Thiol-reactive Fluorescent Probes for Protein Labelling

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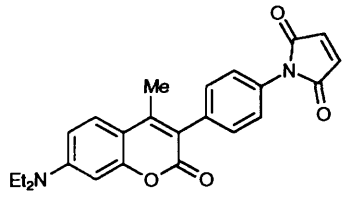
Cyclisation of *N*-alkylmaleamic acids mediated by acetic anhydride in dimethylacetamide in the presence of traces of cobalt naphthenate has been used for efficient assembly of a range of fluorescent maleimide reagents. The fluorescence responses of these reagents to addition of thiol across the maleimide double bond, and to hydrolysis of the maleimide ring, are described.

The bacterium *Escherichia coli* contains in its periplasm a protein which binds inorganic phosphate with high affinity and specificity.[1] The crystal structure of the phosphate-bound form has been solved to high resolution[2] and shows two domains between which the phosphate-binding cleft lies. In a programme to develop a real-time assay for inorganic phosphate in biological preparations, other workers in these laboratories have used oligonucleotide-directed mutagenesis to change to cysteine individual amino acids at various points in the structure, thereby producing a range of single cysteine mutants which could be specifically labelled with fluorescent reagents bearing maleimido or iodoacetyl substituents.[3] When commercially available fluorescent reagents were used to label six different mutant proteins, the best combination found was the A197C mutant labelled with 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin[4] **1**. The labelled protein showed



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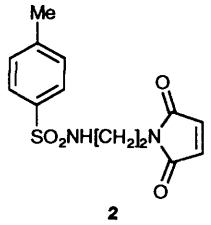

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a maximum 65% increase in fluorescence between the phosphate-free and phosphate-bound forms.[3]

We became interested in this work in the hope that by synthesising new fluorescent labelling reagents, it would be possible to improve the overall performance of the phosphate assay. In the event one of the new reagents, 7-diethylamino-*N*-(2-maleimidoethyl)coumarin-3-carboxamide **11a**, has been found substantially to improve both the fluorescence properties of the labelled A197C protein (5–6-fold fluorescence enhancement depending on conditions of pH and ionic strength) and its binding affinity for phosphate.[3] The present paper describes the synthesis of the new reagent, together with a number of other labelling reagents for use with mutant phosphate-binding proteins. In view of the continued widespread use of fluorescence-based techniques in biology, these reagents may also find other applications.

The functional groups most widely used to facilitate selective alkylation of thiol groups in proteins are *N*-substituted maleimides or iodoacetamides,[5] of which maleimides are the more reactive and this group is used in all but one of the labelling reagents reported here. In most commercially available fluorescent maleimide probes the maleimido group is attached directly to an aromatic ring (see, *e.g.*, ref. 6). However, to facilitate synthesis of analogues with variable distances between the protein and the fluorophore, we wished to use aliphatic spacer arms between the maleimide and the fluorophore. In addition, it seemed possible that the presence of a flexible link in the reagents could be beneficial, since it could allow a probe to take up its most favourable position on the protein more readily than if it were constrained as part of a more rigid structure.

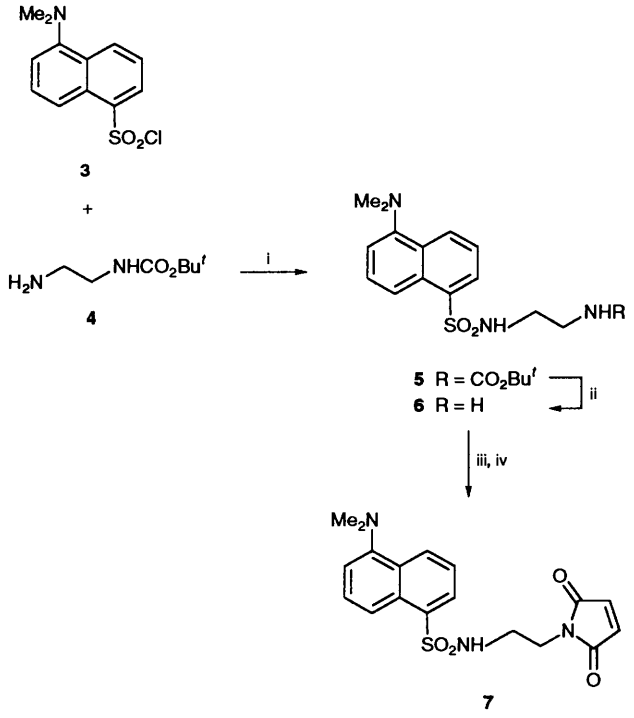
Although synthesis of *N*-arylmaleimides by cyclisation of the corresponding substituted maleamic acid generally proceeds well, synthesis of *N*-alkylmaleimides is less satisfactory, as evidenced by the range of methods which give only poor to moderate yields.[7–9] Therefore at the outset of the present work we were concerned to establish a reasonably efficient means to construct *N*-alkylmaleimides, preferably under mild conditions which would be compatible with a range of fluorophores, and the maleimide **2** was chosen as a convenient model system.

Results and Discussion

Attempts to cyclise the maleamic acid derived from treatment of mono-*N*-tosylethylenediamine[10] with maleic anhydride in acetic acid, using fused sodium acetate in hot acetic anhydride,[7] gave only traces of the maleimide **2**. Better results were obtained using a patented one-pot procedure[11] in which an amine is treated with maleic anhydride in dimethylacetamide (DMA), and the resulting solution of the *N*-substituted maleamic acid is treated first with a small proportion of a metal salt, followed by acetic anhydride, and heated at 70–80 °C to effect cyclisation. The metal salt preferred in the published work was cobalt naphthenate. The role of the metal ion was not discussed, although it presumably acts as a coordinating template to assist the cyclisation. The original publication noted a 4-fold reduction in yield when the metal ion was omitted, and we obtained similar results. However, when the reaction was performed using the suggested proportion of cobalt naphthenate (maleic anhydride : cobalt ~ 70 : 1 molar ratio) the product maleimide **2**, obtained in 42% yield, was contaminated by a deeply pink coloured impurity, which was not readily removed by crystallisation. When the molar proportion of cobalt was decreased ten-fold (*i.e.*, anhydride : cobalt ~ 700 : 1) the yield of crystalline product, still faintly coloured, was improved to 57%. The coloured impurity could be readily removed by chromatography. Cobalt naphthenate has extensive industrial application in catalysis of hydrocarbon oxidation and of polymerisation processes, but seems not to have been widely investigated for other catalytic functions. As a cheap, commercially available and organosoluble form of cobalt it may merit further attention. Recently, Pattenden and co-workers[12] have advocated one-pot reaction of ammonium acetates with maleic anhydride in boiling acetic acid, although use of only unsubstituted and monomethylammonium acetates was described. We briefly compared their method with the modified cobalt naphthenate procedure using benzylamine as a model. Crude yields from the two

procedures were 56 and 82%, respectively, with the cobalt-catalysed procedure giving the higher yield.

With a means in hand to prepare *N*-alkylated maleimides in fair yield and under mild conditions, it was possible to proceed with synthesis of the desired reagents, using a general route exemplified by synthesis of the dansyl maleimide **7** in Scheme 1,

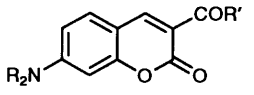


Scheme 1 *Reagents:* i, Et_3N; ii, TFA; iii, maleic anhydride; iv, cobalt naphthenate–Ac_2O

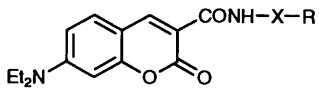
in which the fluorophore was coupled at one end of an α,ω-diamine and the maleimide was constructed on the remaining free amine. Preparation of the required amine **6** has been reported[13] by reaction of dansyl chloride **3** with excess of ethylenediamine, but in our hands the product was consistently contaminated with the compound in which both amino groups had been derivatised. Here and in subsequent related reactions the problem was eliminated by using the mono-*tert*-butoxycarbonyl compound **4**,[14,15] which reacted with dansyl chloride to give the sulfonamide **5**. Deprotection of compound **5** by treatment with trifluoroacetic acid (TFA) gave in pure form the monoamine **6**, which was smoothly converted by sequential treatment with maleic anhydride and cobalt naphthenate–acetic anhydride into the maleimide **7** in 50% yield.

The route shown in Scheme 1 was next used with appropriate modifications to synthesise the coumarin maleimide **11a**. Thus 7-diethylaminocoumarin-3-carboxylic acid **9a** was activated with isobutyl chloroformate and the mixed anhydride was coupled with the ethylenediamine derivative **4** to give the amide **10a**. Deprotection with TFA and conversion into the maleimide **11a** proceeded as previously.

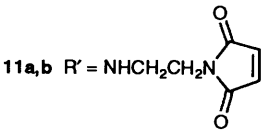
As discussed above, the maleimide **11a** labelled the phosphate-binding protein to great advantage and this result stimulated the synthesis of further analogues, with two aims in view. First, it seemed possible that even better properties (*e.g.* Stokes shift, fluorescence enhancement) of the labelled phosphate-binding protein might be achieved, and secondly we hoped that some insight might be gained into the mechanism underlying the large fluorescence increment observed when phosphate binds to the protein labelled with meleimide **11a**. The analogues initially investigated were those with propane-1,3-diyl or *p*-phenylene

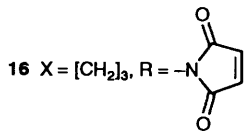


8a,b R′ = OEt
9a,b R′ = OH
10a,b R′ = $NHCH_2CH_2NHCO_2Bu^t$

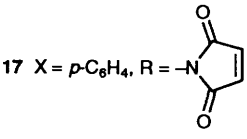


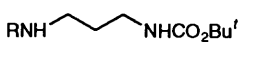
14 X = $[CH_2]_3$, R = $NHCO_2Bu^t$
15 X = *p*-C_6H_4, R = $NHCO_2Bu^t$



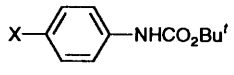


12a R′ = $NHCH_2CH_2NHCOCH_2Cl$
13a R′ = $NHCH_2CH_2NHCOCH_2I$
a R = Et
b R = Me





18 R = CO_2CH_2Ph
19 R = H



20 X = NO_2
21 X = NH_2

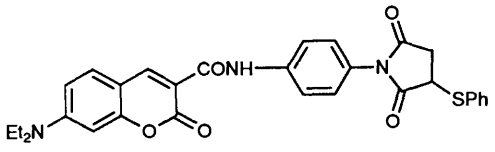
linkers (compounds **16** and **17** respectively). For the propane-1,3-diyl-linked compound, the monoprotected amine **19** was prepared as for its analogue **4** *via* hydrogenolysis of the benzyloxy derivative **18**, while the required phenylene linker **21** was readily prepared by hydrogenation of the nitro compound **20**, itself conveniently prepared by Curtius rearrangement of 4-nitrobenzoyl azide followed by addition of *tert*-butyl alcohol to the intermediate isocyanate.

Construction of the trimethylene compound **16** followed exactly the route for its ethylene analogue **11a**, while the route for the phenylene compound **17** differed only in the conventional use of acetic anhydride–sodium acetate to close the maleimide ring. The phenylene maleimide **17** was very insoluble in most solvents and for purposes of characterisation was converted into its more soluble thiophenol adduct **22**. Somewhat unexpectedly, when the phosphate-binding protein was labelled with either compound **16** or **17**, the labelled proteins both showed only small negative changes in fluorescence (−4 and −22%, respectively) between the phosphate-free and phosphate-bound states.[3] This result suggests that further changes to the length of the spacer arm in these reagents are unlikely to yield significant benefits. However, we have prepared by appropriate modifications of the general scheme a further two analogues which maintain the ethylene spacer as in maleimide **11a**, namely the iodoacetamide **13a** and the maleimide analogue **11b** in which a dimethylamino replaces the diethylamino substituent. When used to label the A197C phosphate-binding protein, the iodoacetamide **13a** showed no fluorescence response to added phosphate, while for the dimethylamino compound **11b** the response was less than half that seen for the diethylamino compound **11a**.[16] The structural implications of the latter result are at present under study.

The final fluorophore investigated in this work was based on a recent report by Parrick and Ragunathan[17] who described the dimethoxyphthalimide **23** as a new fluorescent labelling reagent, with a good fluorescence quantum yield and large Stokes shift. Compound **23** (kindly donated by Dr. Parrick) labelled the A197C mutant phosphate-binding protein well, but the fluorescence was not responsive to phosphate binding.[16] However, by analogy with results already obtained[3] it seemed possible that insertion of a spacer between the maleimide and phthalimide nitrogens might yield a useful reagent.

Initial attempts were focussed on a derivative which

contained a phenylene spacer, and treatment of 3,6-dimethoxyphthalic anhydride **24** with 4-nitroaniline under prolonged reflux in acetic acid gave in high yield the nitrophenylphthalimide **25**. The anhydride **24** has previously been prepared[18] by acid hydrolysis of 3,6-dimethoxyphthalonitrile but was conveniently prepared here by saponification of dimethyl 3,6-dimethoxyphthalate[19] followed by cyclisation in hot acetic anhydride. The nitro compound **25** was smoothly hydrogenated to the amine **26** but when the latter compound was treated with maleic anhydride in acetic acid, the precipitated maleamic acid was extremely insoluble. Attempted cyclisation with acetic anhydride–fused sodium acetate gave no product which contained a recognisable maleimide group and this approach was abandoned. By analogy with the coumarin derivatives described above, we anticipated that compounds containing an aliphatic link between the phthalimide and maleimide rings would prove more tractable. Attempts to get the anhydride **24** to react with benzylamine (used as a model aliphatic amine) in DMA, followed by cyclisation of the presumed phthalamic acid with acetic anhydride, gave complex reaction mixtures which appeared to contain the desired imide **27** in poor yield. When *tert*-butyl-*N*-(2-aminoethyl)carbamate **4** was used in place of benzylamine, no recognisable product was obtained.



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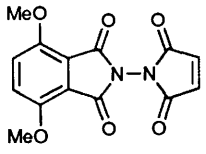

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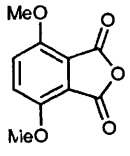

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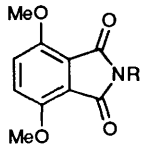
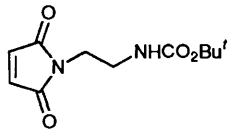


25 R = $C_6H_4NO_2$-*p*
26 R = $C_6H_4NH_2$-*p*
27 R = CH_2Ph
28 R = H



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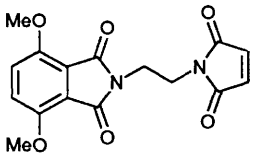

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As an alternative approach, we hoped to alkylate the parent phthalimide **28** with an ω-substituted alkyl halide and then to elaborate the ω-substituent to the required maleimide. However, in our hands the oxidative ring contraction of 5,8-dimethoxy-1,2,3,4-tetrahydrophthalazine-1,4-dione to give the phthalimide **28** as described by Parrick and Ragunathan[17] proceeded in much lower yield than reported. The product had spectral properties in agreement with published values[17] but its melting point was higher than the reported value by more than 30 °C (see Experimental section), and in the face of these problems this approach also was discontinued.

Finally, based on the Pattenden work with maleimides already referred to,[12] the anhydride **24** was treated with benzylamine in glacial acetic acid under prolonged reflux and the imide **27** was crystallised in 72% yield from the cooled reaction mixture. We therefore prepared *N*-[2-(*tert*-butoxycarbonylamino)ethyl]maleimide **29** from the monoamine **4** using the standard cobalt naphthenate procedure. The maleimide **29** has been prepared previously by a different route but its properties were not reported.[20] The compound was treated briefly with TFA to remove the *tert*-butoxycarbonyl (BOC) protecting group and the crude residue, presumably the TFA salt of the free amine, was condensed with 3,6-dimethoxyphthalic anhydride **24** to give the required maleimide **30**. The A197C phosphate-binding protein labelled with compound **30** did not show a fluorescence change in response to phosphate binding.[16]

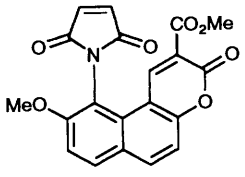
In common with a number of previously described maleimide reagents,[4,6,16,21–24] the compounds synthesised in this work showed significant enhancements in fluorescence after addition of a thiol (2-sulfanylethanesulfonate) to the maleimide double bond. Table 1 shows the relative fluorescence of the new reagents before and after addition of the thiol, all measured in predominantly (≥90%) aqueous solution at pH 7.0. The data show several features of interest. First, all previous maleimide probes which have shown fluorescence enhancement upon addition of thiol have had the nitrogen atom of the maleimide bonded directly to the fluorophore, whereas for all the reagents in Table 1 the maleimide and the fluorophore are separated by several sigma bonds. The quenching efficacy of the maleimide is evidently correlated with the wavelength of fluorescence excitation. The data in Table 1 indicate that the effect declines strongly as the excitation moves to longer wavelength. It is also distance related as shown by its diminution as the distance between the maleimide and fluorophore is increased (*cf.* compounds **11a** and **16**). Finally, the absence of a fluorescence enhancement in the iodoacetamide **13a** when treated with a thiol indicates that the effect is specific to the extended π-system of the maleimide group, which is consistent with previous interpretations[22,25] invoking intersystem crossing from an excited π→π* state of the fluorophore to an n→π* state of the maleimide.

The hydrolysis properties of representative compounds, namely the naphthalene derivative **7**, the coumarin **11b** and the phthalimide **30** were also examined, since opening of the maleimide ring greatly reduces the reactivity towards addition of thiol. Aliquots were withdrawn at various times from solutions of each compound at pH 7 and were treated with a slight excess of the thiol 2-sulfanylethanesulfonate. The unconsumed thiol, *i.e.* that fraction which had not added to the maleimide, was assayed spectroscopically following treatment with the chromogenic reagent bis(2-carboxy-4-nitrophenyl) disulfide.[26] Each compound showed approximately 50% hydrolysis after 24 h at room temperature, which is of the same order of magnitude as the 40 h half-life reported[27] for *N*-ethylmaleimide under similar conditions. In contrast, *N*-arylmaleimides hydrolyse much more rapidly, with reported[27] half-lives in the range 60–330 min at pH 7 for a variety of such compounds. Despite these previous studies of maleimide hydrolysis, the effect of hydrolytic opening of the maleimide ring on the fluorescence of maleimido fluorophores seems to have been little considered. Yang and Langmuir[24] reported that the benzocoumarin **31** did not show a time-dependent change in fluorescence when incubated at pH 7.2, but they did not report the time scale or extent of hydrolysis of the compound. The previous, more extensive study by Machida *et al.*[27] did not report fluorescence measurements.

Table 1 Fluorescence properties of compounds in response to addition of a thiol

Compound	[Fluorescence] Without Thiol λ_{ex}/nm	λ_{em}/nm	With Thiol λ_{ex}/nm	λ_{em}/nm	Quotient of fluorescence intensities before and after addition of thiol ($\lambda_{max}/\lambda_{max}$)
7	342	510	342	530	31
11a	430	477	430	481	4.2
11b	425	475	425	475	4.8
13a	430	477	430	476	1.0
16	430	477	430	476	2.0
30	394	452	394	458	7.4

The three compounds studied here showed large differences in their responses to hydrolysis. For compounds **11b** and **30**, hydrolysis was accompanied by a parallel increase in fluorescence, while for compound **7**, despite a similar extent of hydrolysis, the fluorescence was unchanged. We have not attempted to examine the corresponding fluorescence changes for the multitude of maleimide fluorophores described by other workers. However, the present results do indicate that some attention should be given to this property whenever reagents of this type are used, particularly in quantitative applications.



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In conclusion, among the reagents synthesised here for use to label the A197C mutant phosphate-binding protein, only the maleimide **11a** shows a useful fluorescence increment when inorganic phosphate binds to the labelled protein. However, in the wider context of reagents which show large fluorescence increases in response to reaction with thiols, others in the series may merit attention. In particular the dansyl maleimide **7** is prepared in two steps from commercially available dansyl chloride and shows a 30-fold fluorescence increment on addition of thiol. By contrast the benzocoumarin **31**, which has a comparable (40-fold) fluorescence increment when thiol adds to the maleimide,[24] is the product of a 5-step synthesis from 2,6-dihydroxy-1-naphthaldehyde, which itself is not commercially available. Clearly the search for thiol-reactive fluorophores tailored to particular applications is worthy of continued pursuit.

Experimental

Analyses were carried out by MEDAC Ltd., Brunel University, Uxbridge. NMR spectra were determined on a JEOL FX90Q spectrometer with tetramethylsilane as internal standard for solutions in deuteriochloroform unless otherwise stated. *J* Values are given in Hz. The FAB mass spectrum was run at low resolution on a VG 70-250SE instrument with the sample in a glycerol matrix. IR-spectra were determined as Nujol mulls on a Perkin–Elmer spectrophotometer. UV spectra were determined on a Beckman DU 70 spectrophotometer. Merck 9385 silica gel was used for flash chromatography. Light petroleum was the fraction boiling between 40–60 °C unless stated otherwise. Organic extracts were dried over anhydrous Na_2SO_4. Cobalt naphthenate was purchased from Fluka, Gillingham, Dorset. 4-Diethylamino-2-hydroxybenzaldehyde was from Aldrich, Gillingham, Dorset. *tert*-Butyl *N*-(2-aminoethyl)carbamate **4** was prepared by hydrogenolysis of benzyl *N*-[2-(*tert*-butoxycarbonylamino)ethyl]carbamate as described.[14,15] Phosphate buffer solutions were prepared from NaH_2PO_4 at the molarities specified and adjusted to the required pH value by addition of 1 mol dm^{-3} NaOH.

N-(2-*Maleimidoethyl*)*toluene*-4-*sulfonamide* **2**.—A solution of *N*-tosylethylenediamine[10] (2.14 g, 10 mmol) in DMA (4.5 cm^3) was added dropwise to a stirred solution of maleic anhydride (0.98 g, 10 mmol) in DMA (2.5 cm^3), at such a rate that the temperature did not rise above 40 °C. A solution of cobalt naphthenate (0.01 cm^3) in DMA (0.5 cm^3) was added, followed by acetic anhydride (2.04 g, 20 mmol), and the solution was warmed to 70–80 °C and stirred for 2 h, then was cooled, and diluted with water. The solid which separated after a short time was collected and washed well with water, then was dissolved in $CHCl_3$ and the solution was filtered through a short column of alumina. The eluate was evaporated under reduced pressure and the residue was crystallised from EtOAc–light petroleum to give the *maleimide* **2** as pale pink plates (1.69 g, 57%), m.p. 130–134 °C. For analysis a sample was purified by flash chromatography in EtOAc–light petroleum (65:35) to give plates, m.p. 131–132.5 °C (Found: C, 53.0; H, 4.8; N, 9.4. $C_{13}H_{14}N_2O_4S$ requires C, 53.05; H, 4.8; N, 9.5%); ν_{max}/cm^{-1} 3800, 1770, 1690, 1330, 1150, 830, 810 and 690; δ_H 7.69 (2 H, d, *J* 8, Ar-2- and 6-H), 7.26 (2 H, d, Ar-3- and 5-H), 6.64 (2 H, s, CH=CH), 5.19 (1 H, t, *J* 6, NH), 3.62 (2 H, t, *J* 6, CH_2N), 3.08–3.27 (2 H, m, CH_2NH) and 2.40 (3 H, s, Me).

5-*Dimethylamino*-N-(2-*maleimidoethyl*)*naphthalene*-1-*sulfonamide* **7**.—A solution of dansyl chloride **3** (3.7 g, 13.7 mmol) in $CHCl_3$ (45 cm^3) was added over a period of 20 min to a stirred solution of the monoamine **4** (2.53 g, 15.8 mmol) and triethylamine (4.52 cm^3, 33 mmol) in $CHCl_3$ (20 cm^3). The solution was stirred for a further 15 min, then was washed successively with aq. $NaHCO_3$ and water, dried, and evaporated under reduced pressure. The crude material, predominantly the sulfonamide **5**, was dissolved in TFA (50 cm^3) and kept for 1 h at room temp. TFA was evaporated off under reduced pressure and the residue was shaken with $CHCl_3$ and aq. $NaHCO_3$. The $CHCl_3$ layer was washed with water, dried and evaporated under reduced pressure and the residue was crystallised from aq. MeOH to give the monoamine **6** as needles (2.06 g, 51%), m.p. 149–152 °C (lit.,[15] 148–149 °C).

The total material was dissolved in DMA (4 cm^3) and the solution was added dropwise under N_2 to a stirred solution of maleic anhydride (0.69 g, 7.04 mmol) in DMA (1.4 cm^3), at a rate to keep the temperature below 40 °C. An aliquot (0.33 cm^3) of a solution of cobalt naphthenate (20 mm^3) in DMA (1 cm^3) was added and the mixture was warmed to 60 °C. Acetic anhydride (1.33 cm^3, 14.1 mmol) was added and the solution was stirred at 70–80 °C for 2 h, cooled, and diluted with water. The aqueous mixture was extracted with $CHCl_3$ and the

extracts were washed successively with aq. $NaHCO_3$ and brine, dried, and evaporated under reduced pressure, to leave an oily residue, which was triturated with water to remove residual DMA, then was dissolved in $CHCl_3$ and the solution was filtered through alumina (50 g). The recovered material crystallised on trituration with diethyl ether, and was recrystallised from EtOAc–light petroleum to give the *maleimide* **7** as prisms (1.30 g, 50%), m.p. 109–110 °C (Found: C, 57.8; H, 5.2; N, 11.2. $C_{18}H_{19}N_3O_4S$ requires C, 57.9; H, 5.1; N, 11.25%); λ_{max}(EtOH)/nm 250 (ε/dm^3 mol^{-1} cm^{-1} 14 700) and 334 (4800); λ_{max}[EtOH–50 mmol dm^{-3} sodium phosphate, pH 7.0 (1:1)]/nm 248 (ε/dm^3 mol^{-1} cm^{-1} 15 100) and 330 (4600); ν_{max}/cm^{-1} 1770, 1715, 1590, 1575, 1405, 1315, 1165, 1140, 910, 830, 795, 785 and 695; δ_H 8.50 (1 H, d, *J* 8.5, ArH), 8.18 (2 H, m, ArH), 7.48 (2 H, m, ArH), 7.12 (1 H, d, *J* 8, ArH), 6.35 (2 H, s, CH=CH), 5.37 (1 H, t, *J* 6, NH), 3.04–3.32 (4 H, m, CH_2) and 2.86 (6 H, s, Me).

Ethyl 7-Diethylamino-2-oxo-2H-chromene-3-carboxylate **8a**.—A solution of 4-diethylamino-2-hydroxybenzaldehyde (29.8 g, 154 mmol) and diethyl malonate (23.8 cm^3, 157 mmol) in EtOH (185 cm^3) was treated with piperidine (1.37 cm^3, 13.9 mmol) and heated under reflux for 4 h. The cooled solution was concentrated ~2-fold under reduced pressure, then was diluted with diethyl ether, washed successively with water, aq. NaOH and water, dried, and evaporated under reduced presure. The residue crystallised on trituration with EtOH to give the coumarin **8a** as yellow prisms (18 g, 40%), m.p. 77–78 °C (from EtOAc–light petroleum) (lit.,[28] 87 °C) (Found: C, 66.4; H, 6.7; N, 4.8. Calc. for $C_{16}H_{19}NO_4$: C, 66.4; H, 6.6; N, 4.8%); λ_{max}(EtOH)/nm 255 (ε/dm^3 mol^{-1} cm^{-1} 8900) and 417 (46 400); δ_H 8.42 (1 H, s, 4-H), 7.35 (1 H, d, $J_{5,6}$ 8.8, 5-H), 6.60 (1 H, dd, $J_{6,8}$ 1.6, 6-H), 6.46 (1 H, d, 8-H), 4.37 (2 H, q, *J* 7, OCH_2), 3.45 (4 H, q, *J* 7, NCH_2), 1.39 (3 H, t, Me) and 1.23 (6 H, t, Me). Although the m.p. obtained here and the literature value do not agree, the satisfactory analytical and spectroscopic data for the present sample suggest that the digits of the literature value may accidentally have been transposed.

7-Diethylamino-2-oxo-2H-chromene-3-carboxylic Acid **9a**.—A solution of the ester **8a** (11.6 g, 40 mmol) in MeOH (100 cm^3) was heated under reflux and 0.5 mol dm^{-3} aq. NaOH (100 cm^3) was added rapidly. A yellow solid precipitated within 5 min and the mixture was cooled and acidified with 2 mol dm^{-3} aq. HCl to give an orange solid, which was filtered off, washed successively with 2 mol dm^{-3} aq. HCl, then with water until neutral and finally with MeOH (50 cm^3). The solid was dried *in vacuo* to give the acid **9a** (9.4 g, 90%). A sample crystallised from MeOH–diisopropyl ether as orange laths, m.p. 231–232 °C (lit.,[29] 227–229 °C).

Ethyl 7-Dimethylamino-2-oxo-2H-chromene-3-carboxylate **8b**.—The ester **8b** was prepared from 4-dimethylamino-2-hydroxybenzaldehyde[30] as for the ester **8a**. The product crystallised as orange needles (40% yield), m.p. 170–171 °C (from EtOAc–light petroleum) (lit.,[31] 168–169 °C).

7-Dimethylamino-2-oxo-2H-chromene-3-carboxylic Acid **9b**.—The ester **8b** was hydrolysed with aq. methanolic NaOH as for ester **8a** to give the acid **9b** (98% yield). A sample was crystallised from glacial acetic acid as yellow needles, m.p. 279–280 °C (lit.,[32], 280–283 °C).

tert-*Butyl* N-[2-(7-*Diethylamino-2-oxo-2H-chromene-3-carboxamido)ethyl*]*carbamate* **10a**.—A stirred solution of 7-diethylamino-2-oxo-2*H*-chromene-3-carboxylic acid **9a** (1.15 g, 5 mmol) and tributylamine (1.38 g, 7.5 mmol) in dry dimethylformamide (DMF) (50 cm^3) was cooled in an ice-bath and isobutyl chloroformate (0.715 g, 5.2 mmol) was added. The solution was kept in the ice-bath for 0.5 h, then was treated with a solution of the monoamine **4** (0.8 g, 5 mmol) in dry DMF (5 cm^3). The mixture was allowed to warm to room temp. and was kept for 3 h, then was diluted with EtOAc and washed successively with water, dil. aq. HCl, aq. $NaHCO_3$ and brine, dried, and evaporated under reduced pressure. Trituration of the residue with a little Et_2O gave a yellow solid, which was crystallised from EtOAc–light petroleum to give the *amide* **10a** as yellow prisms (0.55 g, 45%), m.p. 157–158 °C (Found: C, 62.6; H, 7.3; N, 10.4. $C_{21}H_{29}N_3O_5$ requires C, 62.5; H, 7.2; N, 10.4%); ν_{max}/cm^{-1} 3400, 3335, 1715, 1680, 1610, 1580, 1530, 1510, 1230 and 795; δ_H 8.95 (1 H, t *J* 5.5, NH), 8.68 (1 H, s, 4-H), 7.42 (1 H, d, $J_{5,6}$ 8.8, 5-H), 6.64 (1 H, dd, $J_{6,8}$ 2.3, 6-H), 6.49 (1 H, d, 8-H), 5.08 (1 H, br s, NH), 3.23–3.64 (8 H, m, CH_2), 1.44 (9 H, s, CMe_3) and 1.24 (6 H, t, *J* 7, Me).

tert-*Butyl* N-[2-(7-*Dimethylamino-2-oxo-2H-chromene-3-carboxamido)ethyl*]*carbamate* **10b**.—This compound was prepared as for its homologue **10a**, except that a 4-fold larger proportion of DMF was required initially to dissolve the acid **9b**. At the completion of the reaction sequence most of the DMF was removed under reduced pressure before dilution with EtOAc and work-up as before. The *title product* **10b** was crystallised from EtOAc–light petroleum as yellow plates (69% yield), m.p. 215–217 °C (Found: C, 59.55; H, 6.7; N, 11.1. $C_{19}H_{25}N_3O_5{\cdot}\frac{1}{2}H_2O$ requires C, 59.4; H, 6.8; N, 10.9%); ν_{max}/cm^{-1} 3310, 1715, 1700, 1645, 1630, 1590, 1530, 800 and 790; δ_H 8.95 (1 H, br t, NH), 8.69 (1 H, s, 4-H), 7.44 (1 H, d, $J_{5,6}$ 8.7, 5-H), 6.67 (1 H, dd, $J_{6,8}$ 2.2, 6-H), 6.49 (1 H, d, 8-H), 5.05 (1 H, br s, NH), 3.30–3.60 (4 H, m, CH_2), 3.12 (6 H, s, Me) and 1.44 (9 H, s, Me).

7-Diethylamino-N-(2-maleimidoethyl)-2-oxo-2H-chromene-3-carboxamide **11a**.—A solution of carbamate **10a** (0.88 g, 2.18 mmol) in TFA (10 cm^3) was kept at room temp. for 1 h and was then evaporated under reduced pressure. The residue was partitioned between $CHCl_3$ and aq. $NaHCO_3$, and the chloroform layer was dried, and evaporated under reduced pressure to leave a yellow foam (0.60 g), to which were added maleic anhydride (0.19 g, 2 mmol) and DMA (2 cm^3). The mixture was warmed to 60 °C over a period of 20 min and treated with an aliquot (94 mm^3) of a solution of cobalt naphthenate (20 mm^3) in DMA (1 cm^3), followed by acetic anhydride (0.38 cm^3). The mixture was stirred at 70–80 °C for 2 h, cooled, diluted with water and extracted with EtOAc. The extract was washed with water, dried, and evaporated under reduced pressure, and the residue was purified by flash chromatography to give the *maleimide* **11a**, which was crystallised from EtOAc–light petroleum as fine needles (0.22 g, 26%), m.p. 179–181 °C (Found: C, 62.5; H, 5.5; N, 10.8. $C_{20}H_{21}N_3O_5$ requires C, 62.65; H, 5.5; N, 11.0%); λ_{max}(EtOH)/nm 258 (ε/dm^3 mol^{-1} cm^{-1} 13 150) and 418 (45 000); λ_{max}[EtOH–50 mmol dm^{-3} sodium phosphate, pH 7.0 (1:9)]/nm 264 (ε/dm^3 mol^{-1} cm^{-1} 12 800) and 430 (46 800); ν_{max}/cm^{-1} 3335, 1710, 1695, 1645, 1615, 1580, 1360, 1140 and 695; δ_H 8.88 (1 H, t, *J* 5.7, NH), 8.66 (1 H, s, 4-H), 7.41 (1 H, d, $J_{5,6}$ 8.8, 5-H), 6.69 (2 H, s, CH=CH), 6.63 (1 H, dd, $J_{6,8}$ 2.7, 6-H), 6.47 (1 H, d, 8-H), 3.39–3.88 (8 H, m, CH_2) and 1.24 (6 H, t, *J* 7, Me).

7-Dimethylamino-N-(2-maleimidoethyl)-2-oxo-2H-chromene-3-carboxamide **11b**.—This compound was prepared as for its homologue **11a** above, but was isolated in only 6% final yield because of losses due to its poor solubility. The crude material was purified by flash chromatography [$CHCl_3$–EtOAc (2:3)] and was crystallised as needles from $CHCl_3$–light petroleum to give the *maleimide* **11b**, m.p. 229–231 °C (Found: C, 59.3; H, 4.7; N, 11.4. $C_{18}H_{17}N_3O_5{\cdot}\frac{1}{2}H_2O$ requires C, 59.3; H, 5.0; N, 11.5%);

λ_{max}(EtOH)/nm 413 (ε/dm^3 mol^{-1} cm^{-1} 33 400); λ_{max}[EtOH–50 mmol dm^{-3} sodium phosphate, pH 7.0 (1:9)]/nm 425 (ε/dm^3 mol^{-1} cm^{-1} 33 400); ν_{max}/cm^{-1} 3320, 1710, 1695, 1620, 1580, 840 and 700; δ_H[$(CD_3)_2SO$] 8.70 (1 H, br t, NH), 8.61 (1 H, s, 4-H), 7.64 (1 H, d, $J_{5,6}$ 8.8, 5-H), 6.93 (2 H, s, CH=CH), 6.78 (1 H, dd, $J_{6,8}$ 2.2, 6-H), 6.57 (1 H, d, 8-H), 3.38–3.70 (4 H, m, CH_2) and 3.10 (6 H, s, Me).

N-[2-(*Chloroacetamido*)*ethyl*]-7-*diethylamino*-2-*oxo*-2H-*chromene*-3-*carboxamide* **12a**.—The carbamate **10a** (1.27 g, 3.15 mmol) was deprotected with TFA as described above and the crude product (0.90 g) was dissolved in $CHCl_3$ (20 cm^3) together with triethylamine (0.35 g, 3.5 mmol). The solution was stirred and cooled in an ice-bath while a solution of chloroacetyl chloride (0.36 g, 3.2 mmol) in $CHCl_3$ (10 cm^3) was added dropwise. After 1 h, the solution was diluted with EtOAc and washed successively with dil. aq. HCl, aq. $NaHCO_3$, and brine, dried, and evaporated under reduced pressure. A portion (0.1 g) of the yellow solid was purified by flash chromatography [$CHCl_3$–EtOAc–MeOH (30:68:2)] and the recovered material (80 mg) was crystallised from CH_2Cl_2–light petroleum to give the *chloroacetamide* **12a** as fine needles, m.p. 170–171 °C, which resolidified and had a second m.p. 179.5–181 °C (Found: C, 56.7; H, 5.8; N, 10.9. $C_{18}H_{22}ClN_3O_4$ requires C, 56.9; H, 5.8; N, 11.1%); ν_{max}/cm^{-1} 3280, 1700, 1690, 1640, 1620, 1580, 1515, 1375, 1190 and 790; δ_H 9.07 (1 H, br t, NH), 8.69 (1 H, s, 4-H), 7.44 (1 H, d, $J_{5,6}$ 9.2, 5-H) superimposed on 7.54 (1 H, br t, NH), 6.65 (1 H, dd, $J_{6,8}$ 2.4, 6-H), 6.49 (1 H, d, 8-H), 4.04 (2 H, s, CH_2Cl), 3.24–3.70 (8 H, m, CH_2) and 1.25 (6 H, t, J 7.5, Me).

7-*Diethylamino*-N-[2-(*iodoacetamido*)*ethyl*]-2-*oxo*-2H-*chromene*-3-*carboxamide* **13a**.—A solution of the crude chloroacetamide **12a** (0.69 g) and sodium iodide (10.5 g) in acetone (70 cm^3) was stirred overnight at room temp., then was diluted with EtOAc and washed successively with water, dil. aq. sodium ascorbate, and brine, dried, and evaporated under reduced pressure. The residue was purified by flash chromatography [EtOAc–$CHCl_3$-MeOH (30:68:2)] and crystallised from CH_2Cl_2–light petroleum to give the *iodoacetamide* **13a** (0.59 g), m.p. 196–198 °C (Found: C, 45.1; H, 4.6; N, 8.6. $C_{18}H_{22}IN_3O_4 \cdot \frac{1}{4}H_2O$ requires C, 45.4; H, 4.8; N, 8.8%); λ_{max}(EtOH)/nm 257 (ε/dm^3 mol^{-1} cm^{-1} 14 400) and 418 (47 000); λ_{max}[EtOH–50 mmol dm^{-3} sodium phosphate, pH 7.0 (1:9)]/nm 263 (ε/dm^3 mol^{-1} cm^{-1} 10 500) and 430 (44 800); ν_{max}/cm^{-1} 3280, 1705, 1690, 1640, 1620, 1510, 1375 and 795; δ_H 9.08 (1 H, br t, NH), 8.65 (1 H, s, 4-H), 7.42 (1 H, d, $J_{5,6}$ 8.8, 5-H), 7.26 (1 H, br s, NH), 6.65 (1 H, dd, $J_{6,8}$ 2.2, 6-H), 6.48 (1 H, d, 8-H), 3.70 (2 H, s, CH_2I), 3.34–3.64 (8 H, m, CH_2) and 1.25 (6 H, t, J 7, Me).

Benzyl N-[3-(*tert-Butoxycarbonylamino*)*propyl*]*carbamate* **18**.—A solution of 1,3-diaminopropane (9.62 g, 0.13 mol) in water (25 cm^3) was neutralised to Bromocresol Green with a solution of methanesulfonic acid (25 g) in water (27 cm^3) and cooled to ~15 °C. The solution was stirred vigorously while solutions of benzyl chloroformate (20 g) in 1,2-dimethoxyethane (25 cm^3) and 50% aq. potassium acetate (50 cm^3) were simultaneously added dropwise, at rates to maintain the indicator colour at its end point. When the additions were complete, the solution was allowed to warm to room temp. and was kept for 1 h, then was concentrated under reduced pressure. The residue was dissolved in water (250 cm^3), the solution was filtered, and the filtrate was washed with benzene (3 × 75 cm^3), then was basified with 40% aq. NaOH. The basic solution was again extracted with benzene (3 × 100 cm^3) and the combined benzene extracts were washed once with brine (100 cm^3), dried, and evaporated under reduced pressure, to leave a pale oil (7.53 g). This material was dissolved in dry acetone (250 cm^3) with di-*tert*-butyl dicarbonate (8.15 g, 37.4 mmol) and the solution was kept overnight at room temp., then was heated under reflux for 1 h and evaporated under reduced pressure. The ^{1}H NMR spectrum of the crude residue showed two signals corresponding to *tert*-butyl moieties (δ_H 1.21 and 1.29) and the whole material (11.2 g) was dissolved in MeOH (300 cm^3) with 1 mol dm^{-3} aq. NaOH (12 cm^3) and kept for 1 h at room temp., then was neutralised with glacial acetic acid (12 cm^3) and concentrated to a small volume. The residue was dissolved in diethyl ether and washed successively with aq. $NaHCO_3$ and brine, dried, and evaporated under reduced pressure to leave a pale oil (9.1 g), for which the NMR spectrum showed only a single peak assignable to a *tert*-butyl group. The oil solidified on prolonged storage at 4 °C, and a portion was recrystallised from diisopropyl ether to give the *dicarbamate* **18** as needles, m.p. 64–65 °C (Found: C, 62.2; H, 7.9; N, 9.0. $C_{16}H_{24}N_2O_4$ requires C, 62.3; H, 7.8; N, 9.1%); ν_{max}/cm^{-1} 3330, 1680, 1285, 1270, 1255, 1165 and 1140; δ_H 7.35 (5 H, s, Ph), 5.43 (1 H, br s, NH), 5.10 (2 H, s, OCH_2), 4.98 (1 H, br s, NH), 3.20 (4 H, m, CH_2N), 1.58 (2 H, quintet, J 7, CH_2) and 1.21 (9 H, s, Me).

tert-*Butyl* 4-*Nitrophenylcarbamate* **20**.—A mixture of 4-nitrobenzoyl azide (7 g) in dry toluene (70 cm^3) was heated gently to reflux and was then heated for a further 0.5 h. Dry *tert*-butyl alcohol (35 cm^3) was added and the solution was heated under reflux for a further 2 h, then was cooled and filtered, and the filtrate was evaporated under reduced pressure. The residue was crystallised from light petroleum (boiling range 80–100 °C) to give the carbamate **20** (6.7 g, 80%), m.p. 107–109 °C (lit.,[33] 110.5–111.5 °C).

tert-*Butyl* N-[3-(7-*Diethylamino*-2-*oxo*-2H-*chromene*-3-*carboxamido*)*propyl*]*carbamate* **14**.—A solution of dicarbamate **18** (1.6 g, 5.2 mmol) in EtOH (45 cm^3) was shaken with 5% Pd–C (1.0 g) under hydrogen (50 p.s.i.) for 18 h at room temp. The catalyst was filtered off and the filtrate was evaporated under reduced pressure to leave the crude amine **19** as an oil (0.88 g, 98%), which was coupled with 7-diethylamino-2-oxo-2*H*-chromene-3-carboxylic acid **9a** as in the preparation of amide **10a**. The *title product* **14** was obtained as yellow laths (0.90 g, 43%) from EtOAc–light petroleum, m.p. 140–141 °C (Found: C, 63.5; H, 7.6; N, 10.1. $C_{22}H_{31}N_3O_5$ requires C, 63.3; H, 7.5; N, 10.1%); ν_{max}/cm^{-1} 3305, 1715, 1690, 1640, 1620, 1585, 1250 and 795; δ_H 8.85 (1 H, br t, NH), 8.68 (1 H, s, 4-H), 7.42 (1 H, d, $J_{5,6}$ 8.8, 5-H), 6.64 (1 H, dd, $J_{6,8}$ 2.6, 6-H), 6.49 (1 H, d, 8-H), 5.21 (1 H, br s, NH), 3.02–3.59 (8 H, m, CH_2), 1.62–1.83 (2 H, m, CH_2), 1.45 (9 H, s, Me) and 1.25 (6 H, t, J 7, Me).

tert-*Butyl* N-[4-(7-*Diethylamino*-2-*oxo*-2H-*chromene*-3-*carboxamido*)*phenyl*]*carbamate* **15**.—A suspension of the nitro compound **20** (1.2 g, 5 mmol) and 5% Pd–C (0.5 g) in EtOH (30 cm^3) was stirred at room temp. under hydrogen at atmospheric pressure until uptake of gas had ceased. The catalyst was filtered off and the filtrate was evaporated under reduced pressure to give the crude amine **21** (0.99 g, 95%), which was coupled with 7-diethylamino-2-oxo-2*H*-chromene-3-carboxylic acid **9a** (1.15 g, 5 mmol) as for preparation of amide **10a**, to give the *title amide* **15** as small yellow plates (1.10 g, 49%) from CH_2Cl_2–Et_2O, m.p. 234–236 °C (Found: C, 66.5; H, 6.5; N, 9.3. $C_{25}H_{29}N_3O_5$ requires C, 66.5; H, 6.5; N, 9.3%); ν_{max}/cm^{-1} 3340, 1720, 1685, 1615, 1600, 1585 and 825; δ_H[$(CD_3)_2SO$] 9.21 (1 H, s, NH), 8.71 (1 H, s, 4-H), 7.65 (1 H, d, $J_{5,6}$ 8.8, 5-H), 7.56 and 7.39 (4 H, ABq, J 9, ArH), 6.77 (1 H, dd, $J_{6,8}$ 2.2, 6-H), 6.60 (1 H, d, 8-H), 3.47 (4 H, q, J 7, CH_2), 1.46 (9 H, s, Me) and 1.15 (6 H, t, Me).

7-*Diethylamino*-N-(3-*maleimidopropyl*)-2-*oxo*-2H-*chromene*-3-*carboxamide* **16**.—The amide **14** (0.9 g, 2.16 mmol) was deprotected with TFA and the product was allowed to react with maleic anhydride as described for maleimide **11a**. The

crude product was purified by flash chromatography [EtOAc–light petroleum (7:3)] to give the *maleimide* **16** (90 mg, 10%) as yellow prisms from EtOAc–light petroleum, m.p. 177–178 °C (Found: C, 63.3; H, 5.8; N, 10.5. $C_{21}H_{23}N_3O_5$ requires C, 63.5; H, 5.8; N, 10.6%); λ_{max}(EtOH)/nm 258 (ε/dm^3 mol^{-1} cm^{-1} 13 600) and 418 (45 800); λ_{max}[EtOH–10 mmol dm^{-3} sodium phosphate, pH 7 (1:9)]/nm 265 (ε/dm^3 mol^{-1} cm^{-1} 11 800) and 430 (46 000); ν_{max}/cm^{-1} 3350, 1705, 1680, 1645, 1610, 1580, 1510, 825 and 695; δ_H 8.92 (1 H, br t, NH), 8.67 (1 H, s, 4-H), 7.41 (1 H, d, $J_{5,6}$ 8.8, 5-H), 6.69 (2 H, s, CH=CH), 6.64 (1 H, dd, $J_{6,8}$ 2.2, 6-H), 6.49 (1 H, d, 8-H), 3.33–3.72 (8 H, m, CH_2), 1.76–2.03 (2 H, m, CH_2) and 1.24 (6 H, t, J 7, Me).

*7-Diethylamino-*N*-(4-maleimidophenyl)-2-oxo-*2H*-chromene-3-carboxamide* **17** *and its Thiophenol Adduct* **22**.—A solution of the carbamate **15** (1.07 g, 2.37 mmol) in TFA (10 cm^3) was kept at room temp. for 1 h and was then evaporated under reduced pressure. The residue was dissolved by warming it in glacial acetic acid (50 cm^3), and maleic anhydride (0.235 g, 2.4 mmol) was added. A precipitate formed rapidly and the mixture was cooled to room temp. and filtered. The solid was washed with diethyl ether and dried *in vacuo*, then was mixed with acetic anhydride (20 cm^3) and fused sodium acetate (0.1 g), heated for 2 h at 100 °C, and allowed to cool. The mixture was diluted with diethyl ether and filtered, and the solid was washed successively with water and ethanol, and dried *in vacuo* to give the crude maleimide **17** as yellow microcrystals (0.85 g, 83%), m.p. 288–290 °C (decomp.). To enable characterisation of this very insoluble material, a portion (0.10 g) was suspended in a mixture of $CHCl_3$ (25 cm^3), EtOH (25 cm^3) and 25 mmol dm^{-3} aq. sodium phosphate, pH 7.1 (25 cm^3) and was treated with thiophenol (53 mg). The mixture was stirred vigorously for 1 h and the organic layer was separated, washed successively with aq. NaOH and water, dried, and evaporated under reduced pressure. The residue was purified by flash chromatography [EtOAc–light petroleum (3:2)] and was then crystallised from $CHCl_3$–MeOH to give *7-diethylamino-*N*-{4-[(3-phenylsulfanyl)succinimido]phenyl}-2-oxo-*2H*-chromene-3-carboxamide* **22** as bright yellow needles, m.p. 203–204 °C, which resolidified and had a second m.p. 215–217 °C (Found: C, 65.3; H, 4.9; N, 7.6. $C_{30}H_{27}N_3O_5S\cdot\frac{1}{2}H_2O$ requires C, 65.4; H, 5.1; N, 7.6%); λ_{max}(EtOH)/nm 267 (ε/dm^3 mol^{-1} cm^{-1} 19 500) and 430 (59 700); ν_{max}/cm^{-1} 1710, 1690, 1585, 1460, 1205 and 1190; δ_H 8.76 (1 H, s, 4-H), 7.81 (2 H, d, J 8.8, ArH), 7.29–7.69 (6 H, m, 5-H and Ph), 7.04 (2 H, d, ArH), 6.66 (1 H, dd, $J_{5,6}$ 9, $J_{6,8}$ 2.4, 6-H), 6.52 (1 H, d, 8-H), 4.15 (1 H, dd, J 8.8 and 4, C*H*SPh), 3.47 (4 H, q, J 7, NCH_2) superimposed on 3.35 (1 H, dd, J_{gem} 18.4, one H of CH_2CHS), 2.88 (1 H, dd, one H of CH_2CHS) and 1.25 (6 H, t, Me).

3,6-Dimethoxyphthalic Anhydride **24**.—Dimethyl 3,6-dimethoxyphthalate[19] (8 g) was heated for 2 h under reflux in methanol (70 cm^3)–water (8 cm^3) with KOH (4.5 g). The solution was cooled and concentrated to half-volume under reduced pressure, then was washed twice with Et_2O to remove excess of MeOH. The aqueous phase was acidified with conc. HCl and was kept at 4 °C for 2 h. The precipitated solid was filtered off, washed with water, and dried *in vacuo* to give crude 3,6-dimethoxyphthalic acid (4.84 g).

This material was suspended in acetic anhydride, and the mixture was heated under reflux for 1 h and allowed to cool. The yellow crystalline solid was collected, washed with Et_2O, and dried to give 3,6-dimethoxyphthalic anhydride **24** (4.0 g), m.p. 259–260 °C (lit.,[18] 259–261 °C).

*3,6-Dimethoxy-*N*-(4-nitrophenyl)phthalimide* **25**.—A solution of 3,6-dimethoxyphthalic anhydride **24** (115 mg, 0.55 mmol) and 4-nitroaniline (103 mg, 0.75 mmol) in glacial acetic acid (5 cm^3) was heated under reflux for 24 h, then was allowed to cool, and was diluted with Et_2O. The crystalline precipitate was filtered off, washed with Et_2O, and recrystallised from glacial acetic acid to give the *imide* **25** as yellow needles (160 mg, 87%), m.p. 263–264 °C (Found: C, 58.3; H, 3.7; N, 8.4. $C_{16}H_{12}N_2O_6$ requires C, 58.5; H, 3.7, N, 8.5%); ν_{max}/cm^{-1} 1770, 1730, 1530, 1505 and 1350; δ_H[$(CD_3)_2SO$] 8.36 (2 H, d, J 8.2, ArH), 7.71 (2 H, d, ArH), 7.51 (2 H, s, 4- and 5-H) and 3.93 (6 H, s, OMe).

N-(4-Aminophenyl)-3,6-dimethoxyphthalimide **26**.—A suspension of nitro compound **24** (100 mg) and 5% Pd–C (50 mg) in glacial acetic acid (20 cm^3) was stirred under hydrogen at room temp. and pressure until uptake of gas had ceased (*ca.* 1 h). The solution was filtered and the filtrate was evaporated under reduced pressure. The residue was dissolved in warm EtOAc and the solution was washed successively with aq. $NaHCO_3$ and water, dried, and evaporated. The residue was crystallised from EtOH to give the *amine* **26** as yellow plates (50 mg), m.p. 262–263 °C (Found: C, 64.4; H, 4.7; N, 9.4. $C_{16}H_{14}N_2O_4$ requires C, 64.4; H, 4.7; N, 9.4%); ν_{max}/cm^{-1} 3480, 3390, 1760, 1705, 1625, 1520, 1495, 1280 and 1045; δ_H[$(CD_3)_2SO$] 7.44 (2 H, s, 4- and 5-H), 6.92 (2 H, d, J 8.3, ArH), 6.61 (2 H, d, ArH) and 3.90 (6 H, s, OMe).

N-Benzyl-3,6-dimethoxyphthalimide **27**.—Prepared from 3,6-dimethoxyphthalic anhydride **24** and benzylamine as for the imide **25**. The product was recrystallised from glacial acetic acid to give the *imide* **27** (72% yield), m.p. 221–222 °C (Found: C, 68.5; H, 5.0; N, 4.7. $C_{17}H_{15}NO_4$ requires C 68.7; H, 5.1; N, 4.7%); ν_{max}/cm^{-1} 1765, 1705, 1285, 1060, 815, 760 and 710; δ_H 7.24–7.56 (5 H, m, Ph), 7.12 (2 H, s, 4- and 5-H), 4.77 (2 H, s, NCH_2) and 3.94 (6 H, s, OMe).

3,6-Dimethoxyphthalimide **28**.—A stirred suspension of 5,8-dimethoxy-1,2,3,4-tetrahydrophthalazine-1,4-dione[17] (0.32 g) in a mixture of acetonitrile (20 cm^3) and water (10 cm^3) was treated dropwise over a period of 10 min with aq. cerium(IV) ammonium nitrate (2.9 g in 10 cm^3). The mixture was stirred for a further 4 h, then was diluted with water (15 cm^3) and extracted with CH_2Cl_2. The extraction procedure was hampered by the presence of a quantity of poorly soluble material and by the formation of emulsions. The CH_2Cl_2 extract was dried, and evaporated under reduced pressure to leave a yellow solid (80 mg), which was crystallised from water to give the phthalimide **28** as yellow needles (18 mg, 6%), m.p. 256–258 °C (lit.,[17] 221–223 °C) [Found: (M^+ + H), 208. $C_{10}H_9NO_4$ + H requires m/z, 208]; δ_H [$(CD_3)_2SO$] 7.39 (2 H, s, ArH) and 3.87 (6 H, s, OMe).

tert-Butyl N*-(2-Maleimidoethyl)carbamate* **29**.—A mixture of the monoamine **4** (0.80 g, 5 mmol) and maleic anhydride (0.49 g, 5 mmol) was stirred under nitrogen in dry DMA (5 cm^3) and the solution was warmed to 60 °C over a period of 20 min. An aliquot (0.235 cm^3) of a solution of cobalt naphthenate (20 mm^3) in DMA (1 cm^3) was added, followed by acetic anhydride (0.95 cm^3), and the solution was stirred at 70–80 °C for 2 h, then was cooled, and diluted with EtOAc. This solution was washed successively with water and dil. HCl, dried, and evaporated under reduced pressure. The residue was purified by flash chromatography [EtOAc–light petroleum (35:65)] to give the *maleimide* **29** as a solid (0.39 g). A portion was crystallised from EtOAc–light petroleum to give fine needles, m.p. 130–131.5 °C (Found: C, 55.1; H, 6.8; N, 11.6. $C_{11}H_{16}N_2O_4$ requires C, 55.0; H, 6.7; N, 11.7%); ν_{max}/cm^{-1} 3360, 1705, 1680, 1435, 1170 and 690; δ_H 6.70 (2 H, s, CH=CH), 4.74 (1 H, br s, NH), 3.24–3.74 (4 H, m, CH_2) and 1.41 (9 H, s, Me).

N-(2-*Maleimidoethyl*)-3,6-*dimethoxyphthalimide* **30**.—The maleimide **29** (0.24 g, 1 mmol) was dissolved in TFA (3.5 cm^3) and the solution was kept at room temp. for 0.5 h. The TFA was evaporated off under reduced pressure and the residue was kept *in vacuo* for 1 h to remove residual TFA, then was mixed with 3,6-dimethoxyphthalic anhydride **24** (0.153 g, 0.73 mmol) and glacial acetic acid (6.7 cm^3), and the mixture was heated under reflux for 18 h. The cooled solution was concentrated under reduced pressure and the residue was dissolved in $CHCl_3$ and washed successively with aq. $NaHCO_3$ and water, dried, and evaporated under reduced pressure. The crude product retained a coloured impurity and was purified by flash chromatography [$CHCl_3$–EtOAc (2:3)] to give the *phthalimide* **30** as a pale yellow solid (0.16 g, 66%), which crystallised from glacial acetic acid as prisms, m.p. 282–284 °C (decomp.) (Found: C, 57.8; H, 4.3; N, 8.2. $C_{16}H_{14}N_2O_6$ requires C, 58.2; H, 4.3; N, 8.5%); λ_{max}(EtOH)/nm 375 (ε/dm^3 mol^{-1} cm^{-1} 6200); λ_{max} [EtOH–50 mmol dm^{-3} sodium phosphate], pH 7.0 (1:9)/nm 385 (ε/dm^3 mol^{-1} cm^{-1} 6300); ν_{max}/cm^{-1} 3100, 1755, 1710, 1500, 1415, 1275 and 695; δ_H[$(CD_3)_2SO$] 7.20 (2 H, s, ArH), 6.97 (2 H, s, CH=CH), 3.88 (6 H, s, OMe) and 3.63 (4 H, s, CH_2CH_2).

Hydrolysis Rates of Maleimide Reagents.—Ethanolic solutions (~0.20 mmol dm^{-3}) of the maleimides **7**, **11b** and **30** were diluted 10-fold with 20 mmol dm^{-3} aq. sodium phosphate, pH 7.0 to give ~20 μmol dm^{-3} final concentrations of the maleimides. Duplicate aliquots (1 cm^3) were withdrawn at various times (0–24 h) and each was treated with an aliquot (0.10 cm^3) of a solution of sodium 2-sulfanylethanesulfonate (0.3 mmol dm^{-3} in 10 mmol dm^{-3} sodium phosphate–1 mmol dm^{-3} ethylenediaminetetraacetic acid, pH 6.0). After 5 min an aliquot (10 mm^3) of a solution of bis(2-carboxy-4-nitrophenyl) disulfide (10 mmol dm^{-3} in 50 mmol dm^{-3} aq. sodium phosphate, pH 7.0) was added. When the set of samples was complete, all were read at 412 nm and the thiol concentrations were calculated, using ε/dm^3 mol^{-1} cm^{-1} 13 600 for the dianion of 2-sulfanyl-5-nitrobenzoic acid.[26] The chromophores of the coumarin **11b** and the phthalimide **30** both had some absorbance at 412 nm and these values arising from the compounds alone were subtracted from the total 412 nm absorbance before calculation of the thiol concentrations. Results are given in the Discussion section.

Fluorescence Response to Addition of Thiol.—Ethanolic solutions of the compounds shown in Table 1 were diluted 10-fold with 20 mmol dm^{-3} aq. sodium phosphate, pH 7.0 which also contained either 0 or 1 mmol dm^{-3} sodium 2-sulfanylethanesulfonate. Uncorrected fluorescence spectra were recorded for each solution on a Farrand Mark 1 spectrofluorimeter. Results are given in Table 1.

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