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Acridones and Quinacridones: Novel Fluorophores for Fluorescence Lifetime Studies

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Two new families of fluorescent probe, acridones and quinacridones, whose fluorescence lifetime can be altered to produce a range of lifetimes from 3 ns to 25 ns are described. Both families of fluorophore have fluorescence lifetimes which are unaffected by pH in the range of 5 to 9 and show a marked resistance to photobleaching. The probes have been modified to allow them to be attached to biomolecules and the labelling of a neuropeptide (substance P) is described. The labelled peptides have the same fluorescence lifetime as the free fluorophore. Quinacridone, with an emission around 550 nm offers a long fluorescence lifetime, photostable alternative to fluorescein.

KEY WORDS: Fluorescence lifetime; spectroscopic characteristics; photostability; labelled biomolecules.

INTRODUCTION

The study of interactions of biological molecules using fluorescence based techniques is well described in the literature [1,2]. Of particular concern is the background fluorescence from the biomolecules themselves which often reduces the sensitivity and limits the detection levels in such systems.

One method of overcoming this problem is to use fluorophores with an emission at the red end of the spectrum. This allows better spectral separation from the predominantly blue background fluorescence which can emanate from the plastics used to fabricate microtitre plates and autofluorescence from the sample. An alternative method is to employ a technique which does not use color to discriminate signal from background. One such technique is the utilisation of a property unique to all fluorescent molecules, fluorescence lifetime. This method has been used by several groups for applications such as DNA sequencing [3–6], determining the ratio of single to double stranded DNA [7] and confocal fluorescence lifetime imaging microscopy (FLIM) [8]. DNA sequencing can be carried out by multiplexing with four fluorophores each having a different fluorescence lifetime. It is desirable that a single light source be used to excite all four fluorophores and ideally, that the fluorophores emit at similar wavelengths. Usually these criteria can only be met by using fluorophores from different families. This can result in marked differences in extinction coefficient, molecular weight, hydrophobicity and charge between fluorophores and can cause problems with differences in mobility during separation steps. It is therefore desirable to have a range of fluorescent lifetimes within the same class of fluorophore with ideally each having a similar absorption and emission spectrum. In an ideal situation the fluorophores should also have similar molecular weights and carry the same, or better still, no charge to avoid mobility differences in separation steps. The background fluorescence produced by most biomolecules is at the blue end of the spectrum and typically has a fluorescence lifetime of less than 5 ns. A survey of the literature indicates that the fluorescence lifetime of a large number of fluorophores currently used to investigate biological systems also have fluorescent lifetimes in the range of 1–5 ns. This range includes such widely used fluorophores as fluorescein and rhodamine [9], coumarins [10], cyanines [11], BODIPY's [12] and their derivatives. Dye families

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with lifetimes greater than 5 ns include some polyaromatic hydrocarbons such as pyrene which has a lifetime of ~80 ns in hydrocarbon solvents. Nau has described 2,3- diazabicyclo[2.2.2]oct-2-ene [13] with a lifetime approaching 1 μs in deoxygenated solvent, the longest lifetime of any known organic compound. Other compounds with long fluorescence lifetimes include transitional and lanthanide metal complexes such as those of ruthenium described by Lakowicz [14] and the terbium and europium chelates described by Selvin [15]. The polyaromatic hydrocarbons have the disadvantage of being insoluble in aqueous systems. Although this can be overcome by adding water solubilising groups such as sulfonates, this causes a significant decrease in the fluorescence lifetime and these compounds tend to emit in the blue region of the spectrum where problems with differentiation from background are encountered. The metal complexes have been used extensively in biological studies, have red shifted emissions and very long lifetimes, ranging from hundreds of ns into the μs range that allow for excellent discrimination from background. However, the fluorescence lifetime of the ruthenium complexes is sensitive to the presence of dissolved oxygen whilst the lifetimes of the lanthanide chelates are shortened by the presence of water. These types of molecule are in general rather bulky and not easy to prepare in a form which allows them to be attached to biomolecules. An extensive search of the literature revealed a group of fluorophores with lifetimes of greater than 10 ns. This group included the acridines, acridones and acridinium betaines, which have a fluorescence emission in the 400–500 nm region. Indeed, *N*-(3-sulfopropyl) acridinium betaine with a fluorescence lifetime of 31.6 ns in water has been used as a standard for calibrating instruments used to measure fluorescence lifetime [16] and acridinium compounds have been used extensively as reporter molecules in biological systems by using their chemiluminescence properties [17–20].

One of the criteria which we required for fluorescent probes is that their fluorescence lifetimes should be independent of pH in the physiological range 5 to 9. Screening of a variety of commercially available acridines, acridinium salts and acridones showed that the first two groups were sensitive to pH changes, however the acridones fulfilled our criteria and additionally have the advantage of being small uncharged molecules.

The chemistry of the acridines and acridones has been reviewed in the literature [21] and acridone derivatives have found diverse usage such as anti-neoplastic agents [22] and anti- malarial drugs [23]. Additionally certain acridones have been used as detection reagents for biomolecules. 2-Aminoacridone, for example, has been used to detect disaccharides [24] and carbohydrates [25], and the NHS ester of acridone-10-pentanoic acid for labelling peptides [26]. Enzyme activity can be monitored using acridone labelled substrates [27] whilst metal ions can also be detected using acridone derivatives [28].

These techniques however, do not rely on fluorescence lifetime as the detection modality but instead utilise a steady state technique, such as changes in fluorescence intensity. Fluorescence lifetime has been used to distinguish oligosaccharides wherein 2-aminoacridone is reacted with a variety of oligosaccharides giving rise to small changes in fluorescence lifetime [29].

The fluorescent properties of both acridone and some of its derivatives, in particular, their fluorescence lifetimes were extensively studied by Shigorin's group in the 1970's [30]. Acridone itself has a fluorescence lifetime of 14 ns in ethanol. Shigorin's group also measured the fluorescence lifetimes of 2-bromo- and 2-iodo-acridone, at 8 and 4 ns respectively. Their studies showed that the reduction in lifetime of the halogenated derivatives was due to an increase in intersystems crossing. This occurs because introduction of a heavy halogen atom such as bromine, iodine or a metal such as tin [31] alters the energy levels so that the $T_1\pi$–π^* state lies just below that of the $S_1\pi$–π^* state [32]. This increases the rate of intersystem crossing resulting in a decreased fluorescence lifetime and a reduction in fluorescence quantum yield.

This group also measured the lifetime of quinacridone [33,34], a molecule with an emission about 100 nm red shifted with respect to acridone and with a fluorescence lifetime of 22 ns. A similar figure was also obtained with alkylated quinacridones [35].

References to quinacridone and its derivatives are relatively rare in the scientific literature, however, there is extensive coverage in the patent literature. The majority of these refer to the use of quinacridone and its derivatives as pigments for use in manufacturing paints and inks. The highly insoluble quinacridones exist in several different crystal modifications which exhibit different colors [36,37]. These derivatives also show extreme thermal, photo and chemical stability.

The insolubility, multiple crystal structure and high stability of quinacridone is mainly due to the intermolecular hydrogen bonding that occurs between the two imino and two carbonyl groups in the molecule. N-alkylation of the imino groups leads to greatly increased solubility in organic solvents, whilst sulfonation leads to increased hydrophilicity. The same criteria also applies to acridones to a lesser degree and they also display high thermal, chemical and photostability [38]. Reymond has used an amino derivative of quinacridone to detect certain transition metal ions in solution [39], the compound showing reduced

fluorescence in the presence of metal ions. To our knowledge, there are no reports of quinacridone labelled biomolecules being used as reporters in biological systems where fluorescence lifetime is used as the detection mode.

Here we report the development of a range of acridone and quinacridone fluorophores that have been chemically modified to give them different fluorescent lifetimes without substantially altering their absorption and emission characteristics. Additionally, these molecules have been derivatised so that they can be chemically bonded to biological molecules under mild conditions which will not destroy biological integrity. We have used these derivatives to chemically label a neuropeptide (substance P) of biological interest. The problem of insolubility of quinacridone derivatives has been overcome by sulfonation, making the derivatives compatible with an aqueous environment.

We herein describe the synthesis of a range of both acridone and quinacridone fluorophores with differing fluorescence lifetimes and describe the derivatisation, activation and labelling of a peptide by these compounds. We have also investigated the pH dependence of lifetime of these compounds in the physicological range of 5 to 9. Finally, we have investigated the photostability of both acridone and quinacridone and compared them to fluorescein, a commonly use fluorophore with similar spectroscopic properties to quinacridone.

The structures of the acridone derivatives synthesised are shown in Table I and those of the quinacridone derivatives in Table II.

EXPERIMENTAL

^{1}H NMR (200 MHz) were measured on a Bruker AC 200. Deuterated solvents were used as indicated in the text. Chemical shifts (δ) are reported in ppm relative to TMS.

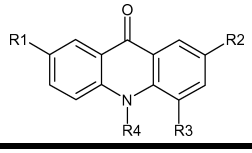
Mass spectra were measured on a Kratos Kompact Maldi Analytical Mass Spectrometer using electrospray in positive mode.

Accurate masses were determined by LC-MS using a Micromass Q-Tof2 with lockspray (Micromass, Manchester, UK), Waters 2790 Liquid chromatography system (Waters, Milford, MA)

A) 0.05% TFA in H_2O
B) 0.05% TFA in acetonitrile.

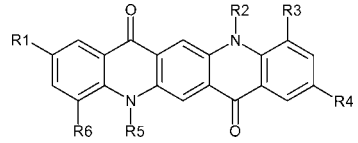
0–100% over 10 min at 300 μL/min. Column: Waters Xterra C18MS 2.1 $\times$ 50 mm, 2.5 μm (Waters, Milford, MA). Lockmass (met)Enkephalin M6638 (Sigma Chemical Co., Poole, Dorset, UK) at 1 μg/mL in H_2O/methanol (1:1) + 0.1% formic acid was used for

Table I. Structures of Acridone Derivatives



Compound	R1	R2	R3	R4
1	H	H	H	$(CH_2)_5CO_2H$
2	H	H	H	CH_2CO_2H
3	Br	H	H	$(CH_2)_5CO_2H$
4	Br	Br	H	$(CH_2)_5CO_2H$
5	I	H	H	$(CH_2)_5CO_2Et$
6	SO_3H	H	H	$(CH_2)_5CO_2H$
7	SO_3H	Br	H	$(CH_2)_5CO_2H$
8	Cl	CH_2CO_2H	H	H
9	H	H	CO_2H	H
10	H	H	$CONH(CH_2)_5CO_2H$	H
11	NO_2	H	H	$(CH_2)_5CO_2Et$
12	NH_2	H	H	$(CH_2)_5CO_2Et$
13	$NHCOCH_3$	H	H	$(CH_2)_5CO_2H$
14	NO_2	NO_2	H	$(CH_2)_5CO_2Et$
15	NH_2	NH_2	H	$(CH_2)_5CO_2Et$
16	$NHCOCH_3$	$NHCOCH_3$	H	$(CH_2)_5CO_2H$
17	F	H	H	$(CH_2)_5CO_2H$
18	CH_3O	H	H	$(CH_2)_5CO_2H$
19	CH_3	H	H	$(CH_2)_5CO_2H$

Table II. Structures of Quinacridone Derivatives



Compound	R1	R2	R3	R4	R5	R6
25	SO_3H	H	SO_3H	SO_3H	H	SO_3H
26	H	$(CH_2)_5CO_2Et$	H	H	$(CH_2)_5CO_2Et$	H
27	I	$(CH_2)_5CO_2Et$	H	H	$(CH_2)_5CO_2Et$	H
28	F	$(CH_2)_5CO_2Et$	H	F	$(CH_2)_5CO_2Et$	H
29	Cl	$(CH_2)_5CO_2Et$	H	Cl	$(CH_2)_5CO_2Et$	H
30	Br	$(CH_2)_5CO_2H$	H	Br	$(CH_2)_5CO_2H$	H
31	CH_3	$(CH_2)_5CO_2Et$	H	CH_3	$(CH_2)_5CO_2Et$	H
32	CH_3O	$(CH_2)_5CO_2Et$	H	CH_3O	$(CH_2)_5CO_2Et$	H
33	NO_2	$(CH_2)_5CO_2Et$	H	NO_2	$(CH_2)_5CO_2Et$	H
34	NH_2	$(CH_2)_5CO_2Et$	H	NH_2	$(CH_2)_5CO_2Et$	H
35	$NHCOCH_3$	$(CH_2)_5CO_2H$	H	$NHCOCH_3$	$(CH_2)_5CO_2H$	H
36	H	C_2H_5	H	H	$(CH_2)_5CO_2Et$	H
37	SO_3H	C_2H_5	H	SO_3H	$(CH_2)_5CO_2H$	H

calibration at 574.2335. Melting points were measured on a Stuart Scientific SMP10 and are uncorrected. Absorption spectra were measured on a Hewlett Pachard 8453 spectrophotometer. Emission spectra were measured on a Perkin Elmer LS 55 fluorimeter.

Peptides were synthesised on an Applied Biosystems 431A peptide synthesiser using FastMoc^{TM} chemistry.

Fluorescence lifetimes were determined by time-correlated single photon counting on an Edinburgh Instruments FL900CDT Time Resolved Fluorimeter using either a pulsed hydrogen arc lamp or a pulsed 405 nm laser. Deconvolution was carried out using a non-linear least squares algorithm.

Materials

Unless otherwise stated, chemicals were obtained from Sigma-Aldrich or Fluka. Anhydrous solvents were purchased stored over molecular sieves and under a nitrogen atmosphere and were used without further purification. Resublimed quinacridone was purchased from Dojindo (Tokyo). Reagents for peptide synthesis were purchased from Applied Biosystems.

SepPak RPC_{18} (10 g) columns were purchased from Waters Ltd. UK.

Thin layer chromatography (TLC) was carried out on either Merck precoated silica gel F_{254} or RP-18 F_{254} plates. Spots were visualised with a 365 nm UV lamp.

HPLC purifications were carried out on semi-preparative reverse phase (C-18) columns, either Phenomenex Jupiter (20 $\times$ 1.0 cm, 7 μm) or a Waters PrepPak (25 mm) column.

Synthesis of Acridones

Synthesis of the Ethyl Ester of 6-(9-Oxo-9H-acridin-10-yl)-hexanoic Acid. 9-oxo-9,10-dihydro-acridine (4.88 g, 25 mM) was stirred with anhydrous dimethyl sulfoxide (20 mL) under a nitrogen atmosphere and then sodium hydride (1.05 g; 60% w/w in oil, 26 mM) was added and the mixture stirred at ambient temperature until effervescence ceased. Ethyl-6-bromohexanoate (4.9 mL, 27.5 mM) was then added and the mixture stirred overnight under a nitrogen atmosphere at ambient temperature when TLC (RP-18, water:methanol,1:9) showed almost complete disappearance of the starting material. The reaction was poured into water (400 mL) and the mixture extracted with ethyl acetate. The organic layer was washed sequentially with water, 1 M hydrochloric acid and dilute sodium hydroxide. The organic layer was dried with anhydrous magnesium sulfate, filtered and the solvent removed by rotary evaporation to leave a yellow solid. This material was purified by flash chromatography (silica, 5–20% ethyl acetate: dichloromethane). The purified material was recrystallised from ethanol to give the ethyl ester of 6-(9-oxo-9H-acridin-10-yl)-hexanoic acid (5.44 gm, 64%) as a pale yellow solid.

^{1}H NMR (CD_3OD) δ 7.96 (dd, $J = 2$, 9Hz, 2H), δ 7.31 (m, 4H), δ 6.85 (t, $J = 5$ Hz, 2H), δ 4.00 (t, $J = 5$ Hz, 2H), δ 3.61 (d, $J = 4$ Hz, 2H), δ 1.88 (t, $J = 5$ Hz, 2H), δ

1.41, 1.23, 1.10 (m, 6H) δ 0.73 (t, $J = 4$ Hz, 3H). $m/z =$ 338.3. $C_{21}H_{23}NO_3$ requires 337.4.

To a 25 mL round bottomed flask was added 170 mg (0.5 mM) of the ethyl ester followed by 40 mg (1 mM) sodium hydroxide, 2 mL water and 5 mL methanol. The mixture was heated with swirling until the solid began to dissolve over a 10 min period to give a yellow solution which showed blue fluorescence. The solution was left to stand for 30 min when TLC (C-18 reverse phase plates, water:methanol,1:9) showed all the starting material had been converted to a faster running spot. The solvent was removed by rotary evaporation and the residue partitioned between dichloromethane (50 mL) and 0.5 M HCl (50 mL). The organic layer was separated and the aqueous phase washed with ethyl acetate (50 mL). The aqueous layer was further washed with 10% v/v methanol:dichloromethane. The combined organic layers were dried with anhydrous magnesium sulfate, filtered and then rotary evaporated to dryness. The residue was re-dissolved in 5% v/v methanol:dichloromethane, filtered and re-evaporated to dryness. The residue was triturated with diethyl ether, the residue filtered off and dried under vacuum to give 150 mg (0.48 mM, 96%) of yellow solid identified as 6-(9-oxo-9H-acridin-10-yl)-hexanoic acid (**1**).

^{1}H NMR (CD_3OD) δ 12.0 (broad s, 1H), δ 8.46 (dd, $J = 4$ Hz, 2H), δ 7.84 (m, 4H), δ 7.35 (t, $J = 5$ Hz, 2H), δ 4.53 (t, $J = 6$ Hz, 2H), δ 2.36 (t, $J = 5.0$ Hz, 2H), δ 1.95, 1.67, 1.17 (m, 6H). $m/z = 310.3$. $C_{19}H_{19}NO_3$ requires 309.4 Melting point = 167°C.

*Synthesis of (9-Oxo-9H-acridin-10-yl)-acetic Acid (**2**).* To an oven dried 25 mL round bottomed flask was added 1.95 g (10 mM) 9-oxo-9,10-dihydro-acridine, sodium hydride (480 mg; 60% w/w dispersed in oil, 12 mM) and 30 mL of anhydrous dimethyl sulfoxide. The mixture was stirred at ambient temperature until effervescence ceased. 2.0 g (12 mM) ethyl bromoacetate was added and stirring continued for 24 hr under a nitrogen atmosphere when TLC (silica, dichloromethane:methanol, 9:1) showed all the starting material had reacted.

Water (150 mL) was added to the reaction mixture and the yellow precipitate filtered off and dried under vacuum. The crude material was purified by flash chromatography (silica, dichloromethane (100%)) to give the ethyl ester of (9-oxo-9H-acridin-10-yl)-acetic acid (1.3 g, 4.6 mM). $m/z = 282$. $M^+ = 281$ for $C_{17}H_{15}NO_3$.

This material was hydrolysed by stirring overnight with 5.5 mL of 1 M hydrochloric acid in 10 mL of glacial acetic acid at 110°C under a nitrogen atmosphere. TLC (RP-18, water:methanol, 2:8) showed that all the starting material had reacted. The solvent was removed by rotary evaporation, the residue dissolved in acetonitrile and again evaporated to dryness to give (9-oxo-9H-acridin-10-yl)-acetic acid (**2**) (0.83 g, 3.2 mM, 92%) as a yellow solid. Accurate mass (M + H) $C_{15}H_{12}NO_3$ requires 254.0817. Found 254.0806 (4.4 ppm). Melting point = 280°C (Lit 280–282°C) [40].

*Synthesis of 6-(2-Bromo-9-oxo-9H-acridin-10-yl)-hexanoic Acid and 6-(2,7-Bromo-9-oxo-9H-acridin-10-yl)-hexanoic Acid (**3** and **4**).* To an oven dried 25 mL round bottomed flask was added 170 mg (0.5 mM) 6-(9-oxo-9H-acridin-10- yl)-hexanoic acid and ethanol (6 mL). The flask was flushed with nitrogen and the mixture stirred until all the solid had dissolved. Benzyltrimethylammonium tribromide (390 mg, 1.0 mM) was added and the mixture stirred at ambient temperature in the dark for 50 hr. TLC (RP-18, water:ethanol, 1:9) showed partial conversion to a slower running component. The solvent was removed from the reaction mixture by rotary evaporation and the residue was partitioned between water and ethyl acetate. The organic layer was washed with dilute sodium thiosulfate solution, then saturated sodium chloride solution and dried with anhydrous magnesium sulfate. After filtration, the solvent was removed by rotary evaporation to give a yellow oil, which was further dried under high vacuum.

The partially crystallised oil was triturated with 5 mL petrol ether (40–60):diethyl ether, 9:1 to give a yellow solid. The solid was filtered off, washed with more solvent and dried under high vacuum in the presence of phosphorus pentoxide. The material was then purified by flash chromatography on a silica column, eluting with ethyl acetate:petrol ether (40–60), 30:70). The combined eluates were rotary evaporated to dryness and the residue dried under vacuum and over phosphorus pentoxide to give the ethyl ester of 6-(2-bromo-9-oxo-9H-acridin-10-yl)-hexanoic acid as a yellow solid (77%). ^{1}H NMR (CD_3OD) δ 8.51 (s, 1H), δ 8.42 (d, $J = 8$ Hz, 1H), δ 7.83 (m, 4H), δ 7.36 (t, $J = 7$ Hz, 1H), δ 4.49 (t, $J = 8$ Hz, 2H), δ 4.11 (ab $J = 7$ Hz, 2H), δ 2.37 (t, $J = 7$ Hz, 2H), δ 1.91, 1.74, 1.58 (m, 6H), δ 1.22 (t, $J = 7$ Hz, 3H).

$m/z = 416.3$, 418.3. $C_{21}H_{22}BrNO_3$ requires 416.3 and the ethyl ester of 6-(2,7-dibromo-9-oxo-9H-acridin-10-yl)-hexanoic acid as a yellow solid (8%). ^{1}H NMR (CD_3OD) δ 8.34 (d, $J = 2$ Hz, 2H), δ 7.93 (ab, $J = 9$ Hz, 2H), δ 7.81 (d, 2H), δ 4.41 (broad t, $J = 7$ Hz, 2H), δ 4.03 (ab, $J = 7$ Hz, 2H), δ 2.31 (t, $J = 7$ Hz, 2H), δ 1.70, 1.54 (t, 6H), δ 1.15 (t, $J = 7$ Hz, 3H).

Hydrolysis of the Ethyl Ester of 6-(2-Bromo-9-oxo-9H-acridin-10-yl)-hexanoic Acid. 170 mg (0.5 mM) of the ethyl ester followed by 40 μl (1 mM) sodium hydroxide, 2 mL water and 5 mL methanol were placed in a round bottomed flask. The reaction was carried out as described above to give 150 mg (0.48 mM, 96%) of yellow solid identified as 6-(2-bromo-9-oxo-9H-acridin-10-yl)-hexanoic acid (**3**).

^{1}H NMR (d_6-DMSO) δ 12.5 (broad s, 1H), δ 8.37 (m, 2H), δ 7.92 (m, 4H), δ 7.35 (m, 1H), δ 4.45 (broad t, $J = 8$ Hz, 2H), δ 2.76 (t, $J = 7$, 2H), δ 1.76 (m, 2H), δ 1.59 (m, 4H). Accurate mass (M + H) $C_{19}H_{19}NO_3Br$ requires 388.0548. Found 388.0540 (2.1 ppm). Melting point = 213°C.

Hydrolysis of the Ethyl Ester of 6-(2,7-Dibromo-9-oxo-9H-acridin-10-yl)-hexanoic Acid. This was carried out as described for in the section Hydrolysis of the Ethyl Ester of 6-(2-Bromo-g-oxo-9H-acridin-10-yl)-hexanoic Acid to give 6-(2,7-dibromo-9-oxo-9H-acridin-10-yl)- hexanoic acid (**4**) as a yellow solid.

^{1}H NMR (d_6-DMSO) δ 12.0 (1H, s), δ 8.31 (d, $J =$ 2 Hz, 2H), δ 7.84 (ab, $J = 9$ Hz, 4H), δ 7.88 (m, 1H), δ 4.40 (t, $J = 7$ Hz, 2H), δ 2.25 (t, $J = 7$ Hz, 2H), δ 1.70 (m, 2H), δ 1.57 (m, 4H). Accurate mass (M + H) $C_{19}H_{18}NO_3Br_2$ requires 465.9653. Found 465.9674 (4.4 ppm). Melting point = 198°C.

*Synthesis of the Ethyl Ester of 6-(2-Iodo-9-oxo-9H-acridin-10-yl)-hexanoic Acid (**5**).* The ethyl ester of 6-(9-oxo-9H-acridin-10-yl)-hexanoic acid (170 mg, 0.5 mmol) was dissolved in glacial acetic acid (5 mL). Benzyltrimethylammonium dichloroiodide (350 mg, 1.0 mmol) and anhydrous zinc chloride (150 mg, 1.1 mmol) were added, the flask was flushed with nitrogen and the mixture stirred at 60°C overnight. TLC (RP-18, methanol:chloroform, 2:8) showed partial conversion to a slower running compound. The solvent was removed by rotary evaporation, the residue dissolved in ethyl acetate, washed with sodium thiosulfate solution, dried over anhydrous magnesium sulfate and the solvent removed by rotary evaporation. The crude product was purified by flash chromatography (silica, ethyl acetate: petrol ether (40–60), 30:70) to give 95 mg (0.20 mmol, 40%) of the ethyl ester of 6-(2-iodo-9-oxo-9H-acridin-10-yl)-hexanoic acid as a yellow solid (**5**).

^{1}H NMR (CD_3OD) δ 8.69 (s, 1H), δ 8.40 (dd, $J =$ 1, 4.2 Hz, 1H), δ 8.02 (dd, $J = 2$, 4 Hz, 1H), δ 7.83 (dd, $J = 1$, 3 Hz, 1H), δ 7.75 (d, $J = 6$ Hz, 1H), δ 7.55 (d, $J =$ 6 Hz, 1H), δ 7.34 (t, $J = 5$ Hz, 1H), δ 4.445 (t, $J = 5$ Hz, 2H), δ 4.10 (ab, $J = 5$ Hz, 2H), δ 2.36 (t, $J = 5$ Hz, 2H), δ 1.87, 1.72, 1.57 (m, 6H), δ 1.22 (t, $J = 5$ Hz, 3H). $m/z =$ 464.28. $C_{21}H_{22}INO_3$ requires 463.32.

*Synthesis of 6-(2-Sulfo-9-oxo-9H-acridin-10-yl)-hexanoic Acid (**6**).* *O*-ethyl-6-(9-oxo-9H-acridin-10-yl)-hexanoate (2.0 g; 6.0 mmol) was dissolved in 98% sulfuric acid (10 mL) and the solution heated to 120°C for 20 hr. The mixture was allowed to cool and added to ~50 gm of crushed ice. The precipitate was collected by centrifugation, washed with 3.0 M hydrochloric acid and dried under vacuum and over phosphorous pentoxide to give 2.1 g (90%) of 6-(2-sulfo-9-oxo-9H-acridin-10-yl)-hexanoic acid (**6**).

^{1}H NMR (DMSO-d_6) δ 12.0 (s, 1H), δ 8.58 (d, $J =$ 2 Hz, 1H), δ 8.37(d, $J = 8$ Hz, 1H), δ 7.9 (m, 4H), δ 7.37 (m, 1H), δ 4.5 (broad t, $J = 8$ Hz, 2H), δ 2.26 (t, $J =$ 7 Hz, 2H), δ 1.7(m, 6H).

$m/z = 390.118$. $C_{19}H_{19}NO_6S$ requires 389.42. Melting point = 269°C decomposes.

*Synthesis of 6-(2-Bromo-7-sulfo-9-oxo-9H-acridin-10-yl)-hexanoic Acid (**7**).* *O*-ethyl-6-(2-bromo-9-oxo-9H-acridin-10-yl)-hexanoate (2.08 g; 5.0 mmol) was dissolved in 98% sulfuric acid (10 mL) and heated to 120°C for 20 hr. The mixture was allowed to cool and added to ~50 gm of crushed ice. The precipitate was collected by centrifugation, washed with 3.0 M hydrochloric acid and dried under vacuum and over phosphorous pentoxide to give 2.2 g (94%) of 6-(2-bromo-7-sulfo-9-oxo-9H-acridin-10-yl)-hexanoic acid (**7**).

^{1}H NMR (DMSO-d_6) δ 12.0 (s, 1H), δ 8.57 (d, $J =$ 2 Hz, 1H), δ 8.42 (d, $J = 2$ Hz, 1H), δ 7.9 (m, 4H), δ 4.5 (m, 2H), δ 2.25 (t, $J = 6$ Hz, 2H), δ 1.7 (m, 6H). $m/z = 468, 470$. $C_{19}H_{18}BrNO_6S$ requires 468.33. Melting point = 275°C decomposes.

*Synthesis of 2-Carboxymethyl-7-chloro-9-oxo-9,10-dihydroacridine (**8**).* To a 100 mL round bottomed flask was added 1.9 g (10 mmol) 2,5-dichlorobenzoic acid, 1.5 g (10 mmol) 4-aminophenylacetic acid, 3.2 gm (26 mmol) anhydrous sodium carbonate, 0.25 g (4 mmol) powdered copper and 50 mL 1-butanol. The flask was fitted with a magnetic stirrer bar, water condenser, silica gel guard tube and heated to reflux for 48 hr. TLC (RP-18, water:methanol, 2:8) showed the formation of a slower moving component. The solvent was removed under reduced pressure with final drying under high vacuum. The residue was dissolved in 50 mL water and heated to reflux, then charcoal was added and the mixture

filtered through celite, washing through with a further 25 mL of hot water. This solution was cooled to 10°C in an ice bath and then acidified to pH 2 with conc. HCl. The resultant oil was extracted into chloroform, the solution dried with anhydrous magnesium sulfate, filtered and the solvent removed by rotary evaporation to leave a gum of *N*-(4-carboxymethylphenyl) (4-chloro-2-carboxy)aniline. $m/z = 304.34, 306.35$. $C_{15}H_{12}ClNO_4$ requires 307.5. λ_{max} (EtOH) 292, 364 nm.

Diphenylamine 500 mg (1.64 mmol) and 5 mL of phosphorus oxychloride was added to a 25 mL round bottomed flask fitted with a magnetic stirrer bar, water condenser, silica gel guard tube and heated to reflux for 1 hr. The excess phosphorus oxychloride was removed from the dark brown mixture under vacuum, then a small amount of ice was added followed by 10 mL 2 M HCl. The mixture was heated to 100°C for 1 hr and allowed to cool. The mixture was evaporated to dryness and dried under vacuum over phosphorus pentoxide. The residue was dissolved in 10% v/v water:methanol and eluted through a column containing 10 g SepPak RPC_{18} with monitoring by TLC. The fluorescent containing fractions were combined, rotary evaporated to dryness and dried under vacuum over phosphorus pentoxide.

This crude material was then purified by reverse phase HPLC (Waters PrepPak 25 mm) eluting in a gradient of 10% v/v methanol:water to 100% methanol over 50 min at 10 mL/min. Detection was at 410 nm. The major component eluted after 38 min.

After removal of solvent by rotary evaporation and drying under vacuum, the combined eluate yielded 19 mg (0.07 mmol) of 2-carboxymethyl-7-chloro-9-oxo-9,10-dihydroacridine (**8**) as a pale yellow solid.

^{1}H NMR (d_6-DMSO) δ 12.0 (s, 1H), δ 8.14 (d, $J =$ 9 Hz, 2H), δ 7.75(d, $J = 3$ Hz, 1H), δ 7.65(d, $J = 5$ Hz, 1H), δ 7.58(d, $J = 5$ Hz, 1H), δ 7.50 (d, $J = 5$ Hz, 1H), δ 2.02 (s, 2H). $m/z = 286.28, 288.30$. $C_{15}H_{10}ClNO_3$ requires 287.7.

*Synthesis of 9-Oxo-9,10-dihydroacridine-4-carboxylic Acid (**9**).* To a 50 mL round bottomed flask was added 2.6 g (10 mmol) 2,2′-imidodibenzoic acid and 10 mL phosphorus oxychloride. The flask was fitted with a magnetic stirrer bar, water condenser, silica gel guard tube and heated to reflux for 2 hr. The excess phosphorus oxychloride was removed under vacuum leaving an orange-yellow residue which was cooled in an ice bath and then 10 g ice added followed by 10 mL 2 M HCl. The mixture was refluxed for 2 hr. After cooling the mixture was diluted with water and the pale yellow solid filtered off. This was washed with water, 10 mL acetone and finally dried over phosphorus pentoxide under vacuum to give 2.26 g (9.5 mmol, 95%) of 9-oxo-9,10-dihydroacridine-4-carboxylic acid (**9**) as a pale yellow solid.

^{1}H NMR (d_6-DMSO) δ 11.9 (s, 1H), δ 8.49 (dd, $J =$ 1, 4 Hz, 1H), δ 8.46 (dd, $J = 1, 4$ Hz, 1H), δ 8.17 (d, $J =$ 4 Hz, 1H), δ 7.73 (m, 2H), δ 7.3 (m 2H). $m/z = 240.14$. $C_{14}H_9NO_3$ requires 239.23. Melting point >300°C.

*Synthesis of 9-Oxo-9,10-dihydroacridine-4-carboxylic Acid (6-Carboxyhexyl)amide (**10**).* 9-oxo-9,10-dihydroacridine-4-carboxylic acid (120 mg, 0.5 mmol), *N*-hydroxybenzotriazole (77 mg, 0.5 mmol), 2-(1H-benzotriazole-1-yl)-1,3,3-tetramethyluronium hexafluorophosphate (190 mg, 0.5 mmol), diisopropylethylamine (100 μL), a catalytic amount of dimethylaminopyridine and dimethylformamide (5 mL) were stirred at ambient temperature. A precipitate formed after 10 min. Stirring was continued for a further 2 hr, then 6-aminohexanoic acid (66 mg, 0.5 mmol) was added. After about 5 min the precipitate disappeared giving a clear, yellow solution. Stirring was continued for a further 15 hr. The solvent was removed by rotary evaporation and the solid residue was taken up as a suspension in 25 mL of 0.5 M HCl. This solution was extracted with 3×50 mL ethyl acetate. The organic layer was dried with anhydrous magnesium sulfate, filtered and the solvent removed by rotary evaporation. The residue was crystallised from DMF/water, the yellow crystals filtered off and recrystallised from ethanol:water. After filtration, the crystals were dried in vacuo to give 53 mg (0.15 mmol, 30%) of 9-oxo-9,10-dihydroacridine-4-carboxylic acid (6-carboxyhexyl)amide (**10**).

This ran as a single spot on TLC (RP-18, water: methanol, 1:9).

^{1}H NMR (d_6-DMSO) δ 12.49 (s, 1H), δ 12.02 (s, 1H), δ 9.00 (broad t, 1H), δ 8.43 (d, $J = 8$ Hz, 1H), δ 8.25 (m, 2H), δ 7.74 (d, $J = 4$ Hz, 2H), δ 7.33 (m, 2H), δ 3.40 (m, 2H), δ 2.24 (t, $J = 7$ Hz, 2H), δ 1.50 (m, 6H). Accurate mass (M + H) $C_{20}H_{21}N2O_4$ requires 353.1501. Found 353.1495 (1.8 ppm). Melting point = 218°C.

*Synthesis of 6-(2-Acetamido-9-oxo-9H-acridin-10-yl)-hexanoic Acid (**13**).* 2-Nitroacridone (2.4 g, 10 mmol) was stirred with anhydrous dimethyl sulfoxide (25 mL) under a nitrogen atmosphere. After 5 min, sodium hydride (60% dispersed in oil, 480 mg, 12 mmol) was added to the yellow solution. Stirring was continued for 90 mins during which time the solution turned magenta. Ethyl 6-bromohexanoate (2.67 mL, 12 mmol) was added and stirring continued overnight. The reaction mixture was

poured into water (300 mL) and the yellow precipitate was collected by filtration, washed with water and dried under vacuum. The solid was dissolved in dichloromethane and anhydrous magnesium sulfate added to the solution. After filtration the solution was evaporated to dryness to leave a yellow-brown solid. The crude product was purified by flash chromatography (silica, 0–5% ethyl acetate:dichloromethane) to give 1.19 g (50%) of *O*-ethyl-6-(2-nitro-9-oxo-9H-acridin-10-yl)-hexanoate (**11**).

^{1}H NMR (d_6-DMSO) δ 9.03 (d, $J = 3$ Hz, 1H), δ 8.50 (dd, $J = 3$, 6 Hz, 1H), δ 8.35 (d, $J = 6$ Hz, 1H), δ 8.03 (d, $J = 6$ Hz, 1H), δ 7.92 (d, $J = 4$ Hz, 2H), δ 7.45 (m, 1H), δ 4.55(broad t, 2H), δ 4.05 (ab, $J = 6$ Hz, 2H), δ 2.3 (t, $J = 7$ Hz, 2H), δ 1.7 (m, 6H), δ 1.2 (t, $J = 8$ Hz, 3H). Melting point = 184°C.

O-ethyl-6-(2-nitro-9-oxo-9H-acridin-10-yl)-hexanoate (1.91 g, 5.0 mmol) and ammonium formate (1.58 g, 25 mmol) were dissolved in ethanol (100 mL) to give a yellow solution. The solution was stirred under nitrogen and a catalytic amount of 5% palladium on carbon was added. Stirring was continued for 5 hr. The solution was then filtered through celite and the solvent removed by rotary evaporation. The residue was dissolved in dichloromethane and extracted with water. The organic layer was dried with anhydrous magnesium sulfate, filtered and the solvent removed by rotary evaporation. The crude product was purified by flash chromatography (silica. 4–6% methanol:dichloromethane) to give 1.66 g (94%) of *O*-ethyl-6-(2-amino-9-oxo-9H-acridin-10-yl)-hexanoate (**12**).

^{1}H NMR (d_6-DMSO) δ 8.3 (d, $J = 8$ Hz, 1H), δ 7.6 (m, 4H), δ 7.2 (m, 2H), δ 5.3 (s, 2H), δ 4.4 (broad t, $J =$ 7 Hz, 2H), δ 4.05 (ab, $J = 7$ Hz, 2H), δ 2.35(t, $J = 7$ Hz, 2H), δ 1.6(m, 6H), δ 1.15 (t, $J = 7$ Hz, 3H).

O-ethyl-6-(2-amino-9-oxo-9H-acridin-10-yl)-hexanoate (350 mg, 1.0 mmol) was dissolved in acetic acid (5 mL) and 1.0 M hydrochloric acid (2 mL) and refluxed for 4 hr. The solvent was removed by rotary evaporation, the residue dissolved in acetic acid and evaporated to dryness and the process repeated twice using acetonitrile as solvent. The residue was dried under vacuum to give 370 mg of 6-(2-amino-9-oxo-9H-acridin-10-yl)-hexanoic acid. This was dissolved in anhydrous pyridine (10 mL) and acetic anhydride (100 μL) followed by diisopropylethylamine (350 μL). The mixture was stirred for 3 hr. The solution was evaporated to dryness under vacuum and the gummy residue dissolved in dichloromethane. This was washed with 1.0 M hydrochloric acid and then saturated sodium chloride solution. The organic phase was dried with anhydrous magnesium sulfate, filtered and the solvent removed by rotary evaporation to leave a sticky solid. Trituration with ether gave a solid which was dried under vacuum to give 360 mg (86%) of 6-(2-acetamido-9-oxo-9H-acridin-10-yl)-hexanoic acid (**13**).

Accurate mass (M + H) $C_{23}H_{27}N_20_4$ requires 395.1971. Found 395.1936 (8.8 ppm). This material was converted to the *N*-hydroxysuccinimide ester (section Synthesis of *O*-(*N*-Succinimidyl)-6-(2-acetamido-9-oxo-9H-acridin-10-yl)-hexanoate (**21**)).

*Synthesis of 6-(2,7-Bis-acetamido-9-oxo-9H-acridin-10-yl)-hexanoic Acid (**16**).* The ethyl ester of 6-(9-oxo-9H-acridin-10-yl)-hexanoic acid (337 mg, 1.0 mmol) was dissolved in 7 mL of ice-cold 98% sulfuric acid to give a pale yellow solution. 69% nitric acid (137 μL, mmol) was added and the mixture stirred under a nitrogen atmosphere for 1 hr at 0°C. The reaction mixture was poured onto ice and the yellow precipitate collected by filtration. The solid was dissolved in dichloromethane, washed with sodium bicarbonate solution, and the organic layer dried over anhydrous magnesium sulfate. The solvent was removed by rotary evaporation to give the ethyl ester of 6-(2,7-dinitro-9-oxo-9H-acridin-10-yl)-hexanoic acid (**14**).

^{1}H NMR (d_6-DMSO) δ 8.91 (d, $J = 3$ Hz, 2H), δ 8.53 (dd, $J = 3$, 6 Hz, 2H), δ 8.11 (d, $J = 7$ Hz, 2H), δ 4.55 (broad t, $J = 8$ Hz, 2H), δ 4.04 (ab, $J = 6$ Hz, 2H), δ 2.34 (t, $J = 6$ Hz, 2H), δ 1.82 (m, 2H), δ 1.62 (m, 4H), δ 1.17 (t, $J = 7$ Hz, 3H). $m/z = 428.15$. $C_{21}H_{21}N_3O_7$ requires 427.42. Melting point = 216°C.

The ethyl ester of 6-(2,7-dinitro-9-oxo-9H-acridin-10-yl)-hexanoic acid (420 mg, 1.0 mmol) was dissolved in 25 mL of ethanol and ammonium acetate (630 mg, 8.2 mmol) was added and the mixture stirred under a nitrogen atmosphere. A catalytic amount of palladium on charcoal (5% w/w) was added and the mixture stirred under a nitrogen atmosphere at ambient temperature for 12 hr. The reaction mixture was filtered through celite and the filtrate evaporated to dryness. The yellow residue was dissolved in dichloromethane and washed with water. The organic layer was dried over anhydrous magnesium sulfate and the solvent removed by rotary evaporation. The crude product was purified by flash chromatography (silica, 5–10% methanol:dichloromethane) to give the ethyl ester of 6-(2,7-diamino-9-oxo-9H-acridin-10-yl)-hexanoic (**15**) acid as an orange solid (300 mg, 80%).

^{1}H NMR (d_6-DMSO) δ 7.46 (dd, $J = 3$, 5 Hz, 4H), δ 7.12 (dd, $J = 2$, 6 Hz, 2H), δ 4.33 (broad t, 2H), δ 4.04 (ab, $J = 7$ Hz, 2H), δ 2.31 (t, $J = 7$ Hz, 2H), δ 1.62 (m, 6H), δ 1.17 (t, $J = 7$ Hz, 3H). Accurate mass (M + H) $C_{21}H_{26}N_3O_3$ requires 368.1974. Found 368.19964 (5.9 ppm). Melting point = 145°C decomposes.

This material was dissolved in glacial acetic acid (5 mL) and 1.0 M hydrochloric acid (3 mL) and the mixture refluxed for 3 hr when TLC (RP-18, water:methanol, 1:9) showed that all the starting material had reacted. After filtration through celite, the solvent was removed by rotary evaporation to give a yellow solid. This was dissolved in glacial acetic acid, evaporated to dryness, then dissolved in acetonitrile and evaporated to dryness to give 6-(2,7-diamino-9-oxo-9H-acridin-10-yl)-hexanoic acid as an orange solid.

6-(2,7-diamino-9-oxo-9H-acridin-10-yl)-hexanoic acid (350 mg, 1.0 mmol) was dissolved in anhydrous pyridine and acetic anhydride (200 μL) added and the mixture stirred at ambient temperature for 3 hr when TLC (RP-18, water:methanol, 2:8) showed all the starting material had reacted. The solvent was removed by rotary evaporation to give a brown oil which on trituration with acetonitrile and diethyl ether gave a yellow solid identified as 6-(2,7-bis-acetamido-9-oxo-9H-acridin-10-yl)-hexanoic acid (**16**).

^{1}H NMR (d_6-DMSO) δ 12.01 (s, 1H) δ 10.164 (s, 2H), δ 8.55 (d, $J = 2$ Hz, 2H), δ 8.02 (d, $J = 8$ Hz, 2H), δ 7.78 (d, $J = 8$ Hz, 2H), δ 4.44 (broad t, 2H), δ 2.25 (t, $J = 4$ Hz, 2H), δ 2.08 (m, 6H), δ 1.91 (m, 6H). Accurate mass $C_{23}H_{26}N_3O_5$ requires 424.1872. Found 424.1891 (4.4 ppm). Melting point = 221°C.

*Synthesis of 6-(2-Fluoro-9-oxo-9H-acridin-10-yl)-hexanoic Acid (**17**).* 4-Fluoroaniline (1.86 g, 20 mmol), 2-chlorobenzoic acid (1.56 g, 10 mmol), ethylene glycol (5 mL) and anhydrous sodium carbonate (1.1 g, 10 mmol) were placed in a reaction vessel and stirred until effervescence ceased. Cupric chloride (100 mg, 0.75 mmol) dissolved in 2 mL of water was added to the reaction mixture which was then heated to 125°C for 6 hr. The reaction was allowed to cool and water (30 mL) and charcoal were added. The mixture was filtered and then acidified to pH 2 with conc. hydrochloric acid. The precipitate was collected by filtration, washed with water and then re-dissolved in 1 M sodium hydroxide solution. Material was re-precipitated by the addition of acetic acid, filtered off, washed with aqueous acetic acid, then water and finally dried under vacuum over phosphorous pentoxide to give 862 mg (37%) of *N*-(4-fluorophenyl)-anthranilic acid. $m/z = 231$. $C_{13}H_{10}FNO_2$ requires 231.23.

The *N*-(4-fluorophenyl)-anthranilic acid (0.70 g, 3 mmol) and phosphorous oxychloride (3 mL) were stirred together and heated to 115°C for 3.5 hr, then allowed to cool. The reaction mixture was placed on ice and small pieces of ice added, a vigorous reaction occurred with the evolution of hydrogen chloride. When the reaction had subsided, water (15 mL) was added and the mixture was boiled for 2 hr. On cooling, a solid precipitated out. This was filtered off and washed with water until the filtrate was colorless. The precipitate was further washed with cold methanol then diethyl ether and dried under vacuum to give 383 mg (59%) of 2-fluoroacridone.

2-fluoroacridone (213 mg, 1.0 mmol) was dissolved in anhydrous DMF (3 mL) under a nitrogen atmosphere. Sodium hydride dispersed in oil (45 mg, 1.1 mmol) was added and the mixture stirred until effervescence ceased. Ethyl 6-bromoacetate (250 μL) was added and the mixture stirred at 70°C overnight. The solvent was removed by rotary evaporation and the yellow residue purified by flash chromatography (silica. 4% ethyl acetate:dichloromethane) to give 230 mg (65%) of *O*-ethyl-6-(2-fluoro-9-oxo-9H-acridin-10-yl)-hexanoate.

^{1}H NMR (d_6-DMSO) δ 8.35 (d, $J = 8$ Hz, 1H), δ 7.9 (m, 5H), δ 7.35 (m, 1H), δ 4.45 (broad t, $J = 8$ Hz, 2H), δ 4.05 (ab, $J = 8$ Hz, 2H), δ 2.35 (t, $J = 7$ Hz, 2H), δ 1.65 (m, 6H), δ 1.20 (t, $J = 7$ Hz, 3H). $m/z = 356.1$. $C_{21}H_{22}FNO_3$ requires 355.41.

The *O*-ethyl-6-(2-fluoro-9-oxo-9H-acridin-10-yl)-hexanoate (71 mg, 0.2 mmol) was dissolved in ethanol (2 mL) and 1.0 M sodium hydroxide solution (0.4 mL) added and the mixture heated to 90°C for 90 min. The mixture was cooled, then water (6 mL) added to give a yellow precipitate. The mixture was cooled on ice and acidified with conc. hydrochloric acid when more material precipitated out. The precipitate was filtered off, washed with water then ethanol and dried under vacuum over phosphorous pentoxide to give 47 mg (72%) of 6-(2-fluoro-9-oxo-9H-acridin-10-yl)-hexanoic acid (**17**).

^{1}H NMR (d_6-DMSO) δ 12.1 (broad s, 1H), δ 8.34 (d, $J = 8$ Hz, 1H) δ 7.85 (m, 5H), δ 7.36 (m, 1H), δ 4.48 (t, $J = 8$ Hz, 2H), δ 2.25 (t, $J = 7$ Hz, 2H), δ 1.68 (m, 6H). Melting point = 198°C. $m/z = 327$. $C_{19}H_{18}FNO_3$ requires 327.36.

*Synthesis of 6-(2-Methoxy-9-oxo-9H-acridin-10-yl)-hexanoic Acid (**18**).* This was synthesised in an analogous manner to (**17**) using 4-methoxyaniline to give 6-(2-methoxy-9-oxo-9H-acridin-10-yl)-hexanoic acid (**18**) a bright yellow solid.

^{1}H NMR (d_6-DMSO) δ 12.0 (broad s, 1H), δ 8.35 (d, $J = 8$ Hz, 1H), δ 7.82 (m, 4H), δ 7.48 (dd, $J = 3$, 6 Hz, 1H), δ 7.31 (m, 1H), δ 4.5 (broad t, $J = 8$ Hz, 2H), δ 3.9 (s, 3H), δ 2.25 (t, $J = 7$ Hz, 2H), δ 1.7 (m, 6H). Accurate mass (M + H) $C_{20}H_{22}NO_3$ requires 340.1549. Found 340.1579 (8.9 ppm). Melting Point = 211°C.

*Synthesis of 6-(2-Methyl-9-oxo-9H-acridin-10-yl)-hexanoic Acid (**19**).* This was synthesised in an analogous manner to (**17**) using 4-methylaniline to give 6-(2-methyl-9-oxo-9H-acridin-10-yl)-hexanoic acid (**19**) a bright yellow solid.

^{1}H NMR (d_6-DMSO) δ 12.0 (broad s, 1H), δ 8.35 (d, $J = 8$ Hz, 1H), δ 8.15 (s, 1H), δ 7.72 (m, 4H), δ 7.31 (m, 1H), δ 4.5 (t, $J = 7$ Hz, 2H), δ 3.9 (s, 3H), δ 2.25 (t, $J = 7$ Hz, 2H), δ 1.70 (m, 6H). Accurate mass (M + H) $C_{20}H_{22}NO_3$ requires 324.1600. Found 324.1605 (1.6 ppm). Melting point = 161°C.

*Synthesis of O-(N-Succinimidyl)-6-(9-oxo-9H-acridin-10-yl)-hexanoate (**20**).* 6-(9-oxo-9H-acridin-10-yl)-hexanoic acid (100 mg, 0.25 mmol) was dissolved in anhydrous dimethylformamide (3 mL) and evaporated to dryness on a rotary evaporator. The process was repeated to remove traces of water. *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (90 mg, 0.3 mmol) was added and the mixture dissolved in anhydrous dimethylformamide (2 mL) and diisopropylethylamine (90 μL). The yellow solution was stirred under nitrogen for 60 min when TLC (RP-18, water:methanol, 50:50) showed all the starting material had been converted to a slower moving component. The solvent was removed by rotary evaporation with final drying under high vacuum to give *O*-(*N*-succinimidyl)-6-(9-oxo-9H-acridin- 10-yl)-hexanoate (**20**).

^{1}H NMR (d_6-DMSO) δ 8.36 (d, $J = 8$ Hz, 2H), δ 7.83 (m, 4H), δ 7.34 (m, 2H), δ 4.47 (t, 2H), δ 2.80 (s, 4H), δ 2.73 (t, $J = 7$ Hz, 2H), δ 1.74 (m, 6H). Accurate mass (M + H) $C_{23}H_{23}N2O_5$ requires 407.1607. Found 407.1597 (2.4 ppm).

*Synthesis of O-(N-Succinimidyl)-6-(2-acetamido-9-oxo-9H-acridin-10-yl)-hexanoate (**21**).* 6-(2-acetamido-9-oxo-9H-acridin-10-yl)-hexanoic acid (360 mg, 1.0 mmol) and *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (350 mg, 1.0 mmol) were dissolved in anhydrous dimethylformamide (5 mL) and diisopropylethylamine (183 μL) added. The yellow solution was stirred under nitrogen for 1 hr. The solvent was removed by rotary evaporation to leave a brown gum. This was purified by flash chromatography (silica. 10% methanol : ethyl acetate) to give 330 mg (50%) of *O*-(*N*-succinimidyl)-6-(2-acetamido-9-oxo-9H-acridin-10-yl)-hexanoate (**21**).

^{1}H NMR (d_6-DMSO) δ 10.2 (s, 1H), δ 8.6 (s, 1H), δ 8.53 (d, 1H), δ 8.05 (d, $J = 7$ Hz, 1H), δ 7.8 (m, 3H), δ 7.3 (m, 1H), δ 4.5 (m, 2H), 2.9 (m, 6H), δ 2.1 (s, 3H), δ 1.8 (m, 6H). Accurate mass (M + H) $C_{25}H_{26}N_3O_6$ requires 464.1822. Found 464.1798 (5.1 ppm).

*Synthesis of N-(Maleimido)-ethyl-6-(9-oxo-9H-acridin-10-yl)-hexanamide (**23**). N-(aminoethyl)-maleimide hydrochloride (**22**).* *N*-butoxycarbonyl-2-(aminoethyl)-maleimide (200 mg, 0.83 mmol) was stirred under nitrogen with 4 M hydrochloric acid in dioxan (4 mL). A white precipitate started to form after a few minutes. Stirring was continued for 40 min and then the solvent was removed by rotary evaporation. The resultant white solid was dried under vacuum to give 180 mg (100%) of *N*-(aminoethyl)-maleimide hydrochloride (**22**).

^{1}H NMR (CD_3OD) δ 6.90 (s, 2H), 3.81 (t, $J = 5$ Hz, 2H), δ 3.14 (t, $J = 5$ Hz, 2H).

*N-(maleimido)ethyl-6-(9-oxo-9H-acridin-10-yl)-hexanamide (**23**).* *O*-(*N*-succinimidyl)-6-(9-oxo-9H-acridin-10-yl)-hexanoate (102 mg, 0.25 mmol) was dissolved in anhydrous dimethylformamide (800 μL) and diisopropylethylamine (53 μL) added. *N*-(aminoethyl)-maleimide hydrochloride (53 mg, 0.30 mmol) was added to the yellow solution which was left to stand overnight. The solvent was removed by rotary evaporation and the residue purified by flash chromatography (silica. 2% methanol:dichloromethane). After removal of the solvent by rotary evaporation a yellow oil remained which slowly crystallised. Trituration with diethyl ether completed the crystallisation. This material was further purified by preparative TLC (silica. 5% methanol:dichloromethane) extracting the required material with 10% methanol:dichloromethane. Solvent was removed under vacuum, the residue triturated with ether to give 65 mg (60%) of *N*-(maleimido)ethyl-6-(9-oxo-9H-acridin-10-yl)-hexanamide (**23**).

^{1}H NMR (d_6-DMSO) δ 8.36 (d, $J = 7$ Hz, 2H), δ 7.85 (m, 5H), δ 7.35 (m, 2H), δ 7.01 (s, 2H), δ 4.45 (broad t, $J = 8$ Hz, 2H), δ 3.45 (t, $J = 6$ Hz, 2H), δ 3.2 (dd, $J = 5$, 6 Hz, 2H), δ 2.02 (t, $J = 6$ Hz, 2H), δ 1.7 (m, 6H). $m/z = 432$. $C_{25}H_{25}N_3O_4$ requires 431.50.

*Labelling of Substance P with 6-(2-Acetamido-9-oxo-9H-acridin-10-yl)-hexanoate (**21**).* Substance P (NH_2-RPKPQQFFGLM-NH_2) was synthesised on a 0.25 mM scale using Rink amide resin and Fmoc protected amino acids and Fastmoc^{TM} coupling. At the end of the synthesis, the N-terminal Fmoc group was removed from the protected resin coupled peptide. After drying under vacuum, 50 mg of the resin ($\sim$0.015 mM peptide) was placed in a 5 mL glass vial and 1.0 mL of anhydrous dimethyl sulfoxide added and the resin left to swell for 30 min. *O*-(*N*-succinimidyl)-6-(2-acetamido-9-oxo-9H-acridin-10-yl)-hexanoate (7.7 mg, 0.017 mM)

dissolved in 1.0 mL of anhydrous dimethyl sulfoxide was added to the resin followed by diisopropylethylamine (80 μL, 0.5 mM). The stoppered vial was placed on rollers for 12 hr at ambient temperature. The resin was filtered of, washed with 3 × 1 mL dimethyl sulfoxide, 3 × 1 mL methanol and 3 × 1 mL dichloromethane, then dried under vacuum. The dried resin was placed in a 5 mL round bottomed flask fitted with a magnetic stirrer bar and 2 mL of an ice-cold mixture of trifluoroacetic acid:water: triisopropylsilane (95:2.5:2.5) was added. The mixture was stirred for 2 hr at ambient temperature. The contents of the flask were filtered through a glass wool plug into 20 mL ice-cold diethyl ether. The plug was washed with 2 × 0.5 mL trifluoroacetic acid. The precipitated peptide was collected by centrifugation and the ether decanted. The pale yellow precipitate was washed twice by re- suspending in ether and collecting by centrifugation. The labelled peptide was then dried under vacuum.

Finally, the peptide was purified by reverse phase HPLC (Phenomenex Jupiter column) using a water to acetonitrile gradient (both containing 0.1% v/v TFA), flow 4 mL/min, detection at 220 and 400 nm.

Pooled material was freeze dried to give 6.0 mg (3.5 μM, 25%) of labelled peptide (**24**) as a pale yellow solid. m/z = 1695.9. $C_{84}H_{118}N_20O_{16}S$ requires 1694.

Synthesis of Quinacridones

*Synthesis of 7,14-Dioxo-5,7,12,14-tetrahydroquino [2,3-b]acridine-2,4,9,11-tetrasulfonic Acid (**25**).* Quinacridone (0.5 g, 1.6 mmol) was dissolved in 20% oleum and heated with stirring at 110°C under a nitrogen atmosphere for 16 hr. TLC (RP-18, methanol:water, 50:50) showed that all the starting material had been converted to a fast moving component. The reaction mixture was cooled and poured onto ice to give a red solution which was carefully neutralised with solid potassium hydrogen carbonate.

An orange precipitate was formed which was collected by centrifugation. The material was recrystallised from water to give the potassium salt of 7,14-dioxo-5,7,12,14-tetrahydroquino[2,3-*b*]acridine-2,4,9,11-tetrasulfonic acid (**25**) as an orange solid.

δ_H (200 MHz, d_6-DMSO) 8.94 (d, J = 2 Hz, 2H), 8.81 (s, 2H), 8.63 (d, J = 2 Hz, 2H). m/z = 632.975. $C_{20}H_{12}IN_2O_{14}S_4$ requires 631.92.

*Synthesis of the Diethyl Ester of 6-[12-(5-Carboxy-pentyl)-7,14-dioxo-7,14-dihydro-12H- quino[2,3-b]acridin-5-yl]-hexanoic Acid (**26**).* Quinacridone (2.35 g, 5.0 mmol) was suspended in anhydrous dimethylformamide (15 mL) under a nitrogen atmosphere. Sodium hydride (60% suspension in oil, 480 mg, 12 mmol) was added and the mixture stirred until effervescence stopped. Anhydrous dimethyl sulfoxide (25 mL) was added. The reaction was heated to 70°C for 2 hr. Ethyl 6-bromohexanoate (2.67 mL, 15 mmol) was added to the dark green solution and the mixture stirred overnight at 50°C. The dark blue solution was allowed to cool, then the mixture was poured into water (200 mL) and acidified with conc. hydrochloric acid. The solid was filtered off, washed with water and air dried. This was purified by flash chromatography (silica. 5–20% ethyl acetate:dichloromethane) to give 1.74 g (46%) of the diethyl ester of 6-[12-(5-carboxy-pentyl)-7,14-dioxo-7,14-dihydro-12*H*-quino[2,3-b]acridin-5-yl]- hexanoic acid (**26**).

^{1}H NMR (CDCl3) δ 8.42 (s, 4H), δ 7.70 (dd, J = 2, 6 Hz, 2H), δ 7.24 (d, J = 8 Hz, 2H), δ 4.39 (broad t, J = 8 Hz, 4H), δ 4.15 (ab, J = 7 Hz, 4H), δ 2.39 (t, J = 7 Hz, 4H), δ 1.80 (m, 12H), δ 1.25 (t, J = 7 Hz, 6H). M−Na$^+$ = 619.28. $C_{36}H_{40}N_2O_6$ requires 596.73.

*Synthesis of the Diethyl Ester of 6-[2-Iodo-12-(5-carboxy-pentyl)-7,14-dioxo-7,14-dihydro-12H-quino[2, 3-b]acridin-5-yl]-hexanoic Acid (**27**).* Diethyl ester of 6-[12-(5-carboxy-pentyl)-7,14-dioxo-7,14-dihydro-12*H*-quino[2,3-b]acridin-5-yl]- hexanoic acid (0.05 g, 0.084 mmol) was suspended in ethanol (25 mL) and then dichloromethane was added dropwise until the suspension dissolved. Benzyltrimethylammonium dichloroiodide (300 mg, 0.86 mmol) was added and the mixture stirred at ambient temperature for 3 days under a nitrogen atmosphere. TLC (RP-18,100% methanol) showed only starting material. A further portion of the dichloroiodide (600 mg, 1.7 mmol) and anhydrous zinc chloride (680 mg, 2.5 mmol) was added and the mixture stirred under reflux for a further 4 days. TLC showed some conversion to a slower running component. The mixture was cooled and the solvent removed by rotary evaporation. The dark residue was dissolved in dichloromethane, washed twice with 1 M sodium metabisulfite solution and the organic layer dried over anhydrous magnesium sulfate. After removal of the solvent, the residue was purified by flash chromatography (silica, 3–10% ethyl acetate:dichloromethane) to give the diethyl ester of 6-[2-iodo-12-(5-carboxy-pentyl)-7,14-dioxo-7,14-dihydro-12 *H*-quino[2,3-*b*]acridin-5-yl]-hexanoic acid (**27**). m/z = 723.170. $C_{36}H_{39}IN_2O_6$ requires 722.63. Melting point >300°C.

*Synthesis of the Diethyl Ester of 6-[2,9-Difluoro-12-(5-carboxy-pentyl)-7,14-dioxo-7,14-dihydro-12H-quino[2,3-b]acridin-5-yl]-hexanoic Acid (**28**).* Dimethyl 1,4-cyclohexanedione-2,5-dicarboxylate (9.12 g, 40 m mol) and methanol (200 mL) were heated to boiling, then 4-fluoroaniline (8.35 mL (9.78 g) 42 mmol) was added followed by conc. hydrochloric acid (400 μL). The mixture was refluxed for 3 hr under a nitrogen atmosphere. On cooling a yellow solid precipitated out which was collected by filtration, washed with methanol and dried under vacuum to give 15.8 g (96%) of dimethyl 2,5-bis[(4-fluorophenyl)amino]cyclohexa-1,4- diene-1,4-dicarboxylate. $m/z = 413$. $C_{22}H_{20}F_2N_2O_4$ requires 414.4.

The dimethyl 2,5-bis[(4-fluorophenyl)amino] cyclohexa-1,4-diene-1,4-dicarboxylate (6.21 g, 15 mmol), the sodium salt of 3-nitrobenzenesulfonic acid (3.6 g, 16 mmol), ethanol (90 mL) and 1.0 M sodium hydroxide (50 mL) were heated to reflux overnight under a nitrogen atmosphere. The bright yellow solution was allowed to cool and water (120 mL) was added. The mixture was acidified with conc. hydrochloric acid when a red solid precipitated out. This material was filtered off, washed with water and dried under vacuum over phosphorous pentoxide to give 5.6 g (97%) of 2,5-bis((4-fluorophenyl)amino)terephthalic acid. λ_{max}(ab) 295 nm, 380 nm (0.1 M sodium hydroxide). $m/z = 383$. $C_{20}H_{14}F_2N_2O_4$ requires 384.34.

The 2,5-bis((4-fluorophenyl)amino)terephthalic acid (5.0 g, 13 mmol) and polyphosphoric acid ($\sim$50 g) were heated at 150°C for 3 hr under a nitrogen atmosphere. The mixture was allowed to cool and then poured into iced water (200 mL) when a magenta solid precipitated out. This was filtered off, washed with water and then methanol, then dried under vacuum over phosphorous pentoxide to give 4.5 g (99%) of 2,9-difluoroquinacridone. $m/z = 349$. $C_{20}H_{10}F_2N_2O_2$ requires 348.31. Melting point >300°C.

2,9-difluoroquinacridone (350 mg, 1.0 mmol) was suspended in anhydrous dimethylformamide (4 mL) under a nitrogen atmosphere. Sodium hydride (60% suspension in oil 100 mg, 2.40 mmol) was added and the mixture stirred until effervescence stopped. The reaction was heated to 70°C for 1 hr. Ethyl 6-bromohexanoate (535 μL, 3.0 mmol) was added to the dark green solution and the mixture stirred overnight at 70°C. The dark orange-red solution was allowed to cool, then the mixture was poured into 150 mL of 1.0 M hydrochloric acid. The solid was filtered off, washed with water and air dried. This was purified by flash chromatography (silica. 20% ethyl acetate:dichloromethane) to give a red oil which crystallised on triturating with diethyl ether to give 171 mg (27%) of the diethyl ester of 6-[2,9-difluoro-12-(5-carboxy-pentyl)-7,14-dioxo-7,14- dihydro-12*H*-quino[2,3-*b*]acridin-5-yl]-hexanoic acid (**28**).

^{1}H NMR ($CDCl_3$) δ 8.61 (s, 2H), δ 8.12 (d, $J = 8$ Hz, 2H), δ 7.46 (dd, $J = 2$, 4 Hz, 4H), δ 4.48 (t, 4H), δ 4.15 (dd, 4H), δ 2.39 (t, 4H), δ 1.80 (m, 12H), δ 1.27 (t, 6H). Accurate mass (M + H) $C_{36}H_{39}F_2N_2O_6$ requires 633.2776. Found 633.2782 (0.9 ppm). Melting point = 219°C.

*Synthesis of the Diethyl Ester of 6-[2,9-Dichloro-12-(5-carboxy-pentyl)-7,14-dioxo-7,14-dihydro-12H-quino [2,3-b]acridin-5-yl]-hexanoic Acid (**29**).* This was synthesised in an analogous manner to (**28**) from dimethyl 1,4-cyclohexanedione-2,5- dicarboxylate and 4-chloroaniline to give dimethyl 2,5-bis[(4-chlorophenyl)amino]cyclohexa-1,4- diene-1,4-dicarboxylate (96%). This was hydrolysed to give 2,5-bis((4-chlorophenyl)amino)terephthalic acid (96%).

λ_{max}(ab) 308 nm, 379 nm. (0.1 M sodium hydroxide) $m/z = 417$. $C_{20}H_{14}C_{12}N_2O_4$ requires 417.25.

This was converted to 2,9-dichloroquinacridone (100%). $m/z = 381$, $C_{20}H_{10}C_{12}N_2O_2$ requires 381.22. Melting point >300°C.

Alkylation of this material with ethyl 6-bromohexanoate gave the diethyl ester of 6-[2,9-dichloro-12-(5-carboxy-pentyl)-7,14-dioxo-7,14-dihydro-12*H*-quino[2,3-*b*]acridin-5-yl]-hexanoic acid (**29**) as an orange powder (31%).

^{1}H NMR ($CDCl_3$) δ 8.60 (s, 2H), δ 8.40 (d, $J = 2$ Hz, 2H), δ 7.64 (dd, $J = 2$, 7 Hz, 2H), δ 7.40 (d, $J = 9$ Hz, 2H), δ 4.46 (broad t, $J = 8$ Hz, 4H), δ 4.15 (ab, $J = 7$ Hz, 4H), δ 2.39 (t, $J = 7$ Hz, 4H), δ 1.80 (m, 12), δ 1.27 (t, $J = 7$ Hz, 6H). Accurate mass (M + H) $C_{36}H_{39}C_{12}N_2O_6$ requires 665.2185. Found 665.2164 (3.2 ppm).

Synthesis of 6-[2,9-Dibromo-12-(5-carboxy-pentyl)-7,14-dioxo-7,14-dihydro-12H-quino[2,3-b]acridin-5-yl]-hexanoic Acid (30). This was synthesised in an analogous manner to (**28**) from dimethyl 1,4-cyclohexanedione-2,5-dicarboxylate and 4-bromoaniline to give dimethyl 2,5-bis[(4-bromophenyl)amino]cyclohexa-1, 4-diene-1,4-dicarboxylate (95%). This was hydrolysed to give 2,5-bis((4- bromophenyl)amino)terephthalic acid (96%). This was converted to 2,9-dibromoquinacridone (99%).

Accurate mass (M + H) $C_{20}H_{11}Br_2N_2O_2$ requires 468.9187. Found 468.9187 (0.1 ppm). Melting point >300°C.

Alkylation of this material with ethyl 6-bromohexanoate gave the diethyl ester of 6-[2,9-dibromo-

12-(5-carboxy-pentyl)-7,14-dioxo-7,14-dihydro-12*H*-quino[2,3-*b*]acridin-5-yl]-hexanoic acid as a red powder (46%).

^{1}H NMR ($CDCl_3$) δ 8.42 (d, $J = 4$ Hz, 2H), δ 7.70 (dd, $J = 2$, 7 Hz, 2H), δ 7.24 (d, $J = 8$ Hz, 2H), δ 4.39 (broad t, $J = 8$ Hz, 4H), δ 4.15 (ab, $J = 7$ Hz, 4H), δ 2.39 (t, $J = 7$ Hz, 4H), δ 1.80 (m, 12H), δ 1.25 (t, $J = 7$ Hz, 6H). $m/z = 753.1609$, 755.1602. $C_{36}H_{38}Br_2N_2O_6$ requires 754.52.

Diethyl ester was dissolved in glacial acetic acid (20 mL) to give a deep magenta solution. 1.0 M hydrochloric acid (10 mL) was added and the mixture heated to reflux for 5 hr. The reaction was allowed to cool, the red precipitate filtered off, washed with acetic acid and then diethyl ether and dried under vacuum over phosphorous pentoxide to give 0.86 g (93%) of 6-[2,9-dibromo-12-(5-carboxy-pentyl)-7,14-dioxo-7,14-dihydro-12*H*-quino[2,3-*b*]acridin-5-yl]-hexanoic acid as a light red solid (**30**).

$m/z = 696$–699. $C_{32}H_{30}Br_2N_2O_6$ requires 698.41. Melting point $>300^\circ$C.

*Synthesis of the Diethyl Ester of 6-[2,9-Dimethyl-12-(5-carboxy-pentyl)-7,14-dioxo-7,14- dihydro-12H-quino[2,3-b]acridin-5-yl]-hexanoic Acid (**31**).* This was synthesised in an analogous manner to (**28**) from dimethyl 1,4-cyclohexanedione-2,5- dicarboxylate and 4-methylaniline to give dimethyl 2,5-bis[(4-methylphenyl)amino]cyclohexa-1,4- diene-1,4-dicarboxylate (97%). This was hydrolysed to give 2,5-bis((4- methylphenyl)amino)terephthalic acid (96%).

λ_{max}(ab) 299 nm, 386 nm. (0.1 M sodium hydroxide). This was converted to 2,9-dimethylquinacridone (92%). $m/z = 381$. $C_{22}H_{16}N_2O_2$ requires 340.39. Melting point $>300^\circ$C.

Alkylation of this material with ethyl 6-bromohexanoate gave the diethyl ester of 6-[2,9-dimethyl-12-(5-carboxy-pentyl)-7,14-dioxo-7,14-dihydro-12*H*-quino [2,3-*b*]acridin-5-yl]-hexanoic acid (**31**) as a light red powder (62%).

^{1}H NMR ($CDCl_3$) δ 8.57 (s, 2H), δ 8.21 (d, $J = 1$ Hz, 2H), δ 7.47 (dd, $J = 2$, 7 Hz, 2H), δ 7.30 (dd, $J = 2$, 9 Hz, 2H), δ 4.45 (broad t, $J = 8$ Hz, 4H), δ 4.15 (ab, $J = 7$ Hz, 4H), δ 2.38 (m, 10H), δ 1.80 (m, 12H), δ 1.24 (t, $J = 2.1$ Hz, 6H). $m/z = 625$. $C_{38}H_{44}N_2O_6$ requires 624.78. Melting point $= 203^\circ$C.

*Synthesis of the Diethyl Ester of 6-[2,9-Dimethoxy-12-(5-carboxy-pentyl)-7,14-dioxo-7,14-dihydro-12H-quino [2,3-b]acridin-5-yl]-hexanoic Acid (**32**).* This was synthesised in an analogous manner to (**28**) from dimethyl-1,4-cyclohexanedione-2,5-dicarboxylate and 4-methoxyaniline to give dimethyl-2,5-bis[(4-methoxyphenyl)amino]cyclohexa- 1,4-diene-1,4-dicarboxylate (98%). This was hydrolysed to give 2,5-bis((4-methoxyphenyl)amino)terephthalic acid (98%).

λ_{max}(ab) 299 nm, 386 nm. (0.1 M sodium hydroxide). This was converted to 2,9-dimethoxyquinacridone (92%). $m/z = 373$. $C_{22}H_{16}N_2O_4$ requires 372.38. Melting point $>300^\circ$C.

Alkylation of this material with ethyl 6-bromohexanoate gave the diethyl ester of 6-[2,9-dimethoxy- 12-(5-carboxy-pentyl)-7,14-dioxo-7,14-dihydro-12*H*-quino[2,3-b]acridin-5-yl]-hexanoic acid (**32**) as a red powder (35%).

^{1}H NMR ($CDCl_3$) δ 8.70 (s, 2H), δ 7.91 (d, $J = 4$ Hz, 2H), δ 7.42 (m, 4H), δ 4.50 (broad t, $J = 8$ Hz, 4H), δ 4.15 (m, 4H), δ 4.00 (s, 6H), δ 2.40 (t, $J = 6$ Hz, 4H), δ 1.80 (m, 12H), δ 1.25 (t, $J = 7$ Hz, 6H). $m/z = 656$. $C_{38}H_{44}N_2O_8$ requires 656.78.

*Synthesis of the Diethyl Ester of 6-[2,9-Dinitro-12-(5-carboxy-pentyl)-7,14-dioxo-7,14- dihydro-12H-quino [2,3-b]acridin-5-yl]-hexanoic Acid (**33**).* 5,12-bis(*O*-ethyl-6-hexanoyl)-5,12-dihydroquino[2,3-*b*]acridin-7,14-dione (300 mg, 0.5 mmol) was cooled in an ice bath and then dissolved in 98% sulfuric acid (3 mL) under a nitrogen atmosphere to give a purple solution. 69% nitric acid (70 μL, 1.08 mmol) was added and the reaction mix removed from the ice bath. After 1 hr, the reaction mix was added to ice when an orange precipitate formed. The mixture was extracted with dichloromethane. The organic phase was washed with dilute sodium bicarbonate solution, then dried with anhydrous magnesium sulfate. After filtration, the solvent was removed by rotary evaporation to give an orange solid. This was purified by flash chromatography (silica. 2–3% methanol:dichloromethane). After removal of solvent, the residue was triturated with diethyl ether to give 240 mg (70%) of the diethyl ester of 6- [2,9-dinitro-12-(5-carboxy-pentyl)-7,14-dioxo-7,14-dihydro-12*H*-quino[2,3-*b*]acridin-5-yl]-hexanoic acid (**33**) as an orange solid.

^{1}H NMR (CD_3OD) δ 9.30 (d, $J = 2$ Hz, 2H), δ 8.66 (s, 2H), δ 8.48 (dd, $J = 3$, 7 Hz, 2H), δ 7.56 (d, $J = 10$ Hz, 2H), δ 4.56 (broad t, $J = 8$ Hz, 4H), δ 4.17 (ab, $J = 7$ Hz, 4H), δ 2.43 (t, $J = 7$ Hz, 4H), δ 1.80 (m, 12H), δ 1.28 (t, $J = 7$ Hz, 6H). Accurate mass (M + H) $C_{36}H_{39}N_4O_{10}$ requires 687.2666. Found 687.2661 (0.8 ppm). Melting point $= 271^\circ$C.

*Synthesis of Diethyl Ester of 6-[2,9-Diamino-12-(5-carboxy-pentyl)-7, 14-dioxo-7, 14-dihydro-12H-quino [2,3-b]acridin-5-yl]-hexanoic Acid (**34**).* The dini-

troquinacridone derivative (**33**) was reduced with ammonium formate as described in section Synthesis of 6-(2-Acetamido-9-oxo-9H-acridin-10-yl)-hexanoic Acid (**13**) and purified by flash chromatography (silica, 5–10% methanol:dichloromethane) to give the diethyl ester of 6-[2,9-diamino-12-(5-carboxy-pentyl)-7,14-dioxo-7,14-dihydro-12*H*-quino[2,3-b]acridin-5-yl]-hexanoic acid (**34**) as a dark blue solid.

^{1}H NMR (CD_3OD) δ 8.58 (s, 2H), δ 7.63 (d, $J =$ 10 Hz, 2H), δ 7.51 (d, $J = 3$ Hz, 2H), δ 7.28 (dd, $J =$ 5, 6 Hz, 2H), δ 7.25 (m, 2H), δ 4.51 (m, 4H), δ 4.04 (ab, $J = 7$ Hz, 4H), δ 2.35 (t, $J = 7$ Hz, 4H), δ 1.75 (m, 4H), δ 1.61 (m, 8H), δ 1.15 (t, $J = 7$ Hz, 6H). $m/z = 626$. $C_{36}H_{42}N_4O_6$ requires 626.76.

*Synthesis of Diethyl Ester of 6-[2,9-Bis-acetamido-12-(5-carboxy-pentyl)-7,14-dioxo- 7,14-dihydro-12H-quino[2,3-b]acridin-5-yl]-hexanoic Acid (**35**).* The diamino compound (**34**) was acetylated with acetic anhydride:pyridine as described in section Synthesis of 6-(2-Acetamido-9-oxo-9H-acridin-10-yl)-hexanoic Acid (**13**) to give the diethyl ester of 6-[2,9-bis-acetamido-12-(5-carboxy-pentyl)-7,14-dioxo-7,14-dihydro- 12*H*-quino [2,3-*b*]acridin-5-yl]-hexanoic acid (**35**) as a pink solid.

^{1}H NMR ($CD_3OD/CDCl_3$) δ 8.70 (s, 2H), δ 8.37 (dd, $J = 2, 8$ Hz, 2H), δ 8.20 (d, $J = 2$ Hz, 2H), δ 7.53 (d, $J =$ 7 Hz, 2H), δ 4.56 (m, 4H), δ 4.15 (ab, $J = 7$ Hz, 4H), δ 2.41 (t, $J = 7$ Hz, 4H), δ 2.23 (s, 6H), δ 1.80 (m, 4H), δ 1.70 (m, 8H), δ 1.26 (t, $J = 7$ Hz, 6H). Accurate mass (M + H) $C_{40}H_{47}N_4O_8$ requires 711.3394. Found 711.3395 (0.2 ppm).

*Synthesis of 6-(12-Ethyl-7,14-dioxo-2,9-disulfo-7,14-dihydro-12H-quino[2,3-b]acridin-5-yl)-hexanoic Acid (**37**).* Quinacridone (1.56 g, 5.0 mmol) was suspended in anhydrous dimethylformamide (15 mL) and anhydrous dimethyl sulfoxide (15 mL) under a nitrogen atmosphere. Sodium hydride (60% suspension in oil; 240 mg, 6.0 mmol) was added and the mixture stirred until effervescence stopped. More sodium hydride (240 mg, 6.0 mmol) was added and the mixture stirred for 10 min when effervescence had ceased. The reaction was heated to 60°C for 1 hr. Ethyl 6- bromohexanoate (890 μL, 5.0 mmol) was added to the dark green solution and the mixture stirred overnight at 60°C. Iodoethane (1.0 mL; 12.5 mmol) was then added and the mixture stirred for 2 hr at 60°C. The dark orange-red solution was allowed to cool, then the mixture was poured into water (300 mL). The solid was filtered off, washed with water and air dried. The solid was then dissolved in dichloromethane (300 mL) and anhydrous magnesium sulfate added. The mixture was filtered and the solvent removed by rotary evaporation to give a red solid. This was purified by flash chromatography (silica. 15% ethyl acetate:dichloromethane) to give 1.04 g (43%) of the ethyl ester of 6-(12-ethyl-7,14-dioxo-7,14-dihydro-12*H*-quino[2,3-*b*]acridin-5-yl)-hexanoic acid (**36**).

^{1}H NMR ($CDCl_3$) δ 8.80 (d, $J = 5$ Hz, 2H), δ 8.59 (d, $J = 8$ Hz, 2H), δ 7.78 (t, $J = 7$ Hz, 2H), δ 7.55 (t $J = 9$ Hz, 2H), δ 7.31 (m, 2H), δ 4.59 (m, 4H), δ 4.15 (ab, $J = 7$ Hz, 2H), δ 2.40 (t, $J = 7$ Hz, 2H), δ 2.05 (m, 2H), δ 1.75 (m, 6H), δ 1.27 (t, $J = 7$ Hz, 4H). $m/z = 484$. $C_{30}H_{30}N_2O_4$ requires 482.58.

The ethyl ester (241 mg, 0.5 mmol) was dissolved in 98% sulfuric acid (5 mL) and the purple solution heated at 110°C overnight under an atmosphere of nitrogen. The reaction was allowed to cool and then poured onto ice ($\sim$20 g). The solution was neutralised with 40% w/v sodium hydroxide solution to give a bright red solution. This was acidified with glacial acetic acid when an orange-red precipitate formed.

This was collected by centrifugation, then dissolved in 0.1% trifluoroacetic acid in water. The solution was purified by reverse phase HPLC (Phenomenex Jupiter column) using a water to acetonitrile gradient (both containing 0.1% v/v TFA), flow 5 mL/min, detection at 530 nm. Purified material was pooled, evaporated to dryness under vacuum and then dried under vacuum over phosphorous pentoxide to give 300 mg (97%) of 6-(12-ethyl-7,14-dioxo-2,9-disulfo-7,14- dihydro-12*H*-quino[2,3-*b*]acridin-5-yl)-hexanoic acid as a dark red solid (**37**).

^{1}H NMR (CD_3OD/D_2O) δ 12.0 (s, 1H), δ 8.89 (d, $J = 2$ Hz, 2H), δ 8.60 (m, 2H), δ 8.10 (d, $J = 9$ Hz, 2H), δ 7.59 (m, 2H), δ 4.52 (m, 4H), δ 2.38 (m, 2H), δ 1.80 (m, 6H), δ 1.48 (t, $J = 7$ Hz, 3H). Accurate mass (M+H) $C_{28}H_{27}N_2O_{10}S_2$, requires 615.1107. Found 615.1089 (2.9 ppm).

*Synthesis of O-(N-Succinimidyl-6-(12-ethyl-7,14-dioxo-2,9-disulfo-7,14-dihydro-12H - quino[2,3-b]acridin-5-yl)-hexanoate (**38**).* 6-(12-ethyl-7,14-dioxo-2,9-disulfo-7,14-dihydro-12H -quino[2,3-b]acridin-5-yl)-hexanoic acid (15 mg, 0.024 mmol), *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (8 mg, 0.027 mmol) anhydrous dimethyl sulfoxide (500 μL) and diisopropylethylamine (17.5 μL) were mixed to give an orange solution. This was left for 30 min when TLC (RP-18 water:methanol 30:70) showed that the starting material had been converted to a slower running component identified as

O-(*N*-succinimidyl-6-(12-ethyl-7,14-dioxo-2,9-disulfo-7,14-dihydro-12H- quino[2,3-*b*]acridin-5- yl)hexanoate by mass spectroscopy (**38**). $m/z = 712.1$. $C_{32}H_{29}N_3O_{12}S_2$ requires 711.73.

*Labelling of Substance P with 6-(12-Ethyl-7,14-dioxo-2,9-disulfo-7,14-dihydro-12H - quino[2,3-b]acridin-5-yl)-hexanoic Acid (**37**).* This was carried out in an analogous manner to that described in section Labelling of Substance P with 6-(2-Acetamido-9-oxo-9H-acridin-ioyl)-hexanoate (**21**). 50 mg of the resin bound peptide (∼0.015 mM peptide) was placed in a 5 mL glass vial and 1.0 mL of anhydrous dimethyl sulfoxide added and the resin left to swell for 30 min. *O*-(*N*-succinimidyl-6-(12- ethyl-7,14-dioxo-2,9-disulfo-7,14-dihydro-12*H*-quino[2,3-*b*]acridin-5-yl)-hexanoate (**38**) (7 mg, 0.01 mM) dissolved in 1.0 mL of anhydrous dimethyl sulfoxide was added to the resin followed by diisopropylethylamine (40 μL, 0.25 mM). After cleavage from the resin, the labelled peptide was purified by reverse phase HPLC (Phenomenex Jupiter column) using a water to acetonitrile gradient (both containing 0.1% v/v TFA), flow 4 mL/min, detection at 220 and 500 nm. Pooled material was freeze dried to give 13.0 mg (6.7 μM, 45%) of labelled peptide (**39**) as a red solid. $m/z = 1943$. $C_{91}H_{120}N_{22}O_{22}S_3$ requires 1942.

RESULTS

The fluorescence lifetimes and absorption and emission maxima for the acridones are shown in Table III and those for the quinacridones in Table IV. Actual absorption and emission spectra of some of the acridones are shown in Figs. 1 and 2, those for some of the quinacridones are in Figs. 3 and 4.

Figs. 5 and 6 show the fluorescence decay curves of the full range of acridones and quinacridones respectively.

The molar extinction coefficients of five of the acridone derivatives and that of the functionalised, water soluble quinacridone derivative are shown in Table V.

DISCUSSION

The strategy used to synthesis the acridone and quinacridone derivatives described in this paper consists of attaching a spacer arm to the fluorophore which terminates in a group which can be linked to a biomolecule. The most extensively used group in this field is the carboxyl group which can be activated as a N-succinimidyl ester and used to label either the N-terminal group of an amino acid, peptide or protein or the ε-amino group of a lysine residue. We have also prepared a maleimido derivative (**23**) (Fig. 8) which can be used to label sulfhydryl groups such as contained in cysteine residues.

Table III. Fluorescence Properties of Some Acridone Derivatives

Compound	Solvent	Lifetime (ns)	λ_{max} nm (ab)		λ_{max} nm (em)	
1	H_2O	14.2	393	410	426	(448)
2	H_2O	14.2	392	409	427	(447)
3	H_2O	8.6	418	439	432	(457)
4	H_2O	6.0	405	425	439	(464)
5	MeOH	1.7	394	414	430	(452)
6	H_2O	13.0	391	409	428	(449)
7	H_2O	7.1	398	416	435	(456)
8	H_2O	16.8	396	415	425	(452)
9	H_2O	3.1	395	413	428	(450)
10	H_2O	4.2	398	409	438	
11	H_2O	N.F.	396	413		
12	H_2O	10.9	(392)	405	543	
13	H_2O	17.1	398		450	
14	EtOH	N.F.	387			
15	H_2O	11.5	449	455	547	
16	H_2O	19.6	421	430	463	(487)
17	H_2O	14.6	399	420	432	(456)
18	H_2O	17.0	405	422	462	
19	H_2O	15.1	399	419	434	(457)
24	H_2O	17.5	408	450		

Figures in parentheses indicate a shoulder on the main peak. N.F. = not fluorescent.

Table IV. Fluorescence Properties of Some Quinacridone Derivatives

Compound	Solvent	Lifetime (ns)	λ_{max} nm (ab)		λ_{max} nm (em)	
25	H_2O	22.8	(487)	517	550	(590)
26	MeOH	21.6	(493)	527	560	(600)
27	MeOH	12.7	535		558	(594)
28	MeOH	22.7	(507)	540	566	(600)
29	MeOH	22.1	(501)	534	556	
30	MeOH	17.3	(500)	533	555	(592)
31	MeOH	22.0	(506)	540	565	(600)
32	EtOH	13.4	(519)	555	592	
33	DCM	5.1	474	506	553 vw	
34	DCM	15.6	(539)	574	608	
35	DCM/ MeOH	16.8	(518)	551	576	
36	MeOH	20.7	(496)	528	550	(585)
37	H_2O	21.7	505	536	559	(595)
39	H_2O	23.7		540	558	(600)

Figures in parentheses indicate a shoulder on the main peak. vw = very weakly fluorescent.

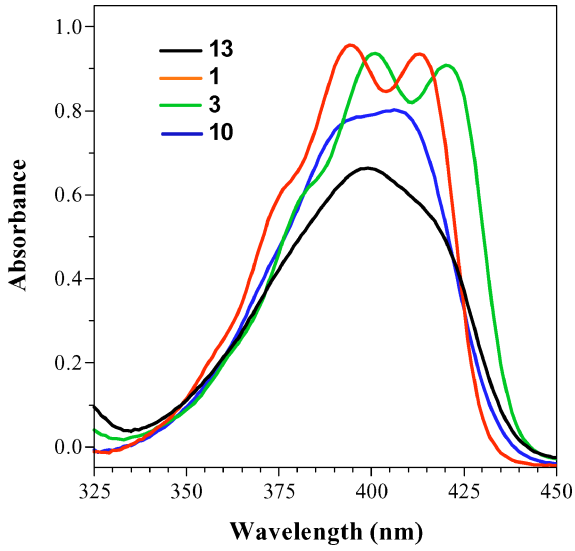


Fig. 1. Absorption spectra of four acridone derivatives in aqueous solution.

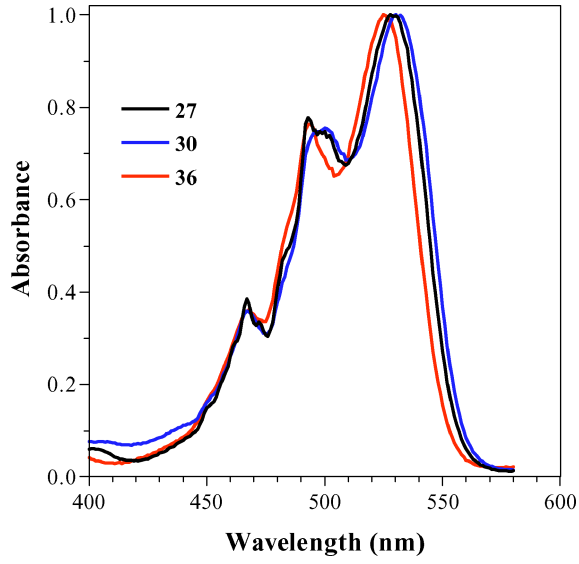


Fig. 3. Absorption spectra of three quinacridone derivatives in methanolic solution.

It is also possible to prepare oligonucleotides containing terminal amino or sulfhydryl groups that can be similarly labelled. In most cases the spacer arm consists of a hexanoic acid residue attached to the imino group of the fluorophore. We have also attached a shorter spacer arm consisting of a carboxymethyl group, again attached to the imino group of the fluorophore. The fluorescence lifetime of the two analogues (**1** and **2** in Table III) were identical to the non- alkylated starting material suggesting that the presence and length of the spacer does not affect the lifetime when attached to the imino group. Two examples of acridones in which the carboxy group has been attached directly to the ring of acridone are described (**8** and **9**). Attachment via a short spacer, a carboxymethyl group (**8**) led to a slight increase in fluorescence lifetime, whilst direct attachment of the carboxy group to the aromatic ring (**9**) produced a marked shortening of the fluorescence lifetime. Attaching a spacer to this carboxy group via an amide bond (**10**) caused only a slight increase in lifetime.

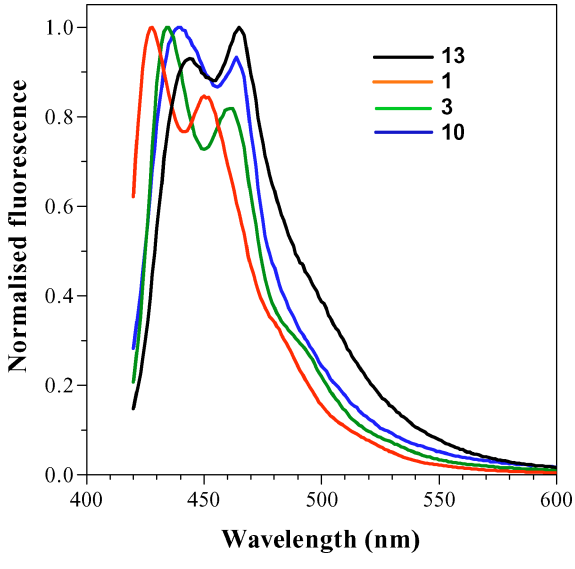


Fig. 2. Normalised emission spectrum of four acridone derivatives in aqueous solution excited at 405 nm.

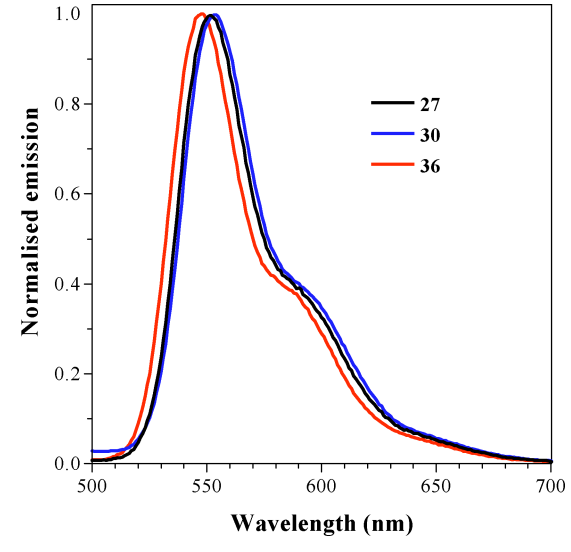


Fig. 4. Normalised emission spectra of three quinacridone derivatives in methanolic solution excited at 480 nm.

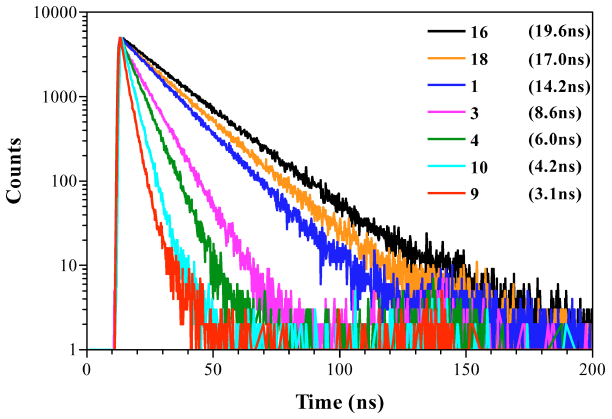


Fig. 5. Fluorescence decays of seven acridone derivatives in aqueous solution.

N-alkylation of both the acridones and quinacridones was used to increase their solubility in organic solvents before attempting to alter the lifetime by introducing other groups such as halogens, alkyl, alkoxy, nitro, amino and acetamido groups into the rings of the fluorophore. Initial attempts to alkylate using a two phase system using potassium hydroxide [41] and the use of potassium t-butoxide as base and ethyl 6-bromohexanoate as the alkylating agent lead to only partial alkylation and it was difficult to separate the unreacted quinacridone from the products. We therefore decided to use sodium hydride in dimethyl sulfoxide to form the anion of the fluorophore, as judged by the cessation of hydrogen evolution, before addition of the alkylating agent. In the case of quinacridone, dimethylformamide proved to be a better solvent for the formation of the anion, dimethyl sulfoxide being added for the alkylation reaction which was carried out at 70°C for quinacridone and ambient temperature for acridone. Both methods gave excellent yields (~90%) of alkylated material.

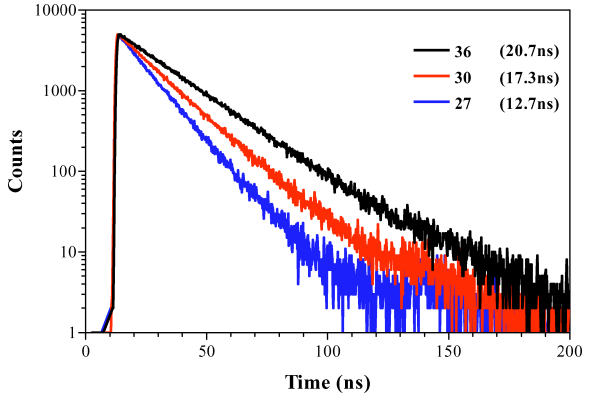


Fig. 6. Fluorescence decays of three quinacridone derivatives in methanolic solution.

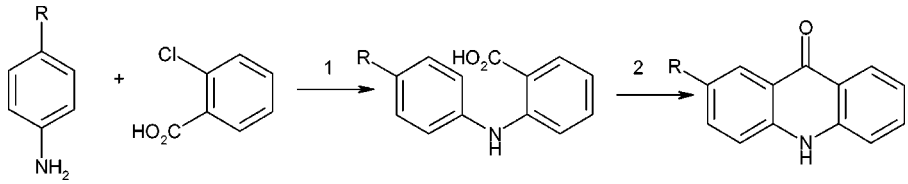
Table V. Molar Extinction Coefficients of Some Acridone and Quinacridone Derivatives

Compound	Absorption (nm)	Extinction coefficient (M^{-1} cm^{-1})
1	413	7190
	394	7580
	257	52700
16	418	5190
	295	35900
	265	37500
17	421	7980
	401	7980
	251	45500
18	428	7150
	408	7150
	255	38100
19	419	7490
	400	7890
	260	54000
37	536	4790
	506	5590
	294	65800

Compounds were dissolved in 1 M sodium hydroxide and then made up to 500 μM (except **17** at 100 μM) with water.

For quinacridone, N-alkylation resulted in the formation of the dicarboxylic acid which is not ideal for single labelling of a biomolecule as the molecule may attach at two sites on the same molecule or cause dimerisation. Although the monocarboxylic acid derivative can be formed by adding a single equivalent of sodium hydride and ethyl 6-bromohexanoate to quinacridone, the solubility of the product is poor due to continued hydrogen bonding from the remaining unfunctionalised imino group. We overcame this problem by alkylating the remaining imino group with ethyl iodide to give (**36**). This material showed good solubility in methanol and after sulfonation with conc. sulfuric acid gave (**37**) which is soluble in water. This compound has been converted to its N-succinimidyl ester and used to label Substance P. The majority of the acridone derivatives that have been synthesised are water soluble if initially dissolved in methanol. However, the quinacridone derivatives are more hydrophobic and only the sulfonated derivatives were water soluble. It would appear that sulfonation does not significantly affect the lifetime of the fluorophore, in agreement with the literature [30].

We initially attempted to make quinacridone more soluble by sulfonation before carrying out the alkylation. Sulfonation with conc. sulfuric acid at 100°C gave the disulfonic acid as described by Jaffe and Marshall [42], however, oleum at the same temperature gave the very hydrophilic tetrasulfonic acid (**25**). Attempts to alkylate this material were unsuccessful, partially due to its insolubility in any solvent other than water.



Scheme 1. Synthesis of 2-substituted acridones (Compounds **17**, **18** and **19**) where R = F, CH_3O or CH_3. 1) Na_2CO_3/ethylene glycol/$CuCl_2$, 125°C; 2) $POCl_3$, 115°C.

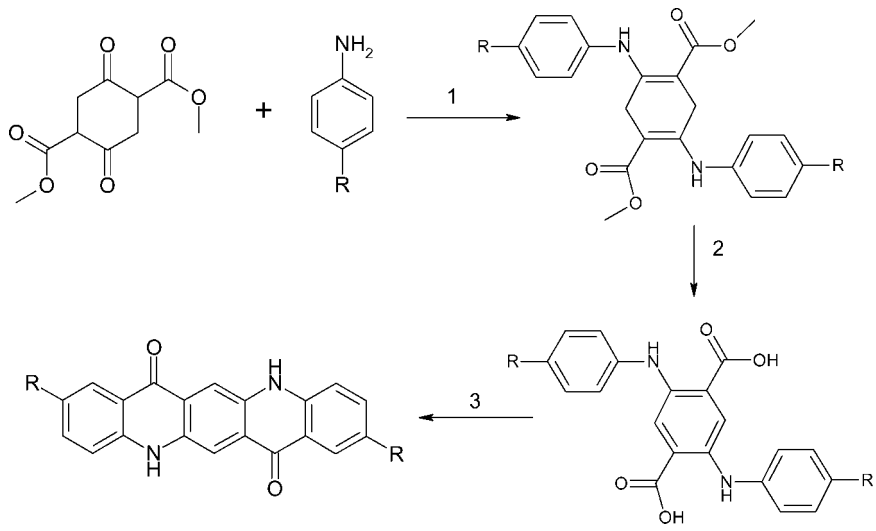
Further examples of substituted acridones and quinacridones were prepared by ring closure reactions [43–45] as shown in Schemes 1 and 2.

The 2-substituted acridones were prepared by condensing 4-substituted anilines with 2- chlorobenzoic acid to give the 2-substituted acridone which was then N-alkylated.

The 2,9-disubstituted quinacridone may be synthesised from dimethyl 1,4-cyclohexanedione-2,5- dicarboxylate and a substituted aniline and then N-alkylated with ethyl 6-bromohexanoate.

Methyl substituted acridone (**19**) and quinacridone (**31**) had similar fluorescence lifetimes to the non- substituted compounds. However, methoxy substitution lengthened the lifetime of acridone (**18**) from 14 to 17 ns, whilst the lifetime of the quinacridone (**32**) was reduced from 22 to 13.4 ns. We initially attempted to reduce the fluorescence lifetime of both acridone and quinacridone by halogenation, in particular, bromination and iodination. Acridones have been successfully halogenated using a variety of reagents [46–48]. Bromination of the ethyl ester of 6-(9-oxo-9H- acridin-10-yl)-hexanoic acid (**1**) (lifetime 14.2 ns) with benzyltrimethylammonium tribromide gave a mixture of mono- and di- substituted material (**3** and **4**) with lifetimes of 8.6 and 6 ns respectively. Iodination of the same compound with benzyl trimethylammonium dichloroiodide required addition of a Lewis acid (anhydrous zinc chloride) to give the mono-iodide (**5**) with a fluorescence lifetime of 1.7 ns. These results are in broad agreement with those reported by Shigorin's group for non- alkylated acridones [30–33].

Attempts to carry out the same reactions on the dialkylated quinacridones resulted in poor yields due to the low reactivity of the quinacridone. Only the diethyl ester of 6-[2-iodo-12-(5-carboxy- pentyl)-7,14-dioxo-7,14-dihydro-12*H*-quino[2, 3-b]acridin-5-yl]-hexanoic acid (**27**) was prepared by direct halogenation in very poor yield. The alternative strategy of synthesising the 2,9-dihaloquinacridone from dimethyl 1,4-cyclohexanedione-2,5-dicarboxylate and 4-haloaniline and then N-alkylating with ethyl 6-bromohexanoate was adopted. Thus the difluoro, dichloro and dibromo derivatives were produced



Scheme 2. Synthesis of 2,9-disubstituted quinacridones (Compounds **28**, **29**, **30**, **31** and **32**) where R = F, Cl, Br, CH_3 or CH_3O. 1) MeOH/HCl, reflux; 2) sodium 3-nitrobenzenesulfonate/NaOH/EtOH, reflux; 3) polyphosphoric acid, 150°C.

by this method. As expected, only substitution with either bromine or iodine produced a shortening of fluorescence lifetime to 17.3 ns for the dibromo (**30**) and 12.7 ns for the monoiodo (**37**) substituted material compared to 22 ns for the non-substituted (**26**), difluoro (**28**) and dichloro-substituted (**29**) compounds.

Dinitroacridones and dinitroquinacridones were synthesised by direct nitration with nitric acid. These compounds are virtually non-fluorescent, however reduction of the nitro groups to amino groups restores the fluorescence. The monoaminoacridone (**12**) shows little change in absorption compared to non-substituted acridone but the diaminoacridone (**15**) shows a 50 nm bathochromic shift in its absorption. Both show a bathochromic shift in the emission spectrum of about 100 nm compared to acridone, the lifetimes being 10.9 and 11.5 ns respectively. This is in agreement with the values reported for 2-aminoacridone [27].

The 2,9-diaminoquinacridone (**34**) shows a red shift in both its absorption and its emission spectrum of about 30 nm. The fluorescence lifetime is reduced to 15.6 ns. These measurements were obtained using dichloromethane as the solvent. Both the 2,9-di-nitro (**33**) and diaminoquinacridone derivatives (**34**) have very poor solubility in methanol and are non- fluorescent in this solvent.

Acetylation of the amino group gives rise to a hypsochromic shift of about 80 nm for both the mono- and di-substituted acridones with only a smaller change in the absorption spectrum. The fluorescence lifetimes are increased to 17.1 and 19.6 ns respectively. The 2,9- diacetamidoquinacridone (**35**) shows a blue shift in its absorption spectrum compared to the di-amino compound (**34**). The fluorescence lifetime is 16.8 ns.

Two other acridones were synthesised which were not N-alkylated, the first, 2-carboxymethyl-7-chloro-9-oxo-9,10-acridine (**8**) has a fluorescence lifetime of 16.8 ns, whilst the second, 9-oxo-9,10-dihydroacridine-4-carboxylic acid (**9**) has a greatly reduced fluorescence lifetime of 3.1 ns. Both compounds have absorption and emission spectra which are very similar to unsubstituted acridone. In order to lengthen the spacer arm on (**9**) the 4-carboxyl group was reacted with 6-aminohexanoic acid to give (**10**) with a slightly increased lifetime of 4.2 ns. Again there was little change in the absorption and emission spectra. The reason for the dramatic shortening of fluorescence lifetime is not clear, however, conversion of the carboxylic acid group to an amide group as in (**10**) increases the fluorescence lifetime.

In a similar manner, acetylation of both mono- and diaminoacridone derivatives and also the diaminoquinacridone derivative lead to an increase in fluorescence lifetime, again the reason for this is not clear. We have not investigated any quinacridones in which a carboxylic acid group is attached directly to the aromatic ring, however, 7,14-dioxo-5,7,12,14-tetrahydroquino[2,3-*b*]acridine-2,9-dicarboxylic acid has previously been synthesised [49] but its fluorescence lifetime has not been reported.

Table VI. Fluorescence Lifetime of an Acridone (**1**) a Quinacridone (**37**) and Fluorescein at Various pHs

	Fluorescence lifetime (ns)		
pH	Acridone	Quinacridone	Fluorescein
5	14.8	22.0	3.7
7	14.6	22.2	4.6
9	14.8	22.1	4.6

Both acridone and quinacridone have fluorescence lifetimes that are independent of pH in the biologically important range of 5 to 9, unlike that of fluorescein which shows a marked pH dependence (Table VI).

Again, unlike fluorescein, both acridone and quinacridone are resistant to photobleaching and showed no change in their absorption spectrum over a period of 1 hr when exposed to an intense white light source. In comparison, fluorescein showed a 70% decrease in absorption under the same conditions (Fig. 7).

CONCLUSIONS

We report two classes of fluorescent dye in which the fluorophore has been chemically modified to alter

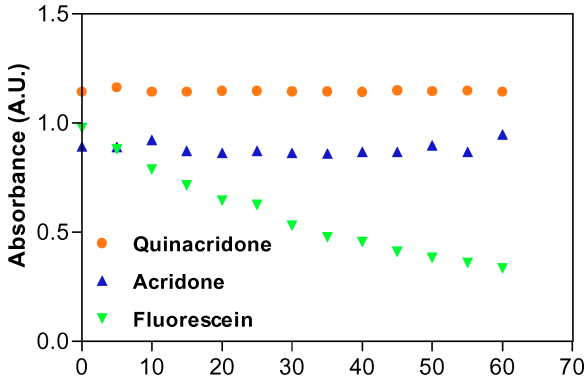


Fig. 7. Photobleaching of an acridone (**1**), a quinacridone (**37**) derivative and fluorescein. Samples were made up at a concentration of 1 AU in pH 7 buffer and placed directly in front of a 20 watt white light source. The absorption at λ_{max} was measured at 5 min intervals. The half life of fluorescein under these conditions was 30 min.

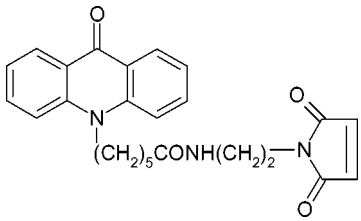


Fig. 8. Structure of maleimido derivative of acridone (**23**).

the fluorescence lifetime without significantly altering the absorption and emission spectra. Additionally, the molecules have been modified in such a way that they can be chemically attached to biomolecules such as proteins and oligonucleotides. We have already labelled a peptide at its N-terminus with both types of fluorophore.

The first set of fluorophores are based on acridone and absorb around the 400 nm region and emit around 450 nm. Nineteen acridone derivatives were investigated giving five distinct fluorescent lifetimes ranging from 3–20 ns, thus allowing the opportunity for temporal multiplexing. Additionally, we report a new class of fluorescent dyes, the quinacridones, for labelling biomolecules.

The quinacridones absorb around 500 nm and emit around 550 nm. Thirteen compounds were investigated giving three distinct fluorescent lifetimes ranging from 12–22 ns, again allowing for the possibility of temporal multiplexing.

Although we have only prepared one quinacridone as a mono-functional N-succimidyl ester, we have shown that it is possible to synthesise several derivatives with different fluorescence lifetimes. It should therefore be possible to modify these into mono-functional and water soluble fluorophores.

Both the acridones and one of the quinacridones have been modified to give them water solubility. Both types of fluorophore have good chemical and photochemical stability (Fig. 7) and their lifetimes are independent of pH in the range 5 to 9 (Table VI). Compounds within each group have similar absorption and emission spectra and their molecular weights fall within a narrow range.

The quinacridones offer a new type of fluorophore for labelling biomolecules and have absorption and emission similar to those of the fluoresceins whilst being much less affected by pH changes. Additionally, unlike fluorescein, quinacridone shows a marked resistance to photobleaching. Whilst we have investigated the fluorescence lifetimes properties of the quinacridone derivatives, this group of compounds could be used as a label for use with other modalities such as fluorescence intensity or polarisation.

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