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Synthesis and properties of novel chemiluminescent biological probes: substituted 4-(2-succinimidyloxycarbonylethyl)phenyl 10-methylacridinium-9-carboxylate trifluoromethanesulphonate

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Abstract

Substituted aryl acridinium esters, 2,6-dimethyl-4-(2-succinimidyloxycarbonylethyl)phenyl 10-methylacridinium-9-carboxylate trifluoromethanesulphonate (**3**), 2,6-dimethoxy-4-(2-succinimidyloxycarbonylethyl)phenyl 10-methylacridinium-9-carboxylate trifluoromethanesulphonate (**4**), 2-methoxy-4-(2-succinimidyloxycarbonylethyl)phenyl 10-methylacridinium-9-carboxylate trifluoromethanesulphonate (**5**) and 2,6-dibromo-4-(2-succinimidyloxycarbonylethyl)phenyl 10-methylacridinium-9-carboxylate trifluoromethanesulphonate (**6**), have been synthesised and coupled to immunoglobulin G (IgG). The chemiluminescent properties and hydrolytic stabilities of the conjugates have also been examined. The different substituents on the phenoxy ring affected the chemiluminescent efficiency and kinetics. All the human IgG conjugates of these substituted acridinium esters demonstrated much higher stability than the conjugate of the corresponding unsubstituted acridinium ester.

Keywords: Acridinium ester; Chemiluminescence; Chemiluminescence immunoassay

1. Introduction

Chemiluminescence immunoassay, based on non-radioactive cyclic arylhydrazides [1], dioxetanes [2] or oxalic acid derivatives [3], has been known for many years. Although the technique offers improvements in terms of reagent stability over the use of radioisotopes, it also suffers disadvantages. Thus, oxalic acid derivatives have poor solubility in water, while immunoassay is performed in aqueous solution. Also, the chemiluminescent reactions of cyclic arylhydrazides proceed only in the presence of a catalyst. However, a wide range of chemical species are capable of catalysing or affecting the reaction, ranging from simple transition metal cations to macromolecules such as peroxidases. This can lead to a substantial background luminescence which consequently limits the sensitivity.

In contrast, chemiluminescence of acridinium esters does not need an additional catalyst. It can be simply triggered by hydrogen peroxide and base and a much lower chemiluminescent background is therefore achieved [4]. The first acridinium ester label **1** (Scheme 1, **1**), which has a succinimidyl ester of a phenylpropanoic acid as the reactive group for coupling to proteins, was reported in 1983 [5]. As a result of its effectiveness in immunoassay, much effort has been devoted to its structural modification, with the aim of improving its stability and chemiluminescent properties. Label **2** (Scheme 1, **2**) is reported to be a more stable acridinium ester. It has been suggested that the methyl groups in the 2- and 6-positions of the phenoxy ring, impart greater stability in pH 7.4 buffer than for its unmethylated analogue. Furthermore, it exhibits a two-fold improvement in the signal-to-noise ratio when used in a solid phase specific binding assay and its IgG conjugate also exhibits a three-fold increase in light emitting efficiency [6]. However, the direct attachment of the activated carboxyl group to the ring results in undesirable effects on both the binding properties and the chemiluminescent kinetics in comparison to the original label **1**.

In order to try to combine some of the advantages of substituted acridinium ester **2** with those of the original label **1**, we decided to synthesise compounds **3**, **4**, **5** and **6**. In these

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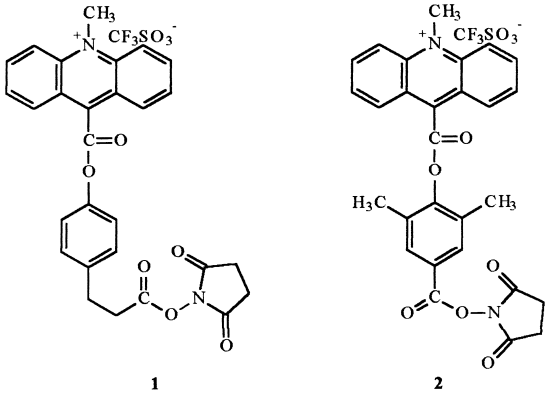


1 2

Scheme 1. Structures of label **1** and label **2**.

compounds, the *ortho*-protons of the phenoxyl ring are substituted by methyl, methoxy or bromo substituents. This is expected to influence both the stability and the chemiluminescent properties of the products by a combination of steric and electronic effects from the substituents. Studies of the chemiluminescent properties of compounds **3**–**6** and of the stability of their conjugates with proteins should, therefore, enable some understanding of the importance of such factors [7–8,21].

2. Experimental

2.1. General methods

Melting points (mp) were recorded on a Griffin Melting Point Apparatus and are reported uncorrected. IR spectra were obtained using a Perkin Elmer FT-IR spectrometer 1725x. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were measured on a Bruker AC 400 spectrometer with tetramethylsilane as an internal standard. Chemical shifts (δ) are expressed in ppm, and coupling constants (J) are in Hz. Mass spectra were recorded on a VG 12/253J mass spectrometer for low resolution electron impact (EI) and chemical ionisation (CI) measurements and a VG AutoSpec mass spectrometer for fast atom bombardment (FAB) mass spectra. The data are presented as *m*/*z* ratios for the molecular ion and several of the most abundant other ions with their percentage relative intensity given in brackets. For dibromo compounds, which give multiple molecular ions, only the ^{79}Br^{81}Br ion is reported in this paper. Microanalyses were obtained from a Carlo Erba 1106 instrument by the microanalysis laboratory at the University of Wales, Cardiff. Column chromatography was carried out with silica gel 60 (230–400 mesh, Merck). Chromatotron separation was carried out on chromatotron Model 7924T from TC Research using 4, 2 or 1 mm thick silica gel 60 GF_{258} (Merck) layers. In order to help in the assignments, expected chemical shifts were calculated using additivity values and model compounds [9]. Assignments of signals having similar chemical shifts have not been rigorously confirmed.

A number of buffer solutions were prepared. (1) labelling buffer: NaH_2PO_4/Na_2HPO_4 (0.1 mol/l, pH=8.0); (2) quench buffer: labelling buffer/lysine monohydrochloride (10 mg/ml); (3) assay buffers: NaH_2PO_4/Na_2HPO_4 (0.1 mol/l, pH=5, 6, 7 and 8)/NaCl (0.15 mol/l)/NaN_3 (0.02%, w/v)/bovine serum albumin (0.2%); (4) column buffer: NaH_2PO_4/Na_2HPO_4 (0.1 mol/l, pH=6.30)/NaCl (0.15 mol/l)/NaN_3 (0.05%, w/v)/bovine serum albumin (0.1%). The chemiluminescent measurements were carried out in a Magic Life Analyser (Ciba–Corning Diagnostics, Medfied, MA 02052, USA).

2.2. Syntheses

The synthetic routes are given in Schemes 2 and 3.

2.2.1. 3-(3,5-Dimethyl-4-hydroxyphenyl)propanenitrile (3*g*)

2,6-Dimethylphenol **3f** (1.220 g, 10 mmol) was dissolved in acrylonitrile (0.65 ml, 0.530 g, 10 mmol). Anhydrous $AlCl_3$ (2.670 g, 20 mmol) and 1,2-dichlorobenzene (2 ml) were added to the solution and dry HCl gas was bubbled through it at 110°C for 1 h. The reaction mixture was added to water (100 ml) and the whole was then extracted with $CHCl_3$ (70 ml×3). The chloroform extract was washed with saturated NaCl aq. (70 ml×3), dried over anhydrous Na_2SO_4, filtered and evaporated. A yellow solid was obtained, which was then recrystallised from $CHCl_3$–hexane to produce a white crystalline solid **3g** (2.801 g, 74%), mp 104–105°C. IR ν_{max} (KBr) 3500, 3000, 2240, 1600, 1480, 1200; ^{1}H NMR ($CDCl_3$) δ 6.88 (s, 2H), 4.60–5.00 (b, 1H), 2.80 (t, 7.2 Hz, 2H), 2.55 (t, 7.2 Hz, 2H), 2.22 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 151.35, 129.62, 128.39, 123.48, 119.42, 30.78, 29.83, 15.97; EI-MS *m*/*z* 175 [M^+, 35%], 135 [(M^+–CH_2CN), 100].

2.2.2. 3-(3,5-Dimethyl-4-hydroxyphenyl)propanoic acid (3*a*)

A solution of **3g** (2.801 g, 7.4 mmol) was dissolved in concentrated hydrochloric acid and refluxed for 1 h. The reaction mixture was added to water (100 ml) and extracted with $CHCl_3$ (70 ml×3) then washed with saturated NaCl aq. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to produce a yellow solid (3.001 g), which was recrystallised from $CHCl_3$ to give a white crystalline solid **3a** (2.80 g, 90%), mp 124–125°C. IR ν_{max} (KBr) 3500, 3000, 1750, 1600; ^{1}H NMR ($CDCl_3$) δ 6.88 (s, 2H), 4.60–5.00 (b, 1H), 2.80 (t, 7.2 Hz, 2H), 2.55 (t, 7.2 Hz, 2H), 2.22 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 172.31, 151.33, 136.56, 128.36, 119.42, 35.79, 29.78, 15.97; EI-MS *m*/*z* 194 [M^+, 20%], 135 [(M^+–CH_2CO_2H), 100].

*2.2.3. Benzyl 3-(3,5-dimethyl-4-hydroxyphenyl)propanoate (**3b**)*

A solution of **3a** (920 mg, 4.70 mmol) in anhydrous benzyl alcohol (4.90 ml, 5.121 g, 47 mmol), and trifluoroacetic anhydride (3.70 ml, 5.480 g, 26.3 mmol) was heated at 60°C for 3 h, then cooled and poured into aqueous sodium hydrogen carbonate (50 ml of 8% aq.). The mixture was exhaustively extracted with chloroform (50 ml×3) and the extract was dried and evaporated to leave a syrup (1.601 g). This residue was subjected to chromatography using a chromatotron (ethyl acetate–hexane, 1:8; 1:4; 1:2 sequentially). After evaporation of the solvent from the appropriate combined fractions, **3b** was obtained as a pure colourless oil (840 mg, 80%). IR ν_{max} (KBr) 3500, 3000, 1750, 1740, 1710, 1500, 1480, 1200; UV λ_{max} ($CHCl_3$) 229 (log ε_1 3.31), 278 (log ε_2 2.84); ^{1}H NMR ($CDCl_3$) δ 7.32 (5H, m, Ph), 6.78 (s, 2H), 5.10 (s, 2H, CH_2Ph), 4.69 (s, 1H, OH), 2.83 (t, 7.2 Hz, 2H), 2.62 (t, 7.2 Hz, 2H), 2.22 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 172.41 (CO), 150.08, 135.37, 131.35, 127.96, 127.81, 127.61, 127.28, 122.49, 65.65, 35.74, 29.54, 15.94; EI-MS *m/z* 284 [M^+, 40%], 193 [(M^+–CH_2Ph), 80], 151 [100], 135 [40], 91 [90]; Anal. Calc. for $C_{18}H_{18}O_3$: C, 76.03%; H, 7.09%; Found: C, 76.15%; H, 7.12%.

2.2.4. Acridine-9-carbonyl chloride

Acridine-9-carboxylic acid (1.730 g, 10 mmol) was dissolved in thionyl chloride (25 ml) and the solution was refluxed for 6 h. The solution was concentrated in vacuo and hexane was added slowly to precipitate the product. The mixture was filtered to give a yellow solid (1.721 g, 94%), mp 218–219°C (lit. [10–13], 218–219°C). The material was used without further purification.

*2.2.5. 2,6-Dimethyl-4-(2-benzyloxycarbonylethyl)phenyl acridine-9-carboxylate (**3c**)*

A mixture of **3b** (170 mg, 0.62 mmol) and 4-dimethylaminopyridine (9 mg, 0.08 mmol) in anhydrous pyridine (10 ml) (stored over 3A molecular sieves) was heated at 90°C for 30 min. The solution was cooled and pipetted into a flask containing dry acridine-9-carbonyl chloride (225 mg, 0.93 mmol). The mixture was stirred at 100°C for 10 h and then at room temperature for 24 h. The solvent was removed under reduced pressure, the crude product was dissolved in $CHCl_3$ (150 ml) and the solution was washed with water (70 ml×3), dried with Na_2SO_4 and evaporated to give a yellow oil (400 mg). This residue was subjected to chromatography using a chromatotron (ethyl acetate–hexane, 1:8; 1:4; 1:2 sequentially). Evaporation of the solvent from the combined fractions containing product gave **3c** as a pure yellow gum (240 mg, 80%). IR ν_{max} (KBr) 3100, 3000, 1750, 1730, 1200, 1000; ^{1}H NMR ($CDC1_3$) δ 8.43 (d, 8.9 Hz, 2H), 8.33 (d, *J*=8.7 Hz, 2H), 7.84 (m, 2H,), 7.65 (m, 2H), 7.33 (m, 5H), 7.02 (s, 2H), 5.14 (s, 2H), 2.95 (t, 7.2 Hz, 2H), 2.72 (t, 7.2 Hz, 2H), 2.39 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 176.61, 165.44, 148.74, 146.88, 138.74, 135.87, 135.62, 130.30, 130.21, 130.09, 129.12, 128.57, 128.24, 127.77, 127.47, 125.18, 127.77, 66.34, 35.79, 30.23, 17.79; FAB-MS *m/z* 490 [($M+1)^+$, 100%], 400 [10], 224 [6], 206 [20], 179 [40]; Anal. Calc. for $C_{32}H_{27}NO_4$: C, 78.51%; H, 5.66%; N, 2.81%; Found: C, 78.31%; H, 5.45%; N, 2.70%.

*2.2.6. 2,6-Dimethyl-4-(2-carboxyethyl)phenyl acridine-9-carboxylate (**3d**)*

A mixture of **3c** (590 mg, 1.21 mmol), glacial acetic acid (16 ml) and 48% hydrobromic acid (4 ml) was heated at 100°C for 3 h and then cooled. The reaction mixture was added to water (150 ml) and extracted with 20% methanol in chloroform (50 ml×3). The organic extracts were combined and evaporated. The residue was suspended in chloroform and neutralised with a slight excess of triethylamine. The mixture was then washed with water (70 ml×3), and the organic layer was dried over sodium sulphate and evaporated to dryness. The residue was purified by recrystallisation from $CHCl_3$ to give **3d** (430 mg, 89%), mp 245–246°C. IR ν_{max} (KBr) 3500, 1740, 1720, 1200; ^{1}H NMR (DMSO-d_6) δ 12.10–12.30 (b, 1H), 8.35 (m, 4H), 8.00 (m, 2H), 7.84 (m, 2H), 7.16(s, 2H), 2.85 (t, 7.2 Hz, 2H), 2.63 (t, 7.2 Hz, 2H), 2.38 (s, 6H); ^{13}C NMR (DMSO-d_6) δ 173.67, 163.64, 148.02, 146.12, 139.12, 134.88, 130.75, 129.81, 129.42, 128.93, 128.11, 124.61, 121.67, 35.04, 29.60, 17.19; FAB-MS *m/z* 400 [($M+H)^+$, 20%], 329 [25], 206 [18], 179 [100]; Anal. Calc. for $C_{25}H_{2l}NO_4$: C, 75.17%; H, 5.23%; N, 3.51%; Found: C, 75.10%; H, 5.22%; N, 3.64%.

*2.2.7. 2,6-Dimethyl-4-(2-succinimidyloxycarbonylethyl)-phenyl acridine-9-carboxylate (**3e**)*

A solution of **3d** (40.0 mg, 0.10 mmol) in dimethylformamide (DMF, 50 ml) was cooled in an ice bath for 10 min, then mixed with a solution of dicyclohexylcarbodiimide (DCC, 16.0 mg, 0.14 mmol) in DMF (2 ml). The mixture was stirred in an ice bath for 30 min, then mixed with a solution of *N*-hydroxysuccinimide (30.0 mg, 0.14 mmol) in DMF (3 ml), stirred at room temperature overnight and evaporated to dryness. The residue was extracted with dichloromethane (50 ml) and filtered. The filtrate was evaporated to obtain the crude product (50 mg). Further purification of product was carried out by chromatography using a chromatotron (chloroform). After evaporation of the solvent from the appropriate combined fractions, the pure desired product **3e** was obtained (35 mg, 70%), mp 236–237°C. IR ν_{max} (KBr) 2920, 1820, 1790, 1740, 1390; ^{1}H NMR ($CDCl_3$) δ 8.43 (d, 8.8 Hz, 2H), 8.33 (d, 8.3 Hz, 2H), 7.85 (m, 2H), 7.68 (m, 2H), 7.08 (s, 2H), 2.94–3.07 (m, 4H), 2.85 (s, 4H), 2.43 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 169.12, 167.95, 165.41, 148.74, 148.18, 137.45, 135.60, 130.30, 130.21, 129.10, 127.54, 125.21, 122.97, 122.80, 32.61, 27.79, 25.60, 17.76; FAB-MS *m/z* 497 [($M+H)^+$, 100%], 496 [M^+, 10], 225 [20], 206 [60], 179 [35]; Anal. Calc. for $C_{29}H_{24}N_2O_6$: C, 70.15%; H, 4.87%; N, 5.46%; Found: C, 69.99%; H, 4.67%; N, 5.51%.

*2.2.8. 2,6-Dimethyl-4-(2-succinimidyloxycarbonylethyl)-phenyl 10-methylacridinium-9-carboxylate trifluoromethanesulphonate (**3**)*

To a solution of **3e** (150 mg, 0.240 mmol) in dichloromethane (5 ml) under argon was added methyl trifluoromethanesulphonate (0.20 ml, 0.290 g, 1.68 mmol). After 1 h stirring, the product precipitated as a bright yellow solid. The reaction was stirred for an additional 2h and the precipitate was then filtered off, washed with benzene and dried. Extensive washing with dichloromethane provided **3** as a yellow solid (174 mg, 92%), mp 360°C. IR ν_{max} (KBr) 2920, 1820, 1750, 1740; ^{1}H NMR (DMSO-d_6) δ 8.98 (d, J=8.9 Hz, 2H), 8.75 (m, 4H), 8.23 (m, 2H), 7.28 (s, 2H), 4.98 (s, 3H), 3.03–3.09 (m, 4H), 2.84 (s, 4H), 2.41 (s, 6H); ^{13}C NMR (DMSO-d_6) δ 170.17, 168.35, 162.53, 146.65, 145.94, 141.85, 139.03, 138.39, 130.03, 129.71, 129.36, 129.09, 127.04, 122.52, 120.04, 39.90, 31.41, 28.93, 25.38, 17.25; FAB-MS *m/z* 511 [(M–SO_3CF_3)$^+$, 100%], 221 [20], 193 [40], 157 [65]; Anal. Calc. for $C_{31}H_{27}N_2F_3O_9S$: C, 56.36%; H, 4.12%; N, 4.24%; Found: C, 56.16%; H, 4.09%; N, 4.25%.

*2.2.9. 3-(3,5-Dimethoxy-4-hydroxyphenyl)propanoic acid (**4a**)*

In a glass bottle for use in a high pressure hydrogenator, a solution of 3-(3,5-dimethoxy-4-hydroxyphenyl)prop-2-enoic acid **4f** (1.110 g, 5.0 mmol)) in methanol (85 ml) was mixed with PtO_2 (0.010 g, 0.044 mmol). The mixture was shaken under H_2 at 56 psi pressure for 20 h. The resulting mixture was filtered and the filtrate was evaporated to dryness. White crystals of **4a** were obtained (1.130 g, 100%), mp 90–91°C. This product was pure enough for the subsequent synthesis but for characterisation recrystallisation from methanol–chloroform was carried out. IR ν_{max} (KBr) 3500, 3300, 3000, 1750, 1600; ^{1}H NMR ($CDCl_3$) δ 12.09 (s, 1H), 8.06 (s, 1H), 6.47 (s, 2H), 3.76 (s, 6H), 2.71 (t, 7.5 Hz, 2H), 2.50 (t, 7.5 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 173.86, 147.78, 133.69, 130.80, 105.58, 55.84, 35.71, 30.51; FAB-MS *m/z* 249 [(M+Na)$^+$, 13], 226 [M$^+$, 100%]. Anal. Calc. for $C_{11}H_{14}O_5$: C, 58.40%; H, 6.24%; Found: C, 58.65%; H, 6.45%.

*2.2.10. Benzyl 3-(3,5-dimethoxy-4-hydroxyphenyl)-propanoate (**4b**)*

To a solution of **4a** (0.800 g, 3.54 mmol) in methanol (20 ml) was added KOH (0.200 g, 3.54 mmol). The solution was stirred at room temperature for 1 h, then the solvent was evaporated and the residue was dried under vacuum for 5 h. The dried potassium salt and dibenzo-18-crown-6 (0.130 g, 0.40 mol) were suspended in DMF–acetonitrile (30 ml, 1:2, v/v). The mixture was stirred at 80–90°C for 30 min, benzyl chloride (0.46 ml, 4 mmol) was then added and stirring was maintained for another 4 h. The solid formed was removed by filtration and the filtrate was evaporated to obtain a thick brown liquid. The crude product was purified on a silica gel column eluted with chloroform–ethyl acetate (5:1), and the fractions with the component having R_F=0.5 (silica gel TLC plate, toluene–ethyl acetate, 4:1) were collected. After evaporation of the solvent, **4b** was obtained as a thick yellow liquid (0.850 g, 76%). IR ν_{max} (KBr) 3500, 3000, 1750, 1500, 1200; ^{1}H NMR ($CDCl_3$) δ 7.25–7.35 (m, 5H), 6.40 (s, 2H, H2′), 5.50 (s, 1H), 5.11 (s, 2H), 3.80 (s, 6H), 2.90 (t, 7.8 Hz, 2H), 2.66 (t, 7.8 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 172.74, 147.00, 135.90, 133.14, 131.50, 128.55, 128.46, 128.23, 128.14, 66.77, 56.20, 36.28, 31.25; FAB-MS *m/z* 316 [M$^+$, 100%], 193 [(M–$CH_2C_6H_5$)$^+$, 56]; Anal. Calc. for $C_{18}H_{20}O_5$: C, 68.38%; H, 6.37%; Found: C, 68.10%; H, 6.56%.

*2.2.11. 2,6-Dimethoxy-4-(2-benzyloxycarbonylethyl)phenyl acridine-9-carboxylate (**4c**)*

A mixture of **4b** (712 mg, 2.25 mmol) and acridine-9-carbonyl chloride (600 mg, 2.5 mmol) in anhydrous pyridine (25 ml) was stirred at room temperature overnight. The resulting mixture was evaporated to dryness and the residue was extracted with chloroform (30 ml). The concentrated extract was chromatographed on a silica gel column (chloroform–ethyl acetate, 4:1). The fractions with the component having R_F =0.4 (silica gel TLC plate, toluene–ethyl acetate, 4:1) were combined. On evaporation, **4c** was obtained as a pale yellow solid (900 mg, 77%), mp 126–127°C. IR ν_{max} (KBr) 2900, 1750, 1730, 1610, 1200, 1150; ^{1}H NMR ($CDC1_3$) δ 8.51 (d, 8.2 Hz, 2H), 8.28 (d, 8.5 Hz, 2H), 7.79 (m, 2H), 7.62 (m, 2H), 7.27–7.34 (m, 5H), 6.55 (s, 2H), 5.13 (s, 2H), 3.88 (s, 6H), 2.99 (t, 7.0 Hz, 2H), 2.73 (t, 7.0 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 172.51, 165.53, 152.02, 148.67, 140.00, 136.83, 135.84, 130.35, 129.23, 125.81, 128.60, 128.29, 128.23, 127.16, 125.15, 122.72, 105.04, 66.41, 56.17, 35.83, 31.50; FAB-MS *m/z* 544 [(M+Na)$^+$, 15%], 522 [(M+H)$^+$, 100], 206 [63], 179 [24]; Anal. Calc. for $C_{32}H_{27}NO_6$: C, 73.69%; H, 5.22%; N, 2.69%; Found: C, 73.65%; H, 5.17%; N, 2.62%.

*2.2.12. 2,6-Dimethoxy-4-(2-carboxyethyl)phenyl acridine-9-carboxylate (**4d**)*

A mixture of **4c** (412 mg, 0.80 mmol), glacial acetic acid (20 ml) and 48% hydrobromic acid (5 ml) was heated at 100°C for 3 h and then cooled. The reaction mixture was added to water (60 ml) and a yellow solid formed. This was collected by filtration and purified by recrystallisation from $CHCl_3$ to give **4d** (337 mg, 98%), mp 224–225°C. IR ν_{max} (KBr) 3200, 2500, 1770, 1720, 1610, 1200; ^{1}H NMR (DMSO-d_6) δ 12.10 (b, 1H), 8.43 (d, 8.6 Hz, 2H), 8.37 (d, 8.5 Hz, 2H), 8.10 (m, 2H), 7.93 (m, 2H), 6.82 (s, 2H), 3.99 (s, 6H), 2.92 (t, 7.5 Hz, 2H), 2.66 (t, 7.5 Hz, 2H); ^{13}C NMR (DMSO-d_6) δ 173.62, 164.02, 151.23, 145.31, 140.85, 139.33, 133.15, 128.44, 126.64, 125.10, 125.40, 121.76, 105.26, 56.32, 35.82, 31.06; FAB-MS *m/z* 454 [(M+Na)$^+$, 7%], 432 [(M+H)$^+$, 100%], 206 [20], 179 [28]; Accurate FAB-MS Calc. for ($C_{25}H_{2l}NO_6$+H)$^+$: 432.1447; Found: 432.1447.

*2.2.13. 2,6-Dimethoxy-4-(2-succinimidyloxycarbonylethyl)-phenyl acridine-9-carboxylate (**4e**)*

To a solution of **4d** (330 mg, 0.77 mmol) in DMF (25 ml) was added *N*-hydroxysuccinimide (148 mg, 1.28 mmol). The mixture was cooled to −20°C for 10 min, then a solution of DCC (246 mg, 1.12 mmol) in DMF (2 ml) was added. The mixture was stirred at −20°C for 2 h and then at room temperature overnight. The solid formed was removed by filtration and the filtrate was evaporated to dryness. The residue was extracted with dichloromethane (30 ml×2) and the extracts were combined, concentrated and then purified on a silica gel column which was eluted with dichloromethane–ethyl acetate (3:1). The fractions with the component having R_F=0.2 (silica gel TLC plate, toluene–ethyl acetate, 4:1) were combined and evaporated to give **4e** (130 mg, 31%), mp 177–178°C. IR ν_{max} (KBr) 2920, 1780, 1650, 1600; ^{1}H NMR ($CDCl_3$) δ 8.48 (d, 8.5 Hz, 2H), 8.26 (d, 8.7 Hz, 2H), 7.86 (m, 2H), 7.68 (m, 2H), 6.69 (s, 2H), 3.14 (t, 7.8 Hz, 2H), 3.03 (t, 7.8 Hz, 2H), 2.88 (s, 4H), 4.02 (s, 6H); ^{13}C NMR (DMSO-d_6) δ 173.90, 165.69, 165.00, 151.43, 148.03, 140.87, 136.20, 131.13, 129.56, 128.03, 127.57, 125.38, 124.99, 105.33, 56.38, 33.38, 31.02, 25.35; FAB-MS *m/z* 529 [$(M+H)^+$, 2%], 449 [M^+, 7], 225 [100]; Accurate FAB-MS for $(C_{29}H_{24}N_2O_8+H)^+$: 529.1611; Found: 529.1582.

*2.2.14. 2,6-Dimethoxy-4-(2-succinimidyloxycarbonylethyl)-phenyl 10-methylacridinium-9-carboxylate trifluoromethanesulphonate (**4**)*

The methylation procedure was similar to that for **3**, but the product was purified by recrystallisation from acetone–diethyl ether instead of washing with benzene and dichloromethane. **4** was obtained in a 32% yield, mp 203–205°C. IR ν_{max} (KBr) 2920, 1820, 1750, 1610; ^{1}H NMR (DMSO-d_6) δ 8.93 (d, 8.5 Hz, 2H), 8.62 (d, 8.5 Hz, 2H), 8.56 (m, 2H), 8.25 (m, 2H), 6.93 (s, 2H), 4.96 (s, 3H), 4.04 (s, 6H), 3.05–3.14 (m, 4H), 2.83 (s, 4H); ^{13}C NMR (DMSO-d_6) δ 170.10, 168.27, 163.43, 151.23, 147.50, 141.92, 140.02, 139.18, 129.46, 127.20, 124.87, 122.52, 119.08, 105.37, 56.34, 39.60, 31.64, 30.33, 25.39; FAB-MS *m/z* 543 [$(M–SO_3CF_3)^+$, 100%], 193 [94]; Anal. Calc. for $C_{31}H_{27}N_2F_3O_{11}S$: C, 53.67%; H, 3.93%; N, 4.04%; Found: C, 53.71%; H, 3.78%; N, 4.19%.

*2.2.15. Benzyl 3-(4-hydroxy-3-methoxyphenyl)propanoate (**5b**)*

The preparation was analogous to that used to prepare **4b**. **5b** was obtained as a thick yellow liquid (87%). IR ν_{max} (KBr) 3510, 3000, 1750, 1500, 1300; ^{1}H NMR ($CDCl_3$) δ 7.27–7.36 (m, 5H), 6.81 (d, 8.0 Hz, 1H), 6.67 (s, 1H), 6.66 (d, 8.0 Hz, 1H), 5.57 (b, 1H), 5.10 (s, 2H), 3.80 (s, 3H), 2.89 (t, 7.4 Hz, 2H), 2.64 (t, 7.4 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 172.80, 146.43, 144.05, 135.93, 132.32, 128.53, 128.20, 128.18, 120.87, 114.38, 110.97, 66.24, 55.81, 36.28, 30.67; FAB-MS *m/z* 309 [$(M+Na)^+$, 14%], 286 [M^+, 100], 195 [$(M–CH_2Ph)^+$, 13]; Anal. Calc. for $C_{17}H_{l8}O_4$: C, 71.31%; H, 6.34%; Found: C, 71.46%; H, 6.08%.

*2.2.16. 2-Methoxy-4-(2-benzyloxycarbonylethyl)phenyl acridine-9-carboxylate (**5c**)*

5c was obtained by a procedure similar to that used to prepare **4c** in a 73% yield, mp 86–88°C. IR ν_{max} (KBr) 3500, 3000, 1760, 1740, 1500, 1175; ^{1}H NMR ($CDC1_3$) δ 8.42 (ddd, 8.7 Hz, 1.2 Hz, 0.7 Hz, 2H), 8.30 (ddd, 8.7 Hz, 1.2 Hz, 0.7 Hz), 7.84 (ddd, 8.7 Hz, 6.6 Hz, 1.2 Hz, 2H), 7.66 (ddd, 8.7 Hz, 6.6 Hz, 1.2 Hz), 7.31–7.34 (m, 5H), 7.22 (d, 8.1 Hz, 1H), 6.97 (d, 1.8 Hz, 1H), 6.90 (dd, 8.1 Hz, 1.8 Hz, 1H), 5.15 (s, 2H), 3.97 (s, 3H), 3.06 (t, 7.6 Hz, 2H), 2.89 (t, 7.6 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 177.52, 165.52, 150.88, 148.65, 140.30, 137.72, 136.25, 135.79, 130.42, 130.39, 129.90, 128.54, 128.21, 128.17, 127.27, 125.45, 122.61, 120.80, 112.90, 66.46, 55.39, 35.82, 30.90; FAB-MS *m/z* 514 [$(M+Na)^+$, 9%], 492 [$(M+H)^+$, 100], 206 [70], 179 [57]; Anal. Calc. for $C_{31}H_{25}NO_5$: C, 75.75%; H, 5.13%; N, 2.85%; Found: C, 78.45%; H, 5.12%; N, 2.50%.

*2.2.17. 2-Methoxy-4-(2-carboxyethyl)phenyl acridine-9-carboxylate (**5d**)*

A procedure analogous to that used to prepare **4d** gave **5d** in a yield of 95%, mp 235–237°C. IR ν_{max} (KBr) 2700, 1760, 1720, 1200; ^{1}H NMR (DMSO-d_6) δ 8.38 (d, 8.6 Hz, 2H), 8.29 (d, 8.7 Hz, 2H), 7.97 (m, 2H), 7.82 (m, 2H), 7.42 (d, 8.0 Hz, 1H), 7.22 (d, 1.7 Hz, 1H), 6.96 (dd, 8.0 Hz, 1.7 Hz, 1H), 4.03 (s, 3H), 2.94 (t, 7.8 Hz, 2H), 2.65 (t, 7.8 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ 173.59, 164.82, 150.30, 147.77, 141.08, 136.25, 135.99, 130.90, 129.24, 127.79, 125.00, 122.42, 121.55, 120.47, 113.03, 55.40, 35.17, 30.31; FAB-MS *m/z* 424 [$(M+Na)^+$, 12%], 402 [$(M+H)^+$, 100], 206 [65], 179 [45]; Anal. Calc. for $C_{24}H_{19}NO_5$: C, 71.81%; H, 4.77%; N, 3.49%; Found: C, 72.06%; H, 4.84%; N, 3.36%.

*2.2.18. 2-Methoxy-4-(2-succinimidyloxycarbonylethyl)-phenyl acridine-9-carboxylate (**5e**)*

5e was obtained in a 42% yield by following a procedure similar to that for **4e**; mp 208–210°C. IR ν_{max} (KBr) 2920, 1820, 1790 1740, 1200; ^{1}H NMR ($CDCl_3$) δ 8.42 (d, 8.7 Hz, 2H), 8.34 (d, 8.8 Hz, 2H), 7.85 (m, 2H), 7.68 (m, 2H), 7.27 (d, 8.0 Hz, 1H), 7.03 (d, 1.9 Hz, 1H), 6.96 (dd, 8.0 Hz, 1.9 Hz, 1H), 3.14 (t, 7.6 Hz, 2H), 3.00 (t, 7.6 Hz, 2H), 2.86 (s, 4H), 4.05 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 169.02, 167.78, 165.32, 151.09, 148.29, 139.09, 138.04, 136.50, 130.67, 129.53, 127.35, 125.47, 122.83, 120.76, 120.46, 112.88, 56.02, 32.68, 30.45, 25.59; FAB-MS *m/z* 521 [$(M+Na)^+$, 16%], 499 [$(M+H)^+$, 100], 206 [55], 179 [28]; Accurate FAB-MS Calc. for $(C_{28}H_{22}N_2O_7+H)^+$: 499.1505; Found: 499.1506.

*2.2.19. 2-Methoxy-4-(2-succinimidyloxycarbonylethyl)-phenyl 10-methylacridinium-9-carboxylate trifluoromethanesulphonate (**5**)*

The preparation was similar to that used to prepare **4**. **5** was obtained as bright yellow crystals in a yield of 60%, mp 114–115°C. IR ν_{max} (KBr) 3500, 2920, 1800, 1750, 1550; ^{1}H NMR (DMSO-d$_6$) δ 8.95 (d, 9.4 Hz, 2H), 8.71 (dd, 9.4 Hz, 0.9 Hz, 2H), 8.54–8.58 (m, 2H), 8.21–8.29 (m, 2H), 7.60 (d, 8.1 Hz, 1H), 7.35 (d, 1.8 Hz, 1H), 7.10 (dd, 8.1 Hz, 1.8 Hz, 1H), 4.98 (s, 3H), 4.09 (s, 3H), 3.07–3.16 (m, 4H), 2.84 (s, 4H); ^{13}C NMR (DMSO-d$_6$) δ 170.10, 168.26, 162.89, 150.16, 147.50, 141.92, 140.35, 139.20, 138.39, 136.49, 129.56, 127.54, 122.68, 122.60, 120.90, 119.79, 113.49, 56.19, 39.69, 31.76, 29.82, 25.49; FAB-MS *m*/*z* 513 [(M–SO_3CF_3)$^+$, 100%], 221 [17], 193 [35]; Anal. Calc. for $C_{30}H_{25}N_2F_3O_{10}S$: C, 54.38%; H, 3.80%; N, 4.23%; Found: C, 54.16%; H, 3.80%; N, 4.35%.

*2.2.20. 3-(3,5-Dibromo-4-hydroxyphenyl)propanoic acid (**6a**)*

To a stirred solution of 3-(4-hydroxyphenyl)propanoic acid **6f** (1.011 g, 6.020 mmol) in glacial AcOH (50 ml) at ambient temperature was added Br_2 (0.620 ml, 1.901 g, 12.03 mmol). The mixture was stirred in the dark for 70 h, after which evaporation of the solution in vacuo left a residue of 2.401 g. The residue was dissolved in diethyl ether (70 ml), and the solution was washed with saturated NaCl aq. (40 ml×3), dried (Na_2SO_4) and evaporated to leave a white powder (1.902 g). The product was recrystallised from benzene/hexane to give white needles of **6a** (1.760 g, 90%), mp 108–109°C (lit. 106–108°C) [7]. IR ν_{max} (KBr) 3450, 3400, 3000, 1700; ^{1}H NMR ($CDCl_3$) δ 10.06 (b, 1H), 8.30 (b, 1H), 7.45 (s, 2H), 2.85 (t, 7.5 Hz, 2H), 2.63 (t, 7.5 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 173.84, 149.75, 136.56, 133.09, 111.27, 35.58, 29.83; CI-MS *m*/*z* 324 [M$^+$, 100%], 278 [9], 265 [98], 244 [15], 198 [12], 185 [40]; EI-MS *m*/*z* 324 [M$^+$, 30%], 278 [10], 265 [100].

*2.2.21. Benzyl 3-(3,5-dibromo-4-hydroxyphenyl)propanoate (**6b**)*

The procedure was analogous to that used to prepare **3b**, but the reaction temperature was 85°C instead of 60°C. **6b** was obtained as a pure colourless oil in an 80% yield after column chromatography purification. IR ν_{max} (KBr) 3500, 3000, 1750, 1700, 1560, 1480, 1200; ^{1}H NMR ($CDCl_3$) δ 7.44 (m, 7H), 5.86 (s, 1H), 5.10 (s, 2H), 2.80 (t, 7.5 Hz, 2H), 2.70 (t, 7.5 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 172.11, 147.82, 134.94, 134.51, 132.24, 131.93, 128.30, 128.24, 109.68, 66.46, 35.55, 29.35; EI-MS *m*/*z* 415 [(M+1)$^+$, 85%]; CI-MS *m*/*z* 432 [(M+NH_4)$^+$, 95%], 91 [100]; Anal. Calc. for $C_{l6}H_{l4}Br_2O_3$: C, 46.40%; H, 3.41%; Found: C, 46.32%; H, 3.39%.

*2.2.22. 2,6-Dibromo-4-(2-benzyloxycarbonylethyl)phenyl acridine-9-carboxylate (**6c**)*

6c was obtained in a yield of 75% from acridine-9-carbonyl chloride and **6b** in pyridine as described for **4c**; mp 114–115°C. IR ν_{max} (KBr) 1770, 1740; ^{1}H NMR ($CDCl_3$) δ 8.76 (d, 9.0 Hz, 2H), 8.32 (d, 8.7 Hz, 2H), 7.85 (m, 2H), 7.70 (m, 2H), 7.54 (s, 2H), 7.35 (m, 5H), 5.14 (s, 2H), 3.00 (t, 7.5 Hz, 2H), 2.74 (t, 7.5 Hz, 2H); ^{13}C NMR ($CDC1_3$) δ 171.82, 164.09, 148.74, 146.15, 142.03, 135.62, 133.89, 132.82, 130.88, 130.33, 128.65, 128.38, 128.30, 127.50, 125.96, 123.30, 117.48, 66.67, 35.14, 29.72; FAB-MS *m*/*z* 619 [M$^+$, 45%], 206 [100], 179 [94]; Anal. Calc. for $C_{30}H_{21}NBr_2O_4$: C, 58.18%; H, 3.42%; N, 2.26%; Found: C, 57.91%; H, 3.45%; N, 2.17%.

*2.2.23. 2,6-Dibromo-4-(2-carboxyethyl)phenyl acridine-9-carboxylate (**6d**)*

The procedure was analogous to that used to prepare **3d**. The pure **6d** was obtained in a 74% yield, mp 240–242°C. IR ν_{max} (KBr) 3500, 3000, 2500, 1750, 1720; ^{1}H NMR (DMSO-d$_6$) δ 12.22 (b, 1H), 8.69 (d, 9.0 Hz, 2H), 8.32 (d, 8.2 Hz, 2H), 7.98 (m, 1H), 7.8 (m, 1H), 7.82 (s, 2H), 2.93 (t, 7.5 Hz, 2H), 2.67 (t, 7.5 Hz, 2H); ^{13}C NMR (DMSO-d$_6$) δ 173.31, 163.64, 148.00, 143.58, 143.38, 132.92, 132.90, 130.79, 129.73, 128.18, 125.13, 121.86, 116.44, 34.37, 28.97; FAB-MS *m*/*z* 529 [M$^+$, 75%], 511 [10], 486 [10], 207 [100]; Anal. Calc. for $C_{23}H_{15}O_4NBr_2$: C, 52.20%; H, 2.86%; N, 2.65%; Found: C, 52.07%; H, 2.82%; N, 2.50%.

*2.2.24. 2,6-Dibromo-4-(2-succinimidyloxycarbonylethyl)-phenyl acridine-9-carboxylate (**6e**)*

6e was obtained in a yield of 64% by a procedure similar to that used to prepare **4e**; mp 175–176°C. IR ν_{max} (KBr) 3000, 2110, 1800, 1730; ^{1}H NMR ($CDCl_3$) δ 8.79 (d, 8.9 Hz, 2H), 8.43 (d, 8.2 Hz, 2H), 7.92 (m, 2H), 7.70 (m, 2H), 7.62 (s, 2H), 3.12 (t, 7.5 Hz, 2H), 3.00 (t, 7.5 Hz, 2H), 2.86 (s, 4H); ^{13}C NMR ($CDCl_3$) δ 168.98, 167.33, 164.03, 148.61, 145.18, 140.59, 133.89, 132.82, 130.44, 129.93, 127.60, 125.91, 122.97, 117.72, 32.09, 29.28, 25.58; FAB-MS *m*/*z* 627 [(M+1)$^+$, 20%], 225 [100], 206 [18], 179 [10], 143 [8]; Anal. Calc. for $C_{27}H_{l9}N_2Br_2O_6$: C, 51.78%; H, 2.90%; N, 4.47%; Found: C, 51.53%; H, 2.99%; N, 4.40%.

*2.2.25. 2,6-Dibromo-4-(2-succinimidyloxycarbonylethyl)-phenyl 10-methylacridinium-9-carboxylate trifluoromethanesulphonate (**6**)*

The procedure was similar to that used to prepare **3**, but the reaction time was 18 h instead of 3 h. **6** was obtained as a yellow solid in a 90% yield, mp 320°C. IR ν_{max} (KBr) 3000, 1820, 1790, 1730; ^{1}H NMR (DMSO-d$_6$) δ 8.91–9.01 (m, 4H), 8.58 (m, 2H), 8.24 (m, 2H), 7.96 (s, 2H), 4.99 (s, 3H), 3.18 (t, 7.5 Hz, 2H), 3.09 (t, 7.5 Hz, 2H), 2.83 (s, 4H); ^{13}C NMR (DMSO-d$_6$) δ 170.09, 168.00, 161.70, 144.86, 142.78, 141.86, 139.48, 139.14, 133.48, 132.45, 129.64, 129.18, 127.45, 120.01, 116.31, 40.09, 30.88, 28.39, 25.38; FAB-MS

m/z 641 [$(M–SO_3CF_3)^+$, 12%], 627 [85], 225 [34], 207 [6], 195 [100], 179 [32]; Anal. Calc. for $C_{29}H_{21}N_2Br_2F_3O_9S$: C, 44.07%; H, 2.68%; N, 3.54%; Found: C, 44.14%; H, 2.78%; N, 3.65%.

2.3. Chemiluminescent kinetics and efficiency

A standard procedure was employed for estimation of chemiluminescent kinetics and efficiency. The samples (compounds **1** and **3**–**6**, 1.0 mg) were separately dissolved in anhydrous acetonitrile (1.0 ml). The solutions were diluted to 1×10^{-10} mol/l using 1.00×10^{-3} M hydrochloric acid. To the test tube containing the diluted sample solution (10 ml), hydrogen peroxide solution (0.3 ml of 0.5% w/v in 0.1 mol/1 HNO_3) was delivered automatically, followed by NaOH solution (0.3 ml of 0.25 mol/l) containing a surfactant. The output of photons was counted for 3 s (compounds **1**, **4**, **6**), 100 s (compound **3**), or 30 s (compound **5**). Chemiluminescent intensity versus time curves were plotted, from which chemiluminescent peak times and life times were measured. The total counts were used to calculate the relative chemiluminescent efficiencies for each compound.

2.4. Labelling

Acridinium ester solution (10 μl, 0.5 mg/ml) in anhydrous acetonitrile was placed in a small glass vial and human IgG solution (200 μl, 250 μg/ml) in labelling buffer was added with mixing. The mixtures were incubated at room temperature for 5 min in the dark. Quench buffer (100 μl) was added to the mixture and incubation was continued for a further 5 min. The solution was transferred to the top of a Sephadex G50 column which was then eluted with column buffer. Fractions with a volume of 0.5 ml were collected. Each fraction (10 μl) was transferred separately to a clean tube and column buffer (1 ml) was added to each tube with mixing. The chemiluminescence intensity of the diluted fractions was measured. For each compound, two active components were found. The first was the labelled IgG conjugate and the second was the free chemiluminescent compound. The four most strongly chemiluminescent fractions of the first component were combined and stored at −20°C.

2.5. Stabilities of the conjugates

The conjugate solutions (10 μl, see Section 2.4) were diluted separately with each of the assay buffers (pH=5, 6, 7 and 8) to a concentration which allowed the counts of the diluted solution to total around 1 million within a convenient measuring period. Samples of the diluted solutions were stored at 4, 25 and 37°C, and the chemiluminescent intensity of each solution was monitored after 2, 4 and 8 days.

3. Results and discussion

3.1. Syntheses of compounds **3**, **4**, **5** and **6**

Scheme 2 outlines the syntheses of the acridinium ester labels from the appropriate 3-(4-hydroxyphenyl)propanoic acid derivatives **3a**–**6a**. Firstly, the carboxylic acid groups were protected by benzylation. The benzyl esters **3b**–**6b** were then coupled to acridine-9-carbonyl chloride, which was prepared by reacting acridine-9-carboxylic acid with thionyl chloride [10–11]. Deprotection of **3c**–**6c** with a mixture of ethanoic acid and HBr yielded acids **3d**–**6d**, which were then esterified with *N*-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide (DCC) to give **3e**–**6e**. Finally, methylation by treatment with methyl trifluoromethanesulphonate under an atmosphere of nitrogen afforded the target molecules **3**–**6**. All of the reactions gave good yields except for the final two steps in the cases with methoxy groups in the phenoxy ring. It is likely that the poorer yields in these steps are due to difficulties with isolation as a result of the physical properties of the compounds rather than to fundamental problems with the reactions themselves.

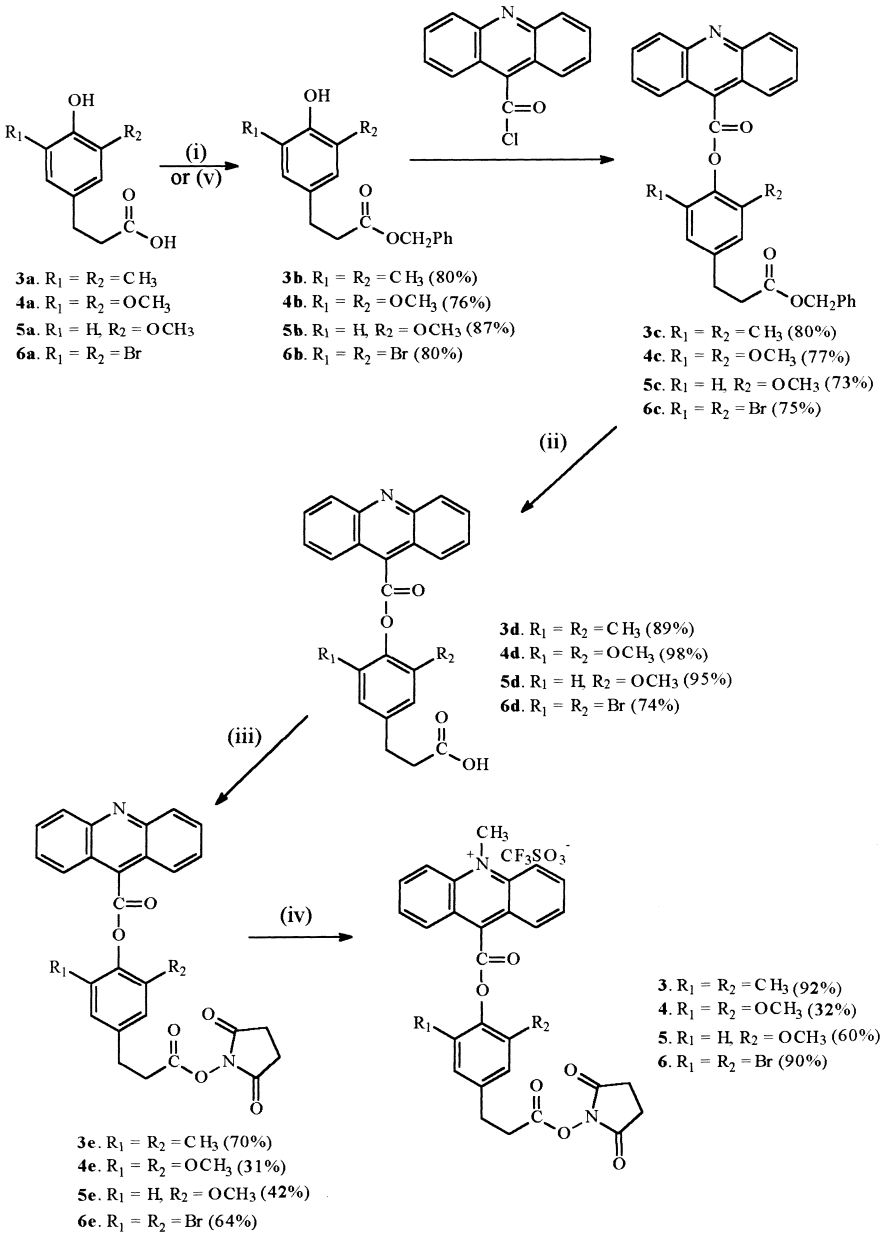
The syntheses of the substituted 3-(4-hydroxyphenyl)-propanoic acids **4a**–**7a**, the starting materials for the syntheses presented in Scheme 2, are summarised in Scheme 3.

The synthesis of 3-(4-hydroxy-3,5-dimethylphenyl)propanoic acid was begun with conversion of 2,6-dimethylphenol (**3f**) to 3-(4-hydroxy-3,5-dimethylphenyl)propanenitrile (**3g**) by reaction with acrylonitrile under catalysis by $AlCl_3$ and HCl. The resulting nitrile was subsequently hydrolysed in acid to **3a** [12–13]. 3-(4-Hydroxy-3,5-dimethoxyphenyl)-propanoic acid (**5a**) was prepared by hydrogenation of 3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid (**5f**) over PtO_2. 3-(3,5-Dibromo-4-hydroxyphenyl)propanoic acid (**6a**) was obtained by direct bromination of 3-(4-hydroxyphenyl)propanoic acid (**6f**) [14–15]. All of the reactions proceeded smoothly and gave good yields. 3-(3-Methoxy-4-hydroxyphenyl)propanoic acid (**5a**) was obtained directly from Aldrich.

3.2. Chemiluminescent properties

3.2.1. Stability of conjugates with human IgG

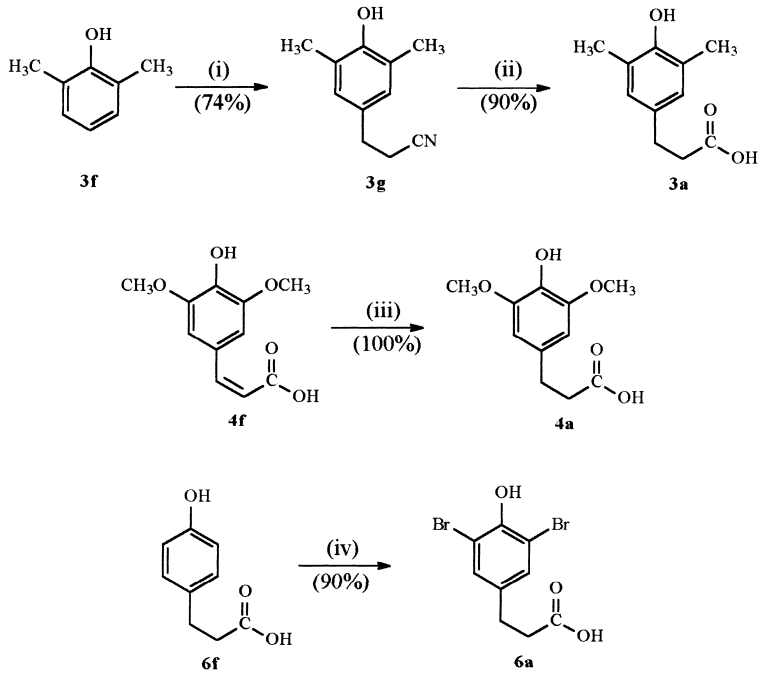
Compound **1**, the first acridinium ester label, has been found to be somewhat unstable during use. The ester bond suffers some hydrolysis in aqueous media, especially during immunoassays when components are incubated at 37°C. This can cause unacceptable reduction of photon output from antibody conjugates. It was hoped that the stability would be improved by steric hindrance around the ester bond in compounds **3**–**6**. Therefore, the compounds were each conjugated to human immunoglobulin G (IgG) using standard techniques, [5] and ready-to-use solutions of the conjugates were prepared. The decay rates of photon output during the



Scheme 2. General strategy for the synthesis of acridinium ester labels.

storage of these solutions in different pH buffers at 4, 25 and 37°C were then investigated. At 4°C, the conjugates of **3**, **4**, **5** and **6** all showed a similar decay rate to that of the conjugate of **1**. However, at 25 and 37°C, the conjugate of **1** demonstrated a quicker decay rate in photon output than the conjugates of **3**, **4**, **5** and **6** in all of the buffers tried (pH=5, 6, 7 and 8). During incubation for 8 days at 37°C in their optimal pH buffers, for example, the conjugates of **3**, **4**, **5** and **6** showed much higher stabilities than that of compound **1** (Fig. 1). (The optimal buffer is that leading to the lowest rate of decay.) Of the new compounds, **6** was significantly less stable than the others.

The steric effects of the substituents presumably account for the fact that all of the new compounds are more stable than compound **1**. However, the electron donating effects of CH_3 and OCH_3 groups also render the appropriately substituted phenoxy moieties less prone to leave and hydrolysis correspondingly more difficult. In contrast, the electron withdrawing Br group would tend to accelerate the hydrolysis. This could explain why the conjugate of compound



Scheme 3. Syntheses of substituted 3-(4-hydroxyphenyl)propanoic acids.

6 is relatively less stable than those of compounds **3**, **4** and **5**.

3.2.2. Chemiluminescence kinetics

The emission of light on oxidation of each of the compounds **1** and **3**–**6** was studied over time under a particular set of conditions (see Section 2). The period of time for which luminescence could be detected (chemiluminescent lifetime) and the time taken to reach maximum intensity of emission (peak time) were determined. The results are recorded in Table 1, which illustrates that the chemiluminescent lifetimes are in the order **3**>**4**>**5**>**1**>**6**.

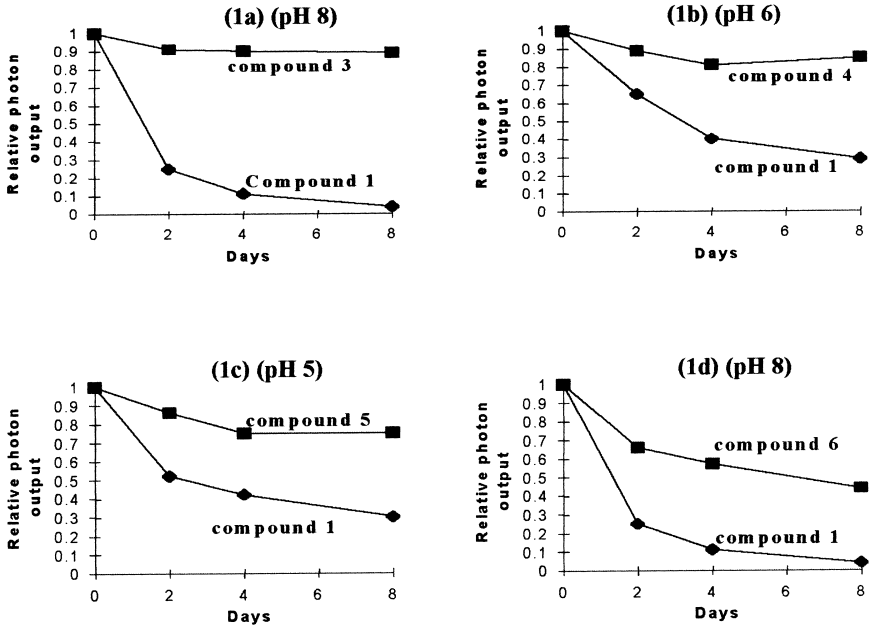
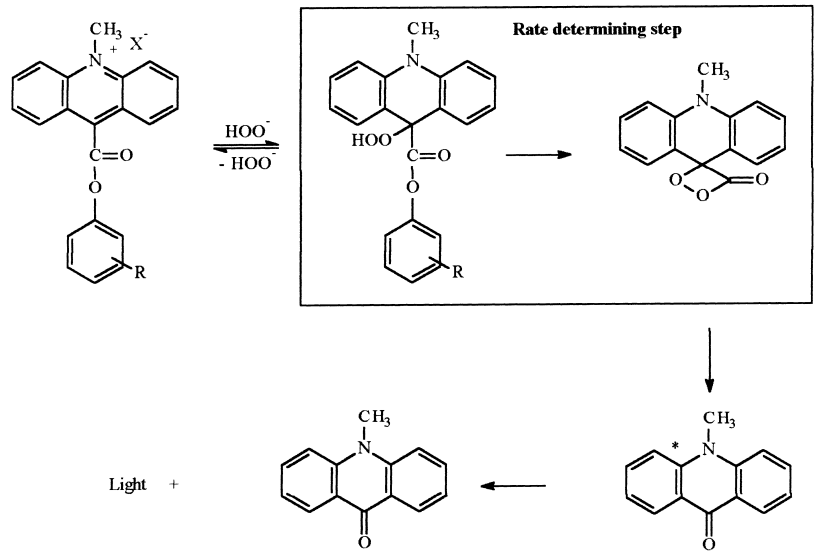


Fig. 1. Stability of the IgG conjugates of **3**, **4**, **5** and **6** in their optimal pH buffers at 37°C in comparison with the stability of the conjugate of **1**: (a) compound **3** in pH 8 buffer; (b) compound **5** in pH 6 buffer; (c) compound **5** in pH 5 buffer; (d) compound **6** in pH 8 buffer.



Scheme 4. Reactions involved in chemiluminescence of acridinium esters.

Table 1
Chemiluminescent lifetimes and peak times

Compound	Lifetime (s)	Peak time (s)
1	3	0.5
3	100	30
4	75	15
5	5	1
6	0.6	0.1

Table 2
Relative chemiluminescent efficiency

Compound	Relative chemiluminescent efficiency
1	100
3	50
4	110
5	94
6	79

The chemical reactions taking place during chemiluminescence of acridinium esters are shown in Scheme 4. The rate determining step involves cleavage of the phenoxy moiety. Electron withdrawing substituents, like Br, would lead to the phenoxy moiety becoming a better leaving group, whereas electron donating groups like CH_3 and OCH_3 would cause the phenoxy moiety to become a relatively poor leaving group. Therefore, on electronic grounds the order of reactivity would be expected to be like the observed order. Indeed, the pK_a values of 2,6-dibromophenol (6.4) [16], phenol (9.89) [17], 2-methoxyphenol (9.98) [18], 2,6-dimethoxyphenol (10.2) [19] and 2,6-dimethylphenol (10.63) [20] are in exactly the reverse order to the rates of the chemiluminescent reactions of compounds **6**, **1**, **5**, **4** and **3**. This suggests that the effects of steric hindrance are less marked in the chemiluminescent reaction than they are in the hydrolysis reaction, where the order for **1** and **6** is inverted. This can be understood because the reactive centre in the chemiluminescent reaction is further away from the hindering groups than that in the hydrolysis reaction. This allows the situation, for the bromo compound, in which the stability to hydrolysis is increased at the same time as the chemiluminescent reaction is speeded up.

3.2.3. Chemiluminescent efficiency

Chemiluminescent efficiency is strongly dependent on the nature of the chemiluminescent molecule. Acridinium esters generally have a chemiluminescent quantum yield of around 5%. This quantum yield could be affected by structural modifications. The relative chemiluminescent efficiencies of **3**, **4**, **5** and **6** have therefore been examined in comparison with that of **1**, and the results are summarised in Table 2. As the results show, the differences in quantum yield are not great. Compound **4** shows a slightly higher chemiluminescent efficiency than compound **1**, while the others are less efficient, with compound **3** having the lowest efficiency, half of that of compound **1**.

4. Conclusions

Several phenyl acridinium ester labelling compounds differing in the substituents on the phenyl group have been successfully prepared in reasonable yields. All exhibit chemiluminescence when reacted with hydrogen peroxide and show minor differences, compared to the unsubstituted analogue, in the quantum yield of luminescence. The presence of substituents at the *ortho*-positions does have the effect of stabilising IgG conjugates of the labels towards hydrolysis at the ester group, irrespective of the electronic nature of the substituents. The electronic effects are more important in determining the rate at which the chemiluminescent reaction occurs, thereby providing a range of labels

which are differentiated in the time over which light is emitted as well as being relatively stable to hydrolysis. This provides opportunities for choosing a label with properties appropriate for a particular application. The dibromo compound flashes quickly while the dimethyl and dimethoxy compounds glow slowly, as a result of the relative ease of expulsion of the corresponding phenoxide anions.

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