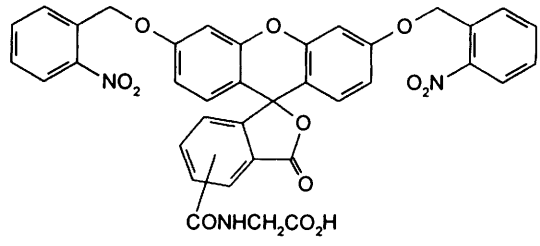
Synthesis of photoactivatable fluorescein derivatives bearing side chains with varying properties

John E. T. Corrie * and David R. Trentham
National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

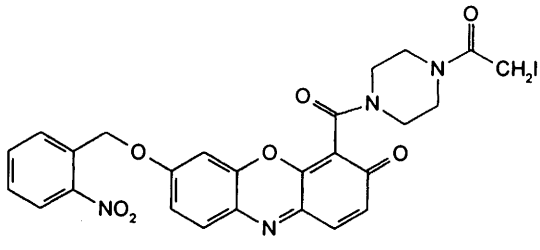
Etherification of both phenolic hydroxyl groups of fluorescein locks the molecule into its non-fluorescent lactone form. Here fluorescein has been unsymmetrically alkylated with a 2-nitrobenzyl ether and one of three variously functionalised alkyl ethers to give compounds **4**, **5** and **6**. The 2-nitrobenzyl group can be removed by photolysis with near-UV light to regenerate a fluorescent species, while the second ether group contains one of a range of functions, maleimido or iodoacetyl for ligation to proteins, or a long alkyl chain to promote association with lipid membranes. Incorporation of the various side chains was achieved by condensation of the common intermediate aldehyde **13** with substituted hydrazides **19** or **23**, leading to compounds **4** and **5**, respectively, or with hexadecanohydrazide to give compound **6**. Photochemical and physical properties of the compounds are described.

Chemically modified fluorophores, in which fluorescence is blocked by the presence of a photocleavable protecting group were introduced by Ware and co-workers, who demonstrated the potential for study of diffusion kinetics and coined the term 'fluorescence photoactivation and dissipation' (FPD) to describe the technique.[1] More recently a number of publications have described the use of these photoactivatable fluorophores to address problems in cell biology.[2] The blocked fluorophore is used to label a cellular protein *in vitro*, and the labelled protein is then microinjected into a living cell and allowed to equilibrate with the endogenous unlabelled protein. Subsequent photolysis using near-UV light over a small region of the cell unmasks the fluorophore and allows movement of the protein bearing the fluorescent tag to be observed. In a recent non-biological example a photoactivatable fluorescein has been used in a similar experimental paradigm to study light distribution within turbid media.[3] The FPD technique is similar in concept to the older method of fluorescence recovery after photobleaching (FRAP)[4] but offers potential advantages, particularly that the observations are made of a bright area against a dark background (the reverse of FRAP) and the possibility of monitoring events over a relatively long time.

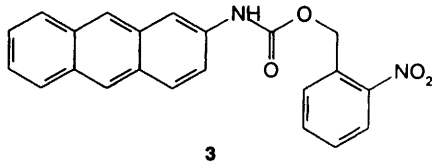
The photoactivatable fluorophores hitherto described for protein labelling have been derivatives of fluorescein, *e.g.* **1**,[2a] or of resorufin, *e.g.* **2**,[2d] and earlier work by Krafft and co-workers[5] had illustrated the potential of this class of compound. The masking of fluorescence can be either total or partial. Thus, for compounds such as **1**, fluorescence is eliminated by etherification of both phenolic groups, which locks fluorescein into its non-fluorescent lactone form. In compounds such as **2** or **3**, fluorescence is only partially blocked, although typically by at least 2 orders of magnitude, since the extended conjugation present in the free fluorophore is diminished but not eliminated by the photolabile blocking groups.



1



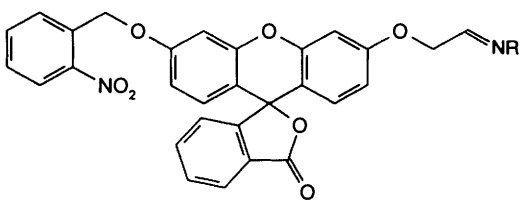
2

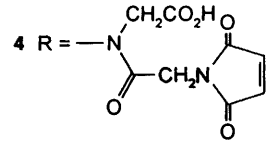


3

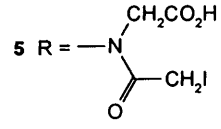
As part of our continuing interest in the application of photoactivatable compounds to problems in biology,[6] we wished to extend the available range of photoactivatable fluorophores. This paper describes two photoactivatable fluorescein derivatives **4** and **5** designed to label cysteine residues and a further variant **6** bearing a lipophilic anchor group. A feature of the synthetic route is its flexibility, as illustrated by the relative ease of assembly of the three compounds from a common intermediate. This property stems from the presence of the allyl group as in compound **7** which, as a latent aldehyde, facilitates convergent incorporation of side chains with differing properties.

As outlined above, the fluorescence of fluorescein can be eliminated by alkylation of the two phenolic oxygen atoms and our initial target species **7** required differential alkylation of these with 2-nitrobenzyl and allyl groups. Remarkably, despite the long history of fluorescein, little is known about formation of ether derivatives but the preliminary report by Krafft *et al.*[5c] indicated that initial monoalkylation and subsequent second (differential) alkylation could be achieved in high overall yield





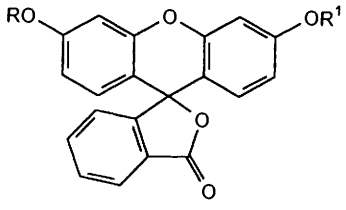
4 R = —N(CH$_2$CO$_2$H)—CH$_2$N(C=O)$_2$



5 R = —N(CH$_2$CO$_2$H)—CH$_2$I

6 R = —NHCOC$_{15}$H$_{31}$

by careful choice of solvent at each stage. Both alkylation steps were promoted by silver(I) oxide. However, our attempts to



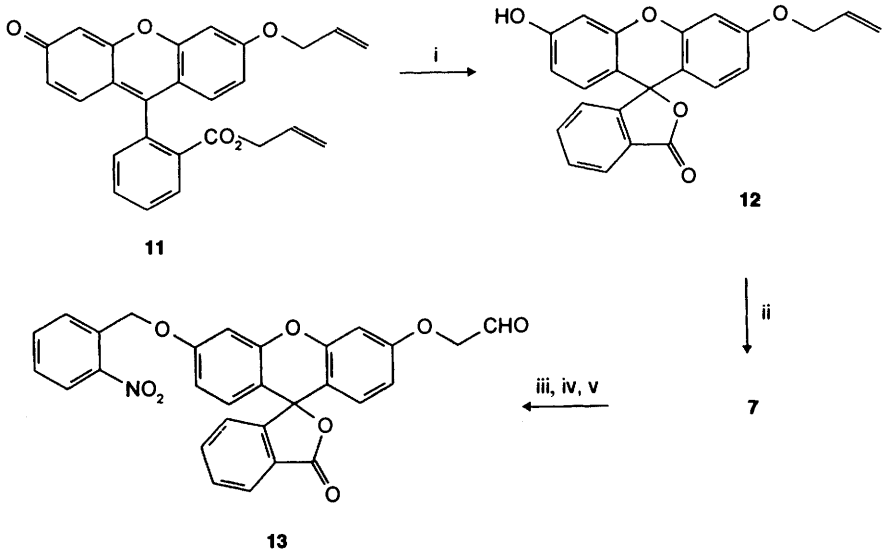
7 R = 2-NO$_2$C$_6$H$_4$CH$_2$, R^1 = allyl
8 R = 2-NO$_2$C$_6$H$_4$CH$_2$, R^1 = H
9 R = MeCO, R^1 = H
10 R = MeC$_6$H$_4$SO$_2$, R^1 = H

prepare the mono(2-nitrobenzyl) ether **8** under apparently similar conditions invariably gave complex mixtures from which the desired compound **8** could be isolated only with difficulty and in *ca.* 15% yield at best. It may be significant that in the reactions outlined by Krafft *et al.*[5c] the alkylation by 2-nitrobenzyl bromide or a substituted variant was always performed as the second stage. We made several efforts to improve the yield by using monoester derivatives such as the acetate **9**[7] or tosylate **10** (prepared from the acetate **9** by tosylation followed by alkaline hydrolysis) but with little overall improvement to the yield or convenience of the overall process (data not shown).

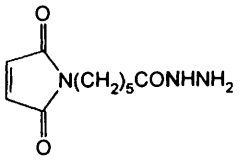
In the light of these unresolved difficulties, we turned to an alternative route (Scheme 1) in which the 2-nitrobenzyl group was introduced in the second alkylation step. The required monoallyl ether **12** was readily obtained by brief alkaline hydrolysis of the monoether ester **11**, which is the major product from alkylation of fluorescein disodium salt with allyl bromide in aqueous acetone.[8] Subsequent alkylation of the monoether **12** with 2-nitrobenzyl bromide and silver(I) oxide in benzene–THF gave the nitrobenzyl ether **7** in satisfactory yield. As expected, compound **7** showed no long wavelength absorption in its UV–visible spectrum apart from the low intensity tail to approx. 400 nm which is associated with the n→π* transition of aromatic nitro compounds. Whereas the monoallyl ether **12** showed strong fluorescence, approx. 15% of the intensity of that of fluorescein itself, compound **7** had no measureable fluorescence. Upon ozonolysis of compound **7** in CH_2Cl_2–MeOH followed by treatment with dimethyl sulfide, the ^{1}H NMR spectrum indicated that the product was a methyl acetal which was cleaved during chromatography on silica gel to give the expected aldehyde **13**. The propensity of aryloxyacetaldehydes to form acetals has been noted previously.[9]

With the desired substituents in place on the fluorescein nucleus, we turned our attention to construction of moieties which could readily be coupled with the aldehyde **13** to enable the photoactivatable fluorescein compound to be linked to proteins. Our aim was to prepare reagents which would be reactive towards cysteine side chains and therefore either a maleimido or iodoacetyl group was required in the final product. In the event, both types of reagent have been synthesised and are discussed below.

Initial experiments in which the known[10] maleimido hydrazide **14** was coupled with the aldehyde **13** (data not shown) indicated that it was desirable to incorporate a charged group into the final product so as to achieve a degree of water solubility and hence facilitate protein labelling protocols. Scheme 2 shows the synthesis of a maleimido hydrazide **19** which fulfilled our requirements. Alkylation of *tert*-butyl carbazate with *tert*-butyl bromoacetate in refluxing benzene



Scheme 1 *Reagents:* i, NaOH–aq. acetone; ii, 2-NO$_2$C$_6$H$_4$CH$_2$Br–Ag$_2$O–C$_6$H$_6$–THF; iii, O$_3$; iv, Me$_2$S; v, silica gel



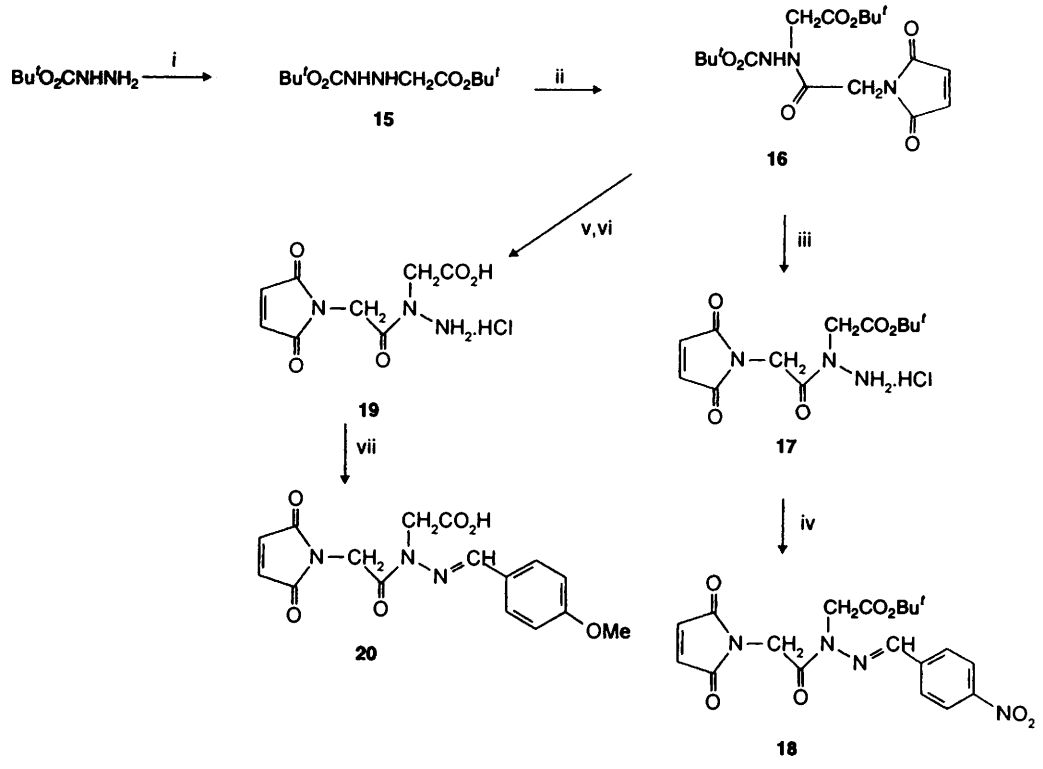
14

was somewhat less efficient than reported[11] for the corresponding reaction using ethyl bromoacetate, but after treatment of the crude reaction product with pyruvic acid to remove unchanged *tert*-butyl carbazate, the substituted hydrazide **15** was readily obtained pure. *N*-Maleylglycine[12] was smoothly converted by treatment with thionyl chloride into its acid chloride, which cleanly acylated the hydrazide **15** in the presence of triethylamine to give the derivative **16**. The only previously reported synthesis of maleylglycyl chloride involved prolonged treatment of *N*-carboxymethylmaleamic acid with a mixture of phosphorus pentachloride and phosphoryl trichloride.[13] When compound **16** was treated at room temperature with anhydrous hydrogen chloride in dioxane,[10] the *tert*-butoxycarbonyl group was cleaved but the *tert*-butyl ester was unaffected. Isolation of the hydrazone **18** after condensation of the initial product **17** with 4-nitrobenzaldehyde confirmed the partial deprotection. However, treatment of compound **16** with trifluoroacetic acid cleanly removed both *tert*-butyl groups and the resulting hydrazide was isolated as its hydrochloride salt **19**. This compound gave the expected hydrazone **20** on reaction with 4-methoxybenzaldehyde.

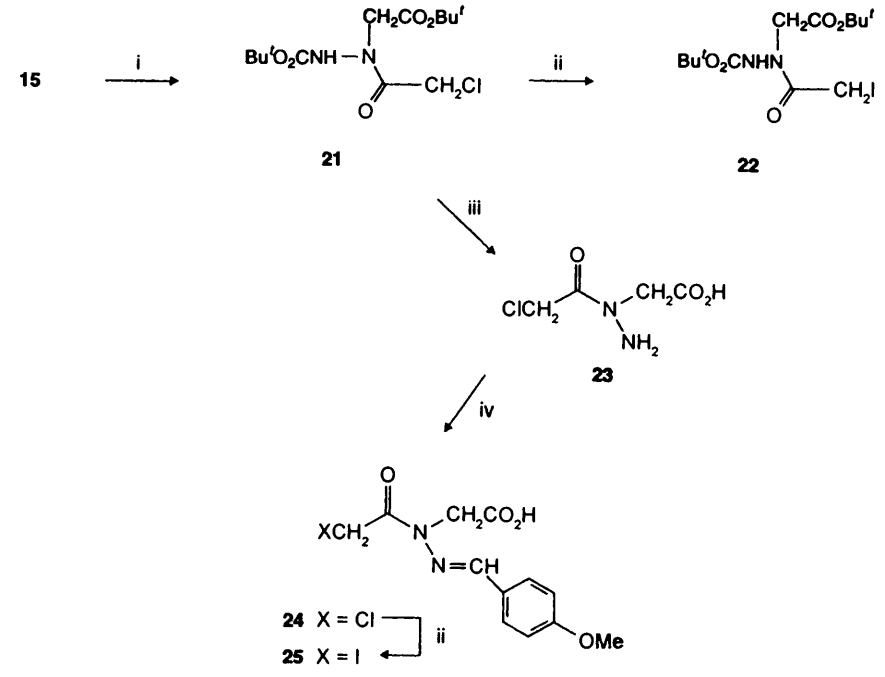
For preparation of an analogous iodoacetyl compound, some modification to the general approach discussed above was required. As shown in Scheme 3, the chloroacetyl compound **21** was readily obtained by acylation of the hydrazide **15** with chloroacetyl chloride, and displacement of the chlorine atom with sodium iodide in acetone cleanly gave the iodoacetyl compound **22**. However, exposure of this compound to trifluoroacetic acid, in the expectation of removing the two *tert*-butyl groups, gave a dark solution contaminated with free iodine. Attempts to isolate a crystalline hydrochloride salt were unsuccessful. We therefore sought an alternative route in which the iodine atom was introduced after deprotection and coupling of the resulting hydrazide with the aldehyde **13**. In model studies, the chloroacetyl compound **21** was deprotected with trifluoroacetic acid and the crude hydrazide **23** was immediately coupled with 4-methoxybenzaldehyde to give the chloroacetyl hydrazone **24**. Displacement with sodium iodide in acetone then gave the iodoacetyl hydrazone **25**. With these results in hand, completion of the synthesis of the target compounds **4** and **5** was straightforward *via* hydrazone formation between the aldehyde **13** and the respective hydrazides. Thus, compound **4** was obtained directly, while for compound **5** initial condensation of the aldehyde **13** and the chloroacetyl hydrazide **23** was followed by displacement with NaI–acetone.

As already mentioned, the effectiveness of the two compounds **4** and **5** in labelling cysteine side chains of proteins is likely to be influenced by their solubility in predominantly aqueous media, and we have assessed this parameter briefly. The two compounds had very different solubilities at pH 7 in a mixture of 10% DMF–90% aqueous buffer (see Experimental section for details). The maleimide **4** was soluble to at least 0.8 mmol dm^{-3}, while the iodoacetamide **5** was soluble only to 66 μmol dm^{-3}. These data suggest that the more soluble maleimide **4** will be preferable as a labelling reagent. Its ability to effect thiol-specific labelling of light chain 1 of rabbit skeletal myosin has been demonstrated[14] but the iodoacetamide **5** has not been used in comparable experiments.

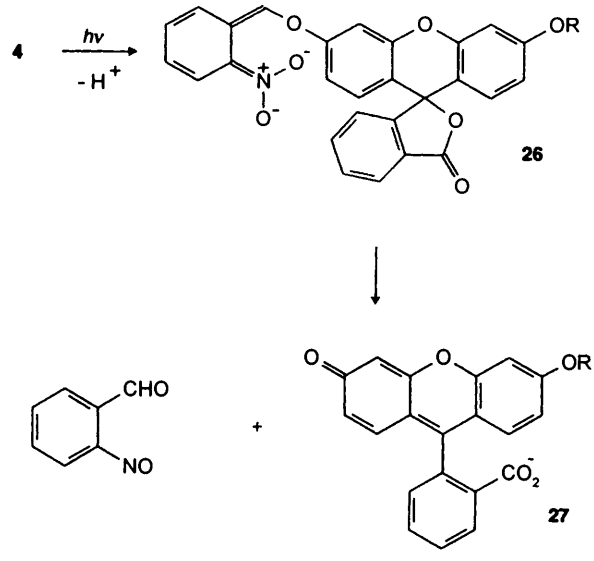
As with any photocleavable compound intended for use in biology, it is desirable to know the rate and efficiency of product formation upon flash photolysis,[6] and for compound **4**, time resolved measurements of fluorescence and absorbance were made following single pulse illumination from a 320 nm



Scheme 2 *Reagents:* i, $BrCH_2CO_2Bu^t$–Et_3N; ii, maleylglycyl chloride–Et_3N; iii, HCl–dioxane; iv, 4-$NO_2C_6H_4CHO$; v, TFA; vi, HCl–MeOH; vii, 4-$MeOC_6H_4CHO$



Scheme 3 *Reagents:* i, $ClCH_2COCl$–Et_3N; ii, NaI–acetone; iii, TFA; iv, 4-$MeOC_6H_4CHO$



Scheme 4 Minimal reaction pathway for photolysis of compound **4** and related derivatives. The functionalised side chain of compound **4** is abbreviated for conciseness

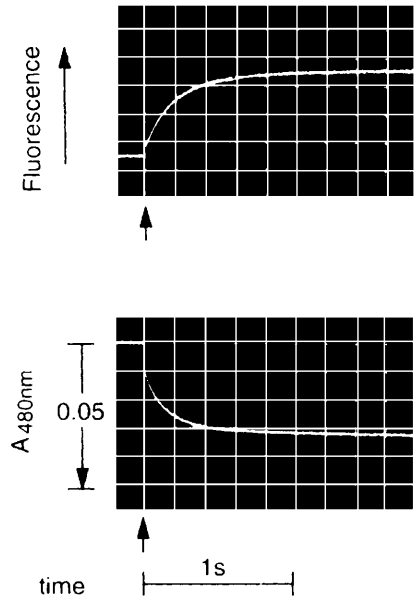


Fig. 1 Time-resolved (a) fluorescence emission and (b) absorbance at 480 nm following flash photolysis of compound **4**. The arrow marks the time of the laser flash

laser. The measurements were made in the presence of an excess of sodium 2-sulfanylethanesulfonate and hence the actual species in solution was that with sulfanylethanesulfonate added to the maleimide double bond of compound **4**. At pH 7.0 and 22 °C, the product quantum yield was 0.71, while the time courses of fluorescence and 480 nm absorbance changes following flash photolysis were as shown in Fig. 1. The 480 nm absorption signal arises from the absorption chromophore of the developing fluorophore. The absorbance transient was fitted by a single exponential with a rate constant of 7 s^{-1} but the fluorescence trace was not a clean exponential, although the initial rate was the same as that derived for the absorbance changes. It may be that internal quenching distorts the fluorescence signal as the concentration of released fluorophore increases. While these traces represent formation of the ultimate product **27**, time-resolved absorbance measurements at shorter wavelengths (406 or 440 nm) in an attempt to monitor the decay of the *aci*-nitro anion **26**, the normal type of intermediate in photolysis of 2-nitrobenzyl compounds, gave complex, multi-phasic time courses which were not readily interpreted in terms of the conventional[6,15] mechanistic pathway. Even in the traces shown, there are initial fast increases in both fluorescence and absorbance, followed by exponential evolution of the signals, and the relationship between the appearance of fluorescence and the chemical reactions of the molecules involved remains obscure. We plan to carry out model studies with simple derivatised phenols with the aim of defining more clearly the rate-determining step(s) of product release.

Finally in our studies of the chemistry of these photoactivatable fluorescein compounds, we have prepared the derivative **6**

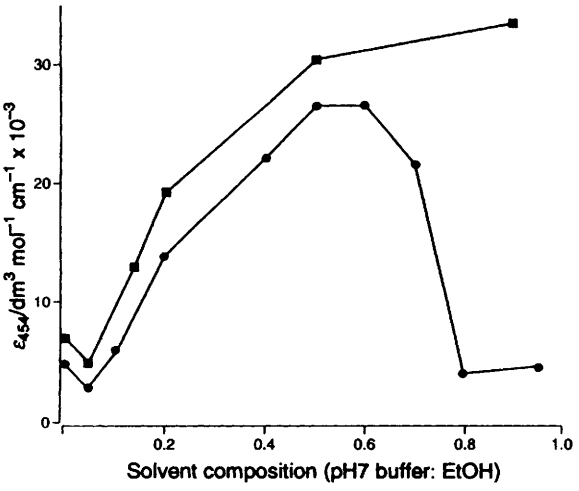
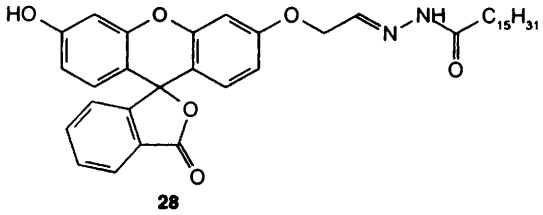


Fig. 2 Variation of extinction coefficient at 454 nm as a function of solvent composition for compounds **12** (■) and **28** (●)

containing an unbranched aliphatic chain, which could be expected to anchor the compound to a lipid membrane even after photocleavage of the nitrobenzyl ether and consequent development of negative charge on the fluorescein nucleus. Lipophilic fluorescent dyes have been extensively used in the related FRAP technique[4] for studies of mobility in cellular lipid membranes (see ref. 18 for examples). Preparation of compound **6** was readily realised by condensation of hexadecano-hydrazide and aldehyde **13**.

In order to characterise the fluorescence properties to be expected upon photocleavage of the nitrobenzyl ether in compound **6**, we have also prepared the expected photoproduct **28** by ozonolysis of the allyl ether **12** and reaction of the



28

resultant aldehyde with hexadecanohydrazide. The spectral properties of compound **28** require some comment. In common with other fluorescein monoethers, its long wavelength absorption in the region 450–480 nm when measured in absolute alcohol is weak but increases substantially upon addition of an aqueous buffer at neutral pH. This behaviour of fluorescein monoethers must be associated with a shift in the equilibrium between the non-absorbing lactone tautomer and the fluorescent open-chain form which has an extended π-system. As shown in Fig. 2, the long wavelength absorption of a simple fluorescein monoether such as the allyl ether **12** rises five-fold as the proportion of water in the solvent increases up to approx. 50% but is little changed thereafter as the solvent is made still more aqueous. The much more hydrophobic compound **28** closely follows this behaviour up to 50–60% aqueous content, but the absorbance declines sharply again beyond this point to a value similar to that seen in 100% ethanol. Addition of ethanol to the weakly absorbing, highly aqueous solution to restore a 1 : 1 aqueous ethanol composition regenerates the strong absorption.

These data suggest that in highly aqueous solutions, compound **28** undergoes a reversion to the lactone tautomer, possibly because of self-association into micelles. We have not further examined this behaviour here, but it indicates that careful control experiments would be needed before embarking upon FPD experiments with the photoactivatable compound **6**. It is noteworthy that the octyl ester of fluorescein has been reported to be so weakly acidic that its anion was not produced in significant concentration in aqueous solution, as observed by absorption spectroscopy.[19] An alternative explanation is that the same mechanism which suppresses the long-wavelength absorption of compound **28** also operates for the lipophilic octyl ester.

In conclusion, we have described the chemistry of a range of photoactivatable fluorescein derivatives with potential for a range of biological labelling applications. The highly functionalised hydrazine derivatives **19** and **23** may also be useful in other situations to mediate linkage of probe molecules to proteins.

Experimental

Elemental analyses were carried out by Butterworth Laboratories, Teddington, Middlesex and by the Chemical Analysis Centre, The University, Canterbury, Kent. ^{1}H NMR spectra were determined at 90 MHz on a JEOL FX90Q spectrometer, in $CDCl_3$ with tetramethylsilane as internal standard unless otherwise stated. *J* Values are given in Hz. Positive ion FAB mass spectra were run at low and high resolution on VG 70-250SE and ZAB-SE instruments respectively. Merck 9385 silica gel was used for flash chromatography. Light petroleum was the fraction boiling in the range 40–60 °C. Phosphate buffer solutions were prepared from NaH_2PO_4 at the molarity required and adjusted to the specified pH value with NaOH.

3'-Allyloxy-6'-hydroxyspiro[isobenzofuran-1(3*H*),9'(9'*H*)-xanthen]-3-one 12

A solution of allyl 2-(3-allyloxy-6-oxo-6*H*-xanthen-9-yl)benzoate[8] **11** (2.0 g, 4.85 mmol) in boiling acetone (100 cm^3) was treated with 1.25 mol dm^{-3} aq. NaOH (60 cm^3) and the mixture was heated under reflux for 12 min and then poured into water (200 cm^3). The solution was acidified with conc. HCl and the precipitated solid was filtered off, washed with water and recrystallised from ethanol to afford the monoether **12** as light yellow crystals (0.83 g, 46%), mp 206–207 °C (lit.,[8] 205 °C); λ_{max}/nm (EtOH) 226, 276, 453 and 479 (ε/dm^3 mol^{-1} cm^{-1} 58 200, 9400, 6600 and 5400); λ_{max}/nm [EtOH–10 mmol dm^{-3} Na phosphate pH 7 (1:9)] 232, 274, 454 and 476 (ε/dm^3 mol^{-1} cm^{-1} 40 900, 16 500, 30 600 and 29 000).

3'-Allyloxy-6'-(2-nitrobenzyloxy)spiro[isobenzofuran-1(3*H*),9'(9'*H*)-xanthen]-3-one 7

A solution of compound **12** (1.10 g, 2.91 mmol) and 2-nitrobenzyl bromide (1.02 g, 4.72 mmol) in dry benzene (22 cm^3) and dry THF (4 cm^3) was mixed with silver(I) oxide (1.40 g, 4.83 mmol) and heated under reflux in the dark for 16 h and then filtered. The solid was washed with EtOAc and the combined filtrate and washings were evaporated. The residue was subjected to flash chromatography [CH_2Cl_2–light petroleum (3:1)] to give the *title compound* **7** as almost colourless plates (0.94 g, 63%) from EtOAc–light petroleum, mp 171–172 °C (Found: C, 71.1; H, 4.2; N, 2.7. $C_{30}H_{21}NO_7$ requires C, 71.0; H, 4.2; N, 2.8%); λ_{max}(EtOH)/nm 225, 274 (ε/dm^3 mol^{-1} cm^{-1} 82 000 and 12 600); ν_{max}(Nujol)/cm^{-1} 1760, 1525, 1500, 1380, 1345, 1250, 1200, 1190 and 1105; δ_H 6.6–

7.3 (14 H, m, ArH), 5.8–6.3 (1 H, m, =CH), 5.53 (2 H, s, ArCH_2), 5.2–5.4 (2 H, m, =CH_2) and 4.76 (2 H, d, *J* 5.5, OCH_2).

***tert*-Butyl N^2-*tert*-Butoxycarbonylhydrazinoacetate 15**

A solution of *tert*-butyl carbazate (15.84 g, 120 mmol) and triethylamine (12.18 g, 120 mmol) in dry benzene (120 cm^3) was treated with *tert*-butyl bromoacetate (23.40 g, 120 mmol) and heated under reflux overnight. The solution was cooled and filtered, and the precipitate was washed with benzene. The combined filtrate and washings were washed with aq. $NaHCO_3$ and brine, and then evaporated. The residue was mixed with a solution of pyruvic acid (12 g) in ethanol (240 cm^3) and 4 mol dm^{-3} aq. NaOAc (30 cm^3) was added to the mixture. After 1 h at room temp. the mixture was diluted with ether and washed extensively with aq. $NaHCO_3$, dried (Na_2SO_4) and evaporated. The residue was distilled to give the *ester* **15** as a viscous, colourless liquid (10.88 g, 37%), bp 98–100 °C (0.07 mmHg) which solidified with time, mp 48–49 °C (Found: C, 54.0; H, 9.3; N, 10.95. $C_{11}H_{22}N_2O_4$ requires C, 53.6; H, 9.0; N, 11.4%); ν_{max}(Nujol)/cm^{-1} 3400, 3350, 3250, 1745, 1730 and 1710; δ_H 6.60 (2 H, br s, NH), 3.54 (2 H, s, CH_2) and 1.45 (18 H, s, Me).

***tert*-Butyl N^2-*tert*-Butoxycarbonyl-N^1-[(2,5-dihydro-2,5-dioxopyrrol-1-yl)acetyl]hydrazinoacetate 16**

Maleylglycine[12] (821 mg, 5.3 mmol) and redistilled thionyl chloride (16 cm^3) were heated under reflux for 0.5 h after which the excess of reagent was removed under reduced pressure. The residual acid chloride was evaporated twice with dry toluene to remove traces of HCl and then dissolved in dry ether (26 cm^3); this solution was then added at 0 °C to a stirred solution of the hydrazide **15** (1.30 g, 5.3 mmol) and triethylamine (588 mg, 5.8 mmol) in dry ether (26 cm^3). The mixture was stirred for 1 h at 0 °C and then diluted with ethyl acetate and washed with dilute hydrochloric acid, aq. $NaHCO_3$ and water, dried (Na_2SO_4) and evaporated under reduced pressure. The residue crystallised from EtOAc–light petroleum to give the *maleimide* **16** as plates (1.67 g, 82%), mp 157–159 °C (Found: C, 53.1; H, 6.7; N, 10.8. $C_{17}H_{25}N_3O_7$ requires C, 53.25; H, 6.6; N, 11.0%); ν_{max}(Nujol)/cm^{-1} 3370, 3085, 1755, 1730, 1710, 1680, 1155, 850 and 695; δ_H 7.15 (1 H, br s, NH), 6.78 (2 H, s, CH=CH), 4.47 (4 H, s, 2 × CH_2), 1.50 (9 H, s, Me) and 1.45 (9 H, s, Me).

Deprotection of the maleimide 16

(a) Compound **16** (393 mg, 1 mmol) was dissolved in a solution of 2 mol dm^{-3} hydrogen chloride in dry dioxane (3.5 cm^3) and the mixture kept at room temp. for 40 min; it was then diluted with diisopropyl ether (5 cm^3) and allowed to stand for a further 2 h. Colourless needles slowly separated and were filtered off, washed with diisopropyl ether and dried *in vacuo* to give the crude *hydrochloride salt* **17** (137 mg, 44%), mp 78–80 °C (decomp.). For characterisation, a portion (40 mg) was dissolved in a mixture of EtOH (0.3 cm^3) and 2 mol dm^{-3} aq. NaOAc (0.06 cm^3) and added to a solution of 4-nitrobenzaldehyde (15 mg) in DMF (0.2 cm^3). After 2 h at room temp., the solution was diluted with EtOAc, washed with dilute hydrochloric acid and brine, dried (Na_2SO_4) and evaporated under reduced pressure. The residue was flash chromatographed [EtOAc–light petroleum (1:3)] to give the *hydrazone* **18** (29 mg) as needles (from EtOAc–light petroleum), mp 192–193 °C (Found: C, 54.6; H, 4.9; N, 13.45. $C_{19}H_{20}N_4O_7$ requires C, 54.8; H, 4.8; N, 13.45%); δ_H 8.27 (2 H, d, *J* 8.5, 3,5-ArH), 7.82 (2 H, d, 2,6-ArH), 7.61 (1 H, s, CH=N), 6.82 (2 H, s, CH=CH), 4.93 (2 H, s, CH_2), 4.68 (2 H, s, CH_2) and 1.46 (9 H, s, CMe_3).

(b) A solution of compound **16** (200 mg, 0.52 mmol) in trifluoroacetic acid (1.5 cm^3) was kept at room temp. for 1 h after which it was evaporated under reduced pressure. The residue was dissolved in a solution of 2 mol dm^{-3} hydrogen chloride in dry dioxane (2 cm^3) and from which colourless crystals separated after a few minutes. The mixture was diluted with diisopropyl ether (5 cm^3) after which the solid present was filtered off and washed with diisopropyl ether to give the crude *hydrochloride salt* **19** as a dioxane solvate (0.26 g, 73%); δ_H(D_2O, acetone ref.) 6.95 (2 H, s, CH=CH), 4.37 (4 H, s, 2 × CH_2) and 3.74 (8 H, s, dioxane). For characterisation, the hydrochloride **19** (137 mg, 0.39 mmol) was dissolved in EtOH (1.2 cm^3) and 2 mol dm^{-3} aq. NaOAc (0.23 cm^3) and the solution mixed with a solution of 4-methoxybenzaldehyde (56 mg, 0.41 mmol) in DMF (1 cm^3). After 2 h at room temp., the solution was diluted with EtOAc and washed with dilute aq. HCl and brine, dried (Na_2SO_4) and evaporated under reduced pressure. The residue crystallised when treated with a little ether, and was recrystallised from EtOAc–light petroleum to give the *hydrazone* **20** as colourless fibrous aggregates (72 mg), mp 183–184 °C (Found: C, 55.6; H, 4.45; N, 12.0. $C_{16}H_{15}N_3O_6$ requires C, 55.65; H, 4.4; N, 12.2%); δ_H([$^2H_6$]-DMSO) 7.89 (1 H, s, CH=N), 7.73 (2 H, d, *J* 8.8, 3,5-ArH), 7.12 (2 H, s, CH=CH), 6.99 (2 H, d, 2,6-ArH), 4.75 and 4.72 (4 H, 2 × s, 2 × CH_2) and 3.81 (3 H, s, OMe).

***tert*-Butyl N^2-*tert*-Butoxycarbonyl-N^1-iodoacetylhydrazinoacetate 22**

A solution of chloroacetyl chloride (226 mg, 2 mmol) in dry ether (5 cm^3) was added at 0 °C to a stirred solution of *tert*-butyl N^2-*tert*-butoxycarbonylhydrazinoacetate **15** (492 mg, 2 mmol) and triethylamine (222 mg, 2.2 mmol) in dry ether (10 cm^3). After 1 h at 0 °C, the mixture was diluted with EtOAc, washed with dilute hydrochloric acid and aq. $NaHCO_3$, dried (Na_2SO_4) and evaporated under reduced pressure, to afford the crude chloroacetyl compound **21** as a pale gum (630 mg, 97%); δ_H 7.14 (1 H, br s, NH), 4.24 (4 H, s, 2 × CH_2), 1.47 (9 H, s, Me), and 1.45 (9 H, s, Me). Without further purification, the chloroacetyl compound **21** (620 mg) was dissolved in a solution of sodium iodide (2.3 g) in acetone (14 cm^3) and kept overnight at room temp. The mixture was diluted with ether, washed with water and aq. sodium thiosulfate, dried (Na_2SO_4) and evaporated under reduced pressure. The residue crystallised from light petroleum to give the *title compound* **22** as colourless needles (0.46 g, 56%), mp 90–91 °C (Found: C, 37.6; H, 5.5; N, 6.7. $C_{13}H_{23}IN_2O_5$ requires C, 37.7; H, 5.6; N, 6.8%); δ_H 6.89 (1 H, br s, NH), 3.83 (4 H, s, 2 × CH_2), 1.50 (9 H, s, Me) and 1.49 (9 H, s, Me).

N^2-(4-Methoxybenzylidene)-N^1-(chloroacetyl)hydrazinoacetic acid 24

A solution of the crude chloroacetyl compound **21** (161 mg, 0.5 mmol), prepared as above, in trifluoroacetic acid (1.5 cm^3) was kept at room temp. for 1 h and then evaporated under reduced pressure to leave the crude compound **23**. This was dissolved in EtOH (1 cm^3) and 2 mol dm^{-3} aq NaOAc (0.03 cm^3) and treated with a solution of 4-methoxybenzaldehyde (68 mg, 0.5 mmol) in DMF (1 cm^3). After 1 h at room temp. the solution was diluted with EtOAc, washed with dilute hydrochloric acid and water, dried (Na_2SO_4) and evaporated under reduced pressure. The residue crystallised from EtOAc–light petroleum to give the *title compound* **24** as colourless plates (77 mg, 54%), mp 171–173 °C (decomp.) (Found: C, 50.5; H, 4.4; N, 9.7. $C_{12}H_{13}ClN_2O_4$ requires C, 50.6; H, 4.6; N, 9.8%); δ_H([$^2H_6$]-DMSO) 7.90 (1 H, s, CH=N), 7.73 (2 H, d, *J* 8.8, 3,5-ArH), 7.00 (2 H, d, 2,6-ArH), 4.91 (2 H, s, CH_2Cl), 4.75 (2 H, s, NCH_2) and 3.80 (3 H, s, OMe).

N^2-(4-Methoxybenzylidene)-N^1-(iodoacetyl)hydrazinoaceticacid 25

A solution of compound **24** (77 mg) and sodium iodide (1.5 g) in

acetone (10 cm^3) was kept overnight at room temp. and then diluted with EtOAc, washed with dilute hydrochloric acid and aq. sodium thiosulfate, dried (Na_2SO_4) and evaporated under reduced pressure. The residue was crystallised from EtOAc–light petroleum to give the title compound **25** as needles (39 mg), mp 155–156 °C (decomp.) (Found: C, 38.0; H, 3.25; N, 7.4. $C_{12}H_{13}IN_2O_4$ requires C, 38.3; H, 3.5; N, 7.45%); δ_H([$^2H_6$]-DMSO) 7.86 (1 H, s, CH=N), 7.72 (2 H, d, *J* 8.8, 3,5-ArH), 7.01 (2 H, d, 2,6-ArH) 4.71 (2 H, s, NCH_2), 4.23 (2 H, s, CH_2I) and 3.80 (3 H, s, OMe).

3′-(2-Nitrobenzyloxy)-6′-(2-oxoethoxy)spiro[isobenzofuran-1(3*H*),9′(9′*H*)-xanthen]-3-one 13

A solution of the allyl ether **7** (102 mg, 0.2 mmol) in dichloromethane (20 cm^3) and methanol (0.4 cm^3) was cooled to −70 °C and treated with a stream of ozonised oxygen until all the starting material was consumed [TLC analysis, EtOAc–light petroleum (1:1)]. The solution was purged with nitrogen, treated with dimethyl sulfide (0.2 cm^3) and allowed to warm to room temp. over 1 h. It was then kept for a further 1 h and then diluted with CH_2Cl_2, washed with water and brine, dried (Na_2SO_4) and evaporated under reduced pressure. The residue was purified by flash chromatography [EtOAc–light petroleum (1:1)] to afford the aldehyde **13** as a gum (82 mg, 80%); ν_{max}($CHCl_3$)/cm^{-1} 1765, 1745, 1635, 1615, 1530, 1500, 1425, 1345, 1250 and 1110; δ_H 9.80 (1 H, t, *J* 0.9, CHO), 6.62–8.24 (14 H, m, ArH), 5.49 (2 H, s, $ArCH_2$) and 4.59 (2 H, d, CH_2CHO).

6′-(2-{*N*-[*N*-Carboxymethyl(2,5-dioxo-2,5-dihydropyrrol-1-yl)-acetamido]imino}ethoxy)-3′-(2-nitrobenzyloxy)spiro[isobenzofuran-1(3*H*),9′(9′*H*)-xanthen]-3-one 4

The aldehyde **13** (82 mg) was dissolved in DMF (0.4 cm^3) and treated with a solution of the maleimide **19** (75 mg, 0.215 mmol) in EtOH (0.65 cm^3) and 2 mol dm^{-3} aq. NaOAc (0.125 cm^3). After 2 h at room temp., the solution was diluted with ethyl acetate and washed with 1 mol dm^{-3} aq. citric acid and brine, dried (Na_2SO_4) and evaporated under reduced pressure. The residue was purified by flash chromatography [EtOAc–MeOH–HOAc (98:2:0.5)] to afford the *title compound* **4** as a gum (84 mg, 58%) (Found: M^+, 719.1622. $C_{37}H_{26}N_4O_{12}$ + H requires *M*, 719.1626); ν_{max}(MeCN)/cm^{-1} 1765, 1740, 1720, 1640, 1615, 1530, 1500, 1345, 1245, 1105 and 695; δ_H([$^2H_6$]-acetone) 7.20–8.23 (8 H, m, ArH), 7.40 (1 H, t, *J* 5.5, CH=N), 6.97 (2 H, d, *J* 7, 1′,8′-H), 6.95 (2 H, s, CH=CH), 6.83 (2 H, dd, *J* 2, 2′,7′-H), 6.76 (2 H, d, 4′,5′-H), 5.59 (2 H, s, $ArCH_2O$), 4.94 (2 H, d, CH_2CH=), 4.74 and 4.69 (4 H, 2 × s, 2 × CH_2N).

6′-(2-{*N*-[*N*-Carboxymethyl(iodo)acetamido]imino}ethoxy)-3′-(2-nitrobenzyloxy)spiro[isobenzofuran-1(3*H*),9′(9′H)-xanthen]-3-one 5

A solution of the protected chloroacetyl compound **21** (71 mg, 0.21 mmol) in trifluoroacetic acid (0.66 cm^3) was kept at room temp. for 1 h and then evaporated under reduced pressure. The residual hydrazide **23** dissolved in ethanol (0.66 cm^3) and 2 mol dm^{-3} aq. sodium acetate (0.13 cm^3) was then added to a solution of the aldehyde **13** (91 mg) in DMF (0.5 cm^3). After 2 h at room temp. the solution was diluted with EtOAc and washed with dilute hydrochloric acid and water. The EtOAc solution was dried (Na_2SO_4) and evaporated under reduced pressure to leave a yellow gum which was purified by flash chromatography [EtOAc–MeOH–HOAc (90:10:0.5)]. The recovered material was dissolved in acetone (5 cm^3) containing sodium iodide (0.75 g) and the solution was kept overnight at room temp. and then diluted with EtOAc, washed with water and aq. sodium thiosulfate, dried (Na_2SO_4) and evaporated under reduced pressure. The residual material was purified by flash chromatography [EtOAc–MeOH–HOAc (90:10:1)] to give the *title compound* **5** as a foam (86 mg) (Found, M^+, 750.0582. $C_{33}H_{24}IN_3O_{10}$ + H requires *M*, 750.0585); ν_{max}($CHCl_3$)/cm^{-1} 1760, 1690, 1635, 1625, 1530, 1500, 1425, 1345, 1250, 1180 and 1100; δ_H 6.63–8.24 (15 H, m, ArH and CH=N), 5.48 (2 H, s, $ArCH_2O$), 4.75 (2 H, d, *J* 4.4, CH_2CH, 4.65 (2 H, s, NCH_2) and 4.15 (2 H, s, CH_2I).

6′-{2-[(*N*-Hexadecamido)imino]ethoxy}-3′-(2-nitrobenzyloxy)-spiro[isobenzofuran-1(3*H*),9′(9′*H*)-xanthen]-3-one 6

The crude aldehyde **13** (40 mg) was mixed with a solution of hexadecanohydrazide[20] (40 mg) in ethanol (0.9 cm^3) and THF (0.3 cm^3) and the mixture heated under reflux for 1 h. After this it was evaporated and the residue purified by flash chromatography [EtOAc–CH_2Cl_2 (15:85)] to afford the title compound **6** as an almost colourless gum (46 mg) (Found: M^+, 762.3751. $C_{45}H_{51}N_3O_8$ + H requires *M*, 762.3754); ν_{max}($CHCl_3$)/cm^{-1} 2920, 2845, 1765, 1680, 1630, 1615, 1525, 1500, 1425, 1345, 1180 and 1110; δ_H 9.92 (1 H, s, NH), 6.61–8.28 (14 H, m, ArH) superimposed on 7.36 (1 H, t, *J* 5.3, CH=N), 5.52 (2 H, s, $ArCH_2O$) 4.69 (2 H, d, CH_2CH=), 1.08–2.71 [28 H, m, $(CH_2)_{14}$] and 0.86 (3 H, t, *J* 7, CH_3).

6′-{2-[(*N*-Hexadecamido)imino]ethoxy}-3′-hydroxyspiro-[isobenzofuran-1(3*H*),9′(9′*H*)-xanthen]-3-one 28

A solution of compound **12** (111 mg, 0.3 mmol) in CH_2Cl_2 (30 cm^3) and MeOH (1 cm^3) was cooled to −70 °C, treated with a stream of ozonised oxygen until all the starting material was consumed [TLC analysis, EtOAc–$CHCl_3$ (60:40)] and then purged with nitrogen and treated with dimethyl sulfide (0.5 cm^3). The mixture was allowed to warm to room temp. over 1 h and kept for a further 1 h, after which it was evaporated under reduced pressure. The residue was subjected to flash chromatography [EtOAc–$CHCl_3$ (55:45)] and the recovered aldehyde was mixed with hexadecanohydrazide[20] (120 mg, 0.44 mmol) in EtOH (2.7 cm^3) and THF (0.9 cm^3). The solution was heated under reflux for 1 h and then cooled and evaporated. The residue was purified by flash chromatography [EtOAc–$CHCl_3$ (30:70)] to afford a powder (93 mg) after trituration with ethanol. Two crystallisations from ethanol gave the title compound **28** as pale yellow prisms, mp 113–114 °C (Found: C, 71.8; H, 8.0; N, 4.1%; M^+, 627.6. $C_{38}H_{46}N_2O_6 \cdot 1/3H_2O$ requires C, 72.1; H, 7.4; N, 4.4%; $C_{38}H_{46}N_2O_6$ + H requires *M*, 627.3); λ_{max}(EtOH)/nm 276, 452, 479 (ε/dm^3 mol^{-1} cm^{-1} 10 400, 6100 and 4900); λ_{max}[EtOH–10 mmol dm^{-3} Na phosphate, pH 7 (1:1)]/nm 276, 454, 479 (ε/dm^{-3} mol^{-1} cm^{-1} 15 500, 26 600 and 22 400); δ_H 9.32 (1 H, s, NH), 6.51–8.05 (11 H, m, ArH and CH=N), 4.67 (2 H, d, *J* 5, CH_2CH), 2.62 (2 H, t, *J* 7, CH_2CO), 1.16–2.33 [26 H, m, $(CH_2)_{13}$] and 0.86 (3 H, t, *J* 7, CH_3).

Aqueous solubility of compounds 4 and 5

Solutions (8 mmol dm^{-3}) of compounds **4** and **5** in DMF were diluted 10-fold with 10 mmol dm^{-3} sodium 3-morpholinopropane-1-sulfonate, pH 7.0 and kept at room temp. for 1 h. The mixtures were centrifuged (13 000 rpm) to pellet precipitated material and aliquots of the supernatants were diluted 20-fold in EtOH. The absorbance of the diluted solutions was measured at 274 nm to determine the concentrations of reagents in the supernatant solutions. For compounds **4** and **5** these concentrations were 0.79 and 0.066 mmol dm^{-3}, respectively, corresponding to recovery of 99 and 8% of the concentrations initially added.

Photolysis kinetics of compound 4

Details of the time-resolved spectrophotometer and its set-up for absorbance and fluorescence measurements were essentially as previously described[15] except that a quartz–iodine lamp was

used as the source for the measuring light, with photolysis being initiated by a light pulse from a Candela 1050 dye laser. For fluorescence measurements the excitation light was passed through a Wratten filter with maximum transmission at 435 nm and a 45 nm bandpass at 50% transmission, while the emitted light was collected *via* a 510 nm Schott cut-off filter. For absorbance measurements, the solution for photolysis contained compound **4** (0.44 mmol dm^{-3}) and sodium 2-sulfanylethanesulfonate (3 mmol dm^{-3}) in 50 mmol dm^{-3} Na phosphate, pH 7.0. For fluorescence measurements the above solution was diluted 100-fold in the same phosphate buffer.

Extent of photolysis of compound 4

A solution containing P^3-1-(2-nitrophenyl)ethyladenosine triphosphate[15] (39 μmol dm^{-3}), compound **4** (46 μmol dm^{-3}) and sodium 2-sulfanylethanesulfonate (2 mmol dm^{-3}) in 30 mmol dm^{-3} Na phosphate, pH 7.0 was exposed for varying times to light from a mercury arc lamp which was passed through a Hoya 340 filter prior to illuminating the optical cuvette. The irradiated solutions were analysed by reversed-phase HPLC [Merck Lichrosphere RP8 column (Cat. No. 50832); mobile phases 10 mmol dm^{-3} Na phosphate (pH 5.5)–MeOH (87:13) and 10 mmol dm^{-3} Na phosphate (pH 5.5)–MeOH (1:1) for analysis of the residual ATP ester and compound **4**, respectively; flow rate 1.5 cm^3 min^{-1}; UV detection at 254 nm]. P^3-1-(2-Nitrophenyl)ethyl adenosine triphosphate was eluted at 1.9 min and compound **4** at 4.6 min in the respective mobile phases. Compound **4** was found to be photoconverted 1.13-fold more efficiently than the ATP derivative (for which the reported[15] product quantum yield is 0.63), corresponding to a product quantum yield for compound **4** of 0.71.

Acknowledgements

We are grateful to Dr S. Howell (NIMR) and Dr D. Carter (School of Pharmacy) for the mass spectrometric measurements and to Dr M. A. Ferenczi for help with the kinetic simulations.

References

1 B. Ware, L. J. Brvenik, R. T. Cummings, R. H. Furukawa and G. A. Krafft, in *Applications of Fluorescence in the Biomedical Sciences*, eds. D. L. Taylor, A. S. Waggoner, R. F. Murphy, F. Lanni and R. R. Birge, Alan R. Liss, New York, 1986, pp. 141–157.
2 (*a*) T. J. Mitchison, *J. Cell Biol.*, 1989, **109**, 637; (*b*) S. S. Reinsch, T. J. Mitchison and M. Kirschner, *J. Cell Biol.*, 1991, **115**, 365; (*c*) K. E. Sawin and T. J. Mitchison, *J. Cell Biol.*, 1991, **112**, 941; (*d*) J. A. Theriot and T. J. Mitchison, *Nature* (*London*), 1991, **352**, 126; (*e*) J. A. Theriot, T. J. Mitchison, L. G. Tilney and D. A. Portnoy, *Nature* (*London*), 1992, **357**, 257; (*f*) S. Okabe and N. Hirokawa, *J. Cell Biol.*, 1992, **117**, 105.
3 L. Lilge, T. J. Flotte, I. E. Kochvar, S. L. Jacques and F. Hillenkamp, *Photchem. Photobiol.*, 1993, **58**, 37.
4 R. Peters, J. Peters, K. H. Tews and W. Bahr, *Biochim. Biophys. Acta*, 1974, **367**, 282; K. Jacobsen, E. Wu and G. Poste, *Biochim. Biophys. Acta*, 1976, **433**, 215; D. Axelrod, E. Schlessinger, E. Elson and W. Webb, *Biophys. J.*, 1976, **16**, 1005; W. Webb, *Ann. N.Y. Acad. Sci.*, 1981, **366**, 300.
5 (*a*) R. T. Cummings and G. A. Krafft, *Tetrahedron Lett.*, 1988, **29**, 65; (*b*) R. T. Cummings, J. P. DiZio and G. A. Krafft, *Tetrahedron Lett.*, 1988, **29**, 69; (*c*) G. A. Krafft, W. R. Sutton and R. T. Cummings, *J. Am. Chem. Soc.*, 1988, **110**, 301.
6 J. E. T. Corrie and D. R. Trentham, in *Bioorganic Photochemistry*, ed. H. Morrison, Wiley, New York, 1993, vol. 2, p. 243.
7 F. Friedrich, K. Kotte and H. Kühnstedt, *DDR Patent* 136 498 (*Chem. Abstr.*, 1980, **92**, 11220).
8 C. D. Hurd and L. Schmerling, *J. Am. Chem. Soc.*, 1937, **59**, 113.
9 L. K. Andreeva, L. G. Derkach and L. A. Kheifits, *Zh. Org. Khim.*, 1978, **14**, 1285.
10 O. Keller and J. Rudinger, *Helv. Chim. Acta*, 1975, **58**, 531.
11 W. Streicher and H. Reinshagen, *Chem. Ber.*, 1975, **108**, 813.
12 D. H. Rich, P. D. Gesellchen, A. Tong, A. Cheung and C. K. Buckner, *J. Med. Chem.*, 1975, **18**, 1004.
13 L. Paul, A. Dittmer and C. Rusch, *Chem. Ber.*, 1967, **100**, 2757.
14 J. E. T. Corrie, Y. E. Goldman and D. R. Trentham *Biophys. J.*, 1991, **59**, 359a.
15 J. W. Walker, G. P. Reid, J. A. McCray and D. R. Trentham, *J. Am. Chem. Soc.*, 1988, **110**, 7170.
16 J. E. T. Corrie, A. DeSantis, Y. Katayama, K. Khodhakah, J. B. Messenger, D. C. Ogden and D. R. Trentham, *J. Physiol.*, 1993, **465**, 1.
17 L. Lindqvist, *Ark. Kemi*, 1960, **16**, 79; M. M. Martin and L. Lindqvist, *J. Luminescence*, 1975, **10**, 381.
18 R. Peters, *Cell. Biol. Int. Rep.*, 1981, **5**, 733; M. L. Greenberg and D. Axelrod, *J. Membr. Biol.*, 1993, **131**, 115; T. Bultmann, W. L. Vaz, E. C. Melo, R. B. Sisk and T. E. Thompson, *Biochemistry*, 1991, **30**, 5573.
19 L. Brown, P. J. Halling, G. A. Johnston, C. L. Suckling and R. H. Valivety, *J. Chem. Soc., Perkin Trans. 1*, 1990, 3349.
20 T. Curtius and F. H. Dellschaft, *J. Prakt. Chem.*, 1901, **64**, 419.

Paper 5/01715G
Received 17*th March* 1995
Accepted 18*th May* 1995