 Hz for 60 min. [c] Thermolysis of 2,2'-azobis(2-methylpropionamidine) dihydrochloride under an Ar atmosphere. [d] Thermolysis of 2,2'-azobis(2-methylpropionamidine) dihydrochloride in air. [e] 100% soy diesel at half load with 4-μM solution of 1. [f] EI-MS on sample isolated from preparatory scale reaction. [g] ESI-MS on sample isolated from preparatory scale reaction. [h] Assignment based on HPLC retention time of authentic sample. [i] Obtained by using a photospray photoionization source.

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cleavage of DMSO to give sulfoxyl (CH₃SO[•]) and methyl ([•]CH₃) radicals.^[19] The radical trapping reactions of sulfoxyl (CH₃SO[•]) radicals with nitroxides have been previously established^[20,21] and proceed through rearrangement of the initial methanesulfinate adduct **8** to give sulfonamide **3**. Aminyl radical **9** can also add to DMSO to form the observed methanesulfinamide **7** in an analogous mechanism to the reactivity of hydroxy radicals.^[16] Secondary amine **6** could result following hydrogen abstraction by aminyl radical **9**. Secondary amines have been detected in materials where nitroxides are involved in radical scavenging reactions,^[22,23] but in this context, where low (ambient) operating temperatures and low (μ M) concentrations are involved, these scavenging reactions are unlikely to operate.^[24]

The isolation of DMSO adducts of nitroxide **1** demonstrates for the first time that radicals formed during the sonication of DMSO can be captured by using a profluorescent nitroxide. Moreover, this finding indicates that a cautious approach should be taken when detecting reactive oxygen species with other probes (such as 2',7'-dichlorofluorescein diacetate: DCFH-DA) by sonication in DMSO or other solvents known to produce radicals when sonicated.

To further explore the chemistry behind the observed fluorescence increase arising from exposure of nitroxide 1 to various sources of PM, we examined reactions between 1 and several model compounds that are common constituents of aerosol pollution and analyzed the results by HPLC/ MS. The reaction of nitroxide 1 with hydrogen peroxide in DMSO produced two main products following analysis by HPLC (Table 1, Entry 6). These were assigned as secondary amine 6 and DMSO adducts 3 and 7 on the basis of their retention times and substantial fluorescence (as no identifiable ions could be obtained by ESI-MS for these compounds). Resulting products 3 and 7 are presumably formed from the reaction of 1 with sulfoxyl (CH₃SO[•]) radicals through the same mechanism as that shown in Scheme 1. In this case, sulfoxyl (CH₃SO[•]) radicals are derived from methanesulfinic acid, which is obtained along with 'CH₃ when hydroxy (HO[']) radicals react with DMSO.^[19]

Reaction of 1 with alkyl radicals produced by the thermolysis of 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) under anaerobic conditions gave a new fluorescent product (assumed to be adduct 10) with a retention time of 14.7 min as well as unreacted nitroxide 1 (Table 1, Entry 7). The reaction of peroxyl radicals (produced by the thermolysis of AAPH under aerobic conditions) with 1 gave two major components after analysis by HPLC (Table 1, Entry 8). These products were assigned as hydroxylamine 2 and DMSO adducts 3 and 7 on the basis of their retention times and strong fluorescence.^[25] Thus, the nitroxide reacted to form fluorescent products when treated with high concentrations of hydrogen peroxide, peroxyl radicals, or carbon-centered radicals.

Following the development of our HPLC/MS method and insight into the reactivity of probe molecule **1** towards common aerosol constituents, we investigated the use of this approach to detect free radicals and related ROS in PM derived from a compression ignition engine fuelled with biodiesel. Aerosol generated from a compression ignition engine employing 100% soy diesel at half-load was bubbled into an impinger containing a solution of nitroxide 1 in DMSO (without sonication). The fluorescence increased linearly over 60 min by spectrofluorimetry (no increase in fluorescence was observed for an untreated solution of 1 in DMSO over the same time period). Analysis of the sample by HPLC/MS gave a surprisingly simple chromatogram with a major component at 5.2 min, which was significantly more fluorescent than starting nitroxide 1 (Table 1, Entry 9; Figures 1 and 2). Notably, only very small amounts of methoxyamine 4 were observed in the HPLC trace. The mass spectrum of the major component at 5.2 min gave a



Figure 1. HPLC chromatograms from the reaction of nitroxide 1 (4 μ M in DMSO) with particulate matter derived from a compression ignition engine employing biodiesel: (a) absorbance at 430 nm, (b) fluorescence detection $\lambda_{ex} = 430$ nm, $\lambda_{em} = 485$ nm.



Figure 2. HPLC chromatograms of nitroxide 1 (10 mM in DMSO): (a) absorbance at 430 nm, (b) fluorescence detection $\lambda_{ex} = 430$ nm, $\lambda_{em} = 485$ nm.

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strong signal at m/z = 554.2 when a photospray photoionization source was used, which corresponds to methanesulfonamide **3** ([M+ H]⁺, Figure 3). An authentic sample of methanesulfonamide **3** (isolated from the sonication of **1** in DMSO) gave an identical mass spectrum with the photoionization source.



Figure 3. Photoionization (+ mode) mass spectrum of the major HPLC component (at 5.16 min) from the reaction of nitroxide 1 (4 μ M in DMSO) with particulate matter derived from a compression ignition engine employing biodiesel.

The species giving rise to the observed fluorescence increase upon exposure of profluorescent nitroxide 1 to PM derived from a compression ignition engine fuelled with biodiesel has now been identified as methanesulfonamide 3. Presumably, the free radicals and related ROS associated with the generated PM can fragment DMSO in a similar manner to that observed during sonication, although the mechanism by which this occurs is currently unclear. In this case, however, the temperature increases associated with sonication are not a factor, as the engine exhaust is cooled to ambient temperature before exposure to nitroxide 1 in DMSO. Moreover, engine exhaust with particles removed does not increase the fluorescence of the DMSO solution of nitroxide 1.

Conclusions

We have identified DMSO adduct methanesulfonamide **3** as the main fluorescent species resulting from the reaction of aerosol-derived biodiesel exhaust with a solution of **1** in DMSO by using HPLC/MS. The fluorescence increase previously observed in our work from the exposure of nitroxide **1** to other pollution sources presumably results from the presence of DMSO-related adducts such as methanesulfonamide **3**. The mechanism by which biodiesel-derived PM interacts with DMSO is currently unclear; however, it is interesting to note that the same product was generated upon exposure of nitroxide **1** to high concentrations of hydrogen peroxide and peroxyl radicals and upon sonication of nitroxide **1** in DMSO. This is also the first example of

the use of a profluorescent nitroxide to detect radicals generated through DMSO sonication. These results will improve our ability to quantify free radicals and related ROS derived from diesel/biodiesel exhaust and are an important step towards our goal of *directly* detecting free radicals and related ROS in PM to aid future improvements in air quality.

Experimental Section

Reaction of Model Aerosol Compounds with Compound 1

Sonication: A solution (10 mL) of 9-(1,1,3,3-tetramethylisoindolin-2-yloxyl-5-ethynyl)-10-(phenylethynyl)anthracene (1, 10 mM in DMSO) was sonicated in an Elma T460H ultrasonic bath and the fluorescence was measured every 5 min by using a spectrofluorimeter. After 60 min, the fluorescence no longer increased and the reaction mixture was analyzed by HPLC/MS.

Hydrogen Peroxide: Hydrogen peroxide (1.0 mL, 70% aqueous solution) was added to a solution of nitroxide 1 (10 mM in DMSO, 10 mL). The solution was stirred at room temperature for 30 min and then analyzed by HPLC/MS.

2,2'-Azobis(2-methylpropionamidine) Dihydrochloride (AAPH)

Aerobic: 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (0.45 g, 1.66 mmol) was added to a quartz cuvette containing a solution of nitroxide **1** (10 mM in DMSO, 15 mL). The cuvette was heated in thermostatted water at 38.5 ± 0.5 °C for 30 min and then the solution was analyzed by HPLC/MS.

Anaerobic: 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (0.45 g, 1.66 mmol) was added to an argon-filled quartz cuvette containing a degassed solution of nitroxide 1 (10 mM in DMSO, 15 mL). The solution was heated in thermostatted water at 38.5 ± 0.5 °C for 30 min and bubbled with argon for the duration of heating. After cooling to room temperature, the reaction mixture was analyzed by HPLC/MS.

Procedure for Biodiesel Sampling: The sample was collected by bubbling the aerosol through an impinger containing a solution of nitroxide 1 (4 µm in DMSO, 20 mL). Custom-made impingers were used, with sintered porosity grade 1 (pore size of 100-160 µm) and a Quickfit dreschel bottle head was modified to fit a Quickfit 75mL test tube. Experiments were conducted on a common rail 6cylinder Cummins Euro 3 diesel engine at the QUT Biofuel Engine Research Facility (BERF). An aerosol stream was generated by employing 100% soy diesel at half-load at 1.0 L/min for 60 min. Impingers were placed after the two-stage dilution system (see Supporting Information for set-up). Fluorescence measurements were taken after 20, 40, and 60-min intervals from both the test sample and a HEPA-filtered control sample. After 60 min, the samples were analyzed by HPLC/MS. An increase in fluorescence upon exposure to biodiesel was normalized with respect to particulate matter (PM) mass. PM mass was measured by using a TSI 8520 (see Supporting Information). The actual mass readings from the Dust-Trak were converted into a gravimetric measurement by using the tapered element oscillating microbalance to DustTrak correlation for DPM obtained by Jamriska.^[26] The amount of particles remaining in the impingers post-sampling was measured, taking into account the impinger collection efficiency correction factor.^[17]

Supporting Information (see footnote on the first page of this article): Experimental procedures and characterization data for compounds **3**, **5**, and **7**; ¹H NMR and ¹³C NMR spectra and HPLC

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chromatograms for compounds **3**, **5**, and **7**; HPLC chromatograms and MS data from the reactions of compound **1**.

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The oxidative potential of particulate pollution derived from a biodiesel engine exhaust stream was evaluated by using a profluorescent nitroxide. A methanesulfonamide adduct arising from the reaction of the nitroxide with DMSO-derived sulfoxyl radicals was identified as the main fluorescent product.

Biodiesel Radical Production

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