Synthesis of 4-(7-Diethylaminocoumarin-3-yl)benzeneisocyanate (DACB-NCO): A Highly Sensitive Fluorescent Derivatization Reagent for Alcohols in High-Performance Liquid Chromatography [1]

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4-(7-Diethylaminocoumarin-3-yl)benzeneisocyanate (DACB-NCO) was synthesized as a new fluorescent derivatization reagent for alcohols for use in high-performance liquid chromatography (hplc). Saturated alcohols (C_6-C_{22}) were derivatized in good yields into the corresponding fluorescent DACB-carbamic esters by treating with DACB-NCO. The DACB-carbamic esters of these alcohols were clearly separated on a reversed-phase hplc column (Inertsil ODS-2, mobile phase: methanol-water, excitation wavelength 402 nm; emission wavelength 488 nm). The detection limit (S/N = 3) of cetyl alcohol, as a test compound, was 5 fmol/10 μl.

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Various fluorescent derivatization reagents have been proposed for the determination of alcohols by high-performance liquid chromatography (hplc) [2-11]. In the previous paper, we have shown that 3-aryl-7-dialkylaminocoumarin was one of the promising candidates as a fluorogenic group, and developed the derivatization reagent, 4-(7-diethylamino-coumarin-3-yl)benzoyl cyanide (DACB-CN) for alcohols [2]. Although DACB-CN was one of the most sensitive fluorescent derivatization reagents (detection limit: 1-2 femtomol per injection volume), this reagent required the derivatization reaction in open vial because of low reactivity to alcohols in the reaction medium of highly diluted solution. The present paper deals with the preparation of 4-(7-diethylaminocoumarin-3-yl)benzeneisocyanate (DACB-NCO, **3**) having an isocyanate moiety as the reacting group, and its fundamental studies on its properties as a new derivatization reagent for hplc analysis of alcohols.

Synthesis of DACB-NCO (**3**).

DACB-NCO (**3**) was synthesized by the route shown in Scheme 1. The absorption maximum and the fluorescence maximum of **3** in acetonitrile appeared at 401 nm (ε = 38,200) and 482 nm, respectively. DACB-NCO is stable in the crystalline state for at least a year in daylight at room temperature.

Preparation of Standard Samples (DACB-Carbamic Esters, **4a-k**) and their Spectroscopic Properties.

The standard samples of DACB-carbamic esters (**4a-k**) were obtained by the reaction of a series of saturated alcohols (C_6-C_{22}) with an equimolar amount of DACB-NCO in acetonitrile (room temperature for 2.0 hours) in the presence of 4-dimethylamino-pyridine (DMAP) (4 equivalents). The structures of DACB-carbamic esters (**4a-k**) were determined on the basis of spectral and analytical data (Table 1). All the DACB-derivatives (**4a-k**) had absorption maxima (λmax) and fluorescence maxima (F.λmax) at 402 nm and 487-489 nm, respectively, with much the same values of molar absorptivity and fluorescence intensity in ethanol (Table 1). DACB-carbamic esters (**4a-k**) were stable in ethanol solution for at least a year in a refrigerator.

The fluorescence properties of DACB-carbamic ester of myristyl alcohol (**4e**) were examined in several solvent systems often used in reversed-phase chromatography

Scheme 1

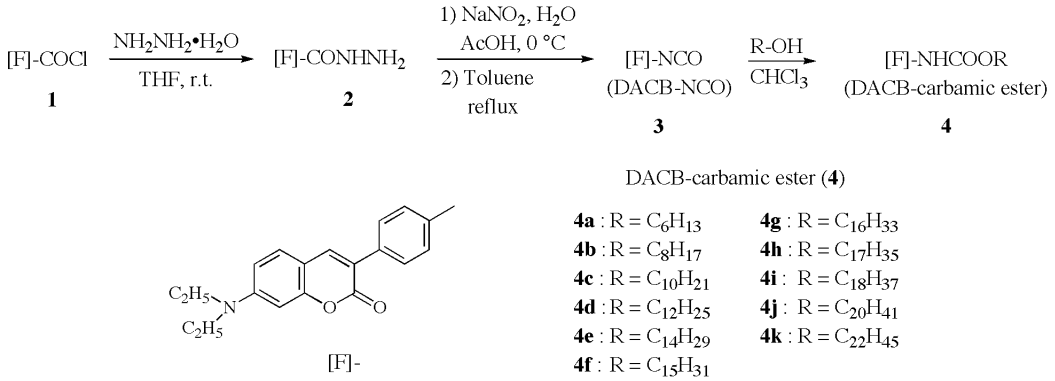


Table 1

Physical Properties of DACB-Carbamic Esters (**4a-k**)

Compound	Mp(°C)	Formula	Molecular Formula Analysis (%) Calcd C	H	N	Found C	H	N	Absorption[a] ε (λmax nm)	RFI[b] (488 nm) (Ex 403 nm)
4a	158-160	$C_{26}H_{32}N_2O_4$	71.53	7.39	6.42	71.54	7.50	6.52	38,600 (402)	1.01
4b	158-160	$C_{28}H_{36}N_2O_4$	72.38	7.81	6.03	72.28	7.71	6.02	38,600 (402)	1.02
4c	146-147	$C_{30}H_{40}N_2O_4$	73.14	8.18	5.69	72.91	8.26	5.65	38,100 (402)	1.00
4d	137-138	$C_{32}H_{44}N_2O_4$	73.81	8.52	5.38	73.77	8.57	5.30	38.900 (402)	1.01
4e	132-134	$C_{34}H_{48}N_2O_4$	74.41	8.82	5.11	74.43	8.85	5.31	38,500 (402)	1.00
4f	134-135	$C_{35}H_{50}N_2O_4$	74.69	8.96	4.98	74.76	9.10	4.84	38,500 (402)	1.00
4g	131-132	$C_{36}H_{52}N_2O_4$	74.96	9.09	4.86	75.05	9.12	5.01	38,900 (402)	0.98
4h	135-136	$C_{37}H_{54}N_2O_4$	75.21	9.21	4.74	75.23	9.40	4.77	38,200 (402)	1.00
4i	129-130	$C_{38}H_{56}N_2O_4$	75.45	9.33	4.63	75.30	9.26	4.74	38,500 (402)	1.02
4j	119-120	$C_{40}H_{60}N_2O_4$	75.90	9.56	4.43	75.98	9.65	4.44	38,600 (402)	1.02
4k	130-131	$C_{42}H_{64}N_2O_4$	76.32	9.76	4.24	76.44	9.82	4.24	38,600 (402)	1.01

[a] Concentration in ethanol: 2.0 X 10^{-5} *M*; [b] Relative fluorescence intensity: the compound **4c** is arbitrarily taken as 1.00. Concentration in ethanol: 4.5 X 10^{-6} *M*

(Table 2). As the solvent polarity decreases, both the absorption maxima and the fluorescence maxima shift slightly toward the blue. However, the variations in the shift values of the F.λmax and fluorescence intensities of **4e** in each solvent are so small that it is possible to separate and detect various alcohols by using a gradient elution technique.

Optimal Conditions for Hplc Analysis of Alcohols.

In order to examine the optimal conditions for hplc analysis, the derivatization yield of cetyl alcohol, a test compound, with DACB-NCO was estimated by comparing the fluorescence intensity of the product (**4g**) at 488 nm with that of an internal standard **4e**. The derivatization reactions [cetyl alcohol (0.01 μmol); DACB-NCO (0.05 μmol); I.S (0.01 μmol)] in chloroform (70 μl), were examined in sealed vials at 40, 60, 80, 100, and 120° for 0-120 minutes, respectively. At 60°, the peak area increased slowly; higher temperatures allowed the fluorescence to develop more rapidly. The peak heights reached a maximum and constant after heating at 120° for 10 minutes. Further, the derivatized products were obtained in good yields in acetonitrile (91%) and chloroform (88%). Chloroform was employed as a solvent due to the insolubility of DACB-NCO. As for base catalysts, DMAP gave good results in the preparative scale reaction at room temperature, but the derivatization reaction at 120° proceeded in the absence of DMAP. Furthermore, the respective various concentrations of DACB-NCO for derivatization of cetyl alcohol were examined. The results indicated that a 3-5-fold molar excess of reagent for cetyl alcohol is suitable. Consequently, the derivatization of alcohols was carried out under the following conditions: heating at 120° for 30 minutes in chloroform with 5-fold molar excess of DACB-NCO, in the absence of base catalysts in sealed vials.

Table 2

Absorption and Fluorescence Properties of **4e** in Various Solvent Systems

Solvent	Absorption[a] λmax (nm)	ε	Fluorescence[b] Ex (nm)	F.λmax (nm)	RFI[c]
ethanol	402	38,500	403	488	1.00
methanol	403	38,800	403	489	1.06
acetonitrile	399	39,100	399	481	1.04
methanol:water (90:10, v/v)	406	38,800	407	491	1.06

[a] Concentration in ethanol: 2.0 X 10^{-5} *M*; [b] Concentration in ethanol: 4.5 X 10^{-6} *M*; [c] Relative fluorescence intensity: the fluorescence intensity in ethanol is arbitrarily taken as 1.00.

Regarding the calibration curve, a linear relationship between the ratio of the peak areas of cetyl alcohol DACB-carbamic ester (**4g**) to that of the internal standard (**4e**) and the amount of cetyl alcohol was observed in the range of 50 fmol - 100 pmol/injection volume (10 µl) of the alcohol (linear correlation coefficient: 0.999), and the detection limit in this case was 5 fmol/10 µl (S/N=3).

Next, the simultaneous determination of saturated alcohols (C_6-C_{22}) was examined. As shown in Figure 1, peaks corresponding to the DACB-derivatives of a series of saturated alcohols (C_6-C_{22}) (**4a-k**) were completely separated by gradient elution with methanol-water as the mobile phase within 30 minutes (Figure 1).

The precision of analysis was established by repeated determinations (n = 7) using a mixture of ten alcohols (5 pmol each per 10 µl). The yields of the fluorescent derivatives under these conditions are 78% (**4a** for hexyl alcohol), 86% (**4b** for octyl alcohol), and 88-98% for long chain alcohols (**4c,d,f-k**; C_{10}-C_{22}). Further, the relative

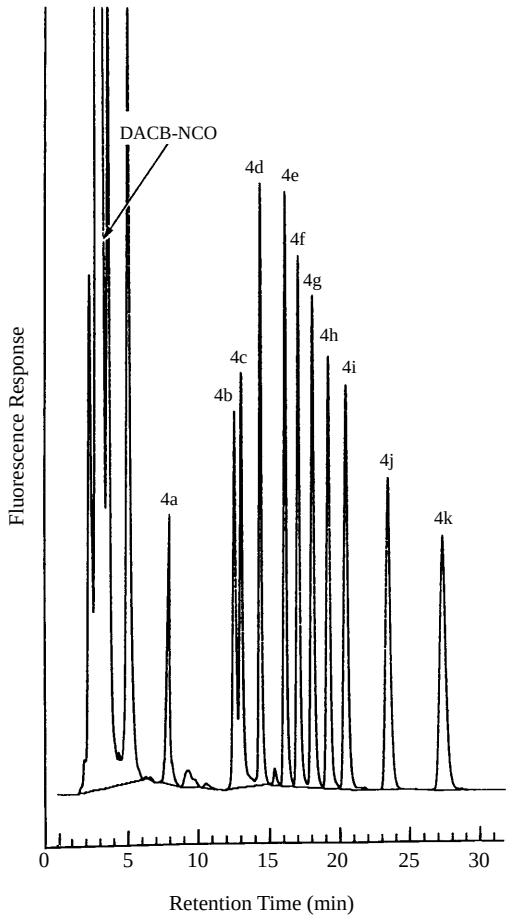


Figure 1. Chromatogram of the DACB-Carbamic Esters of Saturated Alcohols (**4a-k**). The derivatization was carried out as described in the experimental section. Each peak area corresponds to 5 pmol alcohol.

standard deviations were 8% for hexyl alcohol, 6% for octyl alcohol, 3% for decyl alcohol, and did not exceed 1% for long chain alcohols (C_{12}-C_{22}).

In addition, the reactivities of DACB-NCO (**3**) toward secondary and tertiary hydroxy groups were also examined. Dihydrocholesterol (a secondary alcohol) was also derivatized in good yield (88%) under the same condition, however tertiary butyl alcohol was almost inert (< 5%) toward this derivatization reagent.

As shown in a previous paper [2], the reactivity of DACB-CN was somewhat lower for hydroxy group of alcohols, so that derivatization with DACB-CN was carried out under the conditions where the solvent is distilled away during the reaction in open vials. Because at least 20-fold molar excess of DACB-CN is required for a quantitative derivatization of alcohols, tailing is observed on the chromatogram resulting from carboxylic acid generated by the hydrolysis of DACB-CN during the derivatization reaction. Because DACB-NCO (**3**) is more reactive toward the hydroxy group than DACB-CN only a slight excess of reagent is required, rather than the 3-5-fold excess. The reaction of DACB-NCO in sealed vials allowed for the determination of lower molecular weight alcohols (the number of carbons: C_6 or above) than those examined in the case of DACB-CN.

Comparison of the Spectroscopic Properties of DACB-Carbamic Ester (**4a**) with those of DACB-Ester (**5**), MAC-Carbamic Ester (**6**), OBM-Ester (**7**) and 9-AN-Ester (**8**).

To confirm the utility of 3-aryl-7-dialkylaminocoumarin as a fluorogenic group, we compared the spectroscopic properties of both DACB-carbamic ester (**4a**) and DACB-ester (**5**) with those of ethyl 7-dimethylaminocoumarin-3-carbamate (MAC-carbamic ester, **6**) [3b], 2-(5-ethoxycarbonyl-2-oxazolyl)-5,6-methylenedioxybenzofuran (OBM-ester, **7**) [7b], and methyl anthracene-9-carboxylate (9-AN-ester, **8**) [6]. As shown in Table 3, the quantum yields of **4a** (0.70) and **5** (0.75) in ethanol were approximately equal to that of **6** (0.72) and **7** (0.83). Since the fluorescence sensitivity is proportional to both the molar absorptivity (ε) and quantum yield (ϕ), the fluorescence sensitivity is generally defined as $\varepsilon \times \phi$ (Table 3) [12]. The relative fluorescence sensitivity of DACB-ester (**5**) is stronger than that of OMB-ester (**7**) and 24 times as strong as that of 9-AN-ester (**8**) because of the large molar absorptivity (42,200) of **5**. On the other hand, the fluorescence sensitivity of carbamic ester **4a** is nearly equal to that of benzofuran derivative (**7**) and hexyl 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxo-quinoxaline-2-carbamate [8b]. These results show that DACB-CN and DACB-NCO having a 3-phenyl-7-diethylaminocoumarin moiety as a fluorogenic group are excellent derivatization reagents for use in hplc with fluorescence detection.

pH Dependence of Absorption and Fluorescence Spectra of 3-Phenyl-7-diethylaminocoumarin Derivative (**9**).

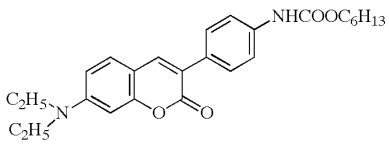
Since 7-diethylamino-3-phenylcoumarin, the fluorogenic group in DACB-ester and DACB-carbamic ester, contains the basic diethylamino functional group, the absorption and fluorescence spectra of these derivatives are dependent on the pH of the solution. To investigate the effect of pH, for this fluorogenic group, 7-(diethylamino)-3-[4-(hydroxymethyl)phenyl]-2*H*-1-benzopyran-2-one (**9**) [13] was used because of its solubility in phosphate buffer solutions. Figure 2 shows the pH dependence of absorbance at 409 nm and the fluorescence intensity at 498 nm of **9**. Apparently the diethylamino group of **9** is not protonated above pH 4, and the results obtained are in agreement with those of *N*-(7-dimethylamino-4-methyl-3-coumarinyl)succinimide (DACS) [14].

Conclusion.

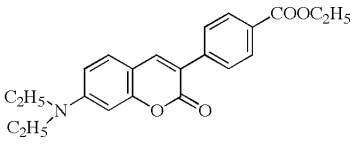
The newly developed fluorescent derivatization reagent, DACB-NCO, for alcohols has proved to be satisfactory with respect to sensitivity and reactivity. Thus, DACB-NCO as well as DACB-CN would be applicable to hplc analysis of trace amounts of primary

Table 3

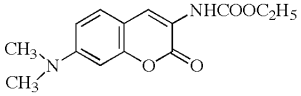
Absorption and Fluorescence Properties of Derivatized Compounds



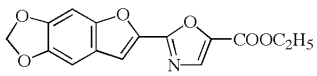
DACB-Carbamic ester (**4a**)



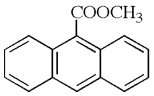
DACB-Ester (**5**)



MAC-Carbamic ester (**6**)



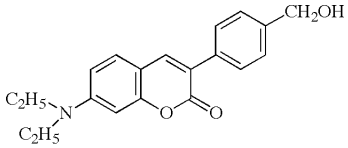
OMB-Ester (**7**)



9-AN-Ester (**8**)

Compound	Absorption[a] λmax (nm)	ε	Fluorescence[b] Ex (nm)	F.λmax (nm)	ϕ[c]	ϕ x ε	RFS[d]
DACB-Carbamic ester (**4a**)	402	38,600	403	489	0.70	27000	(21)
DACB-Ester (**5**)	413	42,200	413	488	0.75	31700	(24)
MAC-Carbamic ester (**6**)	378	23,700	378	480	0.72	17100	(13)
OMB-Ester (**7**)	350	32,900	352	443	0.83	27300	(21)
9-AN-Ester (**8**)	362	7,800	363	447	0.17	1300	(1)

[a] Concentration in ethanol: 2.0 x 10^{-5} *M*; [b] Concentration in ethanol: 4.5 x 10^{-6} *M*; [c] Quantum yield; [d] Relative fluorescence sensitivity: the compound **8** is arbitrarily taken as 1.00.



9

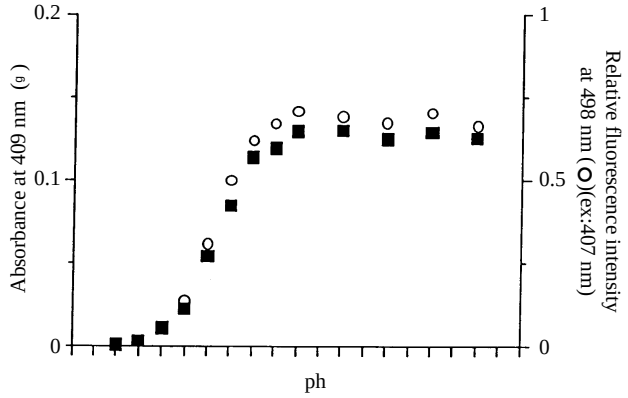


Figure 2. pH Dependence of the Absorption and Fluorescence Spectra of **9** in 0.1 *M* Phosphate Buffer.

alcohols and certain secondary alcohols such as steroids in biological fluids, with satisfactory accuracy and reliability at a femtomol level.

EXPERIMENTAL

All melting points were determined on a Yamato melting point apparatus (Yanaco MP-J3) and are uncorrected. Ir were recorded on JASCO FT/IR-300 spectrometer. The ^{1}H nmr spectra were acquired on either a JEOL JMN-LA-300 or JEOL JNM-EX-400 spectrometer. Ms were determined using a Shimadzu GC MS-9100-MK gas chromatograph-mass spectrometer with a direct inlet system. Absorption and fluorescence spectra were measured with a Hitachi 288 dual-wavelength spectrophotometer and a Hitachi F-4500 fluorescence spectrophotometer, which is equipped with a R928F photomultiplier (200-900 nm). Fluorescence quantum yields were determined according to the method of Parker and Rees [15], as reported previously [16], in which quinine sulfate in 5 x 10^{-2} *M* sulfuric acid was used as a standard. The hplc system consisted of a Hitachi L-6200 pump, a Rheodyne Model 7125 injector valve, a Hitachi F-1040 fluorescence spectrophotometer, a Hitachi D-2500 chromato-integrator and a Gasukuro Kogyo Model-545 degassing unit. The column was Inertsil ODS-2 (150 x 4.6 mm i.d.; particle size, 5 μm; Gasukuro Kogyo, Tokyo).

Synthesis of 4-[7-(Diethylamino)-2-oxo-2*H*-1-benzopyran-3-yl]benzeneisocyanate [4-(7-Diethylaminocoumarin-3-yl)-benzeneisocyanate (DACB-NCO, **3**)].

A mixture of 4-[7-(diethylamino)-2-oxo-2*H*-1-benzopyran-3-yl]benzoyl chloride (**1**, 1.78 g, 5.0 mmoles) prepared from carboxylic acid [2] and oxalyl chloride in the usual manner, and hydrazine monohydrate (0.5 ml, 10 mmoles) in THF (200 ml) was stirred for 1.5 hours at room temperature. The resulting precipitates

were collected and recrystallized from chloroform-hexane to give 4-[7-(diethylamino)-2-oxo-2*H*-1-benzopyran-3-yl]benzenecarbohydrazide (**2**) (1.45 g, 82%). Yellow needles, mp 224-226°; ir (nujol): 3340, 3280, 1705 cm^{-1}; ^1H nmr (dimethyl-d_6 sulfoxide, 400 MHz): δ 1.14 (t, *J* = 7Hz, 6H, $N(CH_2CH_3)_2$), 3.46 (q, *J* = 7Hz, 4H, $N(CH_2CH_3)_2$), 4.75 (bs, 2H, N-NH_2), 6.57 (s, 1H, ArH), 6.75 (d, *J* = 9Hz, 1H, ArH), 7.53 (d, *J* = 9Hz, 1H, ArH), 7.80 (d, *J* = 7Hz, 2H, ArH), 7.87 (d, *J* = 7Hz, 2H, ArH), 8.18 (s, 1H, ArH), 9.82 (s, 1H, CONH); high-resolution ms: m/z Calcd. for $C_{20}H_{21}N_3O_3$: 351.1583 (M^+). Found: 351.1554.

To a suspension of **2** (1.05 g, 3.0 mmoles) in water (24 ml) was added acetic acid (30 ml). Sodium nitrite (207 mg, 3.0 mmoles) in water (15 ml) was then added slowly to the solution under ice-cooling and the reaction mixture was stirred at same temperature for 30 minutes. The precipitated solid was filtered, washed with cold water and dried *in vacuo*. The resulting carbonyl azide was heated at 90° in toluene (120 ml) for 2 hours. The insoluble part was filtered off and the filtrate was concentrated to one-third of its original volume. Subsequently hexane was added to the concentrated solution to give precipitates. The precipitates were collected by filtration and recrystallized from ethyl acetate-hexane to give DACB-NCO (**3**) (0.67 g, 67 %). Yellow prisms, mp 142-143°; ir (nujol): 2265, 1705 cm^{-1}; ^1H nmr (deuteriochloroform, 400 MHz): δ 1.23 (t, *J* = 7Hz, 6H, $N(CH_2CH_3)_2$), 3.43 (q, *J* = 7Hz, 4H, $N(CH_2CH_3)_2$), 6.53 (d, *J* = 2Hz, 1H, ArH), 6.59 (dd, *J* = 9, 2Hz, 1H, ArH), 7.12 (d, *J* = 7Hz, 2H, ArH), 7.31 (d, *J* = 9Hz, 1H, ArH), 7.67 (d, *J* = 7Hz, 2H, ArH), 7.68 (s, 1H, ArH); ms: m/z 334 (M^+).

Anal. Calcd. for $C_{20}H_{18}N_2O_3$: C, 71.84; H, 5.43; N, 8.38. Found: C, 71.59; H, 5.63; N, 8.18.

Syntheses of Alkyl 4-[7-(Diethylamino)-2-oxo-2*H*-1-benzopyran-3-yl]phenyl Carbamates [DACB-Carbamic Esters (**4a-k**)].

General Procedure.

A mixture of DACB-NCO (**3**, 100 mg, 0.3 mmole), an alcohol (C_6-C_{22}) (0.3 mmole), and DMAP (147 mg, 1.2 mmoles) in acetonitrile (9 ml) was stirred at room temperature for 2 hours. The solvent was evaporated *in vacuo* and the residue was chromatographed on silica gel (benzene-hexane-ethyl acetate, 10:3:1, v/v) to give the corresponding DACB-carbamic esters (**4a-k**). Compounds **4a-k** were obtained in 64-86% yields. Physical properties and spectral data for DACB-carbamic esters are listed in Table 1.

Syntheses of Fluorescent Derivatized Compounds (**5-8**).

Ethyl 4-[7-(diethylamino)-2-oxo-2*H*-1-benzopyran-3-yl]-benzoate (DACB-ester, **5**) was prepared according to the reported method [2]. Yellow needles (from ethyl acetate-hexane), mp 144-145°; ^1H nmr (deuteriochloroform, 400 MHz): δ 1.23 (t, *J* = 7Hz, 6H, $N(CH_2CH_3)_2$), 1.41 (t, *J* = 7Hz, 3H, OCH_2CH_3) 3.44 (q, *J* = 7Hz, 4H, $N(CH_2CH_3)_2$), 4.39 (q, *J* = 7Hz, 2H, OCH_2CH_3), 6.54 (d, *J* = 2Hz, 1H, ArH), 6.61 (dd, *J* = 9, 2Hz, 1H, ArH), 7.34 (d, *J* = 9Hz, 1H, ArH), 7.78 (s, 1H, ArH), 7.80 (d, *J* = 9Hz, 2H, ArH), 8.08 (d, *J* = 9Hz, 2H, ArH).

Anal. Calcd. for $C_{22}H_{23}NO_4$: C,72.31; H, 6.34; N, 3.83. Found: C, 72.31; H, 6.30; N, 3.94.

Ethyl 7-Diethylaminocoumarin-3-carbamate (MAC-carbamic ester, **6**).

Compound **6** was prepared according to the reported method [3b]. Pale yellow prisms (from ethanol), mp 183-184°, lit mp 187° [3b].

2-(5-Ethoxycarbonyl-2-oxazolyl)-5,6-methylenedioxybenzofuran (OMB-ester, **7**).

Compound **7** was prepared according to the reported method [7]. Pale yellow prisms (from ethyl acetate-hexane), mp 174-175°, lit mp 186° [7].

Methyl 9-Anthracenecarboxylate (9-AN-ester, **8**).

A mixture of the 9-anthracenecarboxylic acid (225 mg, 1.0 mmole) and (trimethylsilyl)diazomethane (2.5 ml, 5.0 mmoles) in methanol (6 ml) was refluxed overnight. The reaction mixture was evaporated to dryness *in vacuo*, and the residue was treated with ethyl acetate and water. The organic layer was washed with brine, dried over magnesium sulfate and evaporated. The residue was chromatographed on silica gel (ethyl acetate-hexane, 1:4, v/v) to give **8** (223 mg, 95%). Pale yellow prisms (from ethyl acetate-hexane), mp 110-111°; ir (nujol): 1730 cm^{-1}; ^1H nmr (deuteriochloroform, 400 MHz): δ 4.18 (s, 3H, OCH_3), 7.4-7.6 (m, 4H, ArH), 8.0-8.1 (m, 4H, ArH), 8.52 (s, 1H, ArH); ms: m/z 236 (M^+).

Anal. Calcd. for $C_{16}H_{12}O_2$: C, 81.34; H, 5.12. Found: C, 81.50; H, 5.17.

Derivatization Procedure and Hpcl Conditions.

Stock solutions of alcohol (1.0 m*M*), DACB-NCO (**3**, 5.0 m*M*), and internal standard (**4e**, 1.0 m*M*) were prepared in chloroform. To a 10 μl of a test solution of alcohol were sequentially added 50 μl of DACB-NCO and 10 μL of **4e** solutions. The vial was tightly capped and heated at 120° for 30 minutes. After cooling, the reaction mixture was diluted with acetonitrile to 5 ml, and then an aliquot (10 μl) of the mixture was injected into the liquid chromatograph. The eluent from the column was monitored with a fluorophotometric detector at an excitation wavelength of 402 nm and an emission wavelength of 488 nm. The eluent flow-rate was 1.0 ml/minute.

Calibration Curve for Formation of **4g**

Stock solutions of cetyl alcohol (0.25 μ*M*-5.0 m*M*), DACB-NCO (**3**, 5.0 m*M*), and internal standard (**4e**, 1.0 μ*M*-1.0 m*M*) were prepared in chloroform. The derivatization reaction and detection were carried out according to the standard procedure described above.

Simultaneous Determination of Saturated Alcohols (**4a-d,4f-k**).

Stock solutions of ten alcohols (C_6-C_{22}) (0.25 m*M* each), DACB-NCO (**3**, 2.5 m*M*), and internal standard (**4e**, 0.25 m*M*) were prepared in chloroform. The derivatization reaction and detection were carried out according to the standard procedure described above. Simultaneous determination was attained by gradient elution with methanol/water (methanol concentration in the mobile phase: 95%, 0-8 minutes; 95-100%, 8-30 minutes).

Fluorescence Quantum Yields of **4a** and **5-8**.

Absorbances of the solution were kept below 0.2 at the excitation wavelength. The quantum yields of all compounds (**4a, 5-8**) were obtained using a 350, 370, and 390 nm excitation wavelength, respectively. In all compounds, since individual variation of the values measured with different excitation wavelengths was small, each average of their values was taken in Table 3.

pH Dependence of the Absorption and Fluorescence Spectra of **9**.

A stock solution of **9** [13] (1.0 m*M*) was prepared in ethylene glycol dimethyl ether. For measurement of absorption and fluorescence spectra, the stock solution was diluted with various 0.1 *M* phosphate buffer solutions (pH; 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 7.0, 9.0) to 50 μ*M* and 5 μ*M*, respectively. Absorbances and fluorescence intensities were monitored at 409 nm and 498 nm (excitation at 407 nm), respectively.

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