Synthesis and Study of Intramolecularly-quenched Fluorogenic Substrates Containing Aminocoumarin or Aminoquinolinone-type Fluorophores

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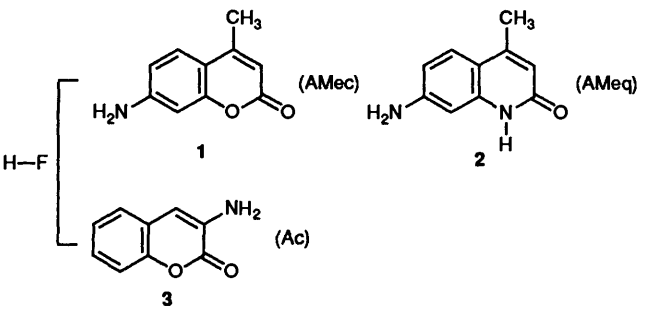
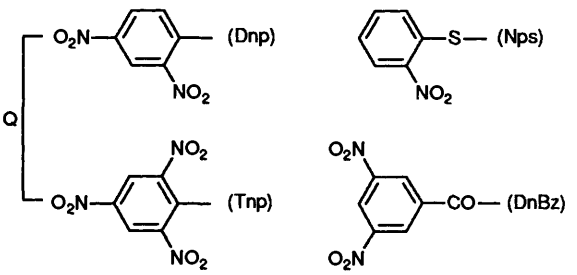
The amines 7-amino-4-methylcoumarin (AMec) (**1**), 7-amino-4-methyl-2-quinolinone (AMeq) (**2**) and 3-aminocoumarin (Ac) (**3**) have been used as fluorophores (H–F) and the aromatic systems 2,4-dinitrophenyl (Dnp), 2,4,6-trinitrophenyl (Tnp), 2-nitrophenylsulphenyl (Nps) and 3,5-dinitrobenzoyl (DnBz) have been used as quenchers (Q) in the synthesis of twelve intramolecularly-quenched fluorogenic substrates of general structure Q–Gly–Phe–F. The study of the fluorescence properties of the substrates synthesized shows that efficient quenching of fluorescence is observed in all cases. The Dnp, Nps and Tnp groups show a higher quenching efficiency and the combination of the Dnp group with the fluorophore Ac gives the best result (99% quenching). The substrates synthesized can be used for the direct specific determination of enzymes which hydrolyse the peptide chain at any point between the interacting groups by measuring the increase in fluorescence.

Synthetic peptide substrates containing a fluorophore at the bond-breaking position have found wide application in the determination of proteases.[1–5] The estimation of enzyme activity is based on the increase of fluorescence observed upon enzymatic liberation of the fluorophore. However, such substrates cannot be used, either for the direct determination of enzymes hydrolysing specifically an internal peptide bond or specificity studies of the P_1', . . ., P_n' positions, since the P_1' position is occupied by the fluorophore. For this purpose intramolecularly quenched fluorogenic substrates, *i.e.* a peptide chain bearing a fluorophore on the one end and a quencher on the other, are needed. Enzymatic cleavage of the peptide chain at any point between the interacting groups destroys the intramolecular quenching, thus resulting in an increase of fluorescence. Although some examples of intramolecularly quenched fluorogenic substrates have been published,[6–12] no systematic study on the quencher–fluorophore combination has been reported. The purpose of this work was to find efficient quenchers for aminocoumarin or aminoquinolinone-type fluorescent markers, which have been widely used as fluorophores because of their excellent fluorescence properties.[1,2,4] We report here the synthesis of twelve substrates of the general structure Q–Gly–Phe–F, containing various quenchers, Q, and fluorescent groups, F, and the study of their fluorescence properties.



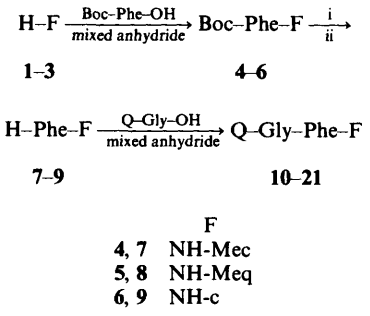


Scheme 1

Results

The structures of the fluorogens H–F and the quenchers Q are shown in Scheme 1. As fluorogens we have chosen the widely used and commercially available 7-amino-4-methylcoumarin (AMec) (**1**)[1] and the amines 7-amino-4-methyl-2-quinolinone (AMeq) (**2**) and 3-aminocoumarin (Ac) (**3**), which have been previously studied by us as fluorescent markers.[4] The amine AMeq has slightly higher fluorescence intensity than AMec and has been successfully used for the assay of various proteases.[4,13] The free amine Ac does not fluoresce in aqueous solutions but its *N*-acyl derivatives do.[4] We have chosen as quenchers the aromatic systems 2,4-dinitrophenyl (Dnp), 2,4,6-trinitrophenyl (Tnp), 2-nitrophenylsulphenyl (Nps) and 3,5-dinitrobenzoyl (DnBz) based on the fact that nitroaromatic derivatives are efficient quenchers for the aminobenzoyl[6,8,9,11] and dansyl[7] groups.

All the substrates were prepared in high yield and purity by conventional methods of peptide synthesis as outlined in Scheme 2. Their analytical data are given in Table 1.



Scheme 2 Synthesis of the substrates. *Reagents:* i, HCl/THF; ii, NMM.

The emission spectra of compounds **7** and **10–13** containing the AMec group, taken at the maximum λ_{ex} of **7** (325 nm) are

Table 1 Analytical data for the substrates Q–Gly–Phe–F (**10–21**)

Compound	Q	F	M.p./°C	$[\alpha]_D$ (*c*, solvent)	Formula	Found (%) (Required) C	H	N	S
10	Dnp	NH-Mec	197–199	−2.8° (0.5, DMF)	$C_{27}H_{23}N_5O_8 \cdot 0.5H_2O$	58.5 (58.7	4.3 4.3	12.6 12.6)	—
11	Nps	NH-Mec	133–135	+32.2° (1.0, DMF)	$C_{27}H_{24}N_4O_6S \cdot 0.5H_2O$	59.9 (60.15	4.6 4.65	10.35 10.3)	—
12	Tnp	NH-Mec	149–152	+41.9° (1.0, DMF)	$C_{27}H_{22}N_6O_{10}$	54.9 (54.8	3.8 3.8	14.2 13.8)	—
13	DnBz	NH-Mec	265–266	+10.1° (1.0, DMF)	$C_{28}H_{23}N_5O_9$	58.6 (58.8	4.0 4.1	12.2 11.8)	—
14	Dnp	NH-Meq	175–178	+ 5.6° (1.0, DMF)	$C_{27}H_{24}N_6O_7 \cdot H_2O$	57.6 (57.9	4.7 4.6	14.9 14.6)	—
15	Nps	NH-Meq	>135	+30.6° (1.0, DMF)	$C_{27}H_{25}N_5O_5S \cdot 0.5C_4H_8O_2$	60.5 (60.3	5.1 5.1	12.2 12.1	5.6 5.8)
16	Tnp	NH-Meq	173–176	+37.0° (0.5, DMF)	$C_{27}H_{23}N_7O_9$	55.0 (55.1	3.9 3.8	16.6 16.3)	—
17	DnBz	NH-Meq	180–182	+19.0° (0.3, DMF)	$C_{28}H_{24}N_6O_8$	58.7 (58.6	4.2 4.1	14.7 14.6)	—
18	Dnp	NH-c	220–224	−34.4° (0.3, DMF)	$C_{26}H_{21}N_5O_8$	58.8 (58.4	4.0 3.75	13.2 13.3)	—
19	Nps	NH-c	134–136	−7.1° (1.0, DMF)	$C_{26}H_{22}N_4O_6S$	60.2 (60.1	4.3 4.25	10.8 10.6	6.2 6.1)
20	Tnp	NH-c	135–138	+12.9° (1.0, EtOAc)	$C_{26}H_{20}N_6O_{10}$	54.2 (54.1	3.5 3.3	14.6 14.65)	—
21	DnBz	NH-c	200–201	−16.2° (1.0, DMF)	$C_{27}H_{21}H_5O_9$	58.0 (57.9	3.8 3.9	12.5 12.2)	—

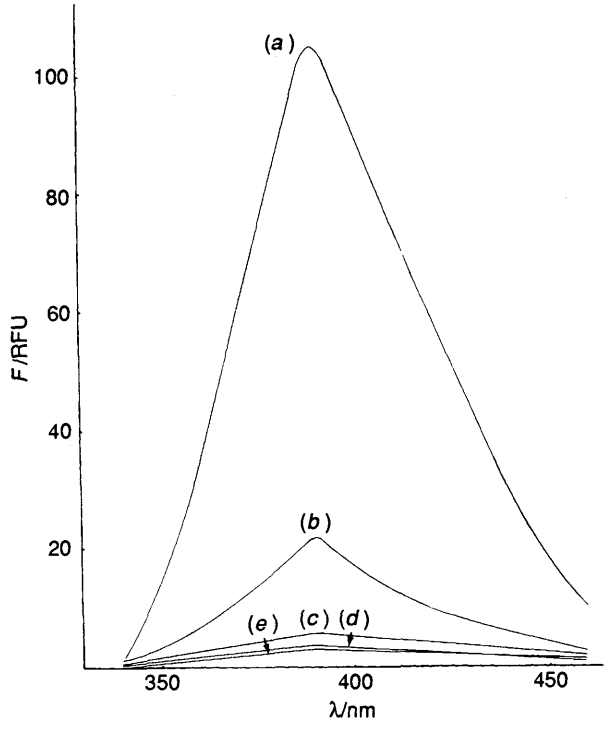


Fig. 1 Fluorescence spectra of (*a*) H-Phe-NH-Mec **7**; (*b*) DnBz–Gly–Phe–NH–Mec **13**; (*c*) Nps–Gly–Phe–NH–Mec **11**; (*d*) Tnp–Gly–Phe–NH–Mec **12** and (*e*) Dnp–Gly–Phe–NH–Mec **10**. 10 μmol dm^{-3} solutions in 0.05 mol dm^{-3} Tris–HCl, pH 7.4, containing 10% DMSO; λ_{ex} = 325 nm.

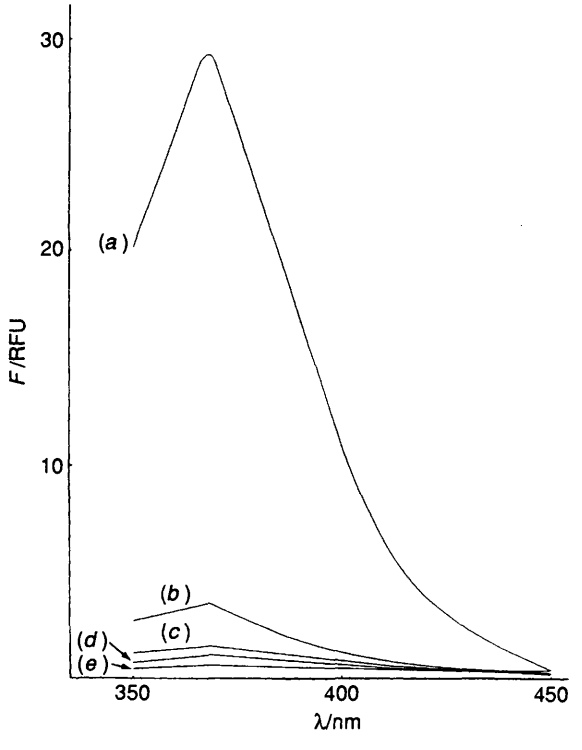


Fig. 2 Fluorescence spectra of (*a*) H–Phe–NH–Meq **8**; (*b*) DnBz–Gly–Phe–NH–Meq **17**; (*c*) Tnp–Gly–Phe–NH–Meq **16**; (*d*) Nps–Gly–Phe–NH–Meq **15**; and (*e*) Dnp–Gly–Phe–NH–Meq **14**. 10 μmol dm^{-3} solutions in 0.05 mol dm^{-3} Tris-HCl, pH 7.4, containing 10% DMSO; λ_{ex} = 333 nm.

shown in Fig. 1. The relative fluorescence intensities *F* of **7**, **10–13** and their quenching efficiencies q.e., measured at the maximum excitation and emission wavelengths of **7** (λ_{ex} = 325 nm, λ_{em} = 390 nm) and of the free amine AMec (λ_{ex} = 345 nm, λ_{em} = 440 nm), are listed in Table 2. The quenching efficiency is expressed by the quotient $F_0 - F/F_0$ where F_0 and *F* are the fluorescence intensities of the donor in the absence and presence of the acceptor. At the maximum wavelengths of **7**, the quenching is 97.3–79.6% efficient following the order Dnp > Tnp > Nps ≫ DnBz.

The emission spectra of compounds **8** and **14–17** containing the AMeq group, taken at the maximum λ_{ex} of **8** (333 nm), are shown in Fig. 2. The relative fluorescence intensities of these

Table 2 Fluorescence properties of substrates containing AMec

	Compound[a]	F(RFU)[b,d]	q.e. (%)[b]	F(RFU)[c,d]	q.e. (%)[c]
7	H–Phe–NH–Mec	105.02	—	5.95	—
10	Dnp–Gly–Phe–NH–Mec	2.89	97.3	1.15	80.7
11	Nps–Gly–Phe–NH–Mec	7.33	93.0	1.47	75.3
12	Tnp–Gly–Phe–NH–Mec	3.47	96.7	0.43	92.8
13	DnBz–Gly–Phe–NH–Mec	21.43	79.6	1.77	70.3

[a] 10 μmol dm^{-3} solution in 0.05 mol dm^{-3} Tris–HCl, pH 7.4, containing 10% DMSO. [b] Maximum excitation (325 nm) and emission (390 nm) wavelengths of **7**. [c] Maximum excitation (345 nm) and emission (440 nm) wavelengths of **1**. [d] A 0.154 μmol dm^{-3} solution of quinine sulphate in 0.05 mol dm^{-3} H_2SO_4 gave 1.0 relative fluorescence unit (RFU).

Table 3 Fluorescence properties of substrates containing AMeq

	Compound[a]	F(RFU)[b,d]	q.e. (%)[b]	F(RFU)[c,d]	q.e. (%)[c]
8	H–Phe–NH–Meq	29.22	—	3.49	—
14	Dnp–Gly–Phe–NH–Meq	0.56	98.1	0.32	90.8
15	Nps–Gly–Phe–NH–Meq	1.04	96.4	0.26	92.6
16	Tnp–Gly–Phe–NH–Meq	1.47	95.0	0.32	90.8
17	DnBz–Gly–Phe–NH–Meq	3.44	88.2	0.58	83.4

[a] 10 μmol dm^{-3} solution in 0.05 mol dm^{-3} Tris–HCl, pH 7.4, containing 10% DMSO. [b] Maximum excitation (333 nm) and emission (368 nm) wavelengths of **8**. [c] Maximum excitation (344 nm) and emission (418 nm) wavelengths of **2**. [d] A 0.154 μmol dm^{-3} solution of quinine sulphate in 0.05 mol dm^{-3} H_2SO_4 gave 1.0 relative fluorescence unit (RFU).

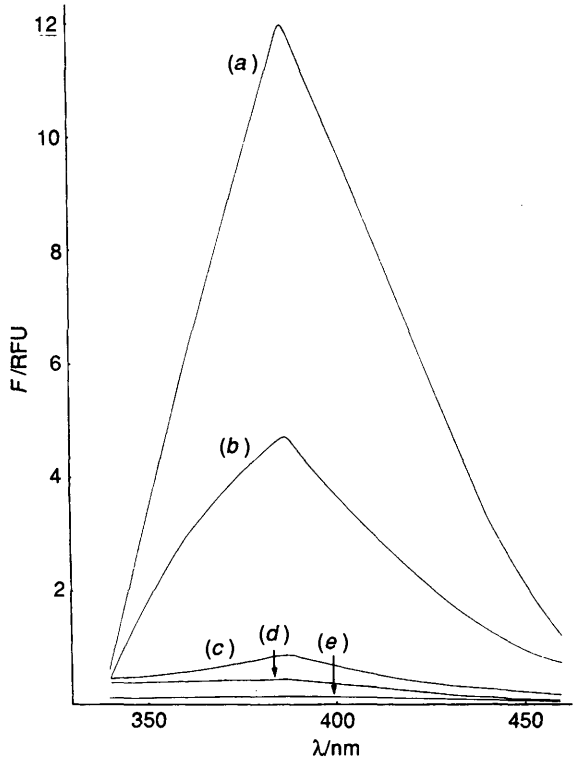


Fig. 3 Fluorescence spectra of (*a*) H–Phe–NH–c **9**; (*b*) DnBz–Gly–Phe–NH–c **21**; (*c*) Tnp–Gly–Phe–NH–c **20**; (*d*) Nps–Gly–Phe–NH–c **19**; and (*e*) Dnp–Gly–Phe–NH–c **18**. 10 μmol dm^{-3} solutions in 0.05 mol dm^{-3} Tris–HCl, pH 7.4, containing 10% DMSO; λ_{ex} = 322 nm.

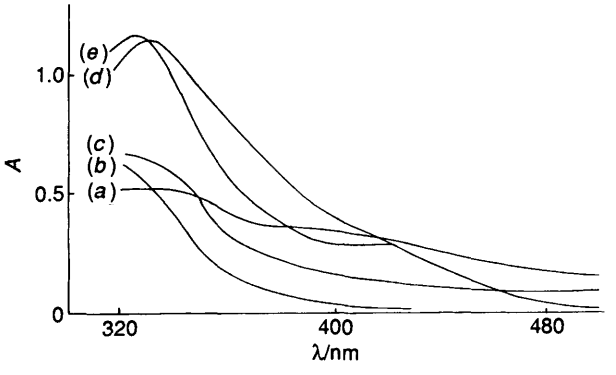


Fig. 4 UV-VIS absorption spectra of (*a*) Nps–Gly–Phe–NH–c **19**; (*b*) H–Phe–NH–c **9**; (*c*) DnBz–Gly–Phe–NH–c **21**; (*d*) Dnp–Gly–Phe–NH–c **18**; and (*e*) Tnp–Gly–Phe–NH–c **20**. 50 μmol dm^{-3} solutions in 0.05 mol dm^{-3} Tris–HCl, pH 7.4, containing 10% DMSO.

Table 4 Fluorescence properties of substrates containing Ac

	Compound[a]	F(RFU)[b,c]	q.e. (%)[b]
9	H–Phe–NH–c	12.06	—
18	Dnp–Gly–Phe–NH–c	0.12	99.0
19	Nps–Gly–Phe–NH–c	0.45	96.3
20	Tnp–Gly–Phe–NH–c	0.86	92.9
21	DnBz–Gly–Phe–NH–c	4.71	61.0

[a] 10 μmol dm^{-3} solution in 0.05 mol dm^{-3} Tris–HCl, pH 7.4, containing 10% DMSO. [b] Maximum excitation (322 nm) and emission (387 nm) wavelengths of **9**. [c] A 0.154 μmol dm^{-3} solution of quinine sulphate in 0.05 mol dm^{-3} H_2SO_4 gave 1.0 relative fluorescence unit (RFU).

compounds at the maximum excitation and emission wavelengths of **8** (λ_{ex} = 333 nm, λ_{em} = 368 nm) and at those of the free amine AMeq (λ_{ex} = 344 nm, λ_{em} = 418 nm), along with the obtained quenching are listed in Table 3. The quenching efficiency at the maximum wavelengths of **8** is 98.1–88.2% following the order Dnp > Nps > Tnp ≫ DnBz.

In Fig. 3 are shown the emission spectra of compounds **9** and **18–21** containing the Ac group, taken at the maximum λ_{ex} of **9** (322 nm). The UV–VIS absorption spectra of these compounds are shown in Fig. 4. Their relative fluorescence intensities at the maximum wavelengths of **9** (λ_{ex} = 322 nm, λ_{em} = 387 nm) and their quenching efficiencies are given in Table 4. At these wavelengths the resultant quenching is 99–61% efficient following the order Dnp > Nps > Tnp ≫ DnBz.

Intermolecular quenching of fluorescence caused by the most efficient quencher, the Dnp group, has been examined and found to be minimal. The compounds H–Phe–F (**7–9**), retain 80–84% of their fluorescence at their maximum wavelengths in the presence of an equimolar amount of Dnp–Gly–OH (10 μmol dm^{-3} solution in 0.05 mol dm^{-3} Tris–HCl, pH 7.41, containing 10% DMSO).

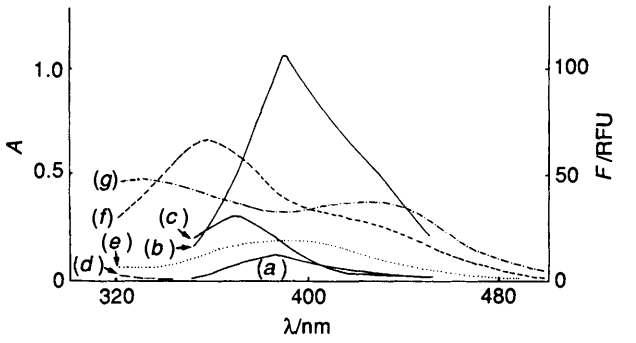


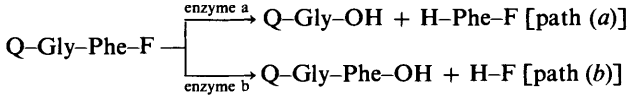
Fig. 5 Fluorescence spectra of (*a*) H–Phe–NH–c **9** (——); (*b*) H–Phe–NH–Mec (**7**) (——) and (*c*) H–Phe–NH–Meq **8** (——). 10 μmol dm^{-3} solutions in 0.05 mol dm^{-3} Tris-HCl, pH 7.4, containing 10% DMSO. UV–VIS absorption spectra of (*d*) DnBz–Gly–OH (– –); (*e*) Nps–Gly–OH (....); (*f*) Dnp–Gly–OH (- - - -); and (*g*) Tnp–Gly–OH (— · — ·). 50 μmol dm^{-3} solutions in 0.05 mol dm^{-3} Tris-HCl, pH 7.4, containing 10% DMSO.

Discussion

A comparison of the fluorescence intensities of substrates Q–Gly–Phe–F (**10–21**) with those of compounds H–Phe–F (**7–9**) at the maximum wavelengths of the latter shows that an efficient quenching of fluorescence is caused by all groups Q. Less efficient quenching is observed at the maximum wavelengths of the free amines H–F (**1–3**). The high quenching efficiency of the Dnp, Tnp and Nps groups is probably a result of a nonradiative long-range resonance energy-transfer mechanism,[6] since there is a spectrum overlap between the absorption bands of Q–Gly–OH and the emission spectra of H–Phe–F (**7–9**) as shown in Fig. 5. The lower quenching efficiency of the DnBz group could be explained by a collisional quenching mechanism,[6] since this quencher does not absorb above λ 340 nm and therefore its absorption band does not overlap the emission bands of the fluorophores.

At the maximum wavelengths of H–Phe–F (**7–9**) the Dnp group proved to be the most efficient quencher for all three fluorophores. The lower the fluorescence intensity of the fluorophore, the higher the quenching efficiency of the Dnp group. Thus, the combination of the Dnp quencher with the fluorophore Ac gives the best result.

All the substrates synthesized **10–21** can be used for the determination of an enzyme hydrolysing the internal peptide bond Gly–Phe, [*e.g.* enkephalinase, path (*a*)], since upon hydrolysis the intramolecular quenching will be interrupted and an increase in fluorescence intensity will be observed. For such an assay, the maximum excitation and emission wavelengths of the substrate H–Phe–F should be used.



The substrates containing AMec, (**10–13**), and AMeq, (**14–17**), can also be used for the determination of those enzymes which hydrolyse the amide bond between the fluorophore and the preceding amino acid, Phe-F, [*e.g.* chymotrypsin, path (*b*)]. Since the fluorescence of these substrates is lower than that of the corresponding non-quenched fluorogenic substrates, the sensitivity of such an assay may be significantly higher. This assay should be monitored at the maximum excitation and emission wavelengths of the free amine H–F.

The results of this study will be of considerable help in the design of intramolecularly quenched fluorogenic substrates containing aminocoumarin- or aminoquinolinone-type fluorophores for the direct and specific determination of various endoproteases such as enkephalinase, collagenase and HIV protease.

Experimental

Materials and Methods.—*N-tert*-Butyloxycarbonylphenylalanine (Boc–Phe–OH) was the L configuration and purchased from Fluka. Isobutyl chloroformate was distilled and stored over $CaCO_3$. *N*-Methylmorpholine (NMM) was distilled from ninhydrine. Dimethyl sulphoxide (DMSO) for UV spectroscopy (Fluka) was used. All other solvents and chemicals were of reagent grade and used without further purification. The purity of the compounds synthesized was checked by TLC, IR spectra and elemental analyses. Solvent systems for TLC on silica gel F-254 plates (Merck) were: chloroform–methanol (5:1) or (9:1) (A); propan-1-ol–25% ammonium hydroxide (67:33) (B); and butan-1-ol–acetic acid–water–pyridine (60:6:24:20) (C). Spots were visualized by UV light, by ninhydrin and by chlorine-4,4-methylenebis-*N*,*N*-dimethylaniline spray. Light petroleum refers to the fraction boiling 40–60 °C.

M.p.s were determined on a Buchi apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 141 polarimeter. The UV-VIS absorption and fluorescence spectra were measured at room temperature using a Hitachi U-2000 double beam spectrophotometer and a Perkin-Elmer 512A spectrofluorometer, respectively. The spectrofluorometer was standardized daily so that a 0.154 μmol dm^{-3} solution of quinine sulphate in 0.05 mol dm^{-3} H_2SO_4 gave 1.0 relative fluorescence unit (RFU). Stock solutions of the various compounds 10^{-2} mol dm^{-3} in dimethyl sulphoxide were prepared and stored at 0 °C. No detectable change in fluorescence intensity was observed after 1 month. The fluorescence and absorption spectra of the substrates were taken in 0.05 mol dm^{-3} Tris–HCl buffer, pH 7.4, containing 10% dimethyl sulphoxide.

Synthesis of the Substrates.—The fluorogens **1**,[14] **2**,[15] and the compounds Dnp–Gly–OH,[16] Tnp–Gly–OH,[17] Nps–Gly–OH,[18] and DnBz–Gly–OH [19] were prepared as described in the literature. The intermediates **4–6** and **7–9** were prepared according to the general procedure, previously reported by us.[4] The new compound Boc–Phe–NH–c (**6**) was obtained in 47% yield, m.p. 159–161 °C; $[\alpha]_D$ −29.5° (*c* = 1 in ethyl acetate).

3-Aminocoumarin (**3**).—A solution of 1 mol dm^{-3} HCl (25 cm^3) was added to a suspension of 3-salicylideneaminocoumarin (0.265 g, 1 mmol) in ethyl acetate (35 cm^3) and the mixture was shaken vigorously for 10 min at room temperature. The organic phase was washed twice with water, dried (Na_2SO_4) and evaporated to a small volume. Following the addition of light petroleum the precipitated solid was filtered and recrystallized from ethanol–water, 1:2 to give **3** (0.125 g, 78%), m.p. 133–135 °C (lit.,[20] 132–133 °C).

General Procedure for the Synthesis of Q–Gly–Phe–F **10–21**.—To a stirred solution of Q–Gly–OH (1 mmol) in ethyl acetate (30 cm^3) at −15 °C, was added *N*-methylmorpholine (0.11 cm^3, 1 mmol) then isobutyl chloroformate (0.13 cm^3, 1 mmol). After 5 min a cooled solution of H–Phe–F (**7–9**) in ethyl acetate (50 cm^3) was slowly added. The reaction mixture was stirred for 30 min at −15 °C then overnight at room temperature. The organic phase was washed consecutively with H_2O, 1 mol dm^{-3} HCl or 0.5 mol dm^{-3} H_2SO_4, H_2O, 5% aqueous $NaHCO_3$, and H_2O then dried (Na_2SO_4) and evaporated to a small volume. On the addition of light petroleum and cooling the product solidified and was filtered and purified by recrystallization from ethyl acetate–light petroleum or by silica gel column chromatography (eluent chloroform–methanol, 95:5).

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References

1 M. Zimmerman, E. Yurewicz and G. Patel, *Anal. Biochem.*, 1976, **70**, 258.
2 R. Smith, E. Bissell, A. Mitchell and K. Pearson, *Thrombosis Res.*, 1980, **17**, 393.
3 P. J. Brynes, P. Bevilacqua and A. Green, *Anal. Biochem.*, 1981, **116**, 408.
4 G. Kokotos and C. Tzougraki, *Int. J. Peptide Protein Res.*, 1986, **28**, 186.
5 E. Sato, A. Matsuhisa, M. Sakashita and Y. Kanaoka, *Chem. Pharm. Bull.*, 1988, **36**, 3496.
6 A. Yaron, A. Carmel and E. Katchalski-Katzir, *Anal. Biochem.*, 1979, **95**, 228.
7 D. Florentin, A. Sassi and B. P. Roques, *Anal. Biochem.*, 1984, **141**, 62.
8 R. Rush, M. Mitas, J. C. Powers, T. Tanaka and L. Hersh, *Arch. Biochem. Biophys.*, 1984, **231**, 390.
9 E. J. Holtzman, G. Pillay, T. Rosenthal and A. Yaron, *Anal. Biochem.*, 1987, **162**, 476.
10 B. Malfroy and J. Burnier, *Biochem. Biophys. Res. Commun.*, 1987, **143**, 58.
11 E. K. Bratovanova and D. D. Petkov, *Anal. Biochem.*, 1987, **162**, 213.
12 M. Ng and D. S. Auld, *Anal. Biochem.*, 1989, **183**, 50.
13 H. P. Maskova, G. Kokotos, C. Tzougraki and T. Barth, *Collect. Czech. Chem. Commun.*, 1989, **54**, 2802; C. Tzougraki, G. Kokotos, H. P. Maskova, E. Anzebacherova and T. Barth, *Collect. Czech. Chem. Commun.*, 1990, **55**, 1112.
14 R. L. Atkins and D. E. Bliss, *J. Org. Chem.*, 1978, **43**, 1975.
15 K. Woods and M. Fooladi, *J. Chem. Eng. Data*, 1968, **13**, 440.
16 J. A. Vinson and L. D. Pepper, *Anal. Chim. Acta*, 1972, **58**, 254.
17 T. Okuyama and K. Satake, *J. Biochem. (Tokyo)*, 1960, **47**, 454.
18 L. Zervas, D. Borovas and E. Gazis, *J. Am. Chem. Soc.*, 1963, **85**, 3660.
19 B. C. Saunders, *Biochem. J.*, 1934, **28**, 580.
20 G. Kokotos and C. Tzougraki, *J. Heterocycl. Chem.*, 1986, **23**, 87.

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