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Rational design and preliminary analytical evaluation of two novel oxamide reagents for aqueous peroxyoxalate chemiluminescence

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The synthesis, characterisation and preliminary analytical evaluation of a novel class of aqueous peroxyoxalate chemiluminescence reagents are described. The two oxamide reagents described incorporate the most desirable features associated with these types of compound, namely, good reactivity and no measurable background emission with analytically useful aqueous solubility. Sequential injection analysis was employed to evaluate the stability characteristics and analytical figures of merit for these compounds. The analytical performances of the new oxamides were assessed using the fluorophore rhodamine B, which realised non-linear calibration functions and detection limits in the range from 1×10^{-7} to 5×10^{-7} M. The non-sulfonated analogues of the two oxamides were also prepared and their performances compared favourably with that of bis (2,4,6-trichlorophenyl) oxalate. During the latter experiments, the calibrations more closely approached linearity with detection limits in the range from 9×10^{-8} to 5×10^{-7} M. Although the development of the novel class of oxamide reagent presented here has not yet realised its full potential, it is felt that this work represents a significant step forward in the development of truly aqueous peroxyoxalate chemiluminescence.

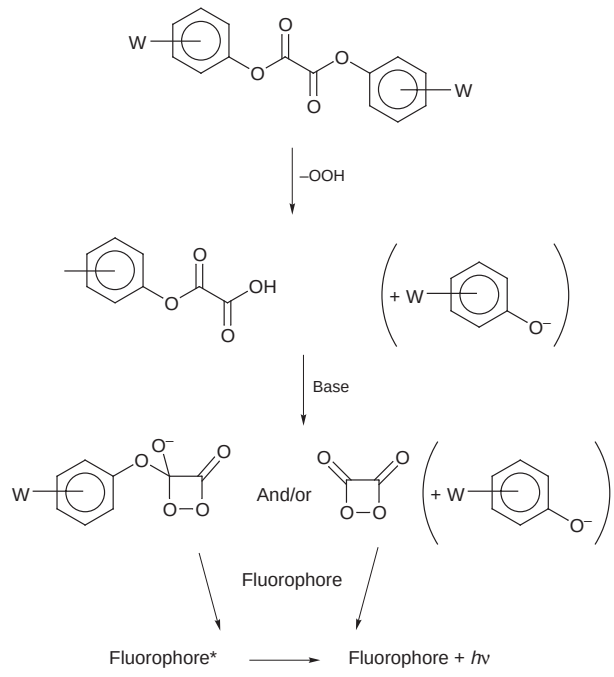
Keywords: *Aqueous peroxyoxalate chemiluminescene; oxamide reagents; sequential injection analysis*

The so-called 'peroxyoxalate' chemiluminescence reaction has been used analytically to detect a wide range of fluorescent compounds, mostly for high performance liquid chromatography and flow injection analysis, and such applications have been discussed in reviews by Bowie *et al.*[1] and Kwakman and Brinkman.[2] Following the pioneering work of Chandross[3] in 1963, who used oxalyl chloride as a peroxyoxalate chemiluminescence reagent, numerous efforts to develop efficient reagents based on the central oxalate moiety (see Scheme 1) have led to a wide variety of compounds that are capable of generating chemiluminescence in the presence of a suitable fluorophore.[4–10] Many of these compounds were originally developed for use as long-life chemical light sources,[4–9] which operate almost exclusively in low polarity solvents such as dibutyl phthalate and dimethyl phthalate. On the basis of earlier work, there would appear to be at least two characteristics that are of significance in the design of useful reagent molecules for peroxyoxalate chemiluminescence.[4–7,9–11] The first criterion is that the substituents surrounding the central oxalate moiety must exhibit a significant degree of electron-withdrawing character. This results in an acyl carbon that is sufficiently activated towards nucleophilic attack by the hydroperoxide anion, which in the presence of further base leads to the generation of reactive intermediates which transfer the excitation energy to a fluorophore compound[12–14] (see Scheme 1). For a more detailed discussion of the postulated mechanisms associated with Scheme 1, the reader is directed to the studies by Hadd and Birks[15] and Orosz *et al.*[16] plus the references therein. Secondly, the selected leaving group must be sufficiently soluble in the solvent for which the reagent was designed. For the central oxalate moiety in diaryl oxalates, these characteristics are provided by substituted phenyl groups, commonly 2,4,6-trichlorophenyl or 2,4-dinitrophenyl, which may be further substituted with additional functional group(s) to enhance solubility in a specific solvent.[10]

For reagents designed to fulfil a role in analytical chemistry, it is also desirable to minimise or eliminate factors that lead to significant emission in the absence of a fluorophore. This, so-called, background emission is considered to be a significant problem in analytical chemistry as it interferes with the detection of fluorophore compounds, especially at low concentrations, as a result of spectral overlap.[17–24]

In a recent investigation,[24] we postulated that the background emission, routinely observed in peroxyoxalate chemiluminescence with diaryl oxalate reagents, was caused by the chemiluminescence of the oxidised phenoxide leaving group (a phenoxyl radical), a feature that we have found to be exacerbated at high pH. Consequently, as the phenol leaving group has now been implicated in the production of background emission,[24] reagents containing this type of moiety should be avoided to increase the likelihood of background free peroxyoxalate chemiluminescence.

Our work has also focused on the synthesis and analytical evaluation[25] of selected reagents (see Fig. 1, compounds **A**, **B**



Scheme 1 W represents the presence of one or more electron withdrawing substituent(s).

and **C**) purported to be suitable for aqueous peroxyoxalate chemiluminescence. These compounds have been previously documented,[26] but to date have not received a great deal of attention in the analytical literature.[27–32] This situation may be attributable to the lack of stability of the most frequently cited reagent (compound **A**),[27–29,31,32] which exhibits a half-life of only 8 min under purely aqueous conditions.[27] During our most recent investigation[25] of the analytical utility of these reagents, we found that whereas compound **A** exhibited useful water solubility (≈10 mM), it was difficult to isolate in high purity. Conversely, compound **B** gave poorer solubility (approximately 5 mM), and compound **C** was essentially insoluble in aqueous solution (< 0.01 mM). Interestingly, neither compound **B** nor compound **C** exhibited measurable background emission under the reaction conditions employed.[24,25] These observations inspired the further investigation into this class of compound as potential reagents for water-soluble peroxyoxalate chemiluminescence.

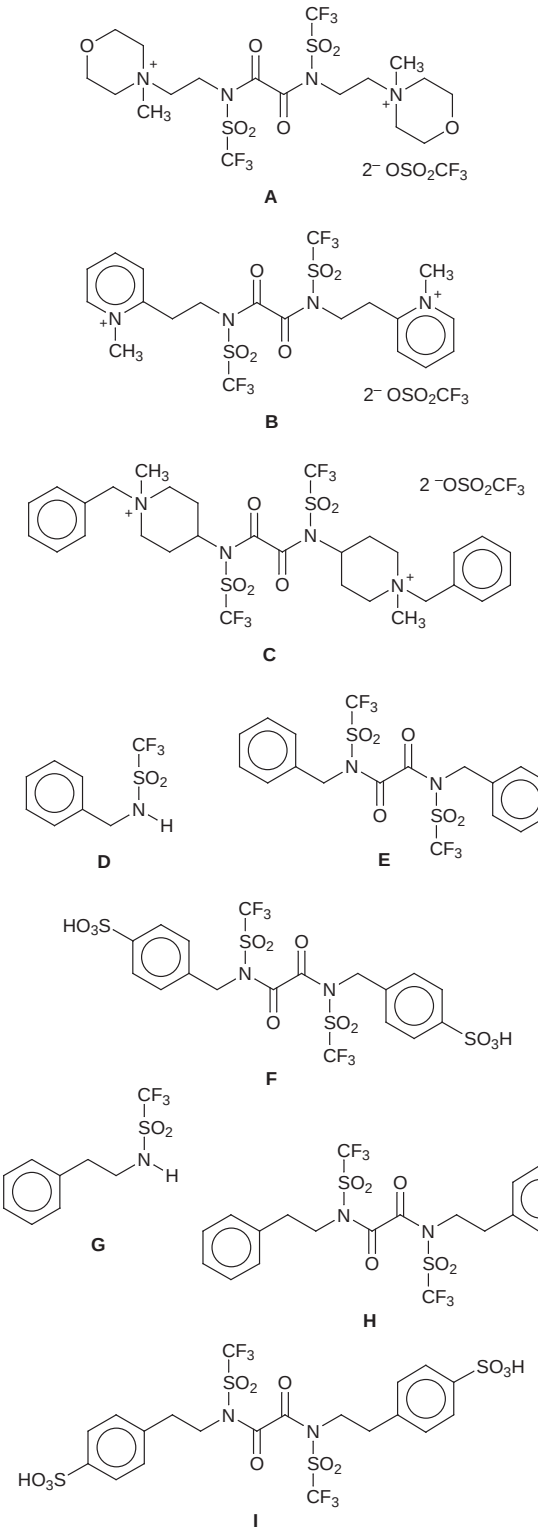


Fig. 1 Structures of the oxamides **A**, **B** and **C** previously reported[25] as aqueous peroxyoxalate reagents and the structures of the novel water-soluble oxamides **F** and **I**, and their synthetic precursor molecules **D** and **E** and **G** and **H**, respectively.

Previous work[26] on facilitating the water-solubility of this class of peroxyoxalate chemiluminescence reagent has focused essentially on the quaternisation of the tertiary amine groups (as shown in Fig. 1, compounds **A**, **B** and **C**) using methyl trifluoromethanesulfonate. An alternative solubilisation strategy was the formation of the hydrochloride salt of the tertiary amine group present in the molecule.[26] This latter approach gave useful solubility of certain compounds[26] in acidic aqueous media (< pH 2.5). However, these hydrochloride salts of the oxamides exhibited only limited analytical utility as water soluble reagents since the leaving groups are insoluble under the basic aqueous reaction conditions commonly employed, which in turn resulted in the formation of precipitates and compromised analytical performance.[33] An additional problem encountered with aqueous peroxyoxalate chemiluminescence is that of premature hydrolysis of the reagent which severely degrades its analytical utility. It is well known that both functionalised oxamides and oxalates used as reagents in this chemistry are susceptible to hydrolysis,[27,34–36] and that the rates of hydrolysis reactions for such compounds may be significantly reduced at neutral pH and/or at low temperatures.[37]

In an attempt to address the inherent problems associated with aqueous peroxyoxalate chemiluminescence, we have used the results of our previous investigations[24,25] to rationally design a new type of oxamide reagent. In this paper, we report the synthesis and analytical performance of two water soluble oxamides for use with peroxyoxalate chemiluminescence.

Experimental

Instrumentation

Nuclear magnetic resonance spectra were obtained using a 300 MHz Varian Unity Plus spectrometer (Varian, Palo Alto, CA, USA). Mass spectra were recorded using a Micromass Platform (II) electrospray (ES) mass spectrometer (Micromass, Altrincham, Cheshire, UK).

The sequential injection experiments were carried out using an Alitea MIS-1B modular injection system (Alitea, Medina, WA, USA) as configured in Fig. 2. Regulation of the pump (direction and flow rate), switching of the multi-position valve and timing was achieved with a computer (Prostar PC, 486 DX2, 66 MHz, Pipeline Computers, Geelong, Australia) fitted

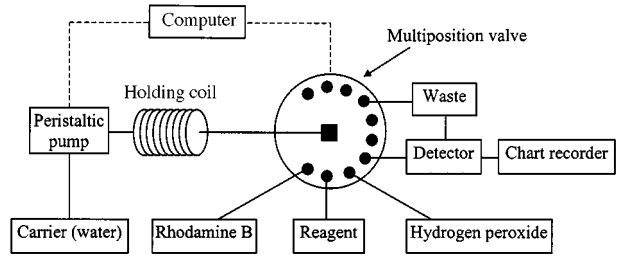


Fig. 2 Schematic diagram of the sequential injection instrument used for the analytical evaluation of the peroxyoxalate chemiluminescence reagents.

with an interface board (Lab-PC+, National Instruments, Austin, TX, USA) using a program developed from the LabView software package (National Instruments). The holding coil connecting the peristaltic pump and the multi-position valve (see Fig. 2) was 0.8 mm id PTFE with a total length of 3 m; all other manifold tubing was 0.5 mm id PTFE (Cole-Parmer, Wantirna South, Victoria, Australia). The peristaltic pump tubing was Viton (Masterflex 13, Extech, Wantirna South, Victoria, Australia) and was lightly oiled externally with silicone oil to minimise wear. The flow-through cell and detector assembly were connected to the multi-position valve using PTFE tubing (70 mm long, 0.5 mm id), in the same manner as described in a previous paper,[38] and the output from this device was measured (as peak heights) using a strip chart recorder (Omniscribe, Houston Instruments, Austin, TX, USA). All pH measurements were made using a model Corning Model 120 pH-meter (Corning, Halstead, Essex, UK).

Reagents

Benzylamine (99%), phenethylamine (99%), trifluoromethanesulfonic anhydride, sulfur trioxide, stabilised (99%), rhodamine B (90%), all from Aldrich, Milwaukee, WI, USA, acetonitrile (99%, Hypersolv grade, BDH, Poole, Dorset, UK), tetrahydrofuran (unstabilised) (99.7%, Hypersolv grade, BDH), and hydrogen peroxide (30 vol., analytical-reagent grade, Rhône Poulenc Chemicals, Clayton, Australia) were used as received. Bis(2,4,6-trichlorophenyl) oxalate (TCPO) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Oxalyl chloride (98%) was obtained from Janssen Chimica, Beerse, Belgium, triethylamine (> 99%) was obtained from Ajax Chemicals (Sydney, Australia). Dichloromethane (analytical-reagent grade, Merck, Crown Scientific, Rowville, Victoria Australia) was dried using calcium hydride (general purpose grade, BDH, Crown Scientific, Rowville, Victoria, Australia).

The buffer solutions employed were made up with de-ionised water obtained from a Waters (Bedford, MA, USA) Milli-Q system. The pH buffers were as follows: pH range 7.0–9.0, disodium hydrogenphosphate–potassium dihydrogenphosphate (0.01 M) (analytical-reagent grade, Ajax Chemicals); pH 10.0–11.0, sodium hydrogencarbonate–sodium carbonate, (0.025 M) (analytical-reagent grade, Ajax Chemicals). The buffers were adjusted to the desired pH with sodium hydroxide solution (5 or 1M) or hydrochloric acid (1 M) as required. The deuteriated solvents used were deuteriochloroform (99.5 at.-%), deuterium oxide (99.9 at.-%) and deuteriodimethyl sulfoxide (99.5 at.-%) (Cambridge Isotopes, Andover, MA, USA).

Procedures

Synthesis

The two oxamides were prepared using the general route summarised in Scheme 2. However, given the novelty of these compounds, the detailed synthesis and definitive characterisation for each appear under results and discussion. The structures of the two disulfonated oxamides and their respective precursor compounds are given in Fig. 1 (see compounds **F**, **I**, **D**, **E**, **G** and **H**).

Analytical

Prior to each set of experiments, each of the reagent/analyte lines (see Fig. 2) was initially filled with the carrier solution (water) and any air bubbles were removed. At which time reagents and fluorophore solutions were aspirated into lines between the respective reservoirs and the multi-position valve by selecting the relevant ports (see Fig. 2), and operating the pump in the reverse direction until a small volume of each solution appeared in the holding coil. The contents of the holding coil were then flushed with the carrier by switching the valve to the waste port and operating the pump in the forward direction. The initial step in an analytical cycle was the aspiration of a volume of rhodamine B solution into the holding coil by selecting the fluorophore port on the valve and reversing the pump for a fixed period of time. This procedure was repeated with the valve switched to the oxamide–TCPO port and then the hydrogen peroxide port in turn, thus stacking the oxamide–TCPO between the fluorophore and the hydrogen peroxide zones. The valve was then switched to the detector port and the stacked zones were flushed to the flow-through cell by the pump operating in the forward mode. During this final step the zones interdispersed, with the resulting emission being monitored at the flowthrough cell by the photomultiplier tube *via* the fibre optic cable. Five analysis cycles were carried out for each of seven standard rhodamine B solutions.

Compounds **E** and **H** plus the TCPO were each made up at 5×10^{-3} M in tetrahydrofuran, as were each of the seven rhodamine B standard solutions (from 1×10^{-7} to 5×10^{-4} M) used for their respective evaluations. The hydrogen peroxide solution, for this part of the study, was made up at 0.7 M in various aqueous buffers and then diluted with tetrahydrofuran (1 + 1) to realise a final hydrogen peroxide concentration of 0.35 M.

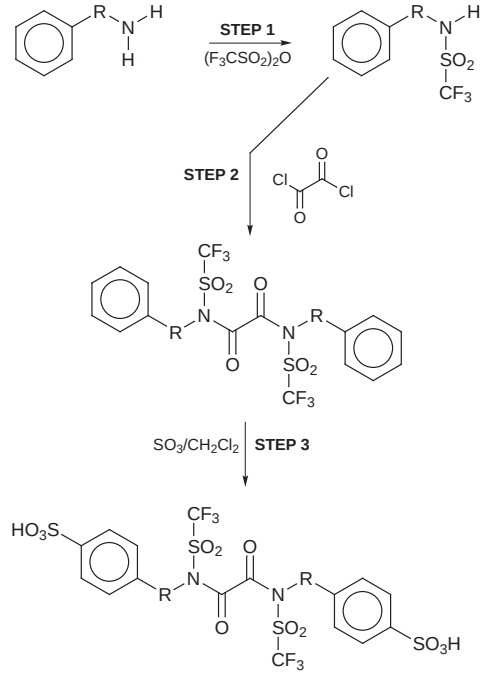
Compounds **F** and **I** were made up at 5×10^{-3} M in aqueous buffer (pH 7.0 and 2×10^{-3} M), whereas the rhodamine B standard solutions (from 1×10^{-7} to 5×10^{-4} M) were prepared in various aqueous buffers.

Results and discussion

Synthesis

Step 1: N-*trifluoromethylsulfonylbenzylamine (compound **D**)*

To a stirred solution of benzylamine (15.2 g, 0.142 mol) in dry dichloromethane (250 ml, dried by refluxing over calcium hydride) was added dropwise a solution of trifluoromethanesulfonic anhydride (20.0 g, 0.071 mol) in dichloromethane (40 ml). The addition was carried out at 0 °C and under an atmosphere of dry nitrogen. Following the addition, the reaction was allowed to warm to room temperature and stirred for a



Scheme 2 R represents either $-CH_2-$ or $-CH_2-CH_2-$

further 4 h. The precipitate obtained (a benzylamine–trifluoromethanesulfonic acid salt) was filtered off and discarded. The resulting solution was evaporated to dryness using a rotary evaporator. The crude solid obtained was then purified by (short) column chromatography using silica gel, with chloroform as the eluent. The yield obtained for compound **D** was 14.8 g, (0.062 mol, 85%).

Step 2: N,N′-*[bis(trifluoromethanesulfonyl)-N,N′-bis(benzyl)]-oxamide, (compound **E**)*

Compound **D** (14.0 g, 0.0585 mol) was dissolved in dry dichloromethane (250 ml), in the presence of triethylamine (5.91 g, 0.0585 mol). Oxalyl chloride (3.71 g, 0.0293 mol), which was dissolved in 20 ml of dry dichloromethane, was then added, dropwise, over a period of 20 min at 0 °C under an atmosphere of dry nitrogen. Following this addition, the reaction mixture was allowed to warm to room temperature, and stirred for a further 24 h. The crude mixture was evaporated to remove the dichloromethane and the resulting solid was extracted twice with hot methylcyclohexane; the non-extracted solid (triethylamine hydrochloride salt) was discarded. Compound **E** was then recrystallised from methylcyclohexane giving a yield of 8.6 g, (0.0162 mol, 55%).

*Step 3: [oxalylbis(trifluoromethanesulfonyl)imino]-[bis(benzyl)]-4,4′disulfonic acid. (compound **F**)*

Sulfonation of the oxamide **E** was performed by condensing sulfur trioxide vapour into a pre-weighed reaction vessel at −90 °C, using an ethanol–liquid nitrogen bath. The mass of condensed sulfur trioxide was then determined (1.05 g, 0.013 mol), and dissolved in cold, dry dichloromethane (100 ml). The oxamide **E** (3.46 g, 0.0065 mol, dissolved in dry dichloromethane, 40 ml) was slowly added to the sulfur trioxide solution. Following the addition, the reaction was allowed to warm to 0 °C, then refrigerated (*ca*. 5 °C) for 48 h. Compound **F** was isolated as a white solid, washed with dry dichloromethane and dried *in vacuo* to give a yield of 1.8 g (0.0026 mol, 40%).

The method employed for the synthesis of the phenethylamine analogue, 2,2′-[oxalylbis(trifluoromethanesulfonyl)-imino]-[bis(phenethyl)]-4″,4‴-disulfonic acid (compound **I**), was identical to that of compound **F**, except that the sulfonation step was performed for 6 h. The yields obtained for each of the three steps of the synthesis were 90% for *N*-trifluoromethylsulfonylphenethylamine, (compound **G**), 55% for *N*,*N*′-[bis(trifluoromethanesulfonyl)-*N*,*N*′-bis(pheneth-2-yl)]-oxamide, (compound **H**), and 50% for compound **I**.

It should be noted that the addition of a sulfonic acid group to a molecule that is sensitive to hydrolysis requires careful manipulation of both the reaction and work-up conditions so as to avoid cleavage of the starting material. The strongly electron-withdrawing nature of the trifluoromethylsulfonyl substituent on the amide nitrogen (see Fig. 1) has the effect of preventing the lone pair of electrons from being available for bonding. Therefore, electrophilic reagents such as sulfur trioxide[39] may be employed to effect the sulfonation of electron rich moieties, such as a phenyl group, within the molecule, without the unwanted side reactions with either the amide nitrogens or the acyl carbons.

Characterisation.

Compound **D:** δ_C ($CDCl_3$), 135.1, 129.1, 128.7, 127.9, 119.7 (q, ^1J 321 Hz), 48.2; δ_H, 7.36, (5 H, m), 5.14, (1 H, br), 4.44 (2 H, d, ^1J 5.9 Hz); δ_F −77.8, white solid, mp 41.5–43 °C. Compound **E:** δ_C ($CDCl_3$), 160.1, 132.5, 129.0, 128.8, 128.7, 119.0 (q, ^1J = 324 Hz), 50.8, δ_H, 7.37, (5 H, m), 5.01, (2 H, s), δ_F, −73.4; white solid, mp 102–104 °C. Compound **F:** δ_C ([$^2H_6$]DMSO), 159.7, 148.2, 133.6, 127.5, 125.8, 118.7, (q, ^1J 325 Hz), 50.5; δ_H (D_2O), 7.58 (2 H, d), 7.30 (2 H, d) 5.16 (2 H, br s); δ_F ([$^2H_6$]DMSO), −72.1; negative ion ES mass spectrum *m/z* = 345.2; white solid, mp 112–114 °C.

Compound **G:** δ_C ($CDCl_3$), 136.8, 128.7, 126.9, 119.6 (q, ^1J 321 Hz), 45.3, 36.3; δ_H 7.34 (3 H, m), 7.22 (2 H, m), 4.92, (1 H, br), 3.57 (2H, dt), 2.92 (2 H, t); δ_F, −78.1; isolated as a light brown oil. Compound **H** δ_C ($CDCl_3$), 159.8, 136.6, 128.9, 128.9, 127.2, 119.2 (q, ^1J 324 Hz), 49.4, 33.8; δ_H 7.34 (5 H, m), 4.16 (1 H, br), 3.88 (1 H, br), 3.13, (2 H, br), δ_F −74.2; white solid, mp 112–113 °C. Compound **I:** δ_C ([$^2H_6$]DMSO) 159.6, 147.2, 137.1, 128.6, 126.4, 119.0 (q, ^1J = 324 Hz), 49.3, 33.3; δ_H (D_2O), 7.55 (2 H, d), 7.22 (2 H, d), 4.21 (1 H, br), 3.90 (1 H, br), 2.94 (2 H, br); δ_F ([$^2H_6$]DMSO) −73.0; negative ion ES mass spectrum, *m/z* 359.0; white solid mp 116–119 °C (with some decomposition).

The NMR data listed above are entirely consistent with the structures of compounds **D**–**I** shown in Fig. 1. Further evidence for the confirmation of compounds **F** and **I** are the respective electrospray mass spectra (negative ion) which both gave molecular ion masses within 0.02 and 0.08%, respectively of the actual values based upon the structures in Fig. 1. Therefore, we are confident that the peroxyoxalate reagents evaluated in this study have the molecular structures of compounds **E**, **F**, **H** and **I** as shown in Fig. 1.

Analytical evaluation

The analytical figures of merit achieved from the performance comparisons between compound **E**, compound **H** and TCPO are summarised in Table 1. The use of sequential injection analysis for these evaluations facilitated both automated operation and minimal reagent consumption. Unlike continuous flow techniques, such as high-performance liquid chromatography or flow injection analysis, the operating principles of sequential injection analysis result in discrete signals for the background emission (or blank) as shown in Fig. 3. It can be clearly seen from Fig. 3 that, as discussed in an earlier paper.[24] the background emission from TCPO in the absence of a fluorophore is significantly greater than that from the oxamide-type reagents. However, in contrast to compounds **B** and **C** (see Fig. 1), which gave no measurable background emission,[24] compounds **E** and **H** appear to have elicited a small blank response. This contradictory behaviour most probably resulted from the presence of concomitant fluorescent impurities and/or the differences in the measurement procedures employed in the two investigations. Our previous studies[23,24] on the nature of

Table 1. Analytical figures of merit for compounds **E**, **H** and TCPO in slightly aqueous tetrahydrofuran

Reagent	pH*	Calibration function†	r^2	Detection limit/M‡
Compound **E**	9	$y = 9 \times 10^6 x + 7$	0.9940	5×10^{-7}
	10	$y = 1 \times 10^7 x + 13$	0.9900	2×10^{-7}
	11	$y = 2 \times 10^7 x + 11$	0.9975	1×10^{-7}
Compound **H**	9	$y = 5 \times 10^6 x + 6$	0.9900	4×10^{-7}
	10	$y = 7 \times 10^6 x + 8$	0.9952	4×10^{-7}
	11	$y = 9 \times 10^6 x + 12$	0.9873	2×10^{-7}
TCPO	9	$y = 3 \times 10^8 x + 110$	0.9948	1×10^{-7}
	10	$y = 3 \times 10^8 x + 210$	0.9947	9×10^{-8}
	11	$y = 4 \times 10^8 x + 260$	0.9863	7×10^{-8}

* This pH is that of the aqueous buffer used to dilute the solution of hydrogen peroxide in tetrahydrofuran, rather than the reaction pH. † Where y = chemiluminescence response (mV) and x = the concentration of rhodamine B (M). ‡ The detection limits were determined as three times the standard deviation of the blank response.

peroxyoxalate chemiluminescence background emissions have not revealed whether the intensity of such signals remains constant, or is proportionally diminished with increasing fluorophore concentration. Therefore, the measured background responses, as shown in Fig. 3, were not subtracted from the signals obtained from the rhodamine B standards. This practise is reflected in the relatively high values of the *y*-intercepts for the TCPO calibration functions compared with those for compounds **E** and **H** given in Table 1. Although the calibration functions (see Table 1) exhibited non-linearity across the extended concentration range of the analyte (from 1 $\times 10^{-7}$ to 5 $\times 10^{-4}$ M), a much closer approximation to a linear relationship was achieved using the mid-range standards. We have seen this type of behaviour with other chemiluminescent systems[40,41] and it probably arises from the large variations in the reagent to analyte ratio which occur over the calibration range. From Table 1, it was noteworthy that TCPO afforded, at best, a five-fold improvement in detectability over the two oxamides under the same reaction conditions. This marginal enhancement is in stark contrast to relative sensitivities between TCPO and oxamide calibrations, which differ by factors between 23 and 90 in favour of TCPO. This latter result is consistent with the improved signal-to-background ratios, at low analyte concentrations, shown by compounds **E** and **H** (see Fig. 3). The slightly improved detection limits and sensitivities for each compound, with increasing pH was expected on the basis of the peroxyoxalate mechanism[12–16] and reflects the essential requirement for basic reaction conditions.[27] It should be borne in mind that the pH values listed in Table 1 were those of the aqueous buffered solutions used to dilute the tetrahydrofuran (1 + 1), and as such do not accurately represent the basicity of the actual reaction.

Our previous experience with water-soluble oxamides used for peroxyoxalate chemiluminescence[25] suggested that the aqueous stability of both compounds **F** and **I** should be measured. This was achieved indirectly, by monitoring the chemiluminescence response from about 20 sequential injection cycles over periods up to 250 min. During these experiments, a single rhodamine B standard solution (1 $\times 10^{-5}$ M) was employed. Both reagent solutions (5 $\times 10^{-3}$ M) were buffered to pH 7 and utilised at 20 and 1.5 °C; the results have been shown graphically in Fig. 4, from which it can be seen that compounds **F** and **I** (lines A and B, respectively, in Fig. 4) show significantly different stability characteristics at 20 °C. Considering that the major use of peroxyoxalate chemiluminescence is for high-performance liquid chromatographic detection,[1,2] the relatively rapid decline in response shown by compound **F** over the first 100 min would appear to render this reagent unsuitable for such application. However, the initial response plateau exhibited by compound **I** over a similar period would be satisfactory for chromatographic detection. The effective reagent half-lives for compounds **F** and **I** at 20 °C were determined from Fig. 4 as approximately 50 and 180 min, respectively. These results represent a significant improvement in the stability of water-soluble oxamide-type reagents.[25] To ensure that the current half-life estimations were directly comparable to those previously obtained,[25] the same type of stability experiments were performed with compounds **A** and **B**. Reagent **A** yielded a chemiluminescence response half-life of 8 min, which was in excellent agreement with hydrolysis data previously reported,[27] whereas compound **B** was found to exhibit a chemiluminescence response half-life of 20 min. Given the structural similarity of these four compounds (**A**, **B**, **F** and **I**) our findings suggest that the nature of the leaving group may be related to the respective rates of hydrolysis of each of the parent oxamides.

Owing to the highly soluble nature of compound **F** and the limited amounts prepared to date, it has not as yet been possible to determine solubility data for this compound. In contrast to compound **F**, compound **I** was found to exhibit rather unusual solubility characteristics. This was difficult to reconcile given their close structural similarities (see Fig. 1). As expected, a rapid drop in the pH of the solution was observed as reagent **I** was added to water, which is commensurate with the sulfonic acid functionality. The same type of behaviour was also seen when reagent **F** was dissolved in water. However, unlike compound **F**, compound **I** does not dissolve completely (at 5 mM), but rather forms a cloudy suspension. At 20 °C, partial clarification occurs on standing (approximately 20 min), this time is considerably extended at lower temperatures (approximately 120 min at 1.5 °C). Aqueous solutions of compound **I** were subjected to filtration (0.45 μm) and solvent extraction (dichloromethane), but neither approach was successful in removing the suspension. It would, appear therefore, that the solubility behaviour of compound **I** at the concentrations used here was kinetically dependent. By placing the flasks containing the oxamide solutions in an ice–water slurry bath, another set of chemiluminescence response *versus* time experiments were conducted while maintaining the reagent reservoirs at 1.5 °C (just above freezing). From Fig. 4, line C, it can be seen that at this reduced temperature, the half-life of the chemiluminescence response for compound **F** was extended to approximately 150 min. Of greater analytical importance is the relatively flat

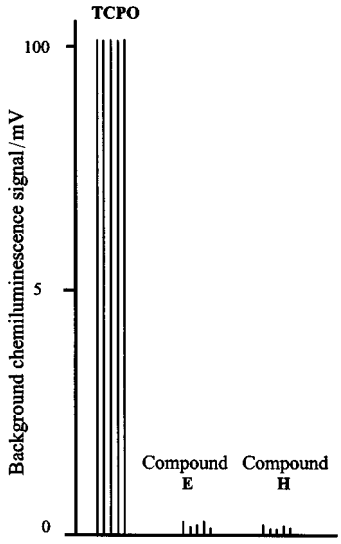


Fig. 3 Typical blank (background) signal responses for the three reagents TCPO, compound **E** and compound **H** in slightly aqueous tetrahydrofuran.

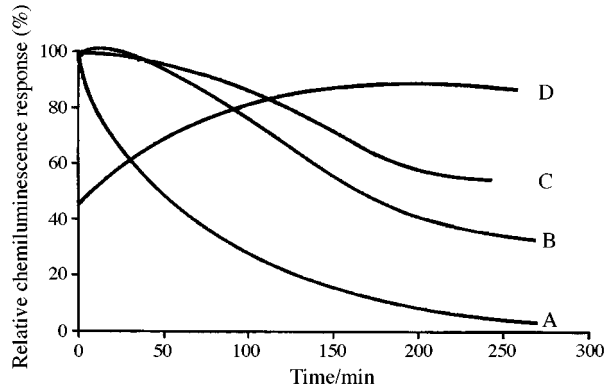


Fig. 4 Relative chemiluminescence response from rhodamine B (1 $\times 10^{-5}$ M) for compounds **F** and **I** stored at 20 °C and for compounds **F** and **I** stored at 1.5 °C, represented by lines **A**, **B**, **C** and **D**, respectively.

Table 2. Analytical figures of merit for compounds **F** and **I** in aqueous solution

Reagent	pH	Calibration function*	r^2	Detection limit/M†
Compound **F**	7.0	$y = -2 \times 10^{11} x^2 + 2 \times 10^7 x + 23$	0.9991	1×10^{-7}
	7.4	$y = -1 \times 10^{11} x^2 + 1 \times 10^7 x + 15$	0.9905	2×10^{-7}
	8.0	$y = -6 \times 10^{10} x^2 + 1 \times 10^7 x + 12$	0.9984	2×10^{-7}
	9.0	$y = -1 \times 10^{11} x^2 + 1 \times 10^7 x + 15$	0.9987	2×10^{-7}
Compound **I**	7.0	$y = -3 \times 10^{11} x^2 + 3 \times 10^7 x - 3$	0.9976	5×10^{-7}
	7.4	$y = -4 \times 10^{11} x^2 + 4 \times 10^7 x - 1$	0.9984	2×10^{-7}
	8.0	$y = -4 \times 10^{11} x^2 + 4 \times 10^7 x + 0.2$	0.9934	5×10^{-7}
	9.0	$y = -4 \times 10^{11} x^2 + 4 \times 10^7 x - 3.6$	0.9939	2×10^{-7}

* Where y = chemiluminescence response (mV) and x = the concentration of rhodamine B (M). † The detection limits were determined as three times the standard deviation of the blank response.

portion of the response curve between 0 and 100 min. This result represents the first report of a truly aqueous peroxyoxalate reagent having analytically useful stability. The steady decline in the response curve beyond 100 min most probably resulted from the production of carbon dioxide (from hydrolysis the oxamide) which, in turn, caused a lowering of the pH and enhanced the acid hydrolysis. Interestingly, compound **F** appears to have a second period of relative stability between 180 and 220 min. The reasons for this are unclear at present, and the limited amount of compound **F** available precluded the extension of the stability experiment. The chemiluminescence response characteristics exhibited by compound **I** (see Fig. 4, line D) may be rationalised in terms of the solubility behaviour of this reagent, as described earlier. It was notable that once the solubility equilibrium appears to be reached at around 120 min the reduction in the analytical response is minimal. Again, limited amount of reagent (compound **I**) curtailed our investigations into the nature of the chemiluminescent response with time at reduced temperature. However, both reagents exhibit improved stability characteristics at reduced temperature. Accordingly, the analytical figures of merit for compounds **F** and **I** were determined with the respective reagent reservoirs maintained at 1.5 °C and these results are summarised in Table 2. All calibration experiments were conducted when each of the reagents exhibited their best respective temporal stabilities, as shown in Fig. 4.

The extent of the non-linear nature of the calibration functions achieved using compounds **F** and **I** can be better appreciated by examining the two typical graphs in Fig. 5. At present, we are unable to offer any molecular level explanations for these observations, but the matter is the subject of an ongoing investigation. What is apparent is that the light producing mechanisms for compounds **F** and **I** in completely aqueous solutions are considerably different to those for compounds **E** and **H** in slightly aqueous tetrahydrofuran. Although the relationship between chemiluminescence intensity and analyte concentration (see Fig. 5) is complex, analytically useful functions were achieved by restricting the calibration range.

The detection limits listed in Table 2 show a slightly better performance from compound **F** under certain pH conditions, however there were no obvious trends in detectability. Interestingly, there is little or no difference between the detection limits in Table 2 and those obtained with the non-sulfonated

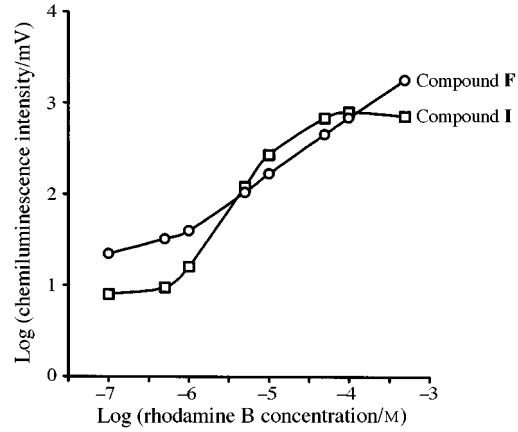


Fig. 5 Typical log–log calibration graph from rhodamine B for compounds **F** and **I** at pH.

analogues (compounds **E** and **H**) listed in Table 1. Such comparisons have limited validity given the likelihood that vastly different chemiluminescent mechanisms are involved. In an attempt to improve the detection limits achievable with compounds **F** and **I**, the reaction pH was increased to ascertain whether higher basicity would realise any enhancement of the detectability as it had with compounds **E** and **H** (see Table 1). Unfortunately, at pH 10 and 11, both reagents gave significant blank signals, which had not been observed with either of these oxamides at pH 9 or below. The background emission caused a serious deterioration in the analytical performance of both reagents, and, consequently, no improvement in detection limits was observed at the higher pH values. The source of the background emission from these compounds is currently being studied and the results will be published in due course.

Conclusion

We believe that the novel reagents described here, designed specifically for aqueous peroxyoxalate chemiluminescence, constitute a significant advancement over the earlier compounds[26] **A** and **B**. We are currently examining reagents **F** and **I** in greater detail with respect to the maximisation of performance and evaluation of their applicability to real analytical problems.

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