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Syntheses of Hindered-Polymethylacridinium Esters with Potential for Biological Probe Nanoarchitectonics

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Abstract: Novel acridinium esters containing several methyl groups, at least one of which is in the 1 or 8-position, have been synthesized and their structures established. The influence of the methyl substituents on the chemiluminescent properties of the synthesized acridinium esters has been investigated.

Key words: synthesis, acridinium ester, chemiluminescence properties, methyl substituents

1 Introduction

Chemiluminescent immunoassay is an important analytical tool for *in-vitro* diagnostics[1-3]. Chemiluminescent compounds can replace radioactive isotopes that are hazardous and expensive to produce[4]. Acridinium esters (AEs) show high chemiluminescent sensitivity and quantum yields and low background. They are safe, stable, can be produced in large quantities, and are easily attached to biological molecules by means of an appropriate linker group. Furthermore, their chemiluminescence can be stimulated by alkaline peroxide without a catalyst. Therefore, the synthesis and use of AEs have gained much attention over the years[5-20].

The AE **1a** (Fig. 1), which contains a succinimidyl (NHS) ester as the active linker group, was developed many years ago[21]. Various studies have been carried out to produce analogues of **1a** that have better chemiluminescent properties. A variety of AEs having different leaving groups has been synthesized[22, 23]. In particular, substitution at the *ortho*-positions of the phenoxy leaving group leads to better stability of immunoglobulin (IgG) conjugates in comparison with those of **1a** by hindering hydrolysis of the AEs[24]. Such substitution also affects chemiluminescent properties such as quantum yield and rate of development of emission. For example, the dimethoxy AE **1b** (Fig. 1) shows slightly better quantum yield than **1a**[24], while the dimethyl and dibromo AEs (**1c** and **1d**, respectively) are slightly less efficient compared with **1a**[24]. We have shown that the introduction of the linker chain at the *ortho*- or *meta*-position of the phenoxy ring also affects some of the chemiluminescent properties[25].

Modification of the leaving group, however, does not affect the emission wavelength or the efficiency of energy transfer to a given acceptor, which depend on the nature of the excited acridone emitter. Therefore, substitution on the acridinium ring is required in order to influence those properties. The recent publication of the syntheses of AEs with methyl groups at the 2, 3, 6 and/or 7 positions of the acridinium ring[6] prompts us to report our own studies of polymethyl AEs, which were studied as part of our long-term interest in the synthesis of AEs[24-28]. In particular, we were interested to see whether introduction of methyl groups in positions 1 and/or 8 would significantly influence the stability towards hydrolysis and the rate of the chemiluminescent reactions. We have successfully synthesized the two novel methylated AEs **2** and **3** and compared their chemiluminescent properties with those of **1a** (Fig. 1), and also prepared the substituted acridine esters **4** and **5**, but were unable to convert those acridines into the corresponding acridinium salts.

2 Experimental

2.1 General methods

Melting points were measured using a Griffin melting

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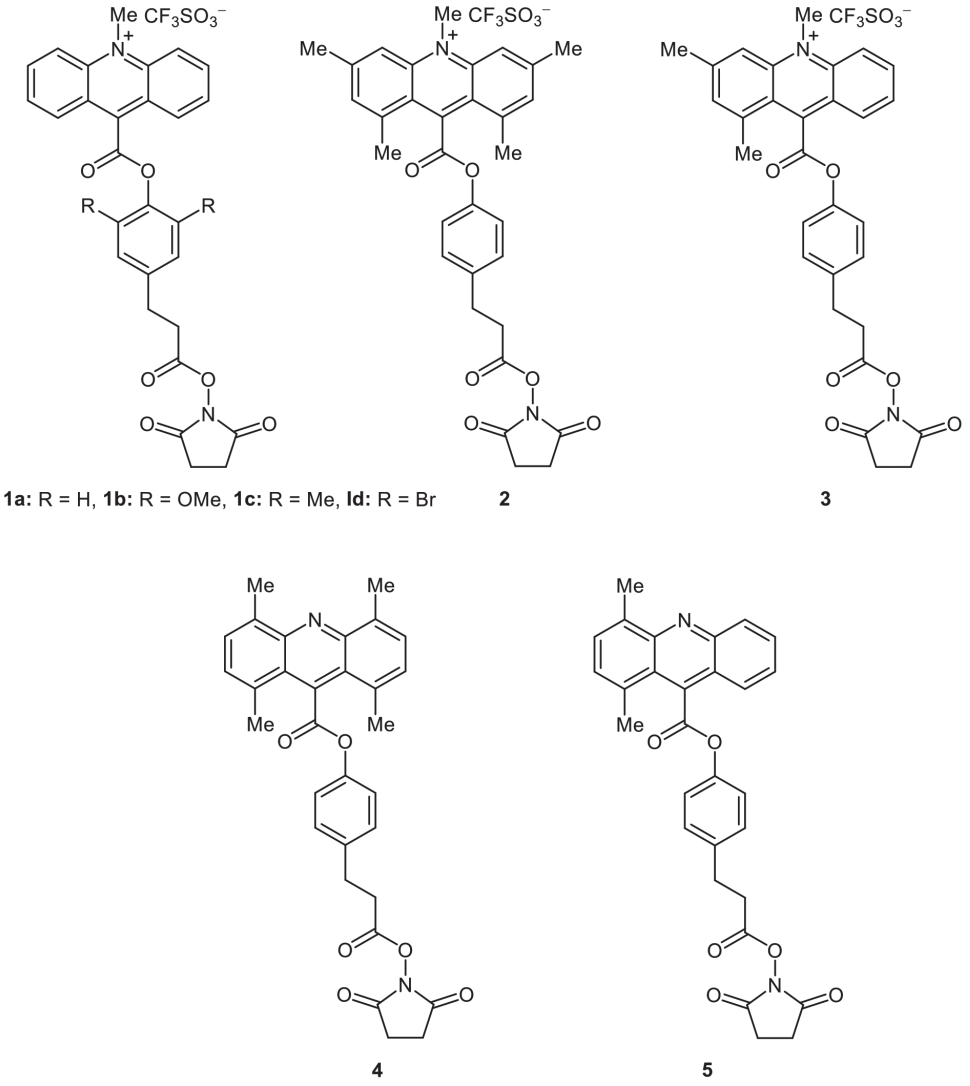


Fig. 1 Structures of acridine and acridinium esters 1–5 discussed in this report.

point apparatus. A Perkin Elmer Fourier Transform Infra-Red (FTIR) Spectrometer was used to record IR spectra (KBr disk). A Bruker AV 400 Spectrometer was used to record ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra; tetramethylsilane (TMS) was used as internal standard. Chemical shifts (δ) are in ppm, and coupling constants (*J*) are in Hz. A VG 12-250 mass spectrometer was used to record the low and high-resolution mass spectra. Chromatographic purifications were performed using Fisons Matrix silica 60. The purity of products was checked using Whatman silica gel plates with the aid of a UV lamp (254 nm). A Ciba-Corning Magic Lite analyzer luminometer was used to measure the chemiluminescence in relative light units (RLU). Reagent A (1 L) contained HNO_3 (70%; 6.3 mL) and H_2O_2 (30%; 16.5 mL) in distilled H_2O. Reagent B (1 L) contained NaOH (10 g) and cetyltrimethylammonium chloride (25%) in distilled H_2O. Both reagents were provided by Molecular Light Technology, Cardiff. The buffer solution (pH = 8) used for the labelling contained sodium dihydrogen orthophosphate (0.1 M) and NaOH (5 M). The quenching buffer contained lysine (10 mg/mL) in addition to the labelling buffer. Dilute solutions of **1**–**3** (1×10^{-4} mg/mL) were made using MeCN for labelling. Standard procedures were used for the labelling of AEs[24, 25].

2.2 Synthesis of 3,5-dimethylacetanilide (7)

A solution of **6** (10.00 g, 82.55 mmol) in H_2O (205 mL) and concentrated HCl (6.90 mL) was treated with activated charcoal and then filtered. To the filtrate, Ac_2O (9.50 mL, 100.7 mmol) and then AcONa (8.10 g, 98.8 mmol in 46 mL water) were added and the mixture was stirred for 15 min. Filtration of the precipitate, washing with water, and drying gave **5** (11.51 g, 85%). Mp 141–142℃ (lit.[29] 140.5℃). ^{1}H NMR ($CDCl_3$; δ): 2.04 (s, 3H, Me), 2.17 (s, 6H, 2Me), 6.58 (s, 1H, Ar), 7.00 (s, 2H, Ar), 8.00 (br s, 1H, NH).

2.3 Synthesis of *N*,*N*-*bis*(3,5-dimethylphenyl)acetamide (8)

A mixture of **7** (10.04 g, 61.6 mmol), 1-bromo-3,5-dimethylbenzene (20.75 mL, 151.7 mmol), K_2CO_3 (8.49 g, 61.4 mmol), and CuI (1.16 g, 6.10 mmol) was refluxed for 17 h. After extraction with dichloromethane (DCM), washing with water, and drying ($MgSO_4$), chromatography (2% EtOAc/DCM) gave **8** (9.53 g, 58%). ^{1}H NMR ($CDCl_3$; δ): 1.95 (s, 3H, Me), 2.20 (s, 12H, 4Me), 6.75 (app. s, 6H, Ar).

2.4 Synthesis of *N*-(3,5-dimethylphenyl)-3,5-dimethylbenzenamine (9)

Reflux of a mixture of **8** (8.68 g, 32.5 mmol) and KOH (8.37 g, 149 mmol) in EtOH (50 mL) followed by removal of the solvent, extraction with DCM, washing with H_2O, drying ($MgSO_4$), evaporation, and distillation (160–162℃, 0.8 mmHg) gave pure **9** (5.37 g, 73%). Mp 52–53℃. FTIR (ν; cm^{-1}): 3346 (NH). ^{1}H NMR ($CDCl_3$; δ): 2.32 (s, 12H, 4Me), 5.54 (s, 1H, NH), 6.63 (s, 2H, Ar), 6.74 (s, 4H, Ar). ^{13}C NMR ($CDCl_3$; δ): 21.6, 115.8, 122.7, 139.0, 143.3. Analysis calculated for $C_{16}H_{19}N$: C, 85.28; H, 8.50; N, 6.22; found: C, 85.14; H, 8.74; N, 6.10%.

2.5 Synthesis of *N*-(3,5-dimethylphenyl)-4,6-dimethylisatin (10)

A boiled solution of $(COCl)_2$ (2.75 mL, 31.5 mmol) in CS_2 (19.5 mL) was added dropwise to **7** (5.13 g, 22.8 mmol) in CS_2 (26 mL). The mixture was refluxed for 1 h, then the excess $(COCl)_2$ and solvent were evaporated and CS_2 (45 mL) was added. The mixture was boiled and $AlCl_3$ (9.13 g, 68.5 mmol) was added in portions over 15 min. After 1 h reflux and removal of the solvent, ice and HCl (1 M) were added. Extraction with DCM, drying ($MgSO_4$), and concentration gave a solid, which was recrystallized (benzene/hexane) to give pure **10** (5.04 g, 79%) as red crystals. Mp 140–142℃. FTIR (ν; cm^{-1}): 1715 (C=O), 1740 (C=O). ^{1}H NMR ($CDCl_3$; δ): 2.31 (s, 3H, Me), 2.38 (s, 6H, 2Me), 2.57 (s, 3H, Me), 6.43 (s, 1H, Ar), 6.72 (s, 1H, Ar), 6.97 (s, 2H, Ar), 7.08 (s, 1H, Ar). ^{13}C NMR ($CDCl_3$; δ): 18.1, 21.3, 22.7, 109.4, 113.5, 124.0, 126.9, 130.5, 132.8, 139.7, 141.2, 149.8, 152.4, 158.1, 182.7. Mass spectrum (MS) (Electron Impact (EI); *m/z*, %): 279 (M^+, 41), 251 (100), 236 (45), 222 (17), 208 (18), 103 (30), 91 (43), 77 (52), 65 (16), 51 (18), 39 (34). Analysis calculated for $C_{18}H_{17}NO_2$: C, 77.40; H, 6.13; N, 5.01; found: C, 77.41; H, 6.36; N, 5.08%.

2.6 Synthesis of 1,3,6,8-tetramethylacridine-9-carboxylic acid (11)

A mixture of **10** (4.97 g, 17.8 mmol) and KOH (9.87 g, 176 mmol) in H_2O (75 mL) was refluxed for 11 days. Cooling and acidification (conc. HCl) provided a solid, which was washed with DCM then dissolved in a solution of K_2CO_3 (10%) and re-precipitated with concentrated HCl. After filtration and drying overnight under vacuum pure **11** (4.31 g, 87%) was obtained. Mp>360℃. FTIR (ν; cm^{-1}): 1733 (C=O), 3100 (OH). ^{1}H NMR (DMSO-d_6; δ): 2.54 (s, 6H, 2Me), 2.85 (s, 6H, 2Me), 7.36 (s, 2H, Ar), 7.84 (s, 2H, Ar). MS (FAB; *m/z*, %): 302 ($[M+Na]^+$, 11), 280 ($[MH]^+$, 60), 235 (16), 192 (100). High-resolution mass spectrometry (HRMS) (FAB; *m/z*): calculated for $C_{18}H_{18}NO_2$ (MH^+): 280.1338; found: 280.1338.

2.7 Synthesis of 4-(2-(benzyloxycarbonyl)ethyl)phenyl 1,3,6,8-tetramethylacridine-9-carboxylate (12)

A mixture of **11** (0.10 g, 0.36 mmol) and Et_3N (0.38 mL, 0.38 mmol) in benzene (1 mL) was heated for 10 min at 80℃, then 4-toluenesulfonyl chloride (0.14 g, 0.73 mmol) was added and heating at 80℃ was continued for a further 30 min. Benzyl 3-(4-hydroxyphenyl)propionate (0.10 g, 0.38 mmol) and Et_3N (0.38 mL, 0.38 mmol) were added and reflux was maintained for 18 h. Removal of solvent, chromatography (5% EtOAc/DCM), and crystallization (EtOAc/hexane) gave pure **12** (21 mg, 12%). Mp 130–131℃. FTIR (ν; cm^{-1}): 1732 (C=O), 1754 (C=O). ^{1}H NMR ($CDCl_3$; δ): 2.54 (s, 6H, 2Me), 2.73 (t, J=7.7 Hz, 2H, CH_2), 2.75 (s, 6H, 2Me), 3.03 (t, J=7.7 Hz, 2H, CH_2), 5.14 (s, 2H, CH_2), 7.23 (s, 2H, Ar), 7.29–7.37 (m, 7H, Ar), 7.48 (d, J=8.6 Hz, 2H, Ar), 7.88 (s, 2H, Ar). ^{13}C NMR ($CDCl_3$; δ): 21.6, 22.5, 30.3, 35.7, 66.4, 120.2, 120.5, 127.3, 128.3, 128.6, 129.6, 132.7, 133.3, 134.6, 135.8, 138.4, 139.8, 149.2, 149.4, 169.1, 172.5. MS (EI; *m/z*, %): 517 (M^+, 2), 262 (100), 236 (53), 235 (35), 234 (51), 219 (13), 165 (90), 123 (17), 91 (30). MS (Chemical Ionization (CI); *m/z*, %): 518 ($[MH^+]$, 100). Analysis calculated for $C_{34}H_{31}NO_4$: C, 78.89; H, 6.04; N, 2.71; found: C, 79.01; H, 6.13; N, 2.58%.

2.8 Synthesis of 4-(2-carboxyethyl)phenyl 1,3,6,8-tetramethylacridine-9-carboxylic acid (13)

A solution HBr (48%; 4 mL) and AcOH (16 mL) was added to **12** (0.660 g, 1.28 mmol) and the whole was heated at 100℃ for 3 h. After cooling, it was poured into water (70 mL). The yellow precipitate was collected, dissolved in hot DCM and hexane was added until cloudiness appeared. The mixture was kept at 20℃ for 18 h, resulting in a precipitate that was collected and dried under vacuum to give pure **13** (0.42 g, 77%). Mp 262℃ (decomposed). FTIR (ν; cm^{-1}): 1708 (C=O), 1753 (C=O), 3000 (NH). ^{1}H NMR (DMSO-d_6; δ): 2.54 (s, 6H, 2Me), 2.60 (t, J=7.6 Hz, 2H, CH_2), 2.75 (s, 6H, 2Me), 2.89 (t, J=7.6 Hz, 2H, CH_2), 7.39 (s, 2H, Ar), 7.43 (d, J=8.6 Hz, 2H, Ar), 7.53 (d, J=8.6 Hz, 2H, Ar), 7.85 (s, 2H, Ar), 12.20 (br s, 1H, OH). ^{13}C NMR (DMSO-d_6; δ): 21.1, 21.7, 29.7, 35.1, 119.3, 120.2, 126.7, 129.6, 132.7, 133.7, 139.1, 139.5, 148.2, 148.7, 168.2, 173.5. MS (EI; *m/z*, %): 428 (MH^+, 4), 263 (24), 262 (100), 235 (28), 234 (61), 219 (18), 107 (13), 91 (12). MS (CI; *m/z*, %): 428 ($[MH^+]$, 100). HRMS (CI; *m/z*): calculated for $C_{27}H_{26}NO_4$ (MH^+): 428.1862; found: 428.1862.

2.9 Synthesis of 4-(2-(succinimidyloxycarbonyl) ethyl) phenyl 1,3,6,8-tetramethylacridine-9-carboxylate (14)

Separate solutions in DMF (0.3 mL) of dicyclohexylcarbodiimide (DCC) (37 mg, 0.18 mmol) and NHS (23 mg, 0.20 mmol) were added to a stirring solution of **13** (51 mg, 0.12 mmol) in DMF (7 mL) and stirring was maintained for 17 h at 20℃. The DMF was distilled out and the residue obtained was washed with DCM. Chromatography (15% EtOAc/DCM) of the concentrated filtrate gave pure **14** (31 mg, 50%). Mp 230–231℃. FTIR (ν; cm^{-1}): 1747 (C=O), 1784 (C=O), 1813 (CO). ^{1}H NMR ($CDCl_3$; δ): 2.54 (s, 6H, 2Me), 2.82 (s, 6H, 2Me), 2.86 (s, 4H, NHS), 2.98 (t, J=7.8, 2H, CH_2), 3.13 (t, J=7.8, 2H, CH_2), 7.24 (s, 2H, Ar), 7.36 (d, J=8.7 Hz, 2H, Ar), 7.54 (d, J=8.7 Hz, 2H, Ar), 7.88 (s, 2H, Ar). ^{13}C NMR ($CDCl_3$; δ): 21.6, 22.5, 25.6, 29.5, 32.6, 120.2, 120.8, 127.3, 129.6, 132.7, 133.3, 134.4, 137.1, 139.8, 149.4, 149.5, 167.8, 169.1. MS (EI; *m/z*, %): 262 (100), 236 (73), 235 (93), 234 (75), 220 (23), 219 (23), 218 (23), 204 (18), 107 (44), 91 (15), 77 (14). MS (CI; *m/z*, %): 428 (100). Analysis calculated for $C_{31}H_{28}N_2O_6$: C, 70.98; H, 5.38; N, 5.34; found: C, 71.04; H, 5.41; N, 5.17%.

2.10 Synthesis of 4-(2-(succinimidyloxycarbonyl) ethyl) phenyl 1,3,6,8,10-pentamethylacridinium-9-carboxylate trifluoromethanesulfonate (2)

To a stirring suspension of **14** (40 mg, 0.08 mmol) in DCM (1.5 mL) was added methyl trifluoromethanesulfonate (0.10 mL, 0.88 mmol) and stirring was maintained for 2.5 h. Addition of diethyl ether gave a yellow solid, which on recrystallization from DCM gave pure **2** (27 mg, 51%). Mp 195–197℃. FTIR (ν; cm^{-1}): 1737 (C=O), 1757 (C=O), 1805 (CO). ^{1}H NMR ($CDCl_3$; δ): 2.72 (s, 6H, 2Me), 2.82 (s, 4H, NHS), 2.88 (s, 6H, 2Me), 3.08 (m, 4H, $2CH_2$), 4.82 (s, 3H, Me), 7.55 (d, J=8.8 Hz, 2H, Ar), 7.62 (d, J=8.8 Hz, 2H, Ar), 7.83 (s, 2H, Ar), 8.58 (s, 2H, Ar). MS (FAB; *m/z*, %): 539 ($[M - CF_3SO_3]^+$, 100), 525 ($[M - CF_3SO_3 - Me]^+$, 15), 277 (21), 249 (43), 235 (8), 165 (10). HRMS (FAB; *m/z*): calculated for $C_{32}H_{31}N_2O_6$ ($[M - CF_3SO_3]^+$): 539.2182; found: 539.2213.

2.11 Synthesis of *N*-(3,5-dimethylphenyl) benzenamine (15)

A mixture of **7** (8.572 g, 52.6 mmol), bromobenzene (10.90 mL, 103.5 mmol), K_2CO_3 (7.283 g, 52.7 mmol), and CuI (0.981 g, 5.15 mmol) was refluxed for 17 h. The solvent was removed and the residue obtained was extracted with DCM, washed with water, dried ($MgSO_4$), and the solvent was evaporated. Distillation (135℃, 0.2 mmHg) gave pure **15** (7.931 g, 77%). Mp 50–51℃ (lit. 51–52℃)[30] FTIR (ν; cm^{-1}): 3385 (NH). ^{1}H NMR ($CDCl_3$; δ): 2.26 (s, 6H, 2Me), 5.58 (br s, 1H, NH), 6.58 (s, 1H, Ar), 6.70 (s, 2H, Ar), 6.90 (m, 1H, Ar), 7.04 (m, 2H, Ar), 7.27 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$; δ): 21.4, 115.6, 117.9, 120.8, 122.9, 129.3, 139.1, 143.1, 143.3. MS (EI; *m/z*, %): 197 (M^+, 100), 196 (24), 180 (28), 179 (17), 167 (13). MS (CI; *m/z*, %): 198 ($[MH]^+$, 100), 197 (M^+, 18), 122 (6).

2.12 Synthesis of *N*-phenyl-4,6-dimethylisatin (16)

The procedure used to produce **10** was also used to produce **16**. It involved the use of **15** (7.504 g, 38.1 mmol), $(COCl)_2$ (3.75 mL, 43.0 mmol), and $AlCl_3$ (16.784 g, 125.9 mmol) and gave pure **16** (5.704 g, 60%). Mp 152–153℃. FTIR (ν; cm^{-1}): 1723 (C=O), 1748 (C=O). ^{1}H NMR ($CDCl_3$; δ): 2.31 (s, 3H, Me), 2.59 (s, 3H, Me), 6.47 (s, 1H, Ar), 6.74 (s, 1H, Ar), 7.38 (m, 2H, Ar), 7.45 (m, 1H, Ar), 7.55 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$; δ): 18.2, 22.8, 109.4, 113.7, 126.3, 127.1, 128.7, 129.9, 133.1, 141.4, 149.8, 152.1, 158.0, 182.5. MS (EI; *m/z*, %): 251 (M^+, 17), 237 (6), 223 (100), 208 (31), 194 (17), 180 (10), 91 (7), 77 (11). MS (CI; *m/z*, %): 269 ($[M + NH_4]^+$, 49), 252 ($[MH]^+$, 100), 238 (45), 223 (14). Analysis calculated for $C_{16}H_{15}NO_2$: C, 76.48; H, 5.21; N, 5.57; found: C, 76.37; H, 5.24; N, 5.48%.

2.13 Synthesis of 1,3-dimethylacridine-9-carboxylic acid (17)

The procedure was identical to that used to produce **11**. It involved the use of **16** (5.570 g, 22.2 mmol) to give pure **17** (4.64 g, 83%). Mp 254–255℃. FTIR (ν; cm^{-1}): 1732 (C=O), 3100 (OH). ^{1}H NMR (DMSO-d_6; δ): 2.53 (s, 3H, Me), 2.81 (s, 3H, Me), 7.38 (s, 1H, Ar), 7.68 (ddd, J=1.3, 6.7, 8.5 Hz, 1H, Ar), 7.85–7.89 (m, 2H, Ar), 7.95 (d, J=8.5 Hz, 1H, Ar), 8.14 (d, J=8.5 Hz, 1H, Ar). MS (EI; *m/z*, %): 251 (M^+, 3), 208 (14), 207 (100), 206 (38), 204 (11), 192 (11), 191 (11). MS (CI; *m/z*, %): 269 ($[M + NH_4]^+$, 49), 252 ($[MH]^+$, 100), 238 (45), 223 (14). HRMS (CI; *m/z*): calculated for $C_{16}H_{14}NO_2$ (MH^+): 252.1025; found: 252.1025.

2.14 Synthesis of 4-(2-(benzyloxycarbonyl) ethyl) phenyl 1,3-dimethylacridine-9-carboxylate (18)

The procedure was identical to that used to produce **12**. It involved the use of **17** (3.816 g, 15.2 mmol), 4-toluenesulfonyl chloride (3.478 g, 18.2 mmol), and benzyl 3-(4-hydroxyphenyl) propionate (3.902 g, 15.2 mmol) and gave pure **18** (2.379 g, 32%). Mp 120–122℃. FTIR (ν; cm^{-1}): 1742 (C=O). ^{1}H NMR ($CDCl_3$; δ): 2.57 (s, 3H, Me), 2.74 (t, J=7.7 Hz, 2H, CH_2), 2.92 (s, 3H, Me), 3.04 (t, J=7.7 Hz, 2H, CH_2), 5.13 (s, 2H, CH_2), 7.31–7.39 (m, 10H, Ar), 7.61 (ddd, J=1.2, 6.6, 8.7 Hz, 1H, Ar), 7.81 (ddd, J=1.2, 6.6, 8.9 Hz, 1H, Ar), 7.95 (s, 1H, Ar), 8.03 (d, J=8.7 Hz, 1H, Ar), 8.25 (d, J=8.9 Hz, 1H, Ar). ^{13}C NMR ($CDCl_3$; δ): 21.7, 21.9, 30.3, 35.8, 66.4, 119.9, 121.1, 122.5, 124.5, 126.9, 127.2, 128.3, 128.6, 129.6, 129.7, 130.2, 132.2, 133.2, 135.8, 138.9, 140.5, 147.8, 148.9, 150.1, 168.2, 172.5. MS (EI; *m/z*, %): 234 (100), 208 (60), 207 (24), 206 (44), 204 (17), 191 (13), 165 (6), 123 (10), 91 (24). MS (CI; *m/z*, %): 490 ($[MH^+]$, 89), 274 (17), 236 (29), 234 (24), 208 (100), 196 (6). Analysis calculated for $C_{32}H_{27}NO_4$: C, 78.51; H, 5.56; N, 2.86; found: C, 78.73; H, 5.70; N, 2.92%.

2.15 Synthesis of 4-(2-carboxyethyl)phenyl 1,3-dimethylacridine-9-carboxylate (19)

The procedure was identical to that used to produce **12**. It involved the use of **18** (0.460 g, 0.94 mmol) and gave pure **19** (0.309 g, 82%). Mp 238–240℃. FTIR (ν; cm^{-1}): 1719 (C=O), 1753 (C=O), 3000 (NH). ^{1}H NMR (DMSO-d_6; δ): 2.60 (s, 3H, Me), 2.64 (t, J = 7.6 Hz, 2H, CH_2), 2.93 (s, 3H, Me), 3.00 (t, J = 7.6 Hz, 2H, CH_2), 7.38–7.42 (m, 5H, Ar), 7.70 (m, 1H, Ar), 7.88 (m, 1H, Ar), 7.93 (s, 1H, Ar), 8.04 (d, J = 8.6 Hz, 1H, Ar), 8.24 (d, J = 8.6 Hz, 1H, Ar). ^{13}C NMR (DMSO-d_6; δ): 21.0, 21.4, 29.8, 35.2, 118.9, 121.2, 121.6, 124.4, 126.8, 127.8, 129.3, 129.9, 130.8, 132.5, 132.8, 135.0, 139.7, 140.6, 147.3, 148.2, 149.5, 167.7, 173.7. MS (EI; *m/z*, %): 399 (M^+, 3), 235 (18), 234 (100), 208 (28), 207 (21), 206 (57), 204 (19), 191 (13), 107 (8). MS (CI; *m/z*, %): 400 ([MH^+], 100), 356 (3), 236 (15), 234 (13), 208 (34). Analysis calculated for $C_{25}H_{21}NO_4$: C, 75.17; H, 5.30; N, 3.51; found: C, 75.58; H, 5.14; N, 3.65%.

2.16 Synthesis of 4-(2-(succinimidyloxycarbonyl)ethyl) phenyl 1,3-dimethylacridine-9-carboxylate (20)

The procedure was identical to that used to produce **14**. It involved the use of **19** (0.275 g, 0.69 mmol), DCC (0.216 g, 1.05 mmol), and NHS (0.122 g, 1.06 mmol) and gave pure **20** (0.191 g, 56%). Mp 137–169℃. FTIR (ν; cm^{-1}): 1736 (C=O), 1781 (C=O), 1814 (CO). ^{1}H NMR ($CDCl_3$; δ): 2.58 (s, 3H, Me), 2.86 (s, 4H, NHS), 2.93 (s, 3H, Me), 2.98 (t, J = 7.6, 2H, CH_2), 3.15 (t, J = 7.6, 2H, CH_2), 7.31 (s, 1H, Ar), 7.39 (d, J = 8.8 Hz, 2H, Ar), 7.43 (d, J = 8.8 Hz, 2H, Ar), 7.62 (m, 1H, Ar), 7.82 (m, 1H, Ar), 7.96 (s, 1H, Ar), 8.03 (d, J = 8.6 Hz, 1H, Ar), 8.26 (d, J = 8.6 Hz, 1H, Ar). ^{13}C NMR ($CDCl_3$; δ): 21.7, 21.9, 25.6, 29.9, 32.6, 119.9, 121.4, 122.5, 124.5, 126.9, 127.3, 129.7, 129.9, 130.2, 132.2, 133.2, 135.6, 137.7, 140.4, 147.8, 149.2, 150.2, 167.8, 168.1, 169.1. MS (EI; *m/z*, %): 234 (100), 208 (38), 207 (26), 206 (54), 204 (17), 191 (14), 115 (8). MS (CI; *m/z*, %): 497 ([MH^+], 18), 400 (28), 382 (14), 382 (14), 356 (3), 281 (6), 236 (31), 208 (100), 166 (3), 133 (15), 107 (6). Analysis calculated for $C_{29}H_{24}N_2O_6$: C, 70.15; H, 4.87; N, 5.64; found: C, 70.34; H, 5.03; N, 5.59%.

2.17 Synthesis of 4-(2-(succinimidyloxycarbonyl)ethyl) phenyl 1,3,10-trimethylacridinium-9-carboxylate trifluoromethanesulfonate (3)

The procedure was identical to that used to produce **2**. It involved the use of **20** (62 mg, 0.12 mmol) and gave pure **3** (53 mg, 64%). Mp 195–197℃. FTIR (ν; cm^{-1}): 1741 (C=O), 1775 (C=O), 1808 (CO). ^{1}H NMR (DMSO-d_6; δ): 2.78 (s, 3H, Me), 2.82 (s, 4H, NHS), 2.99 (s, 3H, Me), 3.09 (m, 4H, $2CH_2$), 4.88 (s, 3H, Me), 7.57 (d, J = 8.8 Hz, 2H, Ar), 7.62 (d, J = 8.8 Hz, 2H, Ar), 7.97 (s, 1H, Ar), 8.15 (m, 1H, Ar), 8.49 (m, 2H, Ar), 8.61 (s, 1H, Ar), 8.88 (d, J = 9.2 Hz, 1H, Ar). MS (FAB; *m/z*, %): 511 ([$M - CF_3SO_3$]$^+$, 30), 273 (4), 249 (10), 221 (17), 120 (35), 107 (53). HRMS (FAB; *m/z*): calculated for $C_{30}H_{27}N_2O_6$ ([$M - CF_3SO_3$]$^+$): 511.1869; found: 511.1899.

2.18 Synthesis of *N*-(2,5-dimethylphenyl)-2,5-dimethylbenzenamine (22)

A mixture of **21** (85.0 mL) and potassium (1.836 g, 47.0 mmol) was refluxed for 1.5 h. 2-Bromo-1,4-dimethylbenzene (10.0 mL, 72.4 mmol) was added dropwise and the mixture was refluxed for 17 h. After cooling, EtOH (75 mL), hexane (75 mL), and Et_2O (150 mL) were added and the mixture was washed with H_2O (3 × 75 mL). The organic extract was dried ($MgSO_4$) and the solvent was removed. Distillation (132–133℃, 0.4 mmHg) gave pure **22** (4.15 g, 39%). Mp 52–54℃ (lit. 51–53℃)[31]. FTIR (ν; cm^{-1}): 3399 (NH). ^{1}H NMR ($CDCl_3$; δ): 2.10 (app. s, 12H, 4Me), 4.90 (br s, 1H, NH), 6.55 (d, J = 7.2 Hz, 2H, Ar), 6.61 (s, 2H), 6.88 (d, J = 7.2 Hz, 2H, Ar).

2.19 Synthesis of *N*-(2,5-dimethylphenyl)-4,7-dimethylisatin (23)

The procedure was similar to that used to obtain **10**. It involved the use of **22** (13.113 g, 58.24 mmol), $(COCl)_2$ (6.60 mL, 75.7 mmol), and $AlCl_3$ (27.131 g, 203.5 mmol) and gave pure **23** (red needles, 13.14 g, 81%). Mp 140–142℃ (lit. 141.5–142℃)[31]. FTIR (ν; cm^{-1}): 1720 (C=O), 1742 (C=O). ^{1}H NMR ($CDCl_3$; δ): 1.58 (s, 3H, Me), 2.19 (s, 3H, Me), 2.35 (s, 3H, Me), 2.60 (s, 3H, Me), 6.82 (d, J = 7.9 Hz, 1H, Ar), 7.07 (s, 1H, Ar), 7.12 (d, J = 7.9 Hz, 1H, Ar), 7.18 (d, J = 7.8 Hz, 1H, Ar), 7.22 (d, J = 7.8 Hz, 1H, Ar).

2.20 Synthesis of 1,4,5,8-tetramethylacridine-9-carboxylic acid (24)

The procedure was identical to that used to produce **11**. It involved the use of **23** (12.81 g, 45.9 mmol) to give pure **24** (10.89 g, 85%). Mp 230–233℃ (lit. 229–233℃)[31]. FTIR (ν; cm^{-1}): 1715 (C=O), 3250 (OH). ^{1}H NMR (DMSO-d_6; δ): 2.80 (s, 6H, 2Me), 2.82 (s, 6H, 2Me), 7.38 (d, J = 7.0 Hz, 2H, Ar), 7.60 (d, J = 7.0 Hz, 2H, Ar). MS (FAB; *m/z*, %): 302 ([$M + Na$]$^+$, 17), 281 (24), 280 ([MH]$^+$, 100), 279 (M^+, 19), 236 (8), 235 (19), 234 (7), 120 (19), 107 (24). HRMS (FAB; *m/z*): calculated for $C_{18}H_{18}NO_2$ (MH^+): 280.1338; found: 280.1338.

2.21 Synthesis of 4-(2-(benzyloxycarbonyl)ethyl)phenyl 1,4,5,8-tetramethylacridine-9-carboxylate (25)

A mixture of **24** (0.503 g, 1.80 mmol) and $SOCl_2$ (6.0 mL) was refluxed for 3 h. The excess reagent was removed and the residue was dissolved in dry pyridine (4 mL). Benzyl 3-(4-hydroxyphenyl) propionate (0.462 g, 1.80 mmol) and 4-dimethylaminopyridine (DMAP) (23 mg, 0.18 mmol) were added and the mixture was stirred overnight at 20℃. The solvent was distilled off and the crude product was chromatographed (10% EtOAc/$CHCl_3$ in 1:9) to give impure **25**

(0.159 g, 17%), which was used in the next step without further purification.

2.22 Synthesis of 4-(2-carboxyethyl)phenyl 1,4,5,8-tetramethylacridine-9-carboxylate (26)

The procedure was identical to that used to produce **12**. It involved the use of **25** (0.208 g, 0.40 mmol) and gave pure **26** (0.110 g, 64%). Mp 209–212℃. FTIR (ν; cm^{-1}): 1708 (C=O), 1749 (C=O), 3100 (OH). ^{1}H NMR (DMSO-d_6; δ): 2.60 (br, 2H, CH_2), 2.76 (s, 6H, 2Me), 2.90–2.92 (m, 8H, 2Me & CH_2), 7.45 (m, 4H, Ar), 7.54 (d, J=8.6 Hz, 2H, Ar), 7.66 (d, J=8.6 Hz, 2H, Ar). MS (EI; *m/z*, %): 427 (M^+, 75), 263 (20), 262 (100), 235 (14), 234 (50), 218 (18), 107 (25), 91 (22). HRMS (EI; *m/z*): calculated for $C_{27}H_{25}NO_4$ (M^+): 427.1784; found: 427.1784.

2.23 Synthesis of 4-(2-(succinimidyloxycarbonyl)ethyl)phenyl 1,4,5,8-tetramethylacridine-9-carboxylate (4)

The procedure was identical to that used to produce **14**. It involved the use of **26** (0.108 g, 0.25 mmol), DCC (95 mg, 0.46 mmol), and NHS (52 mg, 0.45 mmol) and gave pure **4** (76 mg, 57%). Mp 198–199℃. FTIR (ν; cm^{-1}): 1746 (C=O), 1784 (C=O), 1816 (C=O). ^{1}H NMR ($CDCl_3$; δ): 2.81 (s, 6H, 2Me), 2.86 (s, 4H, NHS), 2.91 (s, 6H, 2Me), 2.98 (t, J=7.8, 2H, CH_2), 3.12 (t, J=7.8, 2H, CH_2), 7.30 (d, J=6.9 Hz, 2H, Ar), 7.36 (d, J=8.6 Hz, 2H, Ar), 7.51 (d, J=8.6 Hz, 2H, Ar), 7.54 (d, J=6.9 Hz, 2H, Ar). MS (CI; *m/z*, %): 524 (M^+, 4), 263 (20), 262 (100), 235 (12), 234 (49), 218 (17), 91 (26). Analysis calculated for $C_{31}H_{28}N_2O_6$: C, 70.97; H, 5.38; N, 5.34; found: C, 70.85; H, 5.25; N, 5.25%.

2.24 Synthesis of *N*-(2,5-dimethylphenyl)benzenamine (27)

2,5-Dimethylacetanilide (10.762 g, 80%) was synthesized from **21** (10.00 g, 80.0 mmol) by a procedure similar to that used to obtain **7**. Mp 142–143℃ (lit. 142℃)[29]. ^{1}H NMR ($CDCl_3$; δ): 1.95 (s, 3H, Me), 2.00 (s, 3H, Me), 2.13 (s, 3H, Me), 6.58 (m, 2H, Ar), 7.12 (s, 1H, Ar), 7.60 (br s, 1H, Ar).

N-(2,5-dimethylphenyl)phenylacetamide (12.65 g, 76%) was synthesized from 2,5-dimethylacetanilide (11.42 g, 70.0 mmol) by a procedure similar to that used to obtain **8**. NMR ($CDCl_3$; δ): 1.98 (s, 3H, Me), 2.17 (s, 3H, Me), 2.28 (s, 3H, Me), 6.85–7.25 (m, 8H, Ar).

Compound **27** was synthesized using a procedure similar to that used to obtain **9**. It involved the use of *N*-(2,5-dimethylphenyl)phenylacetamide (12.50 g, 50.0 mmol) and distillation at 112℃, 0.1 mmHg gave pure **27** (8.11 g, 79%). FTIR (ν; cm^{-1}): 3388 (NH). ^{1}H NMR ($CDCl_3$; δ): 2.17 (s, 3H, Me), 2.25 (s, 3H, Me), 5.29 (s, 1H, NH), 6.74 (d, J=1.4 Hz, 1H, Ar), 6.90 (m, 3H, Ar), 7.06 (m, 2H, Ar), 7.18 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$; δ): 17.4, 21.1, 117.3, 119.5, 120.3, 122.8, 125.3, 129.3, 130.7, 136.4, 140.1, 144.1. MS (EI; *m/z*, %): 197 (M^+, 100), 196 (47), 182 (18), 180 (28), 120 (33), 91 (21), 90 (24), 77 (36), 51 (19), 39 (25).

2.25 Synthesis of *N*-phenyl-4,7-dimethylisatin (28)

The procedure was similar to that used to obtain **10**. It involved the use of **27** (8.202 g, 41.6 mmol) and gave **28** (4.176 g, 40%) after crystallization. The mother liquor was concentrated to give *N*-(2,5-dimethylphenyl)isatin (2.942 g, 28%).

Compound **28**: Mp 162–164℃. FTIR (ν; cm^{-1}): 1734 (C=O). ^{1}H NMR ($CDCl_3$; δ): 1.65 (s, 3H, Me), 2.60 (s, 3H, Me), 6.84 (d, J=7.8 Hz, 1H, Ar), 7.15 (d, J=7.8 Hz, 1H, Ar), 7.35 (m, 2H, Ar), 7.49 (m, 3H, Ar). ^{13}C NMR ($CDCl_3$; δ): 18.0, 18.4, 116.6, 119.7, 126.3, 128.1, 129.1, 129.6, 135.7, 139.3, 141.7, 148.7, 158.9, 184.0. MS (EI; *m/z*, %): 252 (12), 251 (M^+, 52), 224 (16), 223 (100), 222 (57), 208 (57), 194 (40), 180 (41), 91 (38), 84 (32), 77 (61), 51 (59). Analysis calculated for $C_{10}H_{13}NO_2$: C, 76.47; H, 5.22; N, 5.58; found: C, 76.62; H, 4.91; N, 5.60%.

N-(2,5-Dimethylphenyl)isatin: Mp 115–116℃. FTIR (ν; cm^{-1}): 1735 (C=O). ^{1}H NMR ($CDCl_3$; δ): 2.17 (s, 3H, Me), 2.37 (s, 3H, Me), 6.55 (d, J=7.9 Hz, 1H, Ar), 7.07 (s, 1H, Ar), 7.15–7.33 (m, 3H, Ar), 7.51 (m, 1H, Ar), 7.69 (d, J=7.4 Hz, 1H, Ar). ^{13}C NMR ($CDCl_3$; δ): 17.4, 20.8, 111.4, 117.4, 124.1, 125.5, 128.0, 130.6, 131.4, 131.6, 133.0, 137.6, 138.5, 152.1, 157.3, 183.2. MS (EI; *m/z*, %): 252 (11), 251 (M^+, 61), 223 (100), 222 (42), 208 (81), 194 (32), 180 (37), 152 (24), 102 (23), 90 (23), 77 (84), 76 (31), 63 (39), 51 (67), 50 (44), 41 (37), 39 (83). Analysis calculated for $C_{10}H_{13}NO_2$: C, 76.47; H, 5.22; N, 5.58; found: C, 76.62; H, 5.37; N, 5.59%.

2.26 Synthesis of 1,4-dimethylacridine-9-carboxylic acid (29)

The procedure was identical to that used to produce **11**. It involved the use of **28** (0.931 g, 3.71 mmol) and a reaction period of 4 days to give pure **29** (0.765 g, 82%). Mp 238–239℃. FTIR (ν; cm^{-1}): 1718 (C=O), 3200 (OH). ^{1}H NMR (DMSO-d_6; δ): 2.79 (s, 3H, Me), 2.82 (s, 3H, Me), 7.37 (d, J=7.0 Hz, 1H, Ar), 7.60 (d, J=7.0 Hz, 1H, Ar), 7.69 (dd, J=7.0, 9.0 Hz, 1H, Ar), 7.87 (dd, J=7.0, 8.6 Hz, 1H, Ar), 7.96 (d, J=8.6 Hz, 1H, Ar), 8.19 (d, J=9.0 Hz, 1H, Ar). MS (FAB; *m/z*, %): 274 ($[M+Na]^+$, 14), 253 (20), 252 ($[MH]^+$, 100), 208 (8), 207 (8), 206 (7). HRMS (FAB; *m/z*): calculated for $C_{16}H_{14}NO_2$ (MH^+): 252.1025; found: 252.1025.

2.27 Synthesis of 4-(2-(benzyloxycarbonyl)ethyl)phenyl 1,4-dimethylacridine-9-carboxylate (30)

The procedure was almost identical to that used to produce **25**. It involved the use of **29** (2.233 g, 8.89 mmol) and the final stage of the reaction was carried out at 100℃ instead of 20℃ to give pure **30** (1.587 g, 35%). Mp 87–89℃. FTIR (ν; cm^{-1}): 1733 (C=O), 1747 (C=O). ^{1}H NMR ($CDCl_3$; δ): 2.76 (t, J=7.7 Hz, 2H, CH_2), 2.93 (s, 3H, Me), 2.94 (s, 3H, Me), 3.06 (t, J=7.7 Hz, 2H, CH_2), 5.16 (s, 2H, CH_2), 7.32–7.41 (m, 10H, Ar), 7.57 (d, J=7.0 Hz, 1H, Ar), 7.65 (ddd, J=1.2, 6.6, 8.7 Hz, 1H, Ar), 7.82 (ddd, J=

1.3, 6.6, 8.7 Hz, 1H, Ar), 8.06 (d, J = 8.7 Hz, 1H, Ar), 8.33 (d, J = 8.7 Hz, 1H, Ar). ^{13}C NMR ($CDCl_3$; δ): 18.9, 21.7, 30.4, 35.8, 66.4, 121.2, 121.4, 122.5, 124.2, 127.0, 128.3, 128.6, 129.1, 129.3, 129.7, 130.4, 131.2, 135.7, 135.8, 136.3, 138.8, 146.9, 148.9, 149.1, 168.4, 172.5. MS (EI; *m/z*, %): 489 (M^+, 14), 234 (100), 206 (63), 205 (26), 91 (22). Analysis calculated for $C_{32}H_{27}NO_4$: C, 78.50; H, 5.56; N, 2.86; found: C, 78.56; H, 5.53; N, 2.75%.

2.28 Synthesis of 4-(2-carboxyethyl) phenyl 1,4-dimethylacridine-9-carboxylate (31)

The procedure was identical to that used to produce **12**. It involved the use of **30** (1.302 g, 2.66 mmol) and gave pure **31** (0.919 g, 87%). Mp 138–140℃. FTIR (ν; cm^{-1}): 1717 (C=O), 1747 (C=O), 3000 (OH). ^{1}H NMR (DMSO-d_6; δ): 2.77 (t, J = 7.5 Hz, 2H, CH_2), 2.91 (s, 3H, Me), 2.93 (s, 3H, Me), 3.05 (t, J = 7.5 Hz, 2H, CH_2), 7.30 (d, J = 6.9 Hz, 1H, Ar), 7.33–7.43 (m, 5H, Ar), 7.63 (ddd, J = 1.3, 6.6, 8.7 Hz, 1H, Ar), 7.80 (ddd, J = 1.3, 6.6, 8.7 Hz, 1H, Ar), 8.06 (d, J = 8.7 Hz, 1H, Ar), 8.33 (d, J = 8.7 Hz, 1H, Ar), 12.20 (br s, 1H, CO_2H). ^{13}C NMR (DMSO-d_6; δ): 18.9, 21.7, 30.0, 35.5, 121.1, 121.4, 122.5, 124.2, 127.2, 129.1, 129.4, 129.7, 129.8, 130.3, 131.2, 135.8, 136.2, 138.6, 146.8, 148.9, 149.0, 168.4, 178.8. MS (CI; *m/z*, %): 400 ([MH^+], 100), 356 (3), 208 (5), 107 (5), 19 (3). Analysis calculated for $C_{25}H_{21}NO_4$: C, 75.16; H, 5.30; N, 3.51; found: C, 74.98; H, 5.20; N, 3.34%.

2.29 Synthesis of 4-(2-(succinimidyloxycarbonyl) ethyl) phenyl 1,4-dimethylacridine-9-carboxylate (5)

The procedure was identical to that used to produce **14**. It involved the use of **31** (0.802 g, 2.01 mmol), DCC (0.629 g, 3.05 mmol), and NHS (0.346 g, 3.01 mmol) and gave pure **5** (0.616 g, 62%). Mp 148–149℃. FTIR (ν; cm^{-1}): 1737 (C=O), 1781 (C=O), 1814 (C=O). ^{1}H NMR ($CDCl_3$; δ): 2.83 (s, 4H, NHS), 2.90 (s, 3H, Me), 2.91 (s, 3H, Me), 2.97 (t, J = 7.6, 2H, CH_2), 3.13 (t, J = 7.6, 2H, CH_2), 7.31 (d, J = 6.9 Hz, 1H, Ar), 7.38 (d, J = 8.8 Hz, 2H, Ar), 7.42 (d, J = 8.8 Hz, 2H, Ar), 7.53 (d, J = 6.9 Hz, 1H, Ar), 7.63 (ddd, J = 1.3, 6.6, 8.7 Hz, 1H), 7.80 (ddd, J = 1.3, 6.6, 8.7 Hz, 1H), 8.04 (d, J = 8.7 Hz, 1H, Ar), 8.30 (d, J = 8.7 Hz, 1H, Ar). ^{13}C NMR ($CDCl_3$; δ): 18.9, 21.7, 25.6, 29.9, 32.6, 121.3, 121.4, 122.5, 124.2, 127.3, 129.1, 129.3, 129.7, 130.4, 131.2, 135.6, 136.3, 137.5, 146.9, 149.1, 149.2, 167.8, 168.3, 169.1. MS (EI; *m/z*, %): 496 (M^+, 6), 381 (8), 234 (100), 206 (69), 204 (63), 191 (15), 178 (10), 91 (26), 65 (17), 55 (23). Analysis calculated for $C_{29}H_{24}N_2O_6$: C, 70.14; H, 4.87; N, 5.64; found: C, 69.99; H, 4.87; N, 5.64%.

2.30 Preparation of acridinium ester-immunoglobulin G conjugates

Immunoglobulin G (IgG) was dissolved in a pH 8 buffer (0.1M NaH_2PO_4 adjusted to pH 8 by addition of 5M NaOH) to give a concentration of 250 μg/mL. A solution of the appropriate AE in dimethyl sulfoxide (5 μL of concentration 1 mg/mL) was added to a portion (200 μL) of the IgG solution, and the mixture was stirred and then left to stand in the dark for 15 min. A quench buffer (0.1M NaH_2PO_4 adjusted to pH 8 by addition of 5M NaOH, plus lysine monohydrochloride [10 mg/mL of buffer]) (100 μL) was added and the mixture was left for a further 5 min. The mixture was subjected to gel column chromatography (Sephadex G50, eluted with a pH 6.3 buffer [0.1M Na_3PO_4, 0.15M NaCl, adjusted to pH 6.3 by addition of 5M NaOH, plus 0.1% NaN_3 and 0.1% bovine serum albumin]) and the fractions containing the first (major) chemiluminescent material were combined. Aliquots (10 μL) of this solution were diluted with the appropriate pH buffer (pH 5, 6, 7, or 8) until the concentration was sufficiently low to allow detection within the limit of the luminometer (ca. 5 million RLU). These solutions were stored at the appropriate temperature and aliquots were extracted and their chemiluminescence was measured at appropriate intervals.

3 Results and Discussion

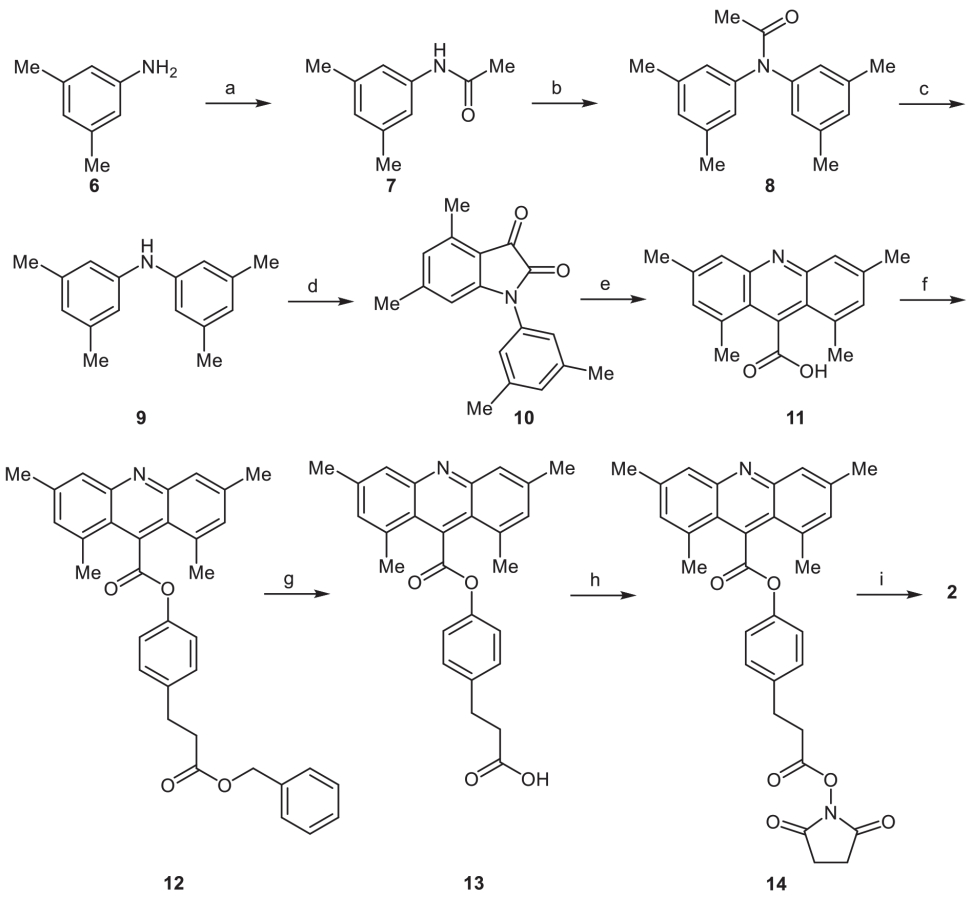
3.1 Chemistry

The synthetic route for the 1,3,6,8-tetramethyl AE **2** is shown in **Scheme 1**. Treatment of 3,5-dimetylaniline (**6**) with acetic anhydride (Ac_2O) gave the corresponding acetanilide **7** in 85% yield. Reaction of this with 1-bromo-3,5-dimethylbenzene, copper iodide (CuI), and potassium carbonate (K_2CO_3) gave a 58% yield of **8**, which on alkaline (KOH) hydrolysis gave **9** in 73% yield. The NMR spectra of **7** and **8** showed the presence of two categories of methyl groups (aryl and acetyl) but those of **9** showed only a single methyl group signal.

Compound **9** in carbon disulfide (CS_2) was refluxed with excess $(COCl)_2$ (oxalyl chloride) and aluminum chloride ($AlCl_3$) to give a 79% yield of isatin **10**, which showed three methyl signals at 2.31 (3 protons), 2.38 (6 protons), and 2.57 ppm (3 protons) in its ^{1}H NMR spectrum. Two different carbonyl groups were evident in both the infra-red (IR) spectrum (at 1715 and 1740 cm^{-1}) and ^{13}C NMR spectrum (at 158.2 and 182.7 ppm) of **10**.

Treatment of **10** with aqueous KOH for a long period under reflux gave the tetramethylacridinecarboxylic acid **11** in 87% yield. The IR spectrum showed absorption bands at 1733 (C=O) and 3100 (OH) cm^{-1}. The FAB mass spectrum of **11** showed pseudo molecular ion peaks at *m/z* = 302 ([M+Na]$^+$) and 280 (MH^+). High-resolution mass spectrometry (HRMS) confirmed the formula of the 280 peak as $C_{18}H_{18}NO_2$.

Attempted conversion of **11** into its corresponding ester **12** in a single step by refluxing it for 17 h with thionyl chloride ($SOCl_2$) and benzyl 3-(4-hydroxyphenyl) propionate in anhydrous pyridine containing 4-dimethylaminopyridine (DMAP) gave only a 2% yield, possibly due to slow



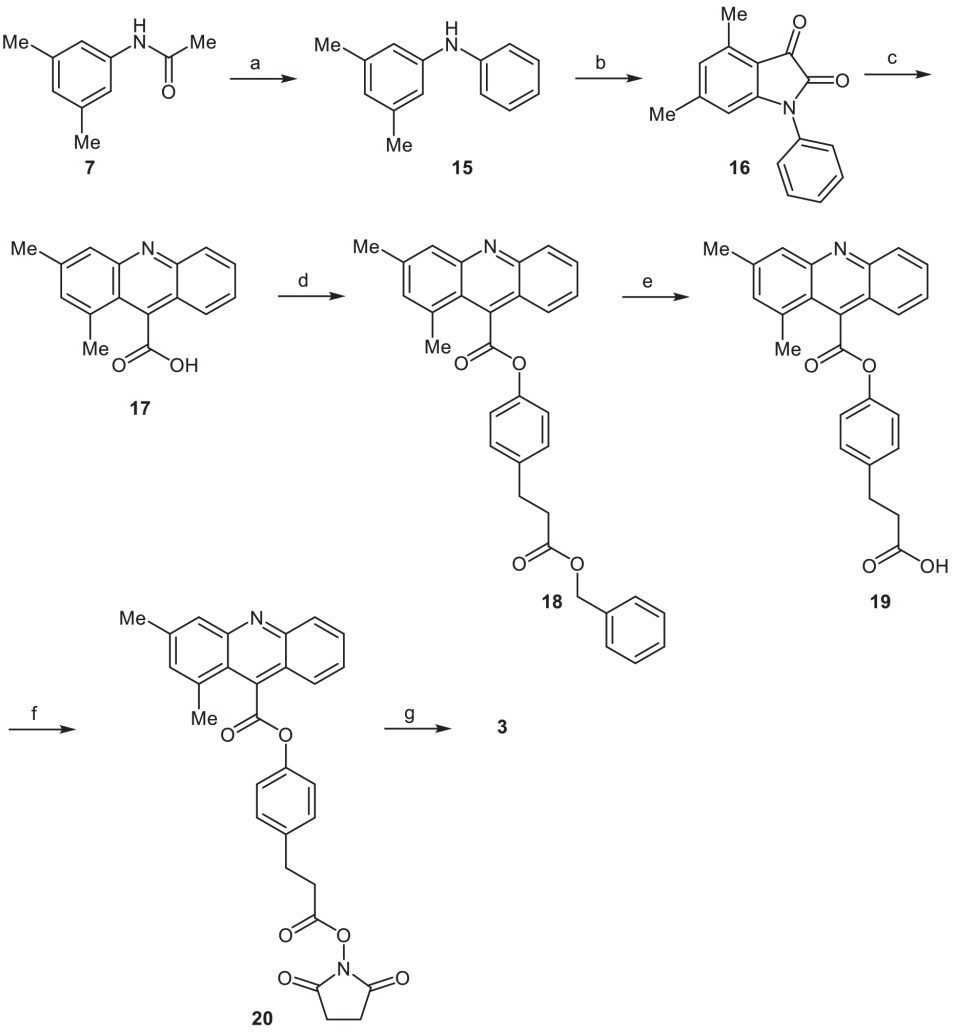
Scheme 1 Synthesis of AE **2**. Reagents and conditions: a: H_2O, HCl, Ac_2O, AcONa, 20℃, 15 min; b: 1-bromo-3,5-dimethylbenzene, K_2CO_3, CuI, reflux, 17 h; c: EtOH, KOH, reflux, 17 h; d: $(COCl)_2$, CS_2, $AlCl_3$, reflux, 2 h; e: KOH, H_2O, reflux, 11 days, HCl; f: Et_3N, 4-$MeC_6H_4SO_2Cl$, benzene, 30 min, benzyl 3-(4-hydroxyphenyl)propionate, reflux, 18 h; g: HBr, AcOH, 100℃, 3 h; h: DCC, NHS, DMF, 20℃, 17 h; i: CF_3SO_3Me, DMF, N_2, 20℃, 2.5 h.

and incomplete formation of the acid chloride intermediate as a result of hindrance by the methyl groups at positions 1 and 8 and competitive reaction of the phenol group of benzyl 3-(4-hydroxyphenyl)propionate with the thionyl chloride. Even when the acid chloride of **11** was prepared in a separate step and then refluxed for 17 h with benzyl 3-(4-hydroxyphenyl)propionate and DMAP in dry pyridine, product **12** was still not produced in significant yield. Use of toluenesulfonyl chloride (TsCl) instead of thionyl chloride was also unsuccessful, producing the toluenesulfonyl ester instead of the tetramethylacridinecarboxylic ester. However, replacing pyridine (which acted as both solvent and base) with benzene as solvent and triethylamine (Et_3N) as base in the reaction of **11** with benzyl 3-(4-hydroxyphenyl)propionate in the presence of 4-toluenesulfonyl chloride for 18 h under reflux gave **12** in 12% yield, and when the reflux period was extended to 2 days the yield was 28%. The chemical ionization (CI) mass spectrum of **12** revealed a pseudo molecular ion (MH^+) peak at $m/z = 518$. The presence of two different carbonyl groups was confirmed by the IR spectrum (1732 and 1754 cm^{-1}) and the ^{13}C NMR spectrum (169.1 and 172.5 ppm). In addition, the ^1H NMR spectrum showed the expected protons from the acridine and phenolic moieties.

Debenzylation of **12** using hydrogen bromide (HBr) in acetic acid (AcOH) for 3 h at 100℃ gave a 77% yield of the acid **13**. Its CI mass spectrum showed a pseudo molecular ion (MH^+) peak at $m/z = 428$. A singlet at 12.20 ppm in its ^1H NMR spectrum confirmed it was a carboxylic acid. In addition, its ^{13}C NMR spectrum showed two signals at low field (168.2 and 173.5 ppm) corresponding to two different carbonyl carbons.

The reaction of **13** and *N*-hydroxysuccinimide (NHS) in the presence of dicyclohexylcarbodiimide (DCC) in dimethylformamide (DMF) at 20℃ gave **14** in 50% yield. The IR and ^{13}C NMR spectra of **14** confirmed the presence of two different carbonyl groups. A singlet integrating for 4 protons at 2.86 ppm in the ^1H NMR spectrum verified that the NHS unit had been incorporated. The purity of **14** was confirmed by elemental analysis.

Finally, *N*-methylation of **14** using methyl trifluoromethanesulfonate in DCM at 20℃ gave the target AE **2** in 51%



Scheme 2 Synthesis of AE **3**. Reagents and conditions: a: bromobenzene, K_2CO_3, CuI, reflux, 17 h; b: $(COCl)_2$, CS_2, $AlCl_3$, reflux, 2 h; c: KOH, H_2O, reflux, 11 days, HCl; d: Et_3N, 4-$MeC_6H_4SO_2Cl$, benzene, 30 min, benzyl 3-(4-hydroxyphenyl)propionate, reflux, 18 h; e: HBr, AcOH, 100℃, 3 h; f: DCC, NHS, DMF, 20℃, 17 h; g: CF_3SO_3Me, DMF, N_2, 20℃, 2.5 h.

yield. A singlet at 4.28 ppm in its ^{1}H NMR spectrum verified the presence of the *N*-methyl protons. The FAB mass spectrum showed a pseudo molecular ion ($[M - CF_3SO_3^-]^+$) peak at $m/z = 539$ and its formula was confirmed by the HRMS as $C_{32}H_{31}N_2O_6$.

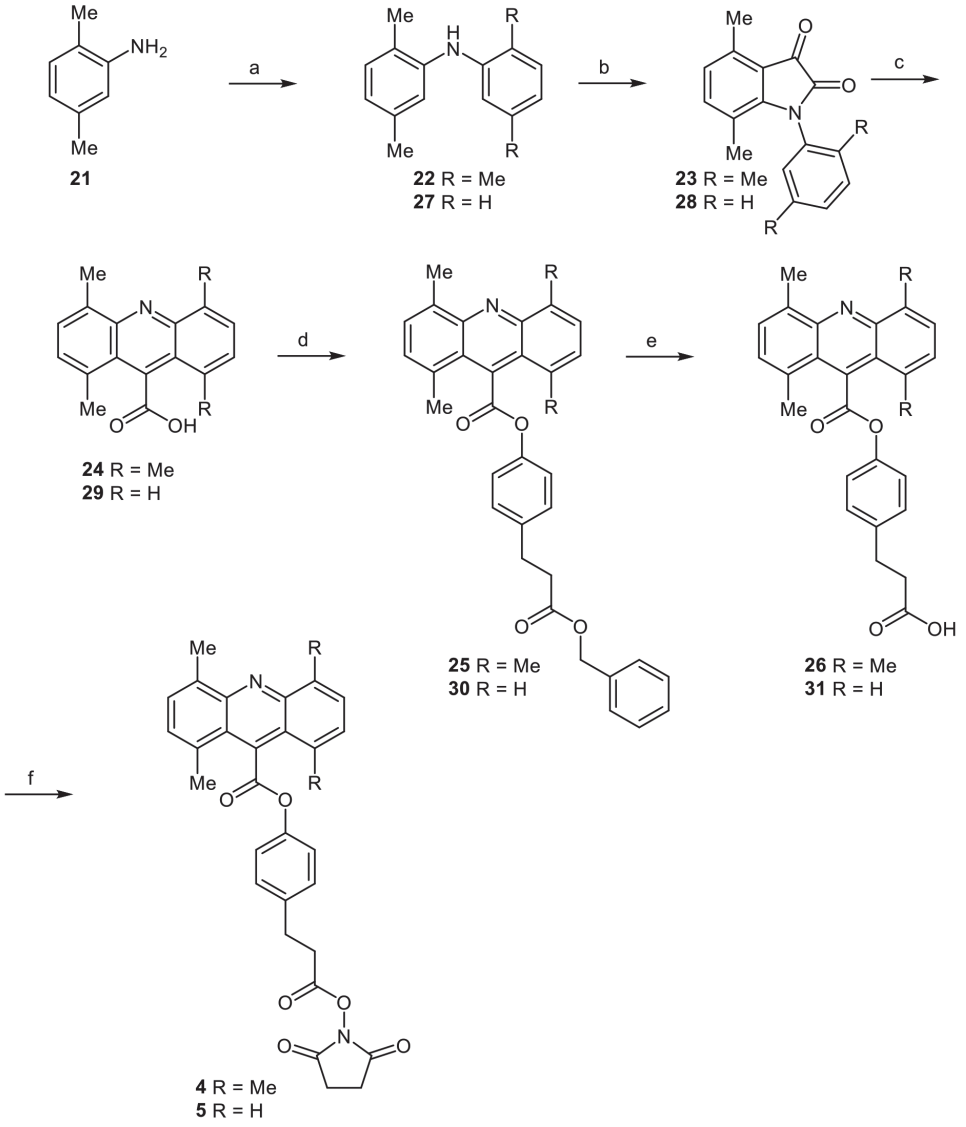
The synthesis of AE **3**, as outlined in Scheme 2, followed the same approach as for the synthesis of **2**. The reaction of **7** and bromobenzene under reflux under basic conditions gave **15** in 77% yield without isolation of the intermediate diarylacetamide (analog of **8**). The structure of **15** was confirmed by a singlet at 5.58 ppm (NH proton) in its ^{1}H NMR spectrum and a molecular ion peak at $m/z =$ 197 in its mass spectrum.

Treatment of **15** with $(COCl)_2$ and $AlCl_3$ gave a 60% yield of **16**. Its IR spectrum showed two carbonyl groups (1723 and 1748 cm^{-1}) and its molecular ion in the mass spectrum was at $m/z = 251$.

Hydrolysis of **16** with boiling aqueous KOH gave carboxylic acid **17** in an 83% yield. The carboxylic acid group was confirmed by its IR spectrum (bands at 1732 and 3100 cm^{-1}) and the molecular formula was indicated by HRMS of the pseudo molecular ion (MH^+) peak in the CI mass spectrum at $m/z = 252$, corresponding to $C_{16}H_{14}NO_2$.

A 32% yield of **18** was obtained from the reaction of **17** with benzyl 3-(4-hydroxyphenyl)propionate under reflux for 18 hours in benzene with 4-toluenesulfonyl chloride and triethylamine. The purity of **18** was confirmed by elemental analysis; there were also two carbonyl carbon signals in its ^{13}C NMR spectrum and its CI mass spectrum showed a pseudo molecular ion (MH^+) peak at $m/z = 490$, confirming its structure.

Debenzylation of **18** gave the corresponding acid **19** in



Scheme 3 Synthesis of compounds **4** and **5**. Reagents and conditions: a: for **22**: K, reflux, 1.5 h, 2,5-dimethylbromobenzene, reflux, 17 h; for **27**: H_2O, HCl, Ac_2O, AcONa, 20℃, 15 min, bromobenzene, K_2CO_3, CuI, reflux, 17 h; b: $(COCl)_2$, CS_2, $AlCl_3$, reflux, 2 h; c: KOH, H_2O, reflux, 13 days (for **24**) or 4 days (for **29**), then HCl; d: $SOCl_2$, reflux, 3 h, pyridine, benzyl 3-(4-hydroxyphenyl) propionate, 17 h, 20℃ (for **25**) or 100℃ (for **30**); e: HBr, AcOH, 100℃, 3 h; f: DCC, NHS, DMF, 20℃, 17 h.

an 82% yield. The IR spectrum showed absorption bands corresponding to the stretching vibrations of the OH (3000 cm^{-1}) and C=O (1719 and 1753 cm^{-1}) groups. The ^{13}C NMR spectrum showed two carbonyl signals at 167.7 and 173.7 ppm, and the molecular ion appeared at $m/z = 399$ in the EI mass spectrum.

The reaction of **19** and NHS in the presence of DCC gave **20** in 56% yield. Its purity was confirmed by elemental analysis. The IR (1736, 1781, and 1814 cm^{-1}) and ^{13}C NMR (167.8, 168.1, and 169.1 ppm) spectra confirmed that there were three different carbonyl groups. The CI mass spectrum showed a pseudo molecular ion (MH^+) peak at $m/z =$ 497 to confirm the molecular mass.

Finally, methylation of **20** gave **3** in a 64% yield. The methyl protons at the 9-position appeared as a singlet at 4.88 ppm in the ^1H NMR spectrum. The IR showed three absorption bands (1741, 1775, and 1808 cm^{-1}) corresponding to the stretching vibrations of three different carbonyl groups, and HRMS confirmed the formula of the pseudo molecular ion ($[M - CF_3SO_3^-]^+$) peak as $C_{30}H_{31}N_2O_6$.

The syntheses of compounds **4** and **5** (Scheme 3) followed similar routes to those of compounds **14** and **20**. However, since compound **24** has been reported previously[31], the approach to that compound followed the literature

route. Also, for the esterification steps it was possible to use a standard method for formation of the appropriate polymethylacridinecarboxylic acid chloride followed by addition of benzyl 3-(4-hydroxyphenyl)propionate, which had not been successful with compounds **12** and **18**. Otherwise the procedures were very similar to those applicable to Schemes **1** and **2**. The experimental details, and the spectroscopic and spectrometric data used to characterize the various compounds are given in the Experimental Section.

It had been intended to convert compounds **4** and **5** into their corresponding AEs, but unfortunately stirring compound **4** with methyl trifluoromethanesulfonate in DCM at room temperature produced no precipitate and the starting material (**4**) was recovered unchanged. Use of more forcing conditions (overnight reflux) still did not produce the desired methylated derivative, but some decomposition of the starting material occurred under such conditions. It has previously been noted that both 4,5-dimethylacridine and 1,4,5,8-tetramethylacridine fail to react with electrophilic boron reagents (BH_3 and BF_3) because of hindrance by the methyl groups at positions 4 and 5[31], so perhaps the failure to methylate **4** is not surprising. Compound **5** was also not methylated with methyl trifluoromethanesulfonate over a period of 2.5 h at room temperature. Under more forcing conditions, some methylation did occur, as evidenced by the emergence of a peak at $\delta = 4.9$ ppm for the N-Me group in the ^{1}H NMR spectrum and characteristic changes in the aromatic region of the spectrum. However, there was also evidence of breakdown of the ester group under such conditions and it was not possible to obtain pure *N*-methylated product suitable for either complete characterization or meaningful study of its chemiluminescent properties.

Following the synthetic studies, therefore, just the two AEs, **2** and **3**, were available for studies of chemiluminescence and hydrolytic stability.

3.2 Chemiluminescence and stability

AEs **2** and **3** were found to give lower light output (31 and 35% of that for AE **1a**, respectively) under typical alkaline conditions, as a result of the presence of the additional methyl groups on the acridine ring. In particular, the methyl groups at the 1- and 8-positions of the acridinium ring of AE **2** probably cause the carboxylic ester group to twist out of planarity and become orthogonal to the acridinium ring, which leads to its loss of conjugation and increased electrophilic character. In addition, attack at position 9 must be highly encumbered, so that the first step of the chemiluminescent reaction, involving attack by peroxide anion at position 9, would be significantly slowed in comparison to attack on **1a**. This would allow greater competition from other, non-radiative, pathways, such as attack at the ester carbonyl group, which is not only more electrophilic than that in **1a** because of the loss of conjugation, but also presents a relatively open face because of its orthogonal orientation. Consequently, attack by a nucleophile such as hydroperoxide anion or hydroxide anion directly at the carbonyl group, leading to non-radiative pathways, becomes more significant. AE **3**, having only one methyl group in such an encumbering situation, will be subject to these same considerations, but to a lesser extent.

If AEs are to be used as biological probes, it is important that their conjugates with biological targets are sufficiently stable to be able to withstand the conditions to which they will be subjected during the monitoring process. In order to assess how AEs **2** and **3** compared to **1a** in this respect, all three AEs were reacted with immunoglobulin G (IgG) and the products were subjected to gel chromatography to give solutions of the corresponding pure AE–IgG conjugates. 10 μL aliquots of these solutions were separately diluted in buffers of different pH values (5, 6, 7, and 8), samples of which were incubated at three different temperatures (0, 20, and 37℃) for different periods of time (1, 2, 4, 8, 16, and 32 days). At the end of the incubation period, the chemiluminescence of each sample was measured and compared with the figure for an identical sample at time 0. The results are shown graphically in Fig. 2.

A few of the readings are clearly outliers (e.g., the 4 day result for IgG–**3** at 0℃ and pH 5, the 32 day reading for IgG–**1a** at 20℃ and pH 5, and the readings for IgG–**2** after 8 days at 20℃ and pH 7 and after 4 days at 0℃ and pH 8), but from the rest of the readings several trends can be deduced. Unsurprisingly, the rate of reduction in the level of emission over time for otherwise similar samples was least for incubation at 0℃. It was also typically the case that incubation at 37℃ led to the greatest rate of reduction, although for some samples the differences between the 20 and 37℃ incubations were quite small.

Under most conditions, IgG–**3** lost performance more rapidly than the other two conjugates, which can probably be understood in terms of the acridine-attached carbonyl group twisting out of conjugation somewhat, leading to greater electrophilicity, while at the same time presenting an open face for attack by a nucleophile. Also, degradation of IgG–**3** was relatively insensitive to the conditions of both temperature and pH. However, IgG–**1a**, which was relatively slowly degraded at pH 5–7 under most conditions, was much more sensitive to degradation at the highest pH studied (pH 8), and this led to its becoming the most rapidly degraded at the higher temperatures at pH 8. Under most conditions, IgG–**2** was at least competitive with the best other conjugate in terms of the slowness of degradation in light output, and at higher temperatures, particularly at higher pH, it was the least rapidly degraded of all. The one case where it was not competitive with the best was for pH 7 at 0℃, where IgG–**1a** was clearly the least rapidly degraded.

There were no conditions under which IgG–**3** would have

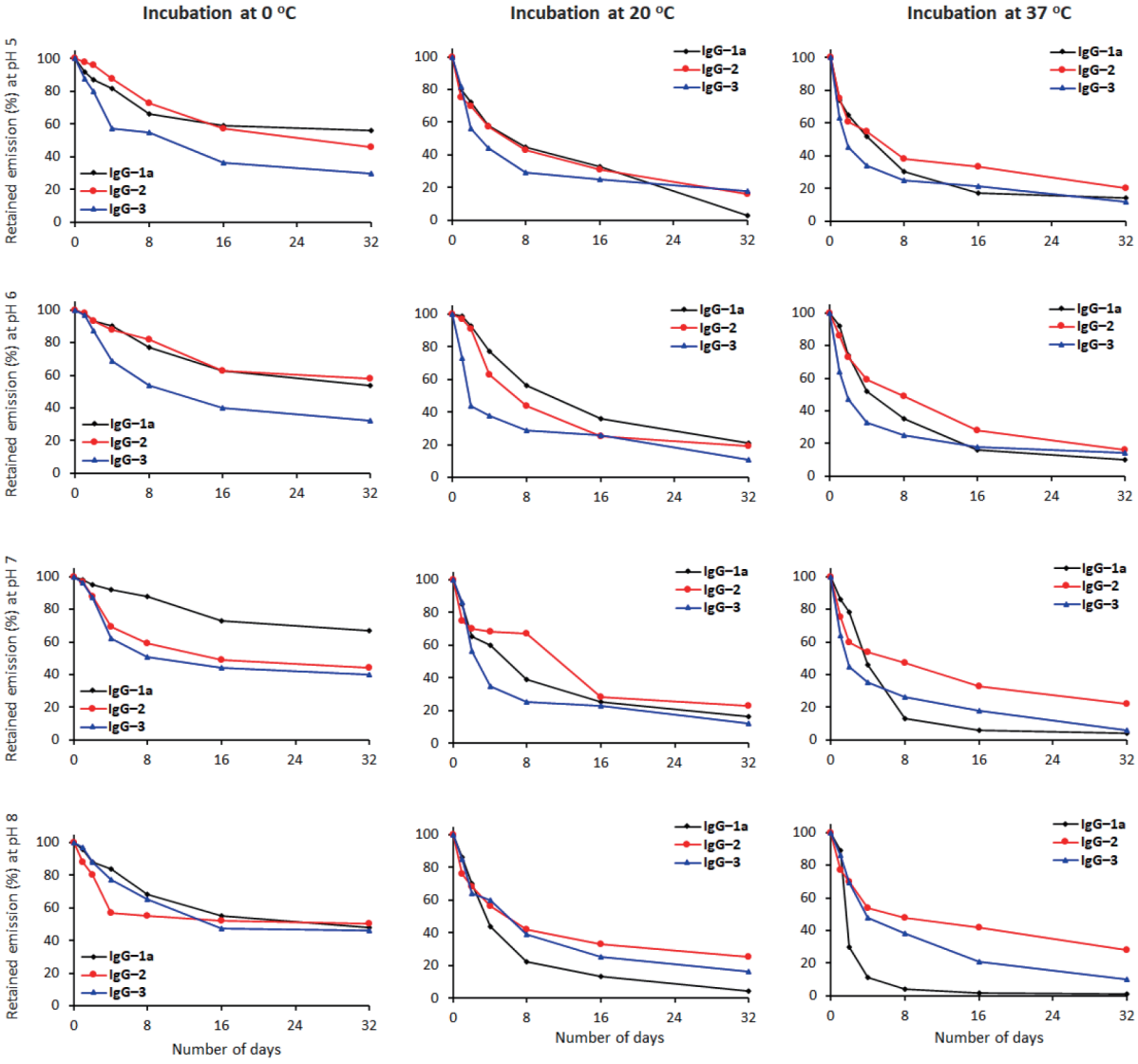


Fig. 2 Percentage of original level of light emission retained after incubation of samples of IgG–**1a** (in black), IgG–**2** (in red), and IgG–**3** (in blue) at different pH values (5, 6, 7, and 8) over time (0–32 days) at different temperatures (0, 20, and 37℃).

advantages over IgG–**1a** in terms of stability, but IgG–**2** was more stable in many circumstances, particularly at higher temperatures and/or higher pH values. Therefore, AE **2** has the potential to be used as an acridinium label in such conditions.

4 Conclusions

Two acridinium esters (**2** and **3**), containing methyl substituents on the acridinium ring, were successfully synthesized and their chemiluminescent properties were measured. Although the synthesized AEs showed substantial chemiluminescence, they displayed lower quantum yields of light output than the corresponding AE that contains no methyl substituents (**1a**). The new AEs also possess an *N*-hydroxysuccinimide ester unit, which enables their easy attachment to many biologically important species, so in principle they could be useful as chemiluminescent labels. For such purposes, the conjugates with biological molecules would need to be relatively stable in buffer solutions for significant amounts of time. However, the 1,3,10-trimethylacridinium ester conjugate with immunoglobulin G (IgG–**3**) showed a more rapid drop in chemiluminescent output than IgG–**1a** under most conditions tried (pH 5–7; 0–37℃), although it was somewhat more stable than IgG–**1a** at pH 8, particularly at the higher temperatures. The 1,3,6,8,10-pentamethylacridinium ester conjugate

IgG–**2** showed greater resistance to loss of chemiluminescent output over time, and was significantly more stable than either IgG–**1a** or IgG–**3** at 37℃ at all pH values from pH 5 to pH 8. It was also more stable at 20℃ in pH 7 or pH 8 buffers. Therefore, acridinium ester **2** has the potential to be useful as a chemiluminescent label under such conditions.

Two acridine esters (**4** and **5**), having methyl groups at positions 4 and/or 5 on the acridine ring, were also synthesized, but it proved impossible to convert them into the corresponding acridinium esters by treatment with methyl trifluoromethanesulfonate, presumably because of the increased steric hindrance around the acridine nitrogen as a result of the proximity of those methyl groups.

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Conflict of Interest

No conflict of interest to declare.

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