Synthesis and Fluorescence Properties of Substituted 7-Aminocoumarin-3-carboxylate Derivatives

John E. T. Corrie* and V. Ranjit N. Munasinghe

National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.

Wolfgang Rettig

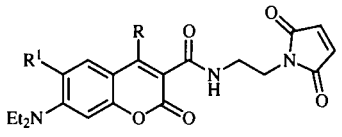
Fachinstitut für Physikalische und Theoretische Chemie, Humbolt-Universität, Bunsenstrasse 1, D-10117 Berlin, Germany

Received March 16, 2000

4-Trifluoromethyl- or 6-bromo-substituted 7-diethylaminocoumarin-3-carboxamide derivatives **2** and **3**, each containing a maleimide have been synthesized as potential fluorescent labeling reagents for thiol groups in proteins and their fluorescence properties have been determined. The 4-trifluoromethyl substituted compound **2** has a significantly greater Stokes shift than the comparable compound lacking this group, but both the new coumarins have low fluorescence quantum yields (ϕ_f). When a 4-trifluoromethyl substituent is present, the 3-carboxamide is unusually labile to hydrolysis. Bromination of ethyl 7-diethylaminocoumarin-3-carboxylate **17** gave the 6- and 8-bromo derivatives **18** and **19** respectively, and also the 8-bromo-7-monoethylamino compound **20**. ϕ_f for the latter compound is 100-fold greater than for its diethylamino analogue **19**. Fluorescence lifetime measurements support an interpretation based on the twisted intramolecular charge transfer (TICT) model to explain these large differences in ϕ_f.

J. Heterocyclic Chem., **37**, 1447 (2000).

We have previously described the fluorescent coumarin maleimide **1** as a reagent for specific labeling of thiol groups on proteins [1]. It was first synthesized for labeling a single-cysteine mutant of the *E. coli* phosphate-binding protein. The labeled protein shows an order of magnitude enhancement of the coumarin fluorescence upon saturation with inorganic phosphate [2] and this probe for inorganic phosphate has enabled measurement of phosphatase activity in real time with millisecond time resolution within working biological systems such as muscle fibers [3]. Further applications of this phosphate probe have also been described [4]. In other work the coumarin **1** has been used to label cysteine mutants of calmodulin. The resultant conjugates showed fluorescence increases up to 2-fold upon saturation with Ca^{2+} [5]. These results imply high environmental sensitivity for the fluorescence of this coumarin and it was of interest to investigate substituent effects, since related compounds might show similar or improved properties. Here we describe the synthesis of two analogues of **1** that bear either a 4-trifluoromethyl or a 6-bromo substituent, compounds **2** and **3** respectively. Compound **2** was chosen because a 4-trifluoromethyl substituent may enhance the Stokes shift and impart greater resistance to photobleaching [6], although there is little available information on compounds bearing an electron-withdrawing substituent at the 3-position. The brominated compound **3** was chosen to explore the effect of the heavy atom substituent. There are few previous examples of halogenated coumarins and apparently none bearing a 7-dialkylamino substituent. During this work we encountered a number of unexpected results, specifically a remarkable lability of the amide bond in compound **2** and related amides, an unusual dealkylation of a diethylamino group during aromatic bromination and a large effect of this dealkylation on the fluorescence quantum yield of the product. These results, together with the synthesis of **2** and **3**, are presented here.

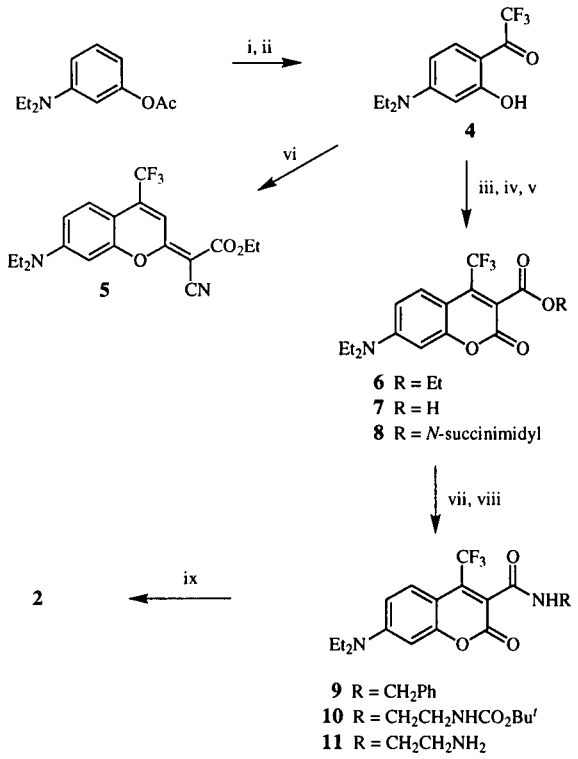


1 R, R^1 = H
2 R = CF_3, R^1 = H
3 R = H, R^1 = Br

Synthesis of **2**, shown in Scheme 1, began with efficient trifluoroacetylation of 3-acetoxy-*N*,*N*-diethylaniline by trifluoroacetic anhydride, as described for other reactive aromatic compounds [7]. Mild acidic hydrolysis of the initial product cleaved the phenolic acetate to give the trifluoromethyl ketone **4** in good overall yield. The *ortho* relationship of the trifluoroacetyl and hydroxy groups was indicated by a strong intramolecular hydrogen bond between the phenol and the carbonyl group in **4**, shown by the low carbonyl frequency in the ir spectrum (1635 cm^{-1} compared to 1665 cm^{-1} for 4-dimethylamino-α,α,α-trifluoroacetophenone [7]) and the appearance of the phenolic hydrogen as a sharp singlet at δ 11.83 in the ^1H nmr spectrum. A striking feature of the ^1H nmr spectrum was the 5-bond coupling of 2.2 Hz between H-6 and the fluorines, that provided additional confirmation of the substitution

pattern. Similar $^5J_{H,F}$ coupling has been reported for 2-hydroxy-α,α,α-trifluoroacetophenone [8] while the $^5J_{H,F}$ value for α,α,α-trifluoroacetophenone itself is only 0.6 Hz [9]. The large H,F coupling in the *o*-hydroxy case has been attributed to through-space effects when internal hydrogen bonding holds the fluorine atoms close to the *ortho*-proton [8]. In the 4-trifluoromethylcoumarins derived from **4**, geometric constraints hold the CF_3 group close to H-5 of the coumarin and the $^5J_{H,F}$ coupling was also large, typically ~2.1 Hz.

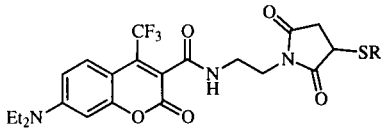
Scheme 1



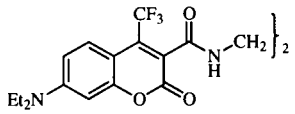
Reagents: (i) trifluoroacetic anhydride; (ii) aqueous H_2SO_4-tetrahydrofuran; (iii) monoethyl malonate-$PhOPOCl_2$; (iv) aqueous NaOH; (v) *N*-hydroxysuccinimide-dicyclohexylcarbodiimide; (vi) EtO_2CCH_2CN-K_2CO_3; (vii) RNH_2-diisopropylethylamine; (viii) trifluoroacetic acid; (ix) maleic anhydride-acetic acid.

Assembly of the coumarin nucleus by conventional reaction of **4** with diethyl malonate and piperidine was unsuccessful, with only the starting materials being recovered. By analogy with a reported reaction [10] of 4-trifluoroacetylresorcinol, that had been shown to give a coumarin in poor yield, ketone **4** was heated with ethyl cyanoacetate and potassium carbonate but the only product obtained was the benzopyran **5**. Its structure and stereochemistry were assigned by analogy with the corresponding 7-hydroxy compound [10]. The mechanism of formation of the 7-hydroxy analogue of **5** has been speculated upon previously [10] and we have no additional mechanistic information. In contrast to the outcome of the above experiment, when **4** was treated with monoethyl malonate, triethylamine and phenyl phosphorodichloridate [11], the required coumarin **6** was readily obtained and subsequent alkaline hydrolysis gave the acid **7**. In a trial experiment to establish conditions for amide formation, **7** was treated with isobutyl chloroformate-tributylamine to form a mixed anhydride, but subsequent addition of benzylamine yielded only a trace of the amide **9**. Instead we prepared and characterized the activated ester **8** with *N*-hydroxysuccinimide-dicyclohexylcarbodiimide, and **8** then gave **9** in moderate yield when treated with benzylamine. Addition of diisopropylethylamine was beneficial and treatment of **8** with the mono-protected *N*-Boc derivative of ethylenediamine under the latter conditions gave the carbamate **10** in 82% yield. In pursuit of a more convergent synthesis of the target maleimide **2**, we attempted to prepare it directly by reaction of **8** with *N*-(2-aminoethyl)-maleimide (prepared as its TFA salt [1]) but obtained only traces of the expected product. Arano *et al.* [12] have reported successful reaction of the same amine, as its TFA salt, with a different *N*-hydroxysuccinimide ester and we were similarly successful during synthesis of **3** (see below). The reasons for failure of the present reaction remain unclear.

Although to this point there were slight variations between reaction conditions in the present work and those previously used for preparation of **1** that lacks a trifluoromethyl substituent [1], the final stage of Scheme 1 revealed a significant difference between the two series. The carbamate protecting group of **10** was removed with trifluoroacetic acid and the amine **11** (as its free base) was treated with maleic anhydride, followed by acetic anhydride-cobalt naphthenate to cyclize the derived maleamic acid as previously described [1,13]. The reaction mixture contained two principal coumarin products in ~1:1 ratio. The ^1H nmr spectrum showed that maleimide **2** was present but its separation from the second product was difficult. However, after treatment of the mixture with thiophenol, the maleimide adduct **12** and the other component could be separated. The latter was assigned as the dimer **14**, confirmed by comparison with an authentic sample prepared by allowing **11**, as its free base, to react with the hydroxysuccinimide ester **8**. The unwanted formation of **14** was avoided by reacting maleic anhydride with the TFA salt of **11**, the latter being obtained directly from **10** by removal of the Boc protecting group. This procedure, in acetic acid at reflux, enabled maleimide **2** to be prepared without contamination by **14**.

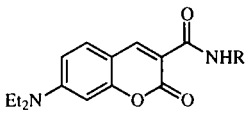


12 R = Ph
13 R = $CH_2CH_2SO_3^-$



14

Formation of **14** under the initial conditions implies that the terminal free amino group of **11** had undergone reaction at the amide carbonyl of a second molecule of **11** to displace ethylenediamine. Such facile amide interchange is remarkable and must have occurred before the free amino group had been trapped by maleic anhydride. This lability of the amide bond was readily confirmed by overnight treatment of **10** with excess benzylamine at room temperature in the presence of diisopropylethylamine. The tlc analysis showed substantial conversion to the benzylamide **9**, without formation of other products, and the ^1H nmr spectrum gave the ratio of **9** and **10** as 1.7:1. A corresponding experiment over the same time scale using **15**, *i.e.* the analogue of **10** without the trifluoromethyl substituent, showed no conversion to the benzylamide **16**. The trifluoromethyl group must be implicated in the unusual reactivity, presumably *via* an inductive effect.



15 R = $CH_2CH_2NHCO_2Bu^t$
16 R = CH_2Ph

As described in the Introduction, a principal reason for undertaking the synthesis of **2** was the anticipation of a large Stokes shift for its fluorescence emission. Table 1 shows excitation and emission maxima measured for an aqueous solution of its thiol adduct **13**. The Stokes shift was 117 nm, more than double the value of 51 nm for compound **1** that lacks the trifluoromethyl group (λ_{ex} 430 nm, λ_{em} 481 nm in aqueous solution; measured as a thiol adduct [1]). Compound **13** was obtained upon addition of a water-soluble thiol (2-sulfanylethanesulfonate) to the maleimide double bond of **2**, and had a 1.6-fold higher fluorescence intensity in aqueous solution than **2**. This relatively small change is consistent with earlier observations that fluorescence quenching by an intramolecular maleimide becomes less efficient as the excitation band moves to longer wavelength [1]. The fluorescence quantum yield of 0.01 for the thiol adduct **13** was comparable to the 0.014 value reported for an adduct of **1** with the same thiol in aqueous solution [2b].

Table 1

Fluorescence Properties of Coumarins **2**, **3** and **17-20**

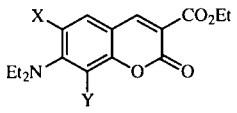
Compound	Solvent [a]	λ_{ex}, nm	λ_{em}, nm	ϕ_f
2	aqueous	436	553	0.01
2 + thiol [b]	aqueous	436	553	0.016
3	EtOH	399	450	0.001
3 + thiol [b]	EtOH	400	451	0.003
17	EtOH	418	461	0.055
18	EtOH	398	446	0.007
19	EtOH	400	447	0.008
20	EtOH	410	450	0.81
20	aqueous	408	461	0.60

[a] The composition of aqueous solutions is defined in the Experimental section. [b] The thiol is 2-sulfanylethanesulfonate (see Experimental section).

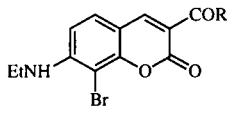
Despite these encouraging fluorescence results, the ease of amide interchange in these trifluoromethylcoumarins raised concern that the amide bond of **2** might also undergo unusually facile hydrolysis, with the consequence that proteins labeled with **2** could gradually lose the fluorescent part of the label. In detailed studies to be reported elsewhere [14] we have shown that the lactone ring of these trifluoromethylcoumarin amides undergoes rapid ring-opening in alkali, with loss of the 436 nm chromophore. Acidification regenerates the lactone ring but is accompanied by quantitative hydrolysis of the amide. Further studies of this process to elucidate the mechanism are in hand but the unexpected lability of the coumarin ring in **2** is likely to limit applications of the compound as a protein labeling reagent.

We required the brominated compound **3** to examine the effect of a heavy atom on the optical properties of a 7-dialkylaminocoumarin labeling reagent. Previous sparse data on 6-halo-7-hydroxycoumarins show relatively little perturbation of fluorescence quantum yields [15] or light absorption [16]. Although direct bromination of 7-hydroxycoumarins has been reported to result in multiple substitution [15], we first examined bromination of the coumarin ester **17** as a means to access the required 6-bromo compound **18**, from which we expected to elaborate the maleimide **3**. In the event, treatment of **17** with a slight molar excess of bromine in glacial acetic acid at room temperature gave the 6- and 8-monobromo species **18** and **19**, readily separated by chromatography, together with third fraction that contained a mixture of unreacted starting

material and a new compound. The latter was readily isolated from this mixture by crystallization and shown to be the dealkylated 8-bromo-7-ethylamino compound **20**. When the reaction was carried out in chloroform solution, the same products were obtained but in different relative proportions (see Experimental section).



17 X, Y = H
18 X = Br, Y = H
19 X = H, Y = Br

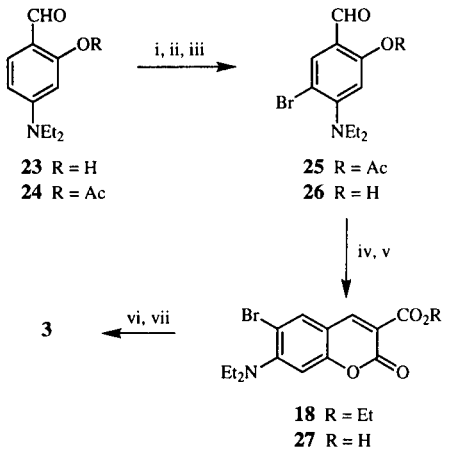


20 R = OEt
21 R = OH
22 R = $NHCH_2Ph$

There are occasional previous examples of *N*-dealkylation during aromatic bromination [17,18]. In each case, dealkylation evidently occurred from an intermediate formed during the bromination rather than simply by action of the released hydrogen bromide on the tertiary amine. In one well-investigated example (bromination of *N,N*-dialkyl-4-bromoanilines), loss of the alkyl group was shown to occur by an oxidative process rather than by nucleophilic displacement [17]. In the present case, the ester **17** was recovered unchanged after exposure to 45% hydrogen bromide in acetic acid, suggesting that dealkylation does not occur by simple displacement.

Preparation of the 6-bromo compound **18** in useful quantities from the direct bromination was impractical, so we adopted the alternative route shown in Scheme 2. Bromination of the aldehyde **24** gave at best ~80% conversion to the monobromo compound **25** that had the required substitution pattern, with the remainder being mainly unreacted starting material. It was pleasing that bromination took place only at the 5-position as, by contrast, nitration of the non-acetylated parent aldehyde **23** has been reported to give a mixture of 3- and 5-mononitration products, in which the 3-nitro isomer was the more abundant [19]. Although **25** could be isolated by chromatography, it was preferable to deacetylate the crude product (aqueous ammonia-dioxane) and use the resultant mixture of phenolic aldehydes **23** and **26** for conversion to the coumarins **17** and **18**. The bromocoumarin **18** was then readily isolated by chromatography and hydrolyzed by alkali to the acid **27**. Unlike the 4-trifluoromethyl-substituted acid **7** described above, **27** underwent straightforward activation by isobutyl chloroformate-tributylamine and subsequent condensation with (2-aminoethyl)-maleimide gave the target maleimide **3**. Each of the new maleimides **2** and **3** was used to label the mutant phosphate-binding protein [2] but neither labeled protein showed significant fluorescence intensity changes upon saturation with phosphate [20].

Scheme 2



23 R = H
24 R = Ac
25 R = Ac
26 R = H
18 R = Et
27 R = H

Reagents: (i) AcCl-Et_3N; (ii) Br_2-acetic acid; (iii) NH_3-dioxane; (iv) diethyl malonate-piperidine; (v) aqueous NaOH; (vi) isobutyl chloroformate-Bu_3N; (vii) *N*-(2-aminoethyl)maleimide.

Table 1 shows fluorescence data for the various brominated coumarins obtained in these experiments. For the diethylamino compounds **18** and **19**, the effect of the bromo-substituent is to reduce the fluorescence quantum yield by an order of magnitude in comparison with the non-brominated **17**. For the maleimide **3**, fluorescence increases ~3-fold upon addition of a thiol as a consequence of removing the quenching effect of the maleimide group. This effect is of similar magnitude to that reported previously for the maleimide **1**, as would be expected from the similar excitation maxima of the two compounds [1]. The most striking result is for monoethyl compound **20**, where the fluorescence quantum yield (ϕ_f, 0.81 in ethanol) is two orders of magnitude greater than for the analogous diethylamino compound **19**. The high quantum yield indicates that the bromine atom cannot significantly promote intersystem crossing. To investigate these properties further, fluorescence lifetimes were measured for the three compounds **17**, **19** and **20**. Table 2 shows these data, together with the derived radiative (k_r) and nonradiative (k_{nr}) rate constants. The results are consistent with twisted intramolecular charge transfer (TICT) theory [21,22]. In 7-dialkylaminocoumarins, fluorescence is considered to occur from an excited state in which the dialkylamino group is essentially co-planar with the coumarin ring system. Twisting around the C-N bond that joins the amino group to the coumarin is accompanied by intramolecular charge transfer and leads to a nonemissive state, *i.e.* fluorescence is quenched. Charge transfer is facilitated by increased strengths of the donor and acceptor (amino group and coumarin ring respectively) and in the present compounds the acceptor strength is enhanced by the 3-carbonyl substituent. Thus compound **17** has a much lower ϕ_f and fluorescence lifetime (τ_f) than for the similar 7-diethyl-

amino-4-methylcoumarin **28** that lacks the electron-withdrawing 3-substituent (ϕ_f and τ_f for **28** in ethanol are 0.73 and 3.1 ns respectively [23]). The high values of k_{nr} for compounds **17** and **19** indicate that the charge transfer state is principally depopulated by nonradiative paths [21-23]. The effect is much more marked for the brominated compound **19** where the bulky bromine substituent would be expected to destabilize the planar conformation of the diethylamino group and promote twisting around the C-N bond. Such ground-state twisting in other compounds has been shown to accelerate the rate of transition to the TICT state [24]. By contrast, in compound **20** where only one ethyl group is present on the amine, the very low value for k_{nr} is consistent with the expectation of much less ground-state twisting of the ethylamino group. Furthermore, the exchange of an ethyl group for a hydrogen atom on the amine will decrease the amine's donor strength, giving additional inhibition of charge transfer. Thus the two effects reinforce one another, leading to the very high fluorescence observed for **20**. The low value for k_{nr} in a compound possessing a bromine heavy atom also supports the inference that intersystem crossing enhanced by bromine is negligible in these compounds.

Table 2

Fluorescence Lifetimes and Rate Constants [a] for Coumarins **17**, **19** and **20**

Compound	τ_f, ns	k_r, nm^{-1}	k_{nr}, ns^{-1}
17	0.21	0.26	4.5
19	<0.05	≥0.16 [b]	>20
20	2.46	0.33	0.08

[a] Rate constants are calculated from the equations $k_r = \phi_f/\tau_f$ and $k_{nr} = (1 - \phi_f)/\tau_f$. [b] The upper limit is estimated to be comparable to the value for **20**, since the two compounds have very similar electronic structure.

Finally, the high fluorescence of **20** makes it a probe of potential interest for attachment to biological molecules. We therefore needed to establish whether reactions intended to link this coumarin to other species *via* an amide bond would be complicated by the presence of the secondary amine. It seemed likely that the secondary amine would have low reactivity because of its relatively crowded steric environment and reduced nucleophilicity, the latter arising from extensive conjugation of the amino group with the carbonyl groups on the coumarin. Therefore **20** was hydrolyzed to the acid **21** and the latter compound was converted to a mixed anhydride (isobutyl chloroformate-tributylamine). Upon subsequent treatment with benzylamine, the amide **22** was obtained in good yield, with no evidence for competing reactivity of the secondary amino function. With this result in hand, we are now assessing the utility of this new fluorescent coumarin as a probe in biological applications.

Acknowledgements.

We thank Dr. S. R. Martin for the fluorescence quantum yield measurements, Dr. K. J. Welham for high resolution mass spectrometry, Professor F. Hibbert and Dr. M. R. Webb for unpublished data and the MRC Biomedical NMR Centre for access to facilities.

EXPERIMENTAL

Elemental analyses were carried out by MEDAC Ltd., Egham, Surrey, U.K. The nmr spectra were determined on JEOL FX90Q, Bruker AM400WB or Varian Unityplus 500 spectrometers for solutions in deuteriochloroform unless otherwise specified and with tetramethylsilane as internal standard; J values are given in Hz. Infrared spectra were determined for Nujol mulls and ultraviolet spectra for solutions in ethanol unless otherwise specified. Merck 9385 silica gel was used for flash chromatography. Petroleum ether was the fraction boiling between 40-60°. Organic extracts were dried over anhydrous Na_2SO_4 and solvents were evaporated under reduced pressure.

4-Diethylamino-2-hydroxy-α,α,α-trifluoroacetophenone (**4**).

A solution of 3-acetoxy-*N*,*N*-diethylaniline [25] (20.4 g, 98.6 mmoles) and trifluoroacetic anhydride (34.7 mL, 246 mmoles) in dry ether (100 mL) was refluxed for 3 hours and the solvent was evaporated. The residue, dissolved in a mixture of tetrahydrofuran (400 mL) and 2 *M* aqueous hydrochloric acid (200 mL), was stirred overnight at room temperature. The solution was concentrated under reduced pressure and the aqueous residue was extracted with ether. The organic extract was washed with water and brine, dried and evaporated. The residue was crystallized (petroleum ether) to give ketone **4** as yellow needles (15.2 g, 59%), mp 51-52°; uv: λ_{max} 366 nm (ε 34 700 $M^{-1}cm^{-1}$; ir: ν_{max} 1635, 1560, 1528 cm^{-1}; ^1H nmr: (400 MHz) δ 1.23 (t, J = 7.2 Hz, 6H, CH_3), 3.43 (q, 4H, CH_2), 6.11 (d, $J_{3,5}$ = 2.5 Hz, 1H, H3), 6.27 (dd, $J_{5,6}$ = 9.6 Hz, 1H, H5), 7.57 (dq, $J_{6,F}$ = 2.2 Hz, 1H, H6), 11.83 (s, 1H, OH).

Anal. Calcd. for $C_{12}H_{14}F_3NO_2$: C, 55.17; H, 5.40; N, 5.36. Found: C, 55.17; H, 5.44; N, 5.35.

Ethyl (*E*)-Cyano-(7-diethylamino-4-trifluoromethyl-2*H*-1-benzopyran-2-ylidene)acetate (**5**).

A mixture of ketone **4** (0.53 g, 2.03 mmoles), ethyl cyanoacetate (0.64 mL, 6.0 mmoles) and potassium carbonate (0.42 g, 3.04 mmoles) was heated at 140° for 0.5 hour, cooled and shaken with ethyl acetate and 0.5 *M* aqueous hydrochloric acid. The ethyl acetate layer was washed with water, dried and evaporated and the major component isolated by flash chromatography [ethyl acetate-petroleum ether (3:7)] was benzopyran **5** as red needles (0.2 g, 26%), mp 193° (ethyl acetate-petroleum ether); uv: λ_{max} 254 (ε 30 200 $M^{-1}cm^{-1}$), 277 (17 400), 485 (26 800), 504 (26 600) nm; ir: ν_{max} 2215, 1700 cm^{-1}; ^1H nmr: (400 MHz) δ 1.24 (t, J = 7.2 Hz, 6H, CH_3), 1.37 (t, J = 7.1 Hz, 3H, CH_3), 3.46 (q, 4H, NCH_2), 4.30 (q, 2H, OCH_2), 6.61 (d, $J_{6,8}$ = 2.6 Hz, 1H, H8), 6.66 (dd, $J_{5,6}$ = 9.2 Hz, 1H, H6), 7.47 (dq, $J_{5,F}$ = 2.0 Hz, 1H, H5).

Anal. Calcd. for $C_{19}H_{19}F_3N_2O_3$: C, 60.00; H, 5.03; N, 7.36. Found: C, 60.20; H, 5.07; N, 7.51.

7-Diethylamino-4-trifluoromethylcoumarin-3-carboxylic Acid (**7**).

A solution of ketone **4** (3.91 g, 15.0 mmoles), triethylamine (6.22 mL, 45.0 mmoles) and monoethyl malonate [26] (1.98 g, 15.0 mmoles) in 1,2-dichloroethane (45 mL) was cooled to 0° and treated dropwise with phenyl phosphorodichloridate (2.24 mL, 15.0 mmoles). The solution was stirred at 0° for 0.5 hour, then warmed to room temperature and refluxed for 3.5 hours. The cooled solution was diluted with ether and washed with water, 10% aqueous sodium hydroxide, 1 *M* aqueous hydrochloric acid and brine, dried and evaporated to give ester **6** as a viscous yellow oil (4.0 g, 75%) that was used without further purification; ^{1}H nmr: (90 MHz) δ 1.23 (t, J = 7 Hz, 6H, CH_3) 1.37 (t, J = 7 Hz, 3H, CH_3), 3.44 (q, 4H, NCH_2), 4.39 (q, 2H, OCH_2), 6.49 (d, $J_{6,8}$ = 2.6 Hz, 1H, H8), 6.64 (dd, $J_{5,6}$ = 9.2 Hz, 1H, H6), 7.52 (dq, $J_{5,F}$ = 2.2 Hz, 1H, H5). A solution of **6** (4.0 g, 11.2 mmoles) in ethanol (225 mL) was mixed with 0.5 *M* aqueous sodium hydroxide (33 mL) and stirred at room temperature for 3 hours, then adjusted to *p*H 6 with glacial acetic acid, concentrated under reduced pressure to ~70 mL, acidified with dilute aqueous hydrochloric acid and diluted with water until a solid began to precipitate. After cooling in ice, the solid was filtered and crystallized to give the carboxylic acid **7** as yellow needles (2.39 g, 63%), mp 170-172° (aqueous ethanol); uv: λ_{max} [ethanol-water (5:95)] 261 (ε 13 500 $M^{-1}cm^{-1}$), 419 (17 400) nm; ir: ν_{max} 3340, 1715, 1685 cm^{-1}; ^{1}H nmr: δ [90 MHz, deuteriochloroform-methanol-d_4 (9:1)] 1.23 (t, J = 7 Hz, 6H, CH_3), 3.44 (q, 4H, CH_2), 6.51 (d, $J_{6,8}$ = 2.6 Hz, 1H, H8), 6.65 (dd, $J_{5,6}$ = 9.2 Hz, 1H, H6), 7.54 (dq, $J_{5,F}$ = 2.2 Hz, 1H, H5).

Anal. Calcd. for $C_{15}H_{14}F_3NO_4 \cdot 1/2H_2O$: C, 53.25; H, 4.47; N, 4.14. Found: C, 53.24; H, 4.51; N, 4.09.

N-Succinimidyl 7-Diethylamino-4-trifluoromethylcoumarin-3-carboxylate (**8**).

N-Hydroxysuccinimide (0.41 g, 3.56 mmoles) and dicyclohexylcarbodiimide (0.91 g, 4.42 mmoles) were added to a solution of carboxylic acid **7** (1.0 g, 2.96 mmoles) in dry acetonitrile (30 mL) and the solution was stirred at room temperature for 2 hours. Glacial acetic acid (172 μl, 3.0 mmoles) was added and after 1 hour the solution was filtered and the precipitate washed with ethyl acetate. The combined filtrates were evaporated and the residue was crystallized (ethyl acetate-petroleum ether) to give *N*-hydroxysuccinimide ester **8** (0.88 g, 70%). This material was suitable for use in subsequent reactions. An analytical sample obtained after flash chromatography [ethyl acetate-petroleum ether (45:55)] had mp 166.5-168° (ethyl acetate-petroleum ether); ^{1}H nmr: δ (400 MHz) 1.25 (t, J = 7.5 Hz, 6H, CH_3), 2.89 (s, 4H, CH_2), 3.47 (q, 4H, CH_2), 6.54 (d, $J_{6,8}$ = 2.6 Hz, 1H, H8), 6.68 (dd, $J_{5,6}$ = 9.4 Hz, 1H, H6), 7.58 (dq, $J_{5,F}$ = 2.1 Hz, 1H, H5).

Anal. Calcd. for $C_{19}H_{17}F_3N_2O_6$: C, 53.53; H, 4.02; N, 6.57. Found: C, 53.30; H, 4.07; N, 6.49.

N-Benzyl-7-diethylamino-4-trifluoromethylcoumarin-3-carboxamide (**9**).

Benzylamine (79 μl, 0.72 mmole) was added to a solution of ester **8** (0.153 g, 0.36 mmole) in dry acetonitrile (5 mL) and the solution was stirred at room temperature for 2 hours, then diluted with ethyl acetate and washed with water, 0.5 *M* aqueous hydrochloric acid, 10% aqueous sodium bicarbonate and brine, dried and evaporated. Flash chromatography [ethyl acetate-petroleum ether (1:4)] gave the *N*-benzyl carboxamide **9** (0.075 g, 50%), mp 210-211° (ethyl acetate); uv: λ_{max} 258 (ε 15 700 $M^{-1}cm^{-1}$), 412 (25 100) nm; ir: ν_{max} 3230, 1735, 1640 cm^{-1}; ^{1}H nmr: (400 MHz) δ 1.23 (t, J = 7.1 Hz, 6H, CH_3), 3.43 (q, 4H, CH_2), 4.63 (d, J = 5.7 Hz, 2H, CH_2Ph), 6.10 (t, 1H, NH), 6.48 (d, $J_{6,8}$ = 2.6 Hz, 1H, H8), 6.63 (dd, $J_{5,6}$ = 9.3 Hz, 1H, H6), 7.26-7.40 (m, 5H, Ph), 7.54 (dq, $J_{5,F}$ = 2.0 Hz, 1H, H5).

Anal. Calcd. for $C_{22}H_{21}F_3N_2O_3$: C, 63.15; H, 5.06; N, 6.69. Found: C, 62.97; H, 4.99; N, 6.70.

tert-Butyl *N*-[2-(7-Diethylamino-4-trifluoromethylcoumarin-3-carboxamido)ethyl]carbamate (**10**).

A solution of ester **8** (0.86 g, 2.0 mmoles) in dry acetonitrile (18.5 mL) was treated with *tert*-butyl *N*-(2-aminoethyl)carbamate [27] (0.48 g, 3.0 mmoles) and diisopropylethylamine (0.37 mL, 2.13 mmoles) and stirred under nitrogen for 20 hours at room temperature, then diluted with ethyl acetate, washed with 0.5 *M* aqueous hydrochloric acid and brine, dried and evaporated. The residue was crystallized (ethyl acetate-petroleum ether) to give carbamate **10** as yellow plates (0.77 g, 81%), mp 194.5-196°; ir: ν_{max} 3310, 3280, 1728, 1685, 1655 cm^{-1}; ^{1}H nmr: (400 MHz) δ 1.22 (t, J = 7.2 Hz, 6H, CH_3), 1.42 (s, 9H, CMe_3), 3.26-3.54 (m, 8H, NCH_2), 5.10 (br s, 1H, NH), 6.49 (d, $J_{6,8}$ = 2.6 Hz, 1H, H8) 6.63 (dd, $J_{5,6}$ = 9.5 Hz, 1H, H6), 7.53 (dq, $J_{5,F}$ = 2.1 Hz, 1H, H5).

Anal. Calcd. for $C_{22}H_{28}F_3N_3O_5$: C, 56.05; H, 5.99; N, 8.91. Found: C, 55.75; H, 5.84; N, 8.75.

N-(2-Maleimidoethyl)-7-diethylamino-4-trifluoromethylcoumarin-3-carboxamide (**2**).

Experiment (a).

A solution of carbamate **10** (0.75 g, 1.59 mmoles) in trifluoroacetic acid (7 mL) was kept at room temperature for 1 hour and evaporated under reduced pressure. The residue was partitioned between chloroform and aqueous sodium bicarbonate, and the organic phase was dried and evaporated to leave a yellow foam (0.43 g), to which maleic anhydride (0.11 g, 1.17 mmoles) and dimethylacetamide (1.2 mL) were added. The mixture was warmed to 60° over 20 minutes and treated with an aliquot (55 μl) of a solution of cobalt naphthenate (20 μl) in dimethylacetamide (1 mL), followed by acetic anhydride (0.22 mL). The mixture was stirred at 70-80° for 2 hours and cooled, then diluted with water and extracted with ethyl acetate. The organic extract was washed with water, dried and evaporated. Flash chromatography [ethyl acetate-petroleum ether (60:40)] gave a yellow foam (0.158 g) that contained two components in ~1:1 ratio. A portion of this mixture (0.08 g) was stirred for 1 hour with thiophenol (41 mg) in a mixture of chloroform (19 mL), ethanol (19 mL) and 25 m*M* aqueous sodium phosphate, *p*H 7.1 (19 mL). The mixture was diluted with chloroform and the organic layer was washed with 0.5 *M* aqueous sodium hydroxide and water, dried and evaporated to give a yellow solid (0.082 g). Flash chromatography [ethyl acetate-petroleum ether (55:45)] gave two components. The less polar was 7-diethylamino-*N*-{2-[(3-phenylsulfanyl)succinimido]ethyl}-4-trifluoromethylcoumarin-3-carboxamide **12** (0.035 g), mp 177.5-179° (ethyl acetate-petroleum ether): ^{1}H nmr: (90 MHz) δ 1.23 (t, J = 7.5 Hz, 6H, CH_3), 2.65 (dd, J = 18.5 Hz and 4.4 Hz, 1H, one H of CH_2CHS), 3.07-3.75 (m, 9H, 4 x NCH_2 and 1H of CH_2CHS), 4.18 (dd, J_{vic} = 9.2 Hz, 1H, CHSPh), 6.14 (br s, 1H, NH), 6.46 (d, $J_{6,8}$ = 2.6 Hz, 1H, H8), 6.61 (dd, $J_{5,6}$ = 9.2 Hz, 1H, H6), 7.63-7.15 (m, 6H, H5 and Ph).

Anal. Calcd. for $C_{27}H_{26}F_3N_3O_5S$: C, 57.75; H, 4.67; N, 7.48. Found: C, 57.75; H, 4.76; N, 7.46.

The more polar component (0.007 g) was shown to be the dimer **14** by comparison with an authentic sample (see below).

Experiment (b).

A solution of carbamate **10** (1.2 g, 2.55 mmoles) in trifluoroacetic acid (13 mL) was kept at room temperature for 1 hour, then evaporated under reduced pressure and the residue was kept *in vacuo* for 0.5 hour to remove residual trifluoroacetic acid. The crude trifluoroacetate salt and maleic anhydride (0.375 g, 3.83 mmoles) were dissolved in glacial acetic acid (20 mL) and the mixture was refluxed for 2 hours and stirred at room temperature for 20 hours. The solvent was evaporated and the residue was dissolved in chloroform and washed with 0.5 *M* aqueous hydrochloric acid, saturated sodium bicarbonate and brine, dried and evaporated. Flash chromatography [ethyl acetate-petroleum ether (55:45)] gave maleimide **2** (0.356 g, 31%), mp 201.5-203° (ethyl acetate-petroleum ether): uv: λ_{max} 257 (ε 14 500 $M^{-1}cm^{-1}$), 415 (21 800) nm; uv: [20 m*M* sodium phosphate, *p*H 7-ethanol (9:1)] λ_{max} 261 (ε 15 000 $M^{-1}cm^{-1}$), 436 (22 600) nm; ir: ν_{max} 3300, 1728, 1700, 1655 cm^{-1}; ^1H nmr: (400 MHz) δ 1.22 (t, J = 7.2 Hz, 6H, CH_3), 3.43 (q, 4H, CH_2Me), 3.66-3.80 (m, 4H, CH_2N), 6.20 (t, J = 4.5 Hz, 1H, NH), 6.48 (d, $J_{6,8}$ = 2.6 Hz, 1H, H8), 6.63 (dd, $J_{5,6}$ = 9.5 Hz, 1H, H6), 6.74 (s, 2H, CH=CH), 7.52 (dq, $J_{5,F}$ = 2.1 Hz, 1H, H5).

Anal. Calcd. for $C_{21}H_{20}F_3N_3O_5$: C, 55.88, H, 4.47; N, 9.30. Found C, 55.88, H, 4.42; N, 9.22.

1,2-Bis(7-diethylamino-4-trifluoromethylcoumarin-3-carboxamido)ethane (**14**).

The carbamate **10** (0.35 g, 0.74 mmole) was deprotected with trifluoroacetic acid and the residue was partitioned between chloroform and aqueous sodium bicarbonate as described above. The dried residue was dissolved in acetonitrile (4.6 mL) together with *N*-hydroxysuccinimide ester **8** (0.213 g, 0.5 mmole) and diisopropylethylamine (92 µl, 0.53 mmole) and the solution was stirred for 20 hours, then filtered and the solid washed with a little acetonitrile. A solution of the crude solid in chloroform-methanol was absorbed on silica gel, that was added to the top of a packed flash chromatography column and eluted with ethyl acetate-petroleum ether (55:45) to give the dimer **14** as a yellow solid (42 mg, 12%), mp 302-304° (chloroform-petroleum ether); ir: ν_{max} 3280, 1730, 1655, 1628, 1600, 1555, 1525 cm^{-1}; ^1H nmr: (400 MHz) δ 1.22 (t, J = 7.1 Hz, 12H, CH_3), 3.43 (q, 8H, CH_2Me), 3.63-3.72 (m, collapsed to s upon irradiation of NH, 4H, CH_2NH), 6.45 (d, $J_{6,8}$ = 2.6 Hz, 2H, H8), 6.50 (dd, $J_{5,6}$ = 9.3 Hz, 2H, H6), 7.01 (br s, 2H, NH), 7.55 (dq, $J_{5,F}$ = 2.1 Hz, 2H, H5).

Anal. Calcd. for $C_{32}H_{32}F_6N_4O_6 \cdot 1/2H_2O$: C, 55.57, H, 4.81; N, 8.10. Found: C, 55.84, H, 4.56; N, 8.02.

N-Benzyl-7-diethylaminocoumarin-3-carboxamide (**16**).

A stirred solution of 7-diethylaminocoumarin-3-carboxylic acid [1] (0.261 g, 1 mmole) and tributylamine (0.357 mL, 1.5 mmoles) in dry dimethylformamide (10 mL) was cooled in an ice-bath and treated with isobutyl chloroformate (0.135 mL, 1.04 mmoles). After 0.5 hour, a solution of benzylamine (0.109 mL, 1 mmole) in dry dimethylformamide (1 mL) was added and the mixture was allowed to warm to room temperature, kept for 3 hours and diluted with ethyl acetate. This solution was washed with water, 1 *M* aqueous hydrochloric acid, 10% aqueous sodium bicarbonate and brine, dried and evaporated. The residue crystallized from ethyl acetate as yellow crystals of amide **16** (0.26 g, 74%), mp 158.5-159.5°; ^1H nmr: (90 MHz) δ 1.24 (t, J = 7.1 Hz, 6H, CH_3), 3.45 (q, 4H, CH_2Me), 4.64 (d, J = 5.7 Hz, 2H, CH_2Ph), 6.49 (d, $J_{6,8}$ = 2.6 Hz, 1H, H8), 6.63 (dd, $J_{5,6}$ = 9.2 Hz, 1H, H6), 7.12-7.48 (m, 6H, H5 and Ph), 8.73 (s, 1H, H4).

Anal. Calcd. for $C_{21}H_{22}N_2O_3$: C, 71.98; H, 6.33; N, 7.99. Found: C, 71.90; H, 6.41; N, 7.94.

Transamidation of **10**.

A solution of carbamate **10** (10 mg, 0.021 mmole), benzylamine (23 µl, 0.21 mmole) and diisopropylethylamine (3.6 µl, 0.021 mmole) in dry acetonitrile (0.40 mL) was stirred at room temperature for 18 hours. The tlc analysis [ethyl acetate-petroleum ether (1:1)] showed ~50% conversion of **10** to benzylamide **9** (R_f values 0.48 and 0.29 for **8** and **9** respectively) and no other products. The solution was diluted with chloroform and washed with aqueous hydrochloric acid and brine, dried and evaporated and the residue was dissolved in trifluoroacetic acid (0.10 mL). After 1 hour at room temperature, the trifluoroacetic acid was evaporated and the tlc analysis, as above, showed a single mobile component, R_f 0.48, identical to benzylamide **9**, together with a yellow spot at the origin, corresponding to the deprotected carbamate **11**. In a separate experiment, the initial reaction mixture was analyzed by ^1H nmr spectroscopy to determine the ratio of **9** and **10** (see Discussion section). For the corresponding compounds **15** and **16** that lacked the trifluoromethyl group [R_f values 0.34 and 0.14 respectively; ethyl acetate-petroleum ether (1:1)], treatment under identical conditions showed no interconversion.

Bromination of Ethyl 7-Diethylaminocoumarin-3-carboxylate (**17**).

A solution of **17** [1] (0.29 g, 1.0 mmole) in glacial acetic acid (2.0 mL) was stirred at room temperature and treated with a solution of bromine (0.19 g, 1.19 mmoles) in glacial acetic acid (2.0 mL) in 5 portions over ~10 minutes. The solution was stirred for 1 hour, poured into ice water and extracted with ethyl acetate. The organic extract was washed with aqueous sodium bicarbonate, water and brine, dried and evaporated to give a yellow gum (0.33 g) that was separated into three components by flash chromatography [ethyl acetate-petroleum ether (3:7)]. The least polar component was ethyl 6-bromo-7-diethylaminocoumarin-3-carboxylate **18** (0.03 g, 8%), mp 74.5-76° (methanol); uv: λ_{max} 257 (ε 8900 $M^{-1}cm^{-1}$), 392.5 (19 300) nm; ^1H nmr: (90 MHz) δ 1.15 (t, J = 7.0 Hz, 6H, CH_3), 1.40 (t, J = 7.0 Hz, 3H, CH_3), 3.31 (q, 4H, NCH_2), 4.39 (q, 2H, OCH_2), 6.88 (s, 1H, H8), 7.74 (s, 1H, H5), 8.38 (s, 1H, H4).

Anal. Calcd. for $C_{16}H_{18}BrNO_4$: C, 52.19; H, 4.93; N, 3.80. Found: C, 52.17; H, 4.88; N, 3.53.

The second component was ethyl 8-bromo-7-diethylaminocoumarin-3-carboxylate **19** (0.10 g, 27%), mp 75.5-77° (methanol); uv: λ_{max} 261 (ε 6200 $M^{-1}cm^{-1}$), 394.5 (19 900) nm; ^1H nmr: (90 MHz) δ 1.15 (t, J = 7.0 Hz, 6H, CH_3), 1.40 (t, J = 7.0 Hz, 3H, CH_3), 3.36 (q, 4H, NCH_2), 4.39 (q, 2H, OCH_2), 6.96 (d, $J_{5,6}$ = 8.8 Hz, 1H, H6), 7.42 (d, 1H, H5), 8.42 (s, 1H, H4).

Anal. Calcd. for $C_{16}H_{18}BrNO_4$: C, 52.19; H, 4.93; N, 3.80. Found: C, 52.25; H, 4.86; N, 3.57.

The most polar component was a mixture of starting material **17** and monoalkylated coumarin **20** (0.14 g, 44:56). An identical experiment but using chloroform as the solvent gave the three components in the following proportions: **18** (0.05 g, 13.5%), **19** (0.03 g, 8%), and a mixture of **17** + **20** (0.11 g, 60:40). To obtain

a pure sample of **20**, the bromination reaction was repeated in acetic acid as above but on a 6-fold larger scale and the reaction products were flash chromatographed [ethyl acetate-petroleum ether (45:55)]. Fractions containing the mixture of **17** + **20** (0.79 g, 3:7) were combined and crystallized to give ethyl 8-bromo-7-ethylaminocoumarin-3-carboxylate **20** (0.44 g, 21%), mp 129.5-131° (methanol); uv: λ_{max} 249 (ε 7900 $M^{-1}cm^{-1}$), 285 (4600), 402 (35 000) nm; uv: [50 m*M* sodium phosphate, *p*H 7-ethanol (19:1)] λ_{max} 258 (ε 10 400 $M^{-1}cm^{-1}$), 410 (34 500) nm; ^1H nmr: (90 MHz) δ 1.37 (t, J = 7.0 Hz, 3H, CH_3), 1.38 (t, J = 7.0 Hz, 3H, CH_3), 3.20-3.46 (br m, 2H, NCH_2), 4.37 (q, 2H, OCH_2), 5.20 (br s, 1H, NH), 6.59 (d, $J_{5,6}$ = 8.4 Hz, 1H, H6), 7.37 (d, 1H, H5), 8.38 (s, 1H, H4).

Anal. Calcd. for $C_{14}H_{14}BrNO_4$: C, 49.43; H, 4.15; N, 4.12. Found: C, 49.42; H, 4.12; N, 3.79.

Ethyl 6-Bromo-7-diethylaminocoumarin-3-carboxylate (**18**).

A solution of 4-diethylaminosalicylaldehyde **23** (2.5 g, 12.95 mmoles) and triethylamine (3.79 mL, 27.20 mmoles) in dry dichloromethane (26 mL) was stirred under nitrogen at 0° and treated dropwise with a solution of acetyl chloride (1.38 mL, 19.43 mmoles) in dry dichloromethane (13 mL). After 1 hour, the solution was stirred overnight at room temperature, then diluted with dichloromethane and washed successively with water, 0.5 *M* aqueous sodium hydroxide and brine, dried and evaporated to give the acetate **24** as a brown oil (2.89 g, 95%) that was used directly in the next step; ^1H nmr: (90 MHz) δ 1.20 (t, J = 7.5 Hz, 6H, CH_3), 2.36 (s, 3H, $COCH_3$), 3.40 (q, 4H, CH_2), 6.26 (d, $J_{3,5}$ = 2.6 Hz, 1H, H3), 6.53 (dd, $J_{5,6}$ = 8.8 Hz, 1H, H5), 7.63 (d, 1H, H6), 9.73 (s, 1H, CHO). A stirred solution of **24** (3.0 g, 12.8 mmoles) in glacial acetic acid (13.5 mL) was treated with a solution of bromine (2.72 g, 17.0 mmoles) in glacial acetic acid (3.6 mL). After 1 hour the solution was poured into ice water and extracted with ethyl acetate. The organic extracts were washed with water, aqueous sodium bicarbonate and brine, dried and evaporated to leave a dark oil (3.52 g) that contained a mixture of acetates **24** and **25** (17:83 based on the ^1H nmr spectrum, see below) and was used directly in the next reaction. A portion purified by flash chromatography [ethyl acetate-petroleum ether (3:7)] gave 2-acetoxy-5-bromo-4-diethylaminobenzaldehyde **25** as a pale oil; ^1H nmr: (90 MHz) δ 1.13 (t, J = 7.0 Hz, 6H, CH_3), 2.36 (s, 3H, $COCH_3$), 3.28 (q, 4H, CH_2), 6.69 (s, 1H, H3), 7.98 (s, 1H, H6), 9.84 (s, 1H, CHO).

The mixed aldehydes **24** and **25** (3.47 g) were dissolved in dioxane-concentrated aqueous ammonia (100 mL; 3:1) and stirred at room temperature for 30 minutes. The solution was concentrated under reduced pressure to remove most of the dioxane and adjusted to *p*H 6 with glacial acetic acid, then partitioned between ethyl acetate and water. The organic extract was washed with water and brine, dried and evaporated to give a mixture of phenolic aldehydes **23** and **26** (27:73 based on the ^1H nmr spectrum, see below). A portion purified by flash chromatography gave 5-bromo-4-diethylaminosalicylaldehyde **26** as a pale oil; ^1H nmr: (90 MHz) δ 1.13 (t, J = 7.0 Hz, 6H, CH_3), 3.30 (q, 4H, CH_2), 6.49 (s, 1H, H3), 7.63 (s, 1H, H6), 9.61 (s, 1H, CHO); hrms (FAB): Calcd. for $C_{11}H_{14}BrNO_2$ + H: 272.0286. Found: 272.0295.

A solution of the crude mixture of **23** and **26** (2.2 g) and diethyl malonate (1.43 g, 8.95 mmoles) in ethanol (10.5 mL) was treated with piperidine (0.08 mL, 0.81 mmole) and refluxed for 4 hours. The cooled solution was concentrated ~2-fold under reduced pressure and diluted with ether, then washed with water, 2 *M* aqueous sodium hydroxide and brine, dried and evaporated. The residue was flash chromatographed [ethyl acetate-petroleum ether (45:55)] to give **18** (0.5 g), identical to the material prepared above.

6-Bromo-7-diethylamino-*N*-(2-maleimidoethyl)coumarin-3-carboxamide (**3**).

A solution of 6-bromo ester **18** (0.45 g, 1.22 mmoles) in methanol (3 mL) was heated under reflux and 0.5 *M* aqueous sodium hydroxide (3 mL) was added rapidly. After 5 minutes the mixture was cooled and acidified with 2 *M* aqueous hydrochloric acid. The precipitate was filtered, washed successively with 2 *M* aqueous hydrochloric acid and water and dried *in vacuo* to give 6-bromo-7-diethylaminocoumarin-3-carboxylic acid **27** (0.22 g, 53%), that was used without further purification. A stirred solution of **27** (0.22 g, 0.65 mmole) and tributylamine (0.23 mL, 0.97 mmole) in dry dimethylformamide (6.5 mL) was cooled in an ice-bath and isobutyl chloroformate (0.08 mL, 0.62 mmole) was added. The solution was kept in the ice-bath for 0.5 hour, when additional tributylamine (0.23 mL, 0.97 mmole) was added, followed by a solution of *N*-(2-aminoethyl)maleimide (trifluoroacetate salt, 0.65 mmole, prepared as previously described [1]) in dry dimethylformamide (0.65 mL). The solution was allowed to warm to room temperature and kept for 3 hours, then diluted with ethyl acetate and washed successively with water, 1 *M* hydrochloric acid, aqueous sodium bicarbonate and brine, dried and evaporated under reduced pressure and purified by flash chromatography [ethyl acetate-petroleum ether (3:1)] to give maleimide **3** (0.2 g, 72%), mp 195-197° (ethyl acetate-petroleum ether); uv: λ_{max} 392 (ε 20 150 $M^{-1}cm^{-1}$) nm; ^1H nmr: (90 MHz) δ 1.14 (t, J = 7.0 Hz, 6H, CH_3), 3.31 (q, 4H, NCH_2CH_3), 3.67-3.84 (m, 4H, CH_2), 6.69 (s, 2H, CH=CH), 6.90 (s, 1H, H8), 7.80 (s, 1H, H5), 8.68 (s, 1H, H4).

Anal. Calcd. for $C_{20}H_{20}BrN_3O_3$: C, 51.96; H, 4.36; N, 9.09. Found: C, 52.12, H, 4.35; N, 9.05.

8-Bromo-7-ethylaminocoumarin-3-carboxylic acid (**21**).

A solution of ester **20** (0.23 g, 0.68 mmole) in methanol (1.7 mL) was heated under reflux and 0.5 *M* aqueous sodium hydroxide (1.7 mL) was added rapidly. After 5 minutes the mixture was cooled and 2 *M* hydrochloric acid (0.5 mL) was added. The reaction mixture was diluted with ethyl acetate and the organic extract was washed with water, dried and evaporated and the solid was crystallized to give carboxylic acid **21** as yellow needles (0.09 g, 60%), mp 210-212° (ethanol); ^1H nmr: (90 MHz, DMSO-d_6) δ 1.18 (t, J = 7.0 Hz, 3H, CH_3), 3.22-3.50 (m, 2H, CH_2), 6.60-6.75 (br m, 1H, NH), 6.80 (d, $J_{5,6}$ = 8.8 Hz, 1H, H6), 7.69 (d, 1H, H5), 8.59 (s, 1H, H4).

Anal. Calcd. for $C_{12}H_{10}BrNO_4$: C, 46.18; H, 3.23; N, 4.49. Found: C, 46.30; H, 3.23; N, 4.44.

N-Benzyl-8-bromo-7-ethylaminocoumrain-3-carboxamide (**22**).

A solution of **21** (0.1g, 0.32 mmole) and tributylamine (0.115 mL, 0.48 mmole) in dry dimethylformamide (3.2 mL) was stirred in an ice-bath and treated with isobutyl chloroformate (0.043 mL, 0.33 mmole). After 0.5 hour, a solution of benzylamine (0.035 mL, 0.32 mmole) in dry dimethylformamide (0.32 mL) was added and the mixture was allowed to warm to room temperature, kept for 3 hours and diluted with ethyl acetate. The solution was washed with water, 1 *M* hydrochloric acid, 10% aqueous sodium

bicarbonate and brine, dried and evaporated. The residue crystallized from methanol to give amide **22** as yellow crystals (0.09 g, 70%), mp 190.5-192°; ^{1}H nmr: (90 MHz) δ 1.37 (t, J = 7.0 Hz, 3H, CH_3), 3.20-3.51 (m, 2H, NCH_2CH_3), 4.65 (d, J = 5.7 Hz, 2H, CH_2Ph), 5.06-5.30 (br m, 1H, $NHCH_2CH_3$) 6.63 (d, $J_{5,6}$ = 8.8 Hz, 1H, H6), 7.32 (s, 5H, Ph), 7.45 (d, J = 8.8 Hz, 1H, H5), 8.73 (s, 1H, H4), 8.96-9.16 (br m, 1H, $NHCH_2Ph$).

Anal. Calcd. for $C_{19}H_{17}BrN_2O_3$: C, 56.87; H, 4.27; N, 6.98. Found: C, 56.74; H, 4.48 N, 6.92.

Fluorescence Spectroscopy and Quantum Yield Determinations.

Fluorescence spectra were measured on a Spex FluoroMax instrument and are uncorrected. Excitation and emission spectra were recorded in a 1 x 1 cm cuvette with 1.7 and 5 nm bandwidths for excitation and emission respectively. Excitation and emission maxima are recorded in Table 1. For determination of the fluorescence quantum yields, the reference standard was an ethanolic solution (0.23 μ*M*) of Coumarin 314 (Eastman Chemicals; ϕ_f = 0.83, ε 45 000 $M^{-1}cm^{-1}$ in ethanol [6]), with excitation at 436 nm. The integrated emission intensities of the reference and test solutions were measured, with excitation maxima as shown in Table 1. For all compounds, stock solutions of known concentration were prepared and diluted in ethanol. For **2** and **20**, dilutions were also made into 20 m*M* sodium phosphate, *p*H 7.0. To measure the effect of thiol addition to the maleimide **2**, an ethanol solution of **2** (0.3 m*M*) was diluted into 20 m*M* sodium phosphate, *p*H 7.0 that contained 1 m*M* sodium 2-sulfanylethanesulfonate to give the thiol adduct **13**. Further dilution with 20 m*M* sodium phosphate gave a final coumarin concentration of 1.6 μ*M*. Similar measurement on the maleimide **3** was complicated by the poor solubility of the compound in aqueous solution prior to addition of a water-soluble thiol. Therefore an ethanol solution of **3** (0.3 m*M*) was diluted 10-fold into 25 m*M* ammonium 3-(*N*-morpholino)propanesulfonate, *p*H 7 that contained 2 m*M* sodium 2-sulfanylethanesulfonate. The solution, now containing the thiol adduct of **3**, was further diluted with ethanol to give a final coumarin concentration of 2 μ*M*. This procedure gave the thiol adduct in ~95% ethanol solution for comparison with the fluorescence in ethanol of maleimide **3** itself.

Fluorescence Lifetime Measurements.

Aerated ethanol solutions of **17**, **19** and **20** (concentrations 2-6 μ*M*, giving optical densities 0.1-0.2 cm^{-1} at the absorption maximum) were irradiated in a 1 x 1 cm cuvette, with instrumentation as previously described [28], except that the excitation source was provided by a train of fs pulses at 352 nm from the third harmonic of a Ti:Sapphire laser (Tsunami, Spectra Physics). Data were collected and processed according to standard single photon counting protocols [29]. Under these conditions, decays of ~50 ps could be resolved.

REFERENCES AND NOTES

[1] J. E. T. Corrie, *J. Chem. Soc., Perkin Trans. 1*, 2975 (1994).

[2a] M. Brune, J. L. Hunter, J. E. T. Corrie and M. R. Webb, *Biochemistry*, **33**, 8262 (1994); [b] M. Brune, J. L. Hunter, S. A. Howell, S. R. Martin, T. L. Hazlett, J. E. T. Corrie and M. R. Webb, *Biochemistry*, **37**, 10370 (1998).

[3] Z. H. He, R. K. Chillingworth, M. Brune, J. E. T. Corrie, D. R. Trentham, M. R. Webb and M. A. Ferenczi, *J. Physiol.*, **501**, 125 (1997).

[4a] S. P. Gilbert, M. R. Webb, M. Brune and K. A. Johnson, *Nature*, **373**, 671 (1995); [b] A. E. Nixon, M. Brune, P. N. Lowe and M. R. Webb, *Biochemistry*, **34**, 15592 (1995); [c] S. Bornemann, D. J. Lowe and R. N. F. Thorneley, *Biochemistry*, **35**, 9907 (1996).

[5] V. Schauer-Vukasinovic, L. Cullen and S. Daunert, *J. Am. Chem. Soc.*, **119**, 11102 (1997).

[6] A. N. Fletcher and D. E. Bliss, *Appl. Phys.*, **16**, 289 (1978).

[7] R. K. Mackie, S. Mhate and J. M. Tedder, *J. Fluorine Chem.*, **10**, 437 (1977).

[8] R. M. Schoth, E. Lork and G. V. Röschenthaler, *J. Fluorine Chem.*, **78**, 187 (1996).

[9] J. Jonas and H. Gutowsky, *J. Chem. Phys.*, **42**, 140 (1965).

[10] Y. V. Voznyi, D. S. Yufit, V. A. Parolov and Y. T. Struchkov, *Izv. Akad. Nauk SSSR, Ser. Khim.*, 913 (1989).

[11] J. Gallastegui, J. M. Lago and C. Palomo, *J. Chem. Res. (S)*, 170 (1984).

[12] Y. Arano, T. Uezono, H. Akizawa, M. Ono, K. Wakisaka, M. Nakayama, H. Sakahara, J. Konishi and A. Yokoyama, *J. Med. Chem.*, **39**, 3451 (1996).

[13] E. T. Corrie, M. H. Moore and G. D. Wilson, *J. Chem. Soc., Perkin Trans. 1*, 777 (1996).

[14] F. Hibbert and A. Hotouras, King's College, London, unpublished data.

[15] T. Furuta, S. S. H. Wang, J. L. Dantzker, T. M. Dore, W. J. Bybee, E. M. Callaway, W. Denk and R. Y. Tsien, *Proc. Natl. Acad. Sci. USA*, **96**, 1193 (1999).

[16] B. N. Mattoo, *Trans. Faraday Soc.*, **52**, 1184 (1956).

[17] F. Effenberger, A. Steinbach, G. Epple and J. Hanauer, *Chem. Ber.*, **116**, 3539 (1983).

[18] H. S. Chiou, P. C. Reeves and E. R Biehl, *J. Heterocyclic Chem.*, **13**, 77 (1976).

[19] D. Billeret, D. Blondeau and H. Sliwa, *Synthesis*, 881 (1993).

[20] M. R. Webb, National Institute for Medical Research, unpublished data.

[21] Z. R. Grabrowski, K. Rotkiewicz, A. Siemiarczuk, D. J. Cowley and W. Baumann, *Nouv. J. Chem.*, **3**, 443 (1979).

[22] W. Rettig, *Angew. Chem., Int. Edn. Engl.*, **25**, 971 (1986).

[23] G. Jones, W. R. Jackson, C. Y. Choi and W. R. Bergmark, *J. Phys. Chem.*, **89**, 294 (1985).

[24] W. Rettig and R. Gleiter, *J. Phys. Chem.*, **89**, 4676 (1985).

[25] F. von Meyenberg, *Chem. Ber.*, **29**, 501 (1896).

[26] R. E. Strube, *Org. Synth. Coll.*, Vol. **4**, 417 (1963).

[27] A. P. Krapcho and C. S. Kuell, *Synth. Commun.*, **20**, 2559 (1990).

[28] M. Vogel and W. Rettig, *Ber. Bunsenges. Phys. Chem.*, **91**, 1241 (1987).

[29] D. V. O'Connor and D. Phillips, Time-correlated Single Photon Counting, Academic Press, London, 1984.