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A Novel Fluorescent Thiol Reagent: Syntheses and Electronic Spectra of N-(7-Dimethylamino-4-methyl-3-coumarinyl)-maleimide (DACM-3) and the Related Compounds[1]

Minoru Machida,[2a] Nobuyuki Ushijima, Tetsuo Takahashi and Yuichi Kanaoka[2b]

Faculty of Pharmaceutical Sciences, Higashi Nippon Gakuen University[2a] *and Faculty of Pharmaceutical Sciences, Hokkaido University*[2b]

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Nitration of 7-dimethylaminocoumarin (**1**) afforded three isomers of the nitrocoumarins, two of which were by way of the amines converted to the corresponding maleimides and succinimides. Their absorption and fluorescence spectra were measured, and it was found that N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (**5a**) has the requisite properties for serving as an useful fluorescence thiol reagent with regard to the fluorescent intensity, excitation wavelength, water solubility and the reactivity. Thus dimethylaminocoumarin is one of candidate fluorogenic groups of choice to be employed in organic fluorescence reagents.

Keywords——fluorogenic group; intrinsic fluorescence; empirical rule; emission maximum; NMR; Pechmann reaction; dimethylaminocoumarin; fluorescence quantum yield; N-acetylcysteine

Fluorescence spectroscopy has rapidly developed in harmony with the progress of physical and analytical chemistry. Because the quantum yield of fluorescence and the emission maximum of a fluorogenic group are sensitive to the biophysical environments and sensitivity for measurement of fluorescent compounds is very high, some fluorescent dyes have been widely used in the studies of biological fields.[3]

We have reported several fluorescent thiol reagents, such as BIPM[4b] and ANM.[4c] These reagents have proved useful in the studies of biological systems particularly because they are fluorescent only when reacted with thiols obeying the empirical rule we had proposed.[4] However, further systematic search was still needed in order to develop new fluorescent reagents which meet various spectroscopic requirements. In a previous paper as one of the systematic screening series of the work, the effects of the nature and the position of substituents on the naphthalene ring were described.[5] It was found that some of

1) *a*) Fluorescent Thiol Reagents. XII. Part XI: Y. Kanaoka, T. Takahashi, M. Machida, K. Yamamoto, and T. Sekine, *Chem. Pharm. Bull.* (Tokyo), **24**, 1417 (1976); *b*) Preliminary communication: M. Machida, N. Ushijima, M.I. Machida, and Y. Kanaoka, *Chem. Pharm. Bull.* (Tokyo), **23**, 1385 (1975); *c*) The abbreviations used are: DACM-3=**5a**, N-(7-Dimethylamino-4-methyl-3-coumarinyl)maleimide; DACM-6=**5b**, N-(7-Dimethylamino-4-methyl-6-coumarinyl)maleimide; DACSA-3=**6a**, N-(7-Dimethylamino-4-methyl-3-coumarinyl)succinamic acid; DACSA-6=**6b**, N-(7-Dimethylamino-4-methyl-6-coumarinyl)succinamic acid; DACS-3=**7a**, N-(7-Dimethylamino-4-methyl-3-coumarinyl)succinimide; DACS-6=**7b**, N-(7-Dimethylamino-4-methyl-6-coumarinyl)succinimide; NAC, N-acetylcysteine.

2) Location: *a*) *Ishikari-Tobetsu, Hokkaido 061-02, Japan*; *b*) *Kita-12, Nishi-6, Kita-ku, Sapporo 060, Japan.*

3) For example, see: *a*) G.G. Guilboult, "Practical Fluorescence; Theory, Methods, and Techniques," Marcel Dekker, Inc., New York, 1973; *b*) R.F. Chen and H. Edelhoch, "Biochemical Fluorescence; Concepts,'Vol. 2 Marcel Dekker, Inc., New York, 1976.

4) *a*) Y. Kanaoka, M. Machida, H. Kokubun, and T. Sekine, *Chem. Pharm. Bull.* (Tokyo), **16**, 1747 (1968); *b*) Y. Kanaoka, M. Machida, K. Ando, and T. Sekine, *Biochim. Biophys. Acta*, **207**, 269 (1970); *c*) Y. Kanaoka, M. Machida, M.I. Machida, and T. Sekine, *Biochim. Biophys. Acta*, **317**, 563 (1973).

5) M. Machida, M. Bando, Y. Migita, M.I. Machida, and Y. Kanaoka, *Chem. Pharm. Bull.* (Tokyo), **24**, 3045 (1976).

naphthalene derivatives have emission maxima distinct from that of the aromatic residues of protein but it was insufficient with regard to their fluorescent intensity. Our purpose of the present work was to find fluorogenic groups which have fluorescent maxima at longer than 400 nm and high fluorescent intensity. Since it is well known that coumarin derivatives strongly fluoresce in a significantly longer wavelength region than fluorescent maxima of the intrinsic fluorescence of protein, and have large molar absorptivity,[6] coumarin derivatives containing dimethylamino group at 7-position have been selected. In the present paper we describe in detail the syntheses and the chemical and spectroscopic properties of certain compounds in which a dimethylaminocoumarin and a maleimide are composed.

Dimethylaminocoumarin **1** synthesized by the Pechmann reaction[7] was nitrated with nitric acid (d=1.42) in acetic anhydride or acetic acid. Since by the nitration of **1** in acetic acid solution formation of dinitro compounds increased, the reaction in acetic anhydride solution was preferred. Three positional isomers of **2** were separated by column chromatography, following products being eluted with methylene chloride in the order cited: dinitro compounds, **2a** and a mixture of **2b** and **2c**. **2b** and **2c** were separated by re-chromatography with benzene–AcOEt (3: 1) as solvent.

The position of a nitro group introduced on the coumarin ring was determined by NMR

Table I. NMR Spectral Assignments of Products ($CDCl_3$)

Compounds	Chemical shift (ppm, TMS=0) of protons attached to					
	C_3–H	C_4–CH_3	C_5–H	C_6–H	C_7–$N(CH_3)_2$	C_8–H
2a	—	2.43(s)	7.48(d) (J=9 Hz)	6.75(d) 6.60(d) (J=9 Hz, J=3 Hz)	3.10(s)	6.44(d) (J=3 Hz)
2b	6.11(b)	2.37(d) (J<1 Hz)	8.02(s)	—	2.96(s)	6.78(s)
2c	6.03(b)	2.38(d) (J<1 Hz)	7.48(d) (J=9 Hz)	6.80(d)	3.00(s)	—

abbreviations: s=singlet, d=doublet, b=broad

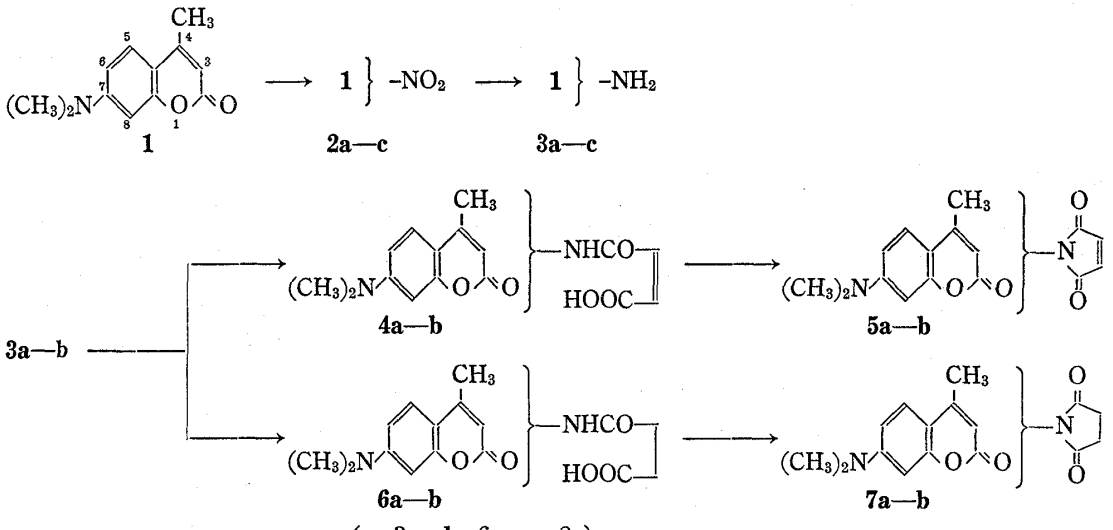

Chart 1

6) O. Takenaka, Y. Nishimura, A. Takenaka, and K. Shibata, *Biochim. Biophys. Acta*, **207**, 1 (1970).
7) H.V. Pechmann and M. Schaal, *Chem. Ber.*, **32**, 3690 (1899).

Table II. Preparation of 7-Dimethylamino-4-methyl-coumarin Derivatives

Compound	Yield (%)	mp (°C)	Recryst. Solvent Appearance	Formula	Analysis Found (Calcd.) C	H	N	IR (cm^{-1})
2a	31	210—212	AcOEt golden needles	$C_{12}H_{12}O_4N_2$	58.15 (58.06)	4.97 (4.87)	11.11 (11.29)	1730
2b	26	193—195	AcOEt orange needles	$C_{12}H_{12}O_4N_2$	58.34 (58.06)	4.90 (4.87)	10.95 (11.29)	1740
2c	13	163—165	EtOH orange yellow needles	$C_{12}H_{12}O_4N_2$	58.15 (58.06)	4.84 (4.87)	11.24 (11.29)	1750
3a	75	147—149	EtOH Yellow prisms	$C_{12}H_{14}O_2N_2$	65.99 (66.03)	6.36 (6.47)	12.74 (12.84)	1680
3b	62	220—222	MeOH Yellow needles	$C_{12}H_{14}O_2N_2$	65.99 (66.03)	6.48 (6.47)	12.79 (12.84)	1690
3c	76	130—132	EtOH Pale yellow needles	$C_{12}H_{14}O_2N_2$	65.99 (66.03)	6.49 (6.47)	12.78 (12.84)	1717

Table III. Preparation of Maleamic Acids, Succinamic Acids, Maleimides and Succinimides

Compound	Yield (%)	mp (°C)	Recryst. Solvent Appearance	Formula	Analysis Found (Calcd.) C	H	N
4a	97	204—207	yellow solids	$C_{16}H_{16}O_5N_2$	60.75 (60.75)	5.16 (5.10)	9.11 (8.86)
4b	94	190—193	yellow needles	$C_{16}H_{16}O_5N_2$	60.65 (60.75)	5.11 (5.10)	8.27 (8.86)
5a	82	218—219.5	AcOEt yellow needles	$C_{16}H_{14}O_4N_2$	64.36 (64.42)	4.65 (4.73)	9.39 (9.39)
5b	66	222—223.5	EtOH orange yellow needles	$C_{16}H_{14}O_4N_2$	64.37 (64.42)	4.78 (4.73)	9.19 (9.39)
6a	64	217—218	EtOH colorless needles	$C_{16}H_{18}O_5N_2$	60.11 (60.37)	5.57 (5.70)	8.96 (8.80)
6b	66	190—191	AcOEt colorless needles	$C_{16}H_{18}O_5N_2$	60.43 (60.37)	5.71 (5.70)	8.61 (8.80)
7a	76	262—265	EtOH colorless needles	$C_{16}H_{16}O_4N_2$	64.02 (63.99)	5.35 (5.37)	9.39 (9.33)
7b	67	220—221 (172.5—174[a])	AcOEt colorless needles	$C_{16}H_{16}O_4N_2$	63.84 (63.99)	5.45 (5.37)	9.16 (9.33)

a) Melting point in parenthese was measured before dryness.

spectroscopy. The chemical shifts of protons are given in Table I. The methyl protons at C_4 of **2b** and **2c** appear as a doublet signal due to long-range coupling with a C_3 proton, on the other hand the C_3 proton appears as a broad signal about 6 ppm due to coupling with the protons of C_4 methyl. In compound **2a** disappearance of the broad band suggests introduction of a nitro group at C_3. Aromatic proton signals of compound **2b** were observed as two singlet peaks indicating that **2b** is the 6-nitrocoumarin derivative. Since NMR spectrum of **2c** shows a typical AB pattern in aromatic protons, a position of nitro group was determined to be at C_8.

The nitro compound (**2a—c**) were hydrogenated to the corresponding amines (**3a—c**) in ethyl acetate with 10% Pd-C as catalyst and the data for the preparation and characterization of **2a—c** and **3a—c** are summerized in Table II. The amines (**3a—b**) were reacted with maleic anhydride in an aprotic solvent to yield the corresponding maleamic acids (**4a—b**), which were cyclized with acetic anhydride and anhydrous sodium acetate to give the

Table IV. NMR and IR Data of DACM and DACS

Compound	Chemical shift[a] (ppm, TMS=0) of protons attached to							IR[b] (cm^{-1})
	C_3–H	C_4–CH_3	C_5–H	C_6–H	C_7–$N(CH_3)_2$	C_8–H	imide ring	
5a	—	2.21(s)	7.40(d) (J=9 Hz)	6.50(d) 6.65(d) (J=9 Hz, J=3 Hz)	3.02(s)	6.41(d) (J=3 Hz)	6.82(s)	1780(w), 1710(s) (imide)
5b	6.09(b)	2.32(d) (J<1 Hz)	7.19(s)	—	2.77(s)	6.85(s)	6.88(s)	1715(s) (imide)
7a	—	2.19(s)	7.42(d) (J=9 Hz)	6.53(d) 6.68(d) (J=9 Hz, J=3 Hz)	3.04(s)	6.44(d) (J=3 Hz)	2.92(s)	1780(w), 1710(s) (imide) 1730(s) (lactone)
7b	6.03(b)	2.26(d) (J<1 Hz)	7.10(s)	—	2.71(s)	6.82(s)	2.87(s)	1790(w), 1710(s) (imide) 1760(s) (lactone)

a) abbreviation: s=singlet, d=doublet, b=broad
b) abbreviations: w=weak, s=strong

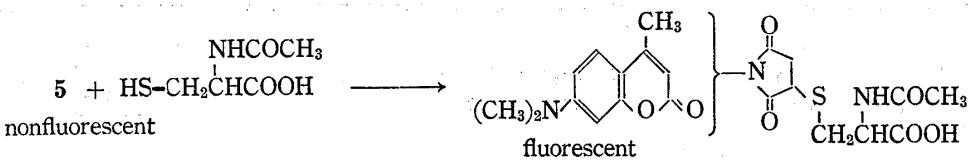

Chart 2

Table V. Absorption and Fluorescence Properties of DACM, DACS and DACSA

	Solvent	Compound					
		DACM–3 (5a)	DACM–6 (5b)	DACS–3 (7a)	DACS–6 (7b)	DACSA–3 (6a)	DACSA–6 (6b)
Absorption maxima (nm)	EtOH	377	350	379	350	377	351
	H_2O[a]	391	351	396	350	382[b]	350
Molar absorptivity	EtOH	25700	17700	26000	17500	23100	14700
	H_2O[a]	23000	13800	24500	13300	20800[b]	13000
Fluorescence maxima (nm) (ex. 350 nm, 380 nm)	EtOH			457	447	464	455
	H_2O			477	447	477	455
Quantum yield	EtOH			0.67	0.02	0.70	0.11
	H_2O			0.11	0.01	0.34	0.01

a) 0.1 M phosphate buffer, pH 7.0 containing 3% ethanol
b) 0.1 M phosphate buffer, pH 7.0 containing 1% ethanol

maleimides (**5a—b**) in good yields. The maleimides (**5a—b**) were purified by recrystallization. The corresponding succinimides (**7a—b**) which lack the double bond of imide ring were similarly prepared as the fluorescent models of the reaction products of **5** with thiols.[4,5] The processes of these reactions are shown in Chart 1. The data for the preparation of the amic acids and the imides are summerized in Table III, and Table IV shows the NMR and IR data. Synthesis of DACM–8 (**5c**) and the related compounds derived from **3c** was not attempted because the amine (**3c**) was obtained only in a poor yield.

As shown in Table V, DACM–3 (**5a**) and DACM–6 (**5b**) were practically nonfluorescent, while DACS–3 (**7a**) and DACS–6 (**7b**), models of addition products with thiol compounds

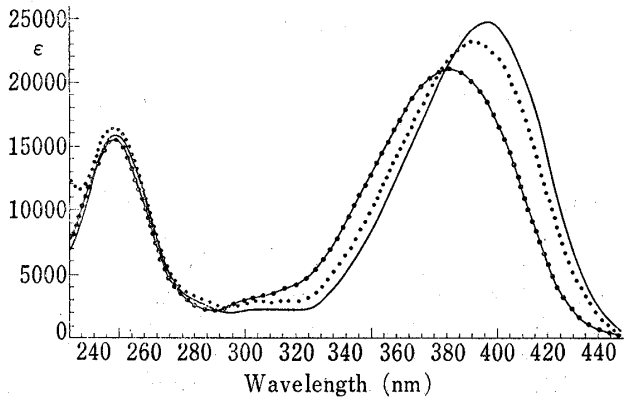

Fig. 1. Absorption Spectra of 3-Substituted-7-dimethylamino-4-methylcoumarin Derivatives in 0.1 M Phosphate Buffer (pH 7.0)

·····: DACM-3 (**5a**)
—●—: DACSA-3 (**6a**)
———: DACS-3 (**7a**)

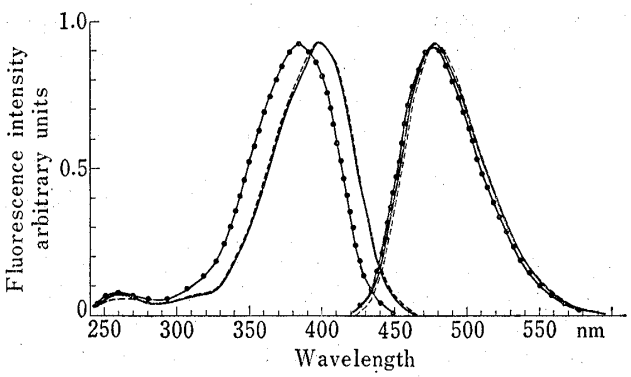

Fig. 2. Fluorescence Emission and Excitation Spectra of 3-Substituted-7-dimethylamino-4-methylcoumarin Derivatives in 0.1 M Phosphate Buffer (pH 7.0)

------: DACM-3-NAC (**5a**+NAC)
—●—: DACSA-3 (**6a**)
———: DACS-3 (**7a**)

(Chart 2), were strongly fluorescent. This observation is in good agreement with the empirical rule which we have proposed in this series of papers.[4] Absorption and fluorescence properties of DACM (**5**) and its related compound are shown in Table V. Compounds derived from **2a** (**5a**, **6a**, **7a**=DAC-3) have absorption maxima and fluorescent maxima at longer wavelength region than those of the compounds derived from **2b** (**5b**, **6b**, **7b**=DAC-6), and the molar absoptivities of the former are greater than those of the latter by a magnitude of ε=8000.

It has been argued that the 3-substituted-7-hydroxycoumarin can form a resonance-stabilizing quinoid structure with extending a conjugated system.[8] Likewise the 3-substituted-7-dimethylaminocoumarin (DAC-3) would take a resonance quinoid form which arises from the 7-dimethylamino group, resulting in the increase of the molar absorptivity of the 3-substituted coumarin. In addition, fluorescence quantum yields of the compounds in the DAC-3 group are larger than those of the DAC-6 group. Fig. 1 shows the absorption spectra of the compounds in the DAC-3 group (**5a**—**7a**) in 0.1 M phosphate buffer solution. Since these compounds have absorption maxima at longer wavelength than 380 nm, DACS-3 (**7a**) and DACSA-3 (**6a**) can be excited at above 400 nm. It has been well established that N-substituted maleimides are group-specific to thiols.[4,9] When DACM-3 (**5a**) was allowed to react with N-acetylcysteine in 0.1 M phosphate buffer at pH 7.0, intense fluorescence immediately developed demonstrating a facile reaction of DACM-3 with a cysteine derivative. As expected, the fluorescence emission and excitation spectra (Fig. 2) of the addition product (DACM-NAC), are superimposable on that of DACS-3 (**7a**).

So far our systematic screening search for useful fluorogenic groups has led to the conclusion that monosubstituted naphthalene derivatives are yet unsuitable.[5] The data described in the present paper prove that the dimethylaminocoumarin ring (**1**) is one of candidate fluorogenic groups of choice. Compound **1** is a bicyclic system which is perhaps of the smallest ring in size that exhibits fluorescence emission of sufficiently strong intensity and long wavelengths and, in addition, has improved water-solubility owing to its polar character due to the presence of three heteroatoms. Indeed, **1** can be selectively excited in the presence of the intrinsic chromophores in natural biopolymers such as proteins and

8) W. Sherman and E. Robins, *Anal. Chem.*, **40**, 803 (1968).
9) M. Friedman, "The Chemistry and Biochemistry of the Sulfhydryl Group in Amino Acids, Peptides and Proteins," Pergamon Press, Ltd., Oxford, 1973, p. 114.

nucleic acids because its absorption bands occur at the longer wavelengths. Finally, DACM-3 (**5a**) has as a good reactivity with a thiol as other maleimides we have examined. Thus DACM-3 (**5a**) has the requisite properties for serving as useful fluorescence thiol reagent. The detailed fluorescence characteristics including solvent-dependency and the application in various biochemical studies will be reported elsewhere.

Experimental

All melting points were determined on a MP-1 melting point apparatus Yamato and uncorrected. NMR spectra were taken in $CDCl_3$ solution at 60 MHz on a Hitachi R-20B spectrometer with TMS as internal standard. Mass spectra were obtained with a Hitachi RMU-6E mass spectrometer. UV and IR spectra were measured with a Shimadzu double beam UV 200 spectrophotometer and a Jasco IR-S spectrophotometer, respectively. Fluorescence spectra were measured with a Hitachi MPF-2A fluorescence spectrophotometer. The samples used for fluorescence studies have absorbances below 0.2 at the exciting wavelength. Quantum yields of the fluorescence spectra were obtained by the method described in a previous paper.[4a)]

7-Dimethylamino-4-methylcoumarin (1)——Compound **1** was prepared from *m*-dimethylaminophenol, ethyl acetoacetate and $ZnCl_2$ in ethanol by the Pechmann reaction. Pale yellow needles from ethanol, mp 144—145° (lit.,[7)] 143°).

Nitration of 7-Dimethylamino-4-methylcoumarin (1): 7-Dimethylamino-4-methyl-nitrocoumarin (2a—c) (Table I and II)——To a solution of **1** (3.033 g, 15 mmole) in 50 ml of Ac_2O under cooling was gradually added a solution of nitric acid (1.8 ml, $d=1.42$) in 25 ml of Ac_2O. After 10 min of additional stirring an ice-cold water was added to this solution and the excess Ac_2O was hydrolyzed. Resulting precipitate was collected by filtration and the aqueous portion was extracted with CH_2Cl_2. The combined organic phases were washed with cold saturated $NaHCO_3$ and water, dried over Na_2SO_4, concentrated *in vacuo* and submitted to silica gel column chromatography. Mononitro and dinitro compounds were separated with CH_2Cl_2 eluent. 6-Nitrocoumarin and 8-nitrocoumarin were separated by re-chromatography on silica gel with benzene–AcOEt, 3: 1 as eluent. The position of nitro group was discussed in the text. Mass spectrum *m/e*: 248 (M^+).

Hydrogenation of Nitrocoumarin Derivatives (2a—c): 7-Dimethylamino-4-methyl-3-aminocoumarin (3a), 7-Dimethylamino-4-methyl-6-aminocoumarin (3b) and 7-Dimethylamino-4-methyl-8-aminocoumarin (3c) (Table II)——The nitro compounds **2** (2 g) were hydrogenated in 700 ml of AcOEt with 10% Pd-C as catalyst for 8 hr. The data for the preparation and characterization of **2** and **3** are summerized in Table I and II.

Maleamic Acids (4a—b) (Table III): General Procedure——To an ice cooled solution of maleic anhydride in $CHCl_3$ was added a solution of equimolar amount of an amine **3a—b** in $CHCl_3$. After the reaction mixture was stood overnight, **4a—b** was filtered, washed with THF and dried. Analytical sample was prepared by washing several times with $CHCl_3$ and THF. The crude acids was used for imide cyclization without purification.

Succinamic Acids (6a—b) (Table III)——Compound **6a** was prepared as in the case of maleamic acids. The reaction mixture was stood overnight, and the solvent was removed *in vacuo*. Compound **6b** was obtained by refluxing in $CHCl_3$ for 1 hr and standing overnight at room temperature.

Imide Cyclization: Syntheses of the Maleimides (5a—b) and the Succinimides (7a—b) (Table III), General Procedure——The mixture of an acid (1 mmole), acetic anhydride (3 mmole) and anhydrous NaOAc (0.3 mmole) was heated on an oil bath at 90° for 15 min. After cooling ice water was added and the reactant was neutralized with $NaHCO_3$ and extracted with CH_2Cl_2. The extract was washed with sat. aq. NaCl, dried over Na_2SO_4 and evaporated *in vacuo*. Resulting imides were purified by recrystallization.

Reaction of DACM-3 (5a) with N-Acetyl-L-cysteine (DACM-NAC)——A mixture of 10^{-3}M DACM-3 (0.01 ml, in monoglyme) and 10^{-3}M N-acetyl-L-cysteine (0.05 ml, in 0.1M phosphate buffer, pH 7.0) was diluted to 3 ml with 0.1M phosphate buffer (pH 7.0), and subjected to measurement of fluorescence spectrum.

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