The synthesis and evaluation of *o*-phenylenediamine derivatives as fluorescent probes for nitric oxide detection

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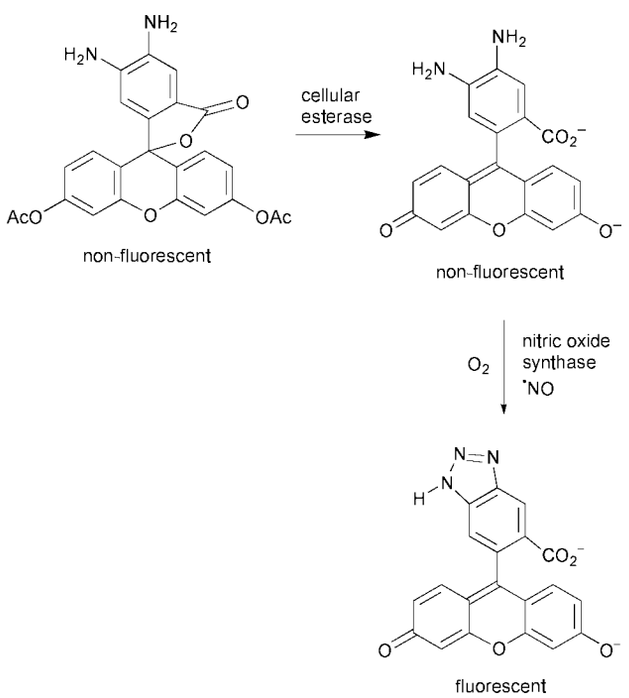
A series of molecular probes for the determination of nitric oxide (NO) have been prepared. Each probe consists of an anthracene, coumarin or acridine fluorophore coupled to an electron rich *o*-phenylenediamine group. The *o*-phenylenediamine group can be substituted with methyl or methoxy groups to enhance its electron rich nature. The fluorophore fluorescence is quenched by photoelectron transfer (PET) from the aromatic amine to the lowest unoccupied orbital of the excited state fluorophore. Reaction with nitrosating species converts the *o*-phenylenediamine group into an electron deficient benzotriazole derivative. This group has a higher oxidation potential and does not quench the fluorophore fluorescence by photoelectron transfer so that these products are highly fluorescent. Some benzotriazole derivatives were made preparatively by alternative synthetic routes. The formation of fluorescent probes was evaluated by treatment of the precursors with nitrous fumes and *S*-nitroso-*N*-acetylpenicillamine (SNAP).

Introduction

Nitric oxide (NO) plays a vital role in human physiology. NO has different, concentration-dependent roles in many tissues, such as in the vasculature, immune system and in neurotransmission.[1–3] We are particularly interested in the role of NO in bone and joint disease. *In vivo* and *in vitro* studies have indicated that NO at low concentrations is an important molecule, essential for normal bone physiology, however, high levels of NO have been demonstrated to be inhibitory for bone and cartilage cell function.[4,5] NO plays an important role in mediating the effect of interleukin-1 in inflammation-induced bone loss and is implicated in the pathology of rheumatoid arthritis.[6,7] To understand more clearly the role of NO in bone and joint pathology, better methods are required to determine the exact concentrations of NO that are beneficial or harmful for specific cell types. We also require more accurate information on the cellular localisation of NO production, rather than just information on the cellular expression of the NO synthase enzymes. Techniques for the detection of NO based on chemiluminescence, or using electrochemical probes have been developed.[8–10] Although these techniques allow detection of NO production from tissues, they do not allow imaging of NO production at the cellular level. We therefore set out to design molecular probes that would allow real time visualisation of NO production within live cells. For studies of intracellular concentrations of small molecules and ions, fluorescent probes are widely used and they also offered the most attractive prospect for this application as a non-invasive and potentially highly sensitive method.

Work from the group of Nagano and co-workers[11–13] has shown the value of fluorophores linked to the *o*-phenylenediamine group for the detection of NO (Scheme 1). The *o*-phenylenediamine group is electron rich and might quench the fluorophores fluorescence by a process known as photoelectron transfer (PET).[14] This involves the transfer of an electron from the HOMO of an electron rich donor (tertiary amine or *o*-phenylenediamine) to the lowest energy vacant orbital of an excited acceptor (fluorophore). *o*-Phenylenediamine and *p*-phenylenediamine derivatives have low oxidation potentials as shown by cyclic voltammetry.[15] Back electron transfer occurs without fluorescent emission restoring the ground state. Reaction of the *o*-phenylenediamine group with NO derived by-products such as N_2O_3 or peroxy nitrite, formed from the reaction of NO with O_2,[13] gives a benzotriazole derivative. Benzotriazole is an acidic, electron deficient heterocycle containing two electron withdrawing pyridine type nitrogens and possesses a higher oxidation potential, so electron transfer to



Scheme 1

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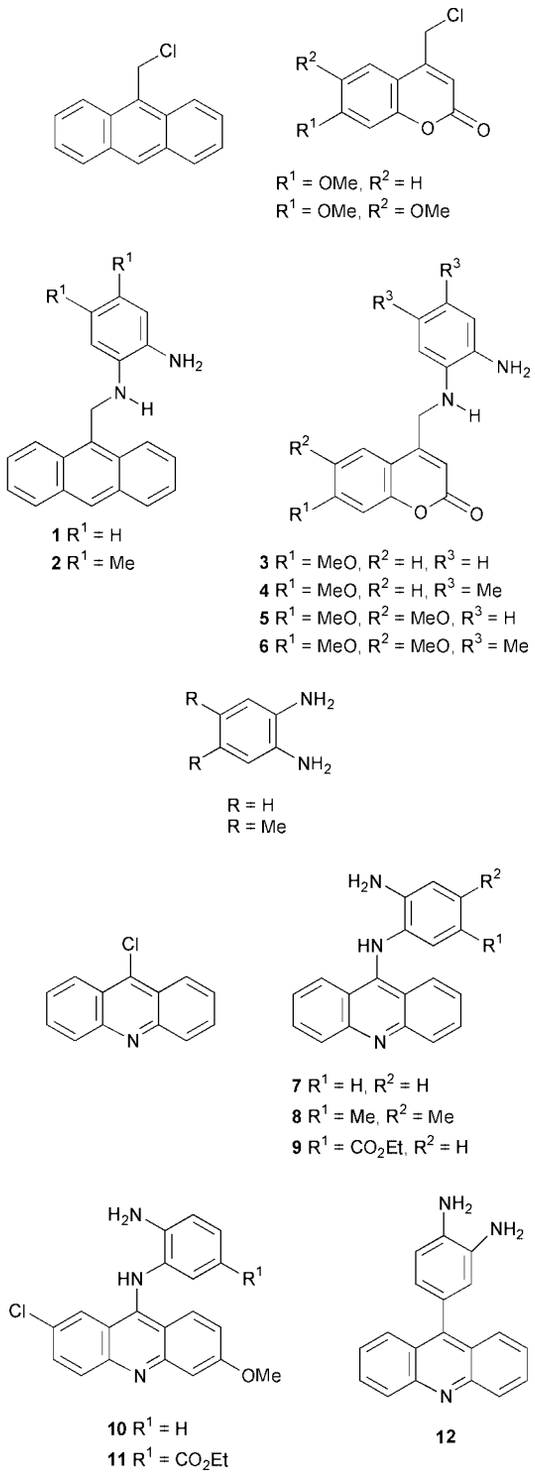
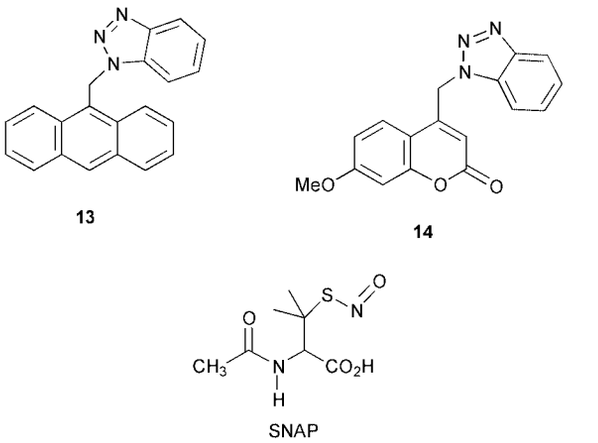
the excited state fluorophore occurs much less readily. It does not quench the fluorophore fluorescence so that a positive increase in fluorescence is observed. The system could be considered to be a chemically irreversible 'Off–On' switch. Diaminofluoresceins,[11] diaminorhodamines[12] and diaminonaphthalenes[13] have been studied as probes for NO. However, we have found these compounds to be toxic for bone cells. The rhodamine derivative is charged so would not be able to penetrate a cell membrane. The fluoresceins and rhodamines also require multistep synthetic preparations. In view of the lengthy syntheses required for their preparation some simpler synthetic alternatives were prepared and studied. It was hoped to fine-tune such structures more easily to find probes with properties that make them suitable for use with live cells. Specific requirements are that the compounds have a suitable long wavelength excitation frequency (above 340 nm) to avoid tissue or cellular damage and to avoid interference from background fluorescence. The compounds should be soluble in aqueous solution (usually phosphate-buffered tissue culture media) with minimal DMSO concentrations (below 0.1%) to avoid having a harmful effect on live cells. Finally, the probe should ideally contain an ester functional group. Ester groups do not prevent compounds passing through the cell plasma membrane, but once inside the cell, esterases (which are present inside bone cells) hydrolyse the ester to give a charged carboxylic acid group,[13,16,17] preventing the exit of the probe from the cell and ensuring that only NO concentrations inside cells would be measured.

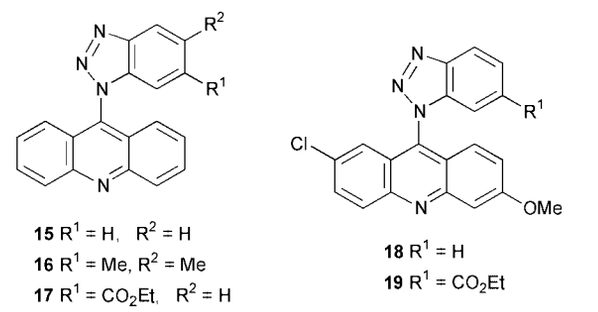
Discussion

In diaminofluoresceins[11] and diaminorhodamines[12] the *o*-phenylenediamine group is conjugated to the fluorophore but is twisted out of plane by steric interactions. The literature on the study of fluorescent sensors[14] provided precedent that new sensors for NO could be designed in which the electron rich amine is tethered to a fluorophore by a saturated linker. A series of *o*-phenylenediamine probes were prepared containing different fluorophores and substituents based on this principle. Probes **1** and **2** contained an anthracene fluorophore, probes **3**–**6** contained a coumarin and probes **7**–**12** contained an acridine. Derivatives were prepared in which the *o*-phenylenediamine group was substituted with either methyl or methoxy groups. These groups make the aromatic ring more electron rich and might enhance its ability to quench fluorescence. The anthracenes and coumarins are easily accessible from their 9-chloromethyl and 4-bromomethyl derivatives respectively by reaction with *o*-phenylenediamine or a derivative of it. The acridine based probes **7**–**11** were prepared by the reaction of an *o*-phenylenediamine and 9-chloroacridine.[18] 9-Chloroacridine was prepared by heating *N*-phenylanthranilic acid with $POCl_3$.[19] They were isolated as their hydrochloride salts. Probes **9** and **11** contain an ester functional group. The primary amine conjugated *para* to the ester was expected to be less nucleophilic than the alternative primary amine which is *meta* to and not conjugated to the ester. Acridine **12** was prepared by heating acridine hydrochloride with *o*-phenylenediamine and sulfur.[20] The interception of NO derived species such as N_2O_3 in cell culture is thought to convert the *o*-phenylenediamine moiety into a benzotriazole which does not quench the fluorophore fluorescence. Therefore, for comparison purposes the corresponding benzotriazole derivatives of probes **1**, **3** and **7**–**11** were prepared. Benzotriazoles **13** and **14** were prepared by treatment of 9-chloromethylanthracene and 4-(bromomethyl)-7-methoxycoumarin with the sodium salt of benzotriazole respectively. Benzotriazoles **15**–**19** were prepared by treatment of probes **7**–**11** with sodium nitrite and hydrochloric acid in aqueous solution. The mechanism is thought to involve diazotisation of the primary aromatic amine followed by a rapid intramolecular cyclisation to give the benzotriazole.



The *o*-phenylenediamine probes **1**–**11** were found, as expected, to be either weakly or non-fluorescent. The corresponding benzotriazole derivatives are however intensely fluorescent. Figs. 1–4 show typical excitation and emission spectra that were obtained for some of the benzotriazole derivatives **13**–**19**. The largest excitation and emission peaks are summarised in Table 1. The solutions were prepared by dilution of standard solutions in DMSO with sodium phosphate buffer (pH 7.4). The acridine derivatives have longer wavelength excitation and emission maxima and therefore show more promise for cell culture studies. Addition of the chloro and methoxy groups in acridines **18** and **19** shifts the emission maxima to over 450 nm. The synthesis of these probes and the observation of enhanced fluorescence output in the corresponding benzotriazole derivatives demonstrates their potential feasibility for the detection of NO.





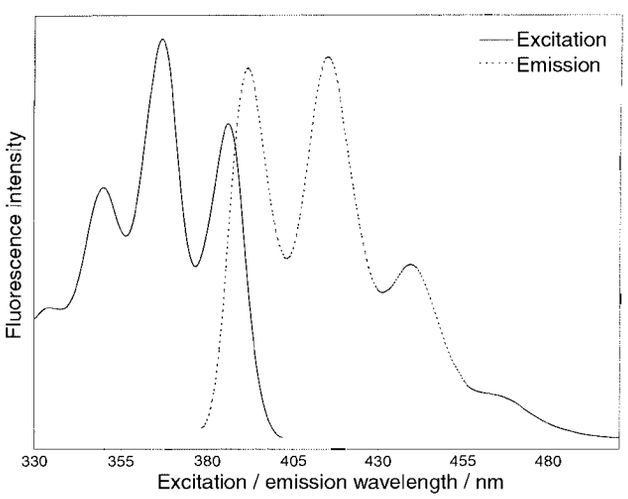


Fig. 1 Excitation spectrum for anthracene **13** taken at emission wavelength of 414 nm and emission spectrum taken at excitation wavelength of 367 nm. Anthracene concentration 5×10^{-7} M.

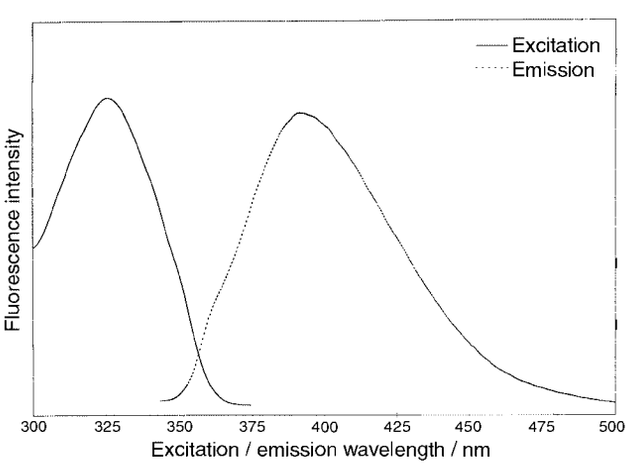


Fig. 2 Excitation spectrum for coumarin **14** taken at emission wavelength of 391 nm and emission spectrum taken at excitation wavelength of 326 nm. Coumarin concentration 5×10^{-7} M.

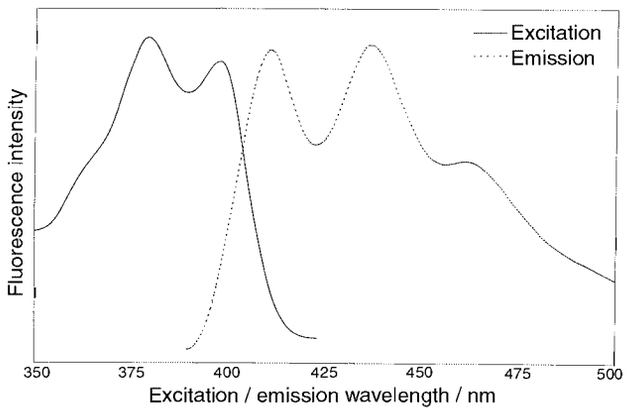


Fig. 3 Excitation spectrum for acridine **15** taken at emission wavelength of 437 nm and emission spectrum taken at excitation wavelength of 380 nm. Acridine concentration 5×10^{-7} M.

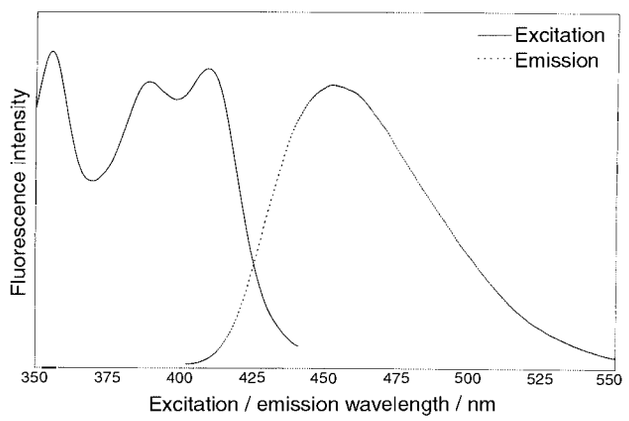


Fig. 4 Excitation spectrum for acridine **18** taken at emission wavelength of 452 nm and emission spectra taken at excitation wavelength of 389 nm. Acridine concentration 5×10^{-7} M.

Table 1 Fluorescence properties of some prepared benzotriazole derivatives

Compound	Excitation maxima/nm	Emission maxima/nm
13	350/367/390	395/418/442
14	327	390
15	383/398	418/438
16	381/397	414/436
17	384/398	417/438
18	358/387/411	454
19	356/386/409	452

From these encouraging results some simple qualitative experiments were performed in which a dilute solution of the probe was treated with a nitrogen gas stream containing some nitrous fumes.[21] Since the reaction of NO with O_2 may generate N_2O_3, which can act as a nitrosating agent, nitrosating fumes provide a simple mimic of this system. These were generated in excess by adding 2–3 drops of conc. HCl to a quantity of $NaNO_2$ (~1 g) in a flask under a flow of nitrogen gas. The N_2 gas was bubbled directly through a dilute solution of the probe for a few seconds. Probes **1**–**6** were not soluble in water so were dissolved up in either ethanol or DMSO. Probes **7**–**11**, as their hydrochloride salts, were dissolved up in DMSO and diluted with phosphate buffer solution. In each case brief treatment with an excess of nitrous fumes gave highly fluorescent solutions indicating the desired *in situ* conversion of the *o*-phenylenediamine group to a benzotriazole.

The ability of probe **12** to monitor NO production was investigated by treating it with *S*-nitroso-*N*-acetylpenicillamine (SNAP).[22,23] SNAP is a hindered nitroso thiol that exists as a crystalline solid making it easy to weigh out and prepare a

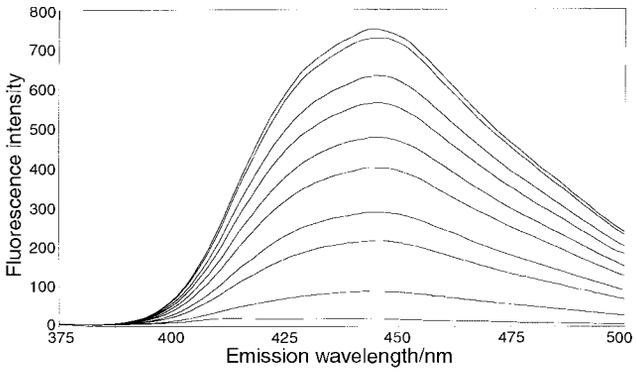


Fig. 5 Emission spectra for 9-(3,4-diaminophenyl)acridine **12** (50 μM) in the presence of SNAP (50 μM). Excitation wavelength 360 nm. Spectra were taken at 30 minute intervals starting at $t = 0$ for the lowest trace.

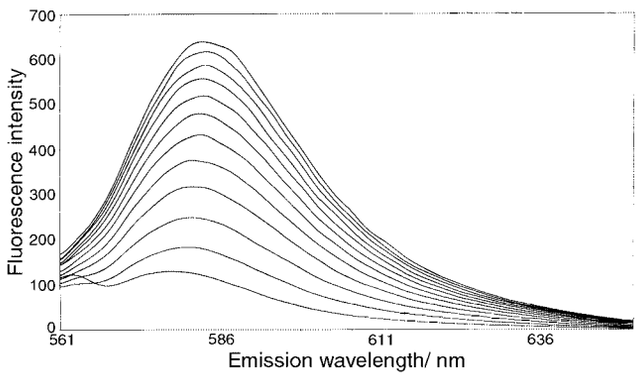
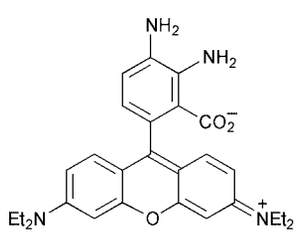


Fig. 6 Emission spectra for diaminorhodamine **20** (10 μM) in the presence of SNAP (50 μM). Excitation wavelength of 565 nm. Spectra were taken at 30 minute intervals starting at $t = 0$ for the lowest trace.

standard solution for immediate use. Under these conditions it probably acts as a source of NO^+ rather than generating NO directly. Fig. 5 shows the increase in fluorescence over time that is observed when equimolar quantities of probe **12** and SNAP are mixed together in ethanol. It takes about 4 h for the maximum fluorescence intensity to be reached. The slow nature of the reaction suggests that probe **12** slowly reacts with the *S*-nitroso group of SNAP which acts as a nitrosating agent (a masked form of NO^+). When an excess of SNAP was used the maximum fluorescence value was the same, indicating that the reaction of probe **12** with SNAP is high yielding. The previously reported diaminorhodamine probe **20**[12] was synthesised for comparison purposes. Fig. 6 shows the emission spectra after treatment with SNAP. The reaction takes several hours to go to completion as for probe **12**. However, probe **20**, after reaction with SNAP, has a longer wavelength of emission centered at 580 nm. These characteristics make it and also the fluorescein based probes[11] more suitable for certain types of cell culture studies.



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In summary, a series of *o*-phenylenediamine probes have been prepared by short synthetic routes from readily available starting materials. Preliminary studies show that they react with the NO related species SNAP and nitrous fumes to give highly fluorescent products. Further studies evaluating the probes for the determination of NO in cell cultures are in progress.

Experimental

Fluorescence measurements were made using a Perkin Elmer Luminescence Spectrometer LS50B running with FL Winlab software. The slit widths for excitation and emission were 5 nm.

9-(2-Aminophenyl)aminomethylanthracene, 1

o-Phenylenediamine (1.1 g, 10 mmol) was dissolved in dry pyridine (50 ml). 9-(Chloromethyl)anthracene (0.57 g, 2.5 mmol) was slowly added and the mixture refluxed overnight. The solvent was evaporated to leave a green–brown residue. Purification by column chromatography (dichloromethane–hexane 1 : 1) gave the *title compound* (0.45 g, 65%) as pale yellow crystals, mp 178–180 °C (from hexane–ethyl acetate) (Found: C, 84.3; H, 6.1; N, 9.1. $C_{21}H_{18}N_2$ requires C, 84.6; H, 6.0; N, 9.4%); λ_{max}(EtOH)/nm 290 (log ε 3.7), 349 (3.7), 367 (4.0) and 386 (3.9); ν_{max}(KBr)/cm^{-1} 339m, 3369m, 3326m, 1622s and 1594s; δ_H (250 MHz; [D_6]DMSO) 4.50 (2H, s, NH_2), 4.62 (1H, s, NH), 5.06 (2H, s, CH_2), 6.56 (2H, m, aryl), 6.65 (1H, m, aryl), 6.94 (1H, d, *J* 7.3, aryl), 7.54 (4H, m), 8.13 (2H, d, *J* 7.0), 8.27 (2H, d, *J* 7.0) and 8.63 (1H, s); δ_C (62.9 MHz; [D_6]DMSO) 40.9, 110.2, 114.0, 117.5, 124.6, 125.2, 126.2, 127.2, 128.9, 130.3, 130.4, 131.2, 135.3 and 136.3 (one resonance is overlapping); *m*/*z* (CI) 299 (M^+ + H, 100%).

9-(2-Amino-4,5-dimethylphenyl)aminomethylanthracene, 2

4,5-Dimethyl-*o*-phenylenediamine (1.0 g, 7.5 mmol) was dissolved in dry pyridine (20 ml). 9-Chloromethylanthracene (0.46 g, 2 mmol) was slowly added and a red solution formed. The reaction was heated to 80 °C for 3 h and allowed to stir at room temperature for 3 days. The solvent was evaporated to give a residue which was purified by chromatography on silica gel. Elution with ethyl acetate gave the *title compound* (0.42 g, 65%) as pale yellow crystals, mp 203–205 °C (from ethyl acetate–light petroleum) (Found: C, 84.9; H, 6.6; N, 8.2. $C_{23}H_{22}N_2$ requires C, 84.7; H, 6.8; N, 8.6%); λ_{max}(EtOH)/nm 303 (log ε 3.7), 347 (3.7), 365 (4.0) and 384 (3.9); ν_{max}(KBr)/cm^{-1} 3548m, 3474m, 3413m, 3233m, 1637s, 1617s and 1517s; δ_C (62.9 MHz; [D_6]DMSO) 18.7, 19.1, 40.9, 112.6, 116.4, 123.9, 124.0, 124.7, 125.2, 126.2, 127.1, 128.9, 130.3, 130.7, 131.2, 132.9 and 134.5; *m*/*z* (CI) 327 (M^+ + H, 100%).

4-(2-Aminophenyl)aminomethyl-7-methoxycoumarin, 3

o-Phenylenediamine (1 g, 9 mmol) was dissolved in dry pyridine (30 ml). 4-Bromomethyl-7-methoxycoumarin (0.54 g, 2 mmol) was added slowly. A precipitate was seen within a few minutes. The mixture was stirred overnight and then evaporated to a gummy solid. The solid was purified by chromatography on silica gel. Elution with ethyl acetate gave the *title compound* (0.36 g, 62%) as a pink powder, mp 174–177 °C (from ethyl acetate–light petroleum) (Found: C, 66.6; H, 5.3; N, 9.3. $C_{17}H_{16}N_2O_3$ requires C, 66.9; H, 5.4; N, 9.5%); λ_{max}(EtOH)/nm 317 (log ε 4.0); ν_{max}(KBr)/cm^{-1} 3494m, 3396m, 3321m, 1701m and 1606m; δ_H (250 MHz; [D_6]DMSO) 3.88 (3H, s, OMe), 4.51 (2H, s, CH_2), 4.60 (2H, s, NH_2), 5.18 (1H, m, NH), 6.17 (1H, s), 6.30–6.60 (4H, m), 6.98–7.04 (2H, m) and 7.88 (1H, d, *J* 8.6); δ_C (62.9 MHz; [D_6]DMSO) 43.5, 56.0, 100.9, 108.6, 110.4, 111.5, 112.2, 114.5, 117.6, 117.7, 125.9, 134.8, 135.4, 154.5, 155.0, 160.5 and 162.4; *m*/*z* (CI) 297 (M^+ + H, 100%).

4-(2-Amino-4,5-dimethylphenyl)aminomethyl-7-methoxycoumarin, 4

1,2-Diamino-4,5-dimethylbenzene (1 g, 7.5 mmol) was dissolved in dry pyridine (20 ml) and 4-bromomethyl-7-methoxycoumarin

(0.54 g, 2 mmol) slowly added. A red solution and a red precipitate was rapidly formed. The suspension was stirred overnight. The solvent was evaporated, the residue dissolved in CH_2Cl_2, washed with H_2O, dried with Na_2SO_4 and evaporated to a brown solid. The product was purified by column chromatography on silica gel. Elution with ethyl acetate gave the *title compound* (0.19 g, 30%) as red needles, mp 191–192 °C (from ethyl acetate) (Found: C, 69.9; H, 6.2; N, 8.3. $C_{19}H_{20}N_2O_3$ requires C, 70.4; H, 6.2; N, 8.7%); λ_{max}(EtOH)/nm 317 (log ε 4.0); ν_{max}(KBr)/cm^{-1} 3371m, 3359m, 3260m, 1717vs, 1607vs and 1523s; δ_H (250 MHz; [D_6]DMSO) 1.97 (3H, s, Me), 2.00 (3H, s, Me), 3.87 (3H, s, OMe), 4.37 (2H, s, NH_2), 4.50 (2H, d, CH_2, *J* 4.9), 4.92 (1H, m, NH), 6.18 (2H, s), 6.42 (1H, s), 7.02 (2H, m) and 7.87 (1H, d, *J* 8.6); δ_C (62.9 MHz; [D_6]-DMSO) 18.6, 19.0, 43.7, 56.0, 100.9, 108.6, 111.6, 112.2, 112.5, 116.7, 124.2, 124.3, 125.8, 132.8, 133.1, 154.8, 155.0, 160.5 and 162.4; *m*/*z* (Found: M^+ + H, 325.1548. $C_{19}H_{21}N_2O_3$ requires 325.1552).

4-(2-Aminophenyl)aminomethyl-6,7-dimethoxycoumarin, 5

o-Phenylenediamine (1 g, 9 mmol) was dissolved in dry pyridine (30 ml). 4-Bromomethyl-6,7-dimethoxycoumarin (0.3 g, 1 mmol) was carefully added and the mixture stirred overnight. During this period a precipitate appeared and the mixture turned from yellow to brown. The solvent was evaporated to give a brown solid. TLC analysis (EtOAc) showed two spots very close to each other. Recrystallisation from ethyl acetate–light petroleum selectively gave the *title compound* (0.16 g, 50%) as pale brown needles, mp 174–177 °C; λ_{max}(EtOH)/nm 295 (log ε 3.7) and 342 (4.0); ν_{max}(KBr)/cm^{-1} 3385m, 3364m, 3307m, 3190m, 1702vs, 1612s and 1593s; δ_H (250 MHz; [D_6]DMSO) 3.86 (3H, s, OMe), 3.87 (3H, s, OMe), 4.59 (4H, m, NH_2 and CH_2), 5.23 (1H, m, NH), 6.16 (1H, m), 6.35 (1H, m), 6.45 (2H, m), 7.10 (1H, s) and 7.34 (1H, s); δ_C (62.9 MHz; [D_6]DMSO) 43.6, 56.1, 56.1, 100.3, 105.7, 108.6, 110.4, 110.5, 114.6, 117.6, 117.7, 134.7, 135.5, 145.9, 149.0, 152.5, 154.6 and 160.8; *m*/*z* (Found: M^+ + H, 327.1351. $C_{18}H_{19}N_2O_4$ requires 327.1345).

4-(2-Amino-4,5-dimethylphenyl)aminomethyl-6,7-dimethoxycoumarin, 6

4,5-Dimethyl-1,2-phenylenediamine (0.54 g, 4 mmol) was dissolved in dry pyridine (40 ml). 4-Bromomethyl-6,7-dimethoxycoumarin (0.3 g, 1 mmol) was added slowly and the mixture left to stir overnight. A precipitate was seen within 10 minutes. The solvent was evaporated to give an orange gum. This was dissolved in CH_2Cl_2, washed with water and dried with Na_2SO_4. Evaporation gave a crystalline solid. The product was purified by chromatography on silica gel. Elution with ethyl acetate gave the *title compound* (0.11 g, 30%) as a yellow solid; mp 208–210 °C (from ethyl acetate–light petroleum) (Found: C, 66.6; H, 6.0; N, 7.4. $C_{20}H_{22}N_2O_4$ requires C, 67.8; H, 6.2; N, 7.9%); λ_{max}(EtOH)/nm 341 (log ε 4.0); ν_{max}(KBr)/cm^{-1} 3396m, 3344m, 1703vs, 1611s, 1566s and 1519s; δ_H (250 MHz; [D_6]DMSO) 1.96 (3H, s), 1.99 (3H, s), 3.87 (6H, s, OMe), 4.36 (2H, s, NH_2), 4.54 (2H, d, *J* 5.2), 4.97 (1H, t, NH, *J* 6.41), 6.16 (2H, d, *J* 7.63), 6.41 (1H, s), 7.09 (1H) and 7.32 (1H, s); δ_C (62.9 MHz; [D_6]DMSO) 18.6, 19.00, 43.8, 56.2, 56.2, 100.2, 105.7, 108.6, 110.4, 112.5, 116.7, 124.1, 124.3, 132.7, 133.1, 145.8, 148.96, 152.5, 154.9 and 160.8; *m*/*z* (Found: M^+ + H, 355.1653. $C_{20}H_{23}N_2O_4$ requires 355.1658).

General methods for the preparation of acridines

Coupling method 1. The phenylenediamine (1.2 g, 11 mmol) was dissolved in dry methanol (50 ml) and heated to reflux. 9-Chloroacridine (1.1 g, 5 mmol)[19] was slowly added and the mixture refluxed for 1 h. After cooling the mixture was poured into ether (200 ml) and the hydrochloride salt precipitated and filtered off. The salt was recrystallised from ethanol–ether to give a red, orange or yellow powder. Yields 75–90%.

Coupling method 2. 3,4-Diaminobenzoic acid (2.3 g, 15 mmol) was added to a solution of conc. H_2SO_4 (6 ml) in ethanol or methanol (60 ml). The mixture was heated to reflux for 5 h and the ethanol evaporated to leave a thick purple–brown liquid. The liquid was poured into ice–water and a purple powder was collected by filtration. The powder was stirred in a saturated solution of $NaHCO_3$ turning a paler shade of purple. The suspension was extracted with CH_2Cl_2, washed with water, turning from brown to yellow and dried with Na_2SO_4. Evaporation gave a pale brown powder. Yield 40–60%. The esterified *o*-phenylenediamine (0.9 g, 5 mmol) was gently heated in freshly distilled phenol (20 ml). 9-Chloroacridine (0.53 g, 2.5 mmol) was slowly added and the mixture heated to reflux for 2 h. After cooling the mixture was poured into ether (200 ml) and a brown hygroscopic solid filtered off. Recrystallisation from ethanol–ether gave the hydrochloride salt as a powder. Yield 80–90%.

9-(2-Aminoanilino)acridine hydrochloride, 7. Red powder (1.32 g, 82%), mp >250 °C decomp. (from ethanol–ether) (lit.[18] 320–322 °C) (Found: C, 70.7; H, 4.8; N, 13.1. $C_{19}H_{16}N_3Cl$ requires C, 70.9; H, 5.0; N, 13.1%); λ_{max}(EtOH)/nm 289 (log ε 4.0), 413 (4.1) and 433 (4.1); ν_{max}(KBr)/cm^{-1} 3553m, 3447m, 3413m, 1633vs, 1579s and 1549vs; δ_H (250 MHz; [D_6]DMSO) 5.75 (2H, s, NH_2), 6.74 (1H, m), 7.00 (1H, d, *J* 6.4), 7.17 (1H, d, *J* 8.2), 7.29 (1H, t, *J* 6.7), 7.44 (2H, m), 8.01 (2H, m), 8.16 (2H, d, *J* 8.2), 8.30 (2H, d, *J* 7.9), 11.26 (1H, s, NH) and 14.74 (1H, s, NH); δ_C (62.9 MHz; [D_6]DMSO) 113.1, 116.2, 116.6, 118.8, 123.3, 124.4, 125.8, 127.1, 129.6, 134.9, 139.9, 144.8 and 156.2; *m*/*z* (CI) 286 (M^+ + H, 100%).

9-(2-Amino-4,5-dimethylanilino)acridine hydrochloride, 8. Orange powder (1.29 g, 74%), mp >250 °C decomp. (from ethanol–ether) (Found: C, 69.5; H, 5.8; N, 11.3. $C_{21}H_{20}N_3Cl$ requires C, 72.1; H, 5.7; N, 12.1%); λ_{max}(EtOH)/nm 294 (log ε 4.0), 412 (4.1) and 433 (4.1); ν_{max}(KBr)/cm^{-1} 3402m, 3317m, 3209m, 2964m, 2870m, 1637vs, 1586s and 1557s; δ_H (250 MHz; [D_6]DMSO) 2.05 (3H, s, Me), 2.21 (3H, s, Me), 4.48 (1H, br s, NH), 5.42 (1H, br s, NH), 6.75 (1H, s), 6.88 (1H, s), 7.37 (2H, dd, *J* 7.9 and 7.6), 7.92 (2H, dd, *J* 7.9 and 7.6), 8.11 (2H, d, *J* 8.2), 8.33 (2H, d, *J* 7.9), 11.18 (1H, br s, NH), 14.76 (1H, br s, NH); δ_C (62.9 MHz; [D_6]DMSO) 18.3, 19.5, 113.1, 117.4, 118.9, 122.2, 123.3, 124.5, 125.8, 127.2, 134.9, 137.5, 139.9, 142.1 and 156.0; *m*/*z* (Found: M^+ + H, 314.1660. $C_{21}H_{20}N_3$ requires 314.1657).

9-(2-Amino-5-ethoxycarbonylanilino)acridine hydrochloride, 9. Dark brown powder (1.42 g, 72%), mp >250 °C decomp. (from ethanol–ether) (Found: C, 66.1; H, 5.3; N, 9.7. $C_{22}H_{20}ClN_3O_2$ requires C, 67.1; H, 5.1; N, 10.7%); λ_{max}(EtOH)/nm 293 (log ε 4.0), 414 (4.1) and 436 (4.1); ν_{max}(KBr)/cm^{-1} 3429m, 3329m, 3195m, 2975m, 2892m, 2796m, 1686vs, 1638s, 1599s, 1585s and 1555s; δ_H (250 MHz; [D_6]DMSO) 1.22 (3H, t, *J* 7.0), 4.17 (2H, q, *J* 7.0), 6.52 (2H, br s, NH_2), 6.94 (1H, d, *J* 8.6), 7.40 (2H, t, *J* 7.0), 7.73 (1H, d, *J* 2.1), 7.80 (1H, dd, *J* 8.6 and 1.8), 7.95 (2H, t, *J* 7.0), 8.12 (2H, d, *J* 8.6), 8.29 (2H, d, *J* 8.8), 11.24 (1H, br s, NH), 14.80 (1H, br s, NH); δ_C (62.9 MHz; [D_6]DMSO) 14.3, 60.0, 113.1, 114.8, 116.8, 118.8, 123.3, 123.5, 125.5, 129.0, 131.0, 135.0, 139.8, 149.4, 156.2 and 165.2; *m*/*z* (Found: M^+ + H, 358.1551. $C_{22}H_{20}N_3O_2$ requires 358.1555).

9-(2-Aminoanilino)-2-chloro-6-methoxyacridine hydrochloride, 10. Yellow powder (1.76 g, 91%), mp >250 °C decomp. (from ethanol–ether); λ_{max}(EtOH)/nm 295 (log ε 4.1), 329 (3.4),

344 (3.5), 430 (4.0) and 448 (4.0); ν_{max}(KBr)/cm^{-1} 3415m, 3348m, 3230m, 2780m, 1632vs, 1583s and 1559s; δ_H (250 MHz; [D$_6$]DMSO) 3.65 (3H, s, OMe), 6.66 (1H, dd, *J* 7.9 and 7.0), 6.94 (1H, d, *J* 7.9), 7.11 (1H, d, *J* 7.0), 7.21 (1H, dd, *J* 7.9 and 7.0), 7.38 (1H, d, *J* 9.5), 7.68 (1H, d, *J* 9.2), 7.70 (1H, s), 8.00 (1H, d, *J* 9.2), 8.10 (1H, s) and 8.19 (1H, d, *J* 9.5); δ_C (62.9 MHz; [D$_6$]DMSO) 55.7, 103.8, 111.4, 114.3, 116.0, 116.6, 117.4, 120.5, 123.6, 124.2, 127.3, 127.3, 127.9, 129.6, 135.5, 138.7, 139.5, 144.8, 154.4 and 155.3; *m*/*z* (Found: M$^+$ + H, 350.1059. $C_{20}H_{17}ClN_3O$ requires 350.1060).

9-(2-Amino-5-ethoxycarbonylanilino)-2-chloro-6-methoxyacridine, 11. Yellow powder (1.9 g, 84%), mp >250 °C decomp. (from ethanol–ether); λ_{max}(EtOH)/nm 291 (log ε 4.1), 344 (3.4) and 428 (4.0); ν_{max}(KBr)/cm^{-1} 3369m, 3296m, 3201m, 3173m, 1705vs, 1629s, 1606s, 1581s and 1547s; δ_H (250 MHz; [D$_6$]-DMSO) 1.23 (3H, t, *J* 7.3, Et), 3.70 (3H, s, OMe), 4.18 (2H, q, *J* 7.3, Et), 6.49 (1H, d, *J* 8.6), 7.90 (4H, m), 8.02 (1H, d, *J* 8.6) and 8.10 (3H, m); δ_C (62.9 MHz; [D$_6$]DMSO) 14.3, 55.7, 60.1, 103.6, 111.4, 114.5, 114.8, 116.9, 117.5, 120.6, 123.3, 123.9, 127.3, 127.9, 129.1, 130.9, 135.5, 138.8, 139.6, 149.4, 154.5, 155.5 and 165.2; *m*/*z* (Found: M$^+$ + H, 422.1280. $C_{23}H_{21}ClN_3O_3$ requires 422.1271).

9-(3,4-Diaminophenyl)acridine (DAA), 12. Acridine was dissolved in ethanol and anhydrous HCl passed through the solution for 5 min. Most of the solvent was removed under vacuum and the remainder of the solution left to stand at 4 °C. The hydrochloride precipitated as pale brown crystals. The hydrochloride (5.7 g, 26 mmol) and *o*-phenylenediamine (5.7 g, 53 mmol) were placed in a flask with sulfur (2.5 g, 78 mmol) and the mixture of solids slowly heated to 130 °C with vigorous stirring. The mixture turned black and formed a tar with stirring no longer possible. H_2S was given off. Heating was continued for 1 h by which time the mixture had thinned and stirring was again possible. The melt was cooled and washed with ether (2 × 50 ml). The black solid was extracted with 10% HCl (3 × 100 ml) to give a dark brown solution. The solution was basified with NH_3 (aq) and a brown solid precipitated. The solid is filtered and dried under vacuum at 40 °C to give yellow brown crystals (4.8 g, 65%), mp >260 °C (from xylene) (lit.[20] 279–281 °C) (Found: C, 79.8; H, 5.3; N, 14.7. $C_{19}H_{15}N_3$ requires C, 80.0; H, 5.3; N, 14.7%); λ_{max}(EtOH)/nm 332 (log ε 3.7), 358 (3.8); ν_{max}(KBr)/cm^{-1} 3419s, 3357s, 1618vs, 1577m, 1515vs; δ_H (250 MHz; [D$_6$]DMSO) 4.78 (4H, s, NH_2), 6.51 (1H, dd, *J* 7.6 and 2.1), 6.65 (1H, d, *J* 2.1), 6.78 (1H, d, *J* 7.6), 7.50 (2H, ddd, *J* 8.5, 7.6 and 1.2), 7.80 (1H, dd, *J* 6.4 and 1.2), 7.82 (1H, dd, *J* 6.4 and 1.2), 7.89 (2H, d, *J* 8.5) and 8.16 (2H, d, *J* 8.5); δ_C (62.9 MHz; [D$_6$]DMSO) 114.1, 116.5, 119.8, 123.2, 124.9, 125.4, 127.3, 129.2, 130.0, 134.8, 135.5, 148.4 and 148.7; *m*/*z* 286 (M$^+$ + H, 100%).

9-(Benzotriazol-1-yl)methylanthracene, 13. Sodium hydride (0.67 g, 16.7 mmol, 60% suspension in oil) was stirred in light petroleum (2 × 40 ml). The solvent was decanted and dry DMF (50 ml) added. Benzotriazole (2 g, 16.8 mmol) was slowly added with vigorous stirring. Once effervescence had ceased, 9-chloromethylanthracene (0.23 g, 1 mmol) was carefully added. The mixture was stirred overnight. Water was added to the mixture and the product extracted into ether. The product was purified by chromatography on silica gel. Elution with dichloromethane give the *title compound* (0.13 g, 42%) as a yellow powder, mp 156–158 °C (from ethyl acetate–light petroleum) (Found: C, 80.1; H, 5.0; N, 14.0. $C_{21}H_{15}N_3$ requires C, 81.5; H, 4.8; N, 13.6%); λ_{max}(EtOH)/nm 289 (log ε 3.7), 348 (3.7), 366 (4.0) and 386 (3.9); ν_{max}(KBr)/cm^{-1} 3552m, 3472m, 3418m, 1638s, 1617s, 1585s and 1572s; δ_H (250 MHz; [D$_6$]DMSO) 6.80 (1H, d), 6.81 (2H, s, CH_2), 7.03 (1H, t, *J* 8.2), 7.16 (1H, t, *J* 8.2), 7.46–7.60 (4H, m), 7.96 (1H, d, *J* 8.2), 8.04 (2H, d, *J* 7.6), 8.47 (2H, d, *J* 8.2) and 8.54 (1H, s); δ_C (62.9 MHz; $CDCl_3$) 46.0, 110.3, 115.0, 119.9, 123.4, 123.8, 125.3, 126.1, 127.3, 127.5, 129.6, 129.9, 131.1, 131.4 and 146.4; *m*/*z* (CI) 310 (M$^+$ + H, 100%).

4-(Benzotriazol-1-yl)methyl-7-methoxycoumarin, 14. NaH (0.67 g, 16.8 mmol, 60% suspension in oil) was stirred in light petroleum (2 × 40 ml). The ether was decanted and dry DMF was added (50 ml). Benzotriazole (2 g, 16.8 mmol) was slowly added with vigorous stirring. Once effervescence had ceased, 4-bromomethyl-7-methoxycoumarin (0.54 g, 2 mmol) was carefully added. The mixture was stirred overnight. Water was added to the mixture and the product extracted into ether. The ether was dried with Na_2SO_4 and evaporated to a runny liquid. Recrystallisation gave the *title compound* (0.12 g, 20%) as fine needles, mp 212 °C (from ethyl acetate–light petroleum) (Found: C, 66.0; H, 4.2; N, 13.4. $C_{17}H_{13}N_3O_3$ requires C, 66.5; H, 4.2; N, 13.7%); λ_{max}(EtOH)/nm 324 (log ε 4); ν_{max}(KBr)/cm^{-1} 1713vs and 1619vs; δ_H (250 MHz; [D$_6$]DMSO) 3.86 (3H, s, OMe), 5.52 (1H, s, coumarin -CH), 6.33 (2H, s, coumarin -CH_2), 7.04 (2H, m), 7.45 (1H, dd, *J* 8.2 and 7.0), 7.59 (1H, dd, *J* 8.2 and 7.0), 7.91 (2H, m) and 8.11 (1H, d, *J* 8.2); δ_C (62.9 MHz; [D$_6$]DMSO) 47.6, 56.1, 101.1, 109.9, 110.6, 112.4, 119.5, 124.5, 126.2, 128.1, 133.2, 145.2, 150.2, 155.2, 159.7 and 162.8 (one overlapping resonance); *m*/*z* (Found: M$^+$ + H, 308.1035. $C_{17}H_{14}N_3O_3$ requires 308.1035).

Diazotisation reactions

The aminoanilino acridine **7**–**11** (0.8 g, 2.5 mmol) was suspended in 2 M HCl (25 ml) and chilled in an ice bath. A solution of $NaNO_2$ (0.35 ml, 5 mmol) was added dropwise and the mixture stirred for 1 h. The suspension was cooled with ice and made basic with conc. aq. NH_3. At neutralisation it became notably easier to stir. A brown powder was collected by filtration. The wet solid was dissolved in hot DMF and crystallisation occurred upon cooling. A pale yellow powder was collected by filtration. Yield 85–95%.

9-(Benzotriazol-1-yl)acridine, 15. Pale yellow powder (0.67 g, 90%), mp 250 °C decomp. (from DMF–water); lit.[18] 250 °C (Found: C, 76.9; H, 4.0; N, 19.0. $C_{19}H_{12}N_4$ requires C, 77.0; H, 4.0; N, 18.9%); λ_{max}(EtOH)/nm 289 (log ε 3.8), 345 (4.0), 362 (4.1) and 386 (4.0); ν_{max}(KBr)/cm^{-1} 3414m, 3048m, 1676s, 1629s, 1607s and 1554vs; δ_H (250 MHz; $CDCl_3$) 7.07 (1H, d, *J* 6.7), 7.33 (2H, d, *J* 8.8), 7.50 (4H, m), 7.8 (2H, t, *J* 7.9), 8.31 (1H, m) and 8.39 (2H, d, *J* 8.8); δ_C (62.9 MHz; $CDCl_3$) 110.1, 120.5, 122.8, 124.8, 128.0, 128.9, 130.0, 130.9, 135.6, 136.6, 144.0, 145.6 and 149.6; *m*/*z* (CI) 297 (M$^+$ + H, 100%).

9-(5,6-Dimethylbenzotriazol-1-yl)acridine, 16. Pale yellow powder (0.69 g, 85%), mp 233–237 °C (from DMF–water) (Found: C, 77.6; H, 4.8; N, 17.1. $C_{21}H_{16}N_4$ requires C, 77.8; H, 4.9; N, 17.3%); λ_{max}(EtOH)/nm 291 (log ε 3.8), 346 (4.0), 361 (4.1) and 385 (4); ν_{max}(KBr)/cm^{-1} 3548m, 3475m, 3414m, 1618vs, 1557s and 1517s; δ_H (250 MHz; $CDCl_3$) 2.28 (3H, s, Me), 2.46 (3H, s, Me), 6.82 (1H, s), 7.34 (2H, d, *J* 8.5), 7.48 (2H, m), 7.84 (2H, m), 8.02 (1H, s) and 8.37 (2H, d, *J* 8.8); δ_C (62.9 MHz; $CDCl_3$) 20.5, 20.9, 109.4, 119.5, 122.8, 122.9, 127.9, 130.0, 130.8, 134.8, 139.0 and 149.6; *m*/*z* (CI) 325 (M$^+$ + H, 100%).

9-(6-Ethoxycarbonylbenzotriazol-1-yl)acridine, 17. Pale yellow powder (0.81 g, 88%), mp 200–202 °C (from DMF–water) (Found: C, 71.2; H, 4.1; N, 14.9. $C_{22}H_{16}N_4O_2$ requires C, 71.7; H, 4.3; N, 15.2%); λ_{max}(EtOH)/nm 300 (log ε 4.0), 346 (4.1), 362 (4.1) and 386 (4.1); ν_{max}(KBr)/cm^{-1} 3546m, 3478m, 3416m, 3055m, 2993m, 1709vs, 1628vs and 1554s; δ_H (250 MHz; $CDCl_3$) 1.27 (3H, t, *J* 7.3), 4.29 (2H, q, *J* 7.3), 7.25

(2H, d, *J* 8.8), 7.50 (2H, dd, *J* 8.6 and 7.7), 7.77 (1H, s), 7.86 (2H, ddd, *J* 7.9, 6.7 and 1.2), 7.98 (1H, s), 8.18 (1H, dd, *J* 8.8 and 1.2), 8.34 (1H, d, *J* 8.8) and 8.39 (1H, d, *J* 8.8); δ_C (62.9 MHz; $CDCl_3$) 14.2, 61.7, 112.3, 120.4, 122.3, 122.8, 125.6, 128.4, 130.1, 130.9, 131.2, 135.4, 147.5, 149.6 and 165.6; *m*/*z* (Found: $M^+ + H$, 369.1355. $C_{22}H_{17}N_4O_2$ requires 369.1351).

9-(Benzotriazol-1-yl)-2-chloro-6-methoxyacridine, 18. Green–blue powder (0.8 g, 89%), mp 207–209 °C (from DMF–water) (Found: C, 66.3; H, 3.8; N, 15.2. $C_{20}H_{13}ClN_4O$ requires C, 66.6; H, 3.6; N, 15.5%); λ_{max}(EtOH)/nm 290 (log ε 3.8), 338 (3.8), 358 (4.0), 389 (4.0) and 408 (4.0); ν_{max}(KBr)/cm^{-1} 3555m, 3469m, 3412m, 3056m, 1632vs and 1560s; δ_H (250 MHz; $CDCl_3$) 3.63 (3H, s, OMe), 6.39 (1H, d, *J* 2.4), 7.07 (1H, m), 7.22 (1H, t, *J* 9.2), 7.41 (1H, dd, *J* 9.2 and 1.8), 7.52 (3H, m), 8.23 (1H, d, *J* 9.5) and 8.30 (2H, m); δ_C (62.9 MHz; $CDCl_3$) 55.6, 97.7, 110.1, 120.6, 121.4, 123.7, 124.0, 125.0, 127.0, 128.5, 129.0, 129.3, 131.7, 134.4, 135.0, 135.6, 145.7, 147.6, 147.8 and 159.2; *m*/*z* (CI) 361 ($M^+ + H$, 100%).

9-(6-Ethoxycarbonylbenzotriazol-1-yl)-2-chloro-6-methoxyacridine, 19. Pale yellow powder (1.0 g, 93%), mp 228–230 °C (from DMF–water) (Found: C, 63.2; H, 3.9; N, 12.8. $C_{23}H_{17}ClN_4O_3$ requires C, 63.8; H, 3.9; N, 13.0%); λ_{max}(EtOH)/nm 291 (log ε 3.8), 338 (3.8), 354 (4.0), 389 (4.0) and 409 (4.0); ν_{max}(KBr)/cm^{-1} 3408m, 3067m, 3040m, 2991m, 2968m, 2932m, 2906m, 2830m, 1713vs, 1676vs, 1632s and 1559s; δ_H (250 MHz; $CDCl_3$) 1.31 (3H, t, *J* 7.3, Et), 3.62 (3H, s, OMe), 4.33 (2H, q, *J* 7.3, ethyl), 6.30 (1H, d, *J* 2.4, benz), 7.10 (1H, d, *J* 9.2, acr), 7.41 (1H, dd, *J* 9.2 and 2.0, benz), 7.53 (1H, dd, *J* 9.5 and 2.4, benz), 7.78 (1H, s, acr), 8.22 (2H, m, acr) and 8.35 (2H, m, acr); δ_C (62.9 MHz; $CDCl_3$) 14.3, 55.7, 61.8, 112.2, 120.5, 121.5, 123.2, 124.2, 125.7, 127.1, 128.7, 129.6, 131.3, 131.8, 134.9, 135.9, 147.6, 147.8, 151.3 and 159.4; *m*/*z* (Found: $M^+ + H$, 433.1063. $C_{23}H_{18}ClN_4O_3$ requires 433.1067).

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