4-(7-Diethylaminocoumarin-3-yl)benzoyl Cyanide (DACB-CN): A Highly Sensitive Fluorescent Derivatization Reagent for Alcohols in High-Performance Liquid Chromatography[1)]

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4-(7-Diethylaminocoumarin-3-yl)benzoyl cyanide (DACB-CN) was synthesized as a new fluorescent derivatization reagent for alcohols for use in high-performance liquid chromatography. Saturated alcohols (C_8—C_{22}) were derivatized in good yields to give the corresponding fluorescent DACB-esters by treatment with DACB-CN. The DACB-esters of these alcohols were clearly separated on a reversed-phase HPLC column. The detection limit ($S/N=3$) of cetyl alcohol, a test compound, was 1—2 fmol/10 μl.

Key words 4-(7-diethylaminocoumarin-3-yl)benzoyl cyanide; derivatization reagent; HPLC; alcohol; fluorescence detection

A number of fluorescent derivatization reagents have been reported for the determination of alcohols by high-performance liquid chromatography (HPLC).[1b-10)] Among these reagents, only 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxo-quinoxaline-2-carbonyl chloride (DMEQ-COCl)[7a)] or its azide (DMOQ-CON_3),[7b)] and 2-(5-chlorocarbonyl-2-oxazolyl)-5,6-methylenedioxybenzofuran (OMB-COCl)[6)] permit the determination of alcohols as low as 1—10 femtomol per injected volume.

We have previously shown that 3-aryl-7-dialkylaminocoumarin was one of the most promising candidates as a fluorogenic group, and developed 3-(4-bromomethyl)-7-diethylamino-2*H*-1-benzopyran-2-one (MPAC-Br) which is one of the most sensitive and practically useful fluorescent derivatization reagents for carboxylic acids in HPLC.[1b,11)] The present paper deals with the preparation of 4-(7-diethylaminocoumarin-3-yl)benzoyl cyanide (**3**, DACB-CN) having acyl nitrile as the reacting group, and fundamental studies of its properties as a new derivatization reagent for HPLC analysis of alcohols.

Results and Discussion

Synthesis of DACB-CN (3) The synthesis of DACB-CN was carried out as shown in Chart 1. The conversion of the mixed anhydride (**2**) prepared from carboxylic acid (**1**)[1b)] and chloroformate to DACB-CN (**3**) proceeded rapidly and the desired DACB-CN (**3**) was separated from the reaction mixture in 86% yield. The absorption maximum and the fluorescence maximum of **3** in acetonitrile appeared at 452 nm ($\varepsilon=38700$) and 498 nm, respectively. DACB-CN was stable in the crystalline state for at least one year in daylight at room temperature.

Preparation of Standard Samples (DACB-Esters, 4a—p) and Their Spectroscopic Properties Standard samples of DACB-esters (**4a—p**) were obtained quantitatively by the esterification of a series of saturated primary alcohols (C_5—C_{22}) with an equimolar amount of DACB-CN in acetonitrile (refluxed for 1.5 h) in the presence of 4-dimethylaminopyridine (DMAP) (4 eq). The structures of the DACB-esters (**4a—p**) were determined from spectral and analytical data (Table 1). All the DACB-derivatives (**4a—p**) had absorption maxima (λ_{max}) and fluorescence maxima (F. λ_{max}) at 412—413 nm and 489 nm, respectively, with similar molar absorptivities and fluorescence intensities in ethanol (Table 1).

Table 2 shows the spectroscopic data for the DACB-ester of myristyl alcohol (**4f**) in several solvent systems often used in reversed-phase chromatography. The varia-

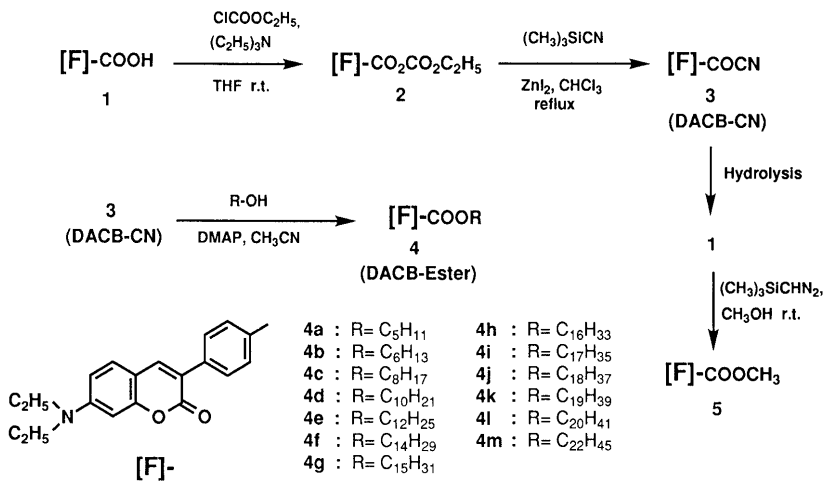

Chart 1

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Table 1. Physical Properties of DACB-Esters (**4a—p**)

Compd.	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found)			Absorption[a] ε (λ_{max} nm)	RFI[b] (489 nm) (Ex 413 nm)
				C	H	N		
4a	100	103.5—105	$C_{25}H_{29}NO_4$	73.68 (73.60)	7.17 7.21	3.44 3.35)	41600 (413)	1.03
4b	98	98—99	$C_{26}H_{31}NO_4$	74.08 (73.83)	7.41 7.41	3.32 3.12)	42000 (412)	1.03
4c	99	89.5—91	$C_{28}H_{35}NO_4$	74.80 (74.76)	7.85 7.87	3.12 3.03)	42000 (412)	1.02
4d	98	94.5—96	$C_{30}H_{39}NO_4$	75.44 (75.40)	8.23 8.25	2.93 2.85)	42200 (412)	1.00
4e	99	83—84	$C_{32}H_{43}NO_4$	76.00 (75.87)	8.57 8.52	2.77 2.70)	41900 (412)	1.00
4f	98	86.5—88	$C_{34}H_{47}NO_4$	76.51 (75.55)	8.88 8.87	2.62 2.54)	42100 (413)	1.00
4g	92	96—97	$C_{35}H_{49}NO_4$	76.74 (76.78)	9.02 9.06	2.56 2.74)	42500 (413)	1.02
4h	98	92.5—93.5	$C_{36}H_{51}NO_4$	76.96 (77.01)	9.15 9.18	2.49 2.34)	41800 (412)	0.98
4i	90	99.5—100.5	$C_{37}H_{53}NO_4$	77.17 (77.20)	9.28 9.39	2.43 2.46)	42000 (413)	1.03
4j	100	96—97.5	$C_{38}H_{55}NO_4$	77.37 (77.50)	9.40 9.41	2.37 2.29)	42100 (413)	0.98
4k	97	102—103	$C_{39}H_{57}NO_4$	77.57 (77.76)	9.51 9.56	2.32 2.32)	42300 (413)	1.00
4l	98	98—100	$C_{40}H_{59}NO_4$	77.75 (77.85)	9.63 9.61	2.27 2.19)	42000 (413)	0.96
4m	88	101—102	$C_{42}H_{63}NO_4$	78.09 (78.28)	9.83 9.91	2.17 2.19)	41700 (412)	0.99
4n	90	269—271	$C_{47}H_{63}NO_4$	79.96 (79.86)	9.00 9.07	1.98 2.20)	43000 (413)	1.00[c]
4o	95	233—236	$C_{47}H_{61}NO_4$	80.18 (80.25)	8.73 8.74	1.99 1.86)	43600 (413)	1.01[c]
4p	82	283—285	$C_{42}H_{65}NO_4$	79.73 (79.71)	9.25 9.29	1.98 1.97)	43100 (412)	0.99[c]

a) Concentration in ethanol: 2.0×10^{-5} M. *b*) Relative fluorescence intensity: the compound **4d** is arbitrarily taken as 1.00. Concentration in ethanol: 4.5×10^{-6} M. *c*) Relative fluorescence intensity: the compound **4n** is arbitrarily taken as 1.00. Concentration in ethanol: 4.5×10^{-6} M.

Table 2. Absorption and Fluorescence Properties of **4f** in Various Solvent Systems

Solvent	Absorption[a]		Fluorescence[b]		
	λ_{max} (nm)	ε	Ex (nm)	F. λ_{max} (nm)	RFI[c]
C_2H_5OH	412	42200	413	489	1.00
CH_3OH	412	43000	414	490	1.02
CH_3CN	409	42300	411	492	0.97
$CH_3OH:H_2O$ (90:10, v/v)	415	42700	417	492	1.01

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

tion in shift value in the spectrum of the DACB-ester (**4f**) in each solvent is characteristically smaller than those of fluorescent derivatized compounds derived from the alcohols using reagents such as OMB-COCl,[6] DMEQ-COCl,[7a] and DMOQ-CON_3.[7b]

Optimal Conditions for HPLC Analysis of Alcohols The reaction conditions for the precolumn derivatization of alcohols with DACB-CN were optimized using cetyl alcohol as a representative compound. The derivatization yield of cetyl alcohol with DACB-CN was estimated by comparing the fluorescence intensity of the product (**4h**) at 489 nm with that of an internal standard **4f** at regular time intervals. At first, the derivatization reaction in a sealed vial was examined. However, the esterification yield of cetyl alcohol was less than 10%, probably because the reaction medium was very dilute. To improve the derivatization yield, the reaction in an open vial was examined. At 85 °C, acetonitrile was distilled from the reaction mixture within 15 min, and the peak area for cetyl alcohol was almost maximal (94%). By using tetrahydrofuran (THF) in place of acetonitrile as a solvent, an even better result (97%) was obtained. As far as a base catalyst was concerned, DMAP gave good results. Furthermore, the effect of various concentrations of DACB-CN and DMAP for the derivatization of cetyl alcohol was examined. The results indicated that a 20- to 30-fold molar excess of reagent for cetyl alcohol (0.5-fold molar or above of catalyst *vs.* DACB-CN) was suitable. Consequently, the derivatization of alcohols was carried out under the following conditions: heating at 85 °C for 30 min in THF with a 20-fold molar excess of DACB-CN, in the presence of DMAP (0.5-fold molar *vs.* reagent) in an open vial.

Regarding the calibration curve, a linear relationship between the ratio of the peak areas of cetyl alcohol DACB-ester (**4h**) to that of the internal standard (**4f**) and

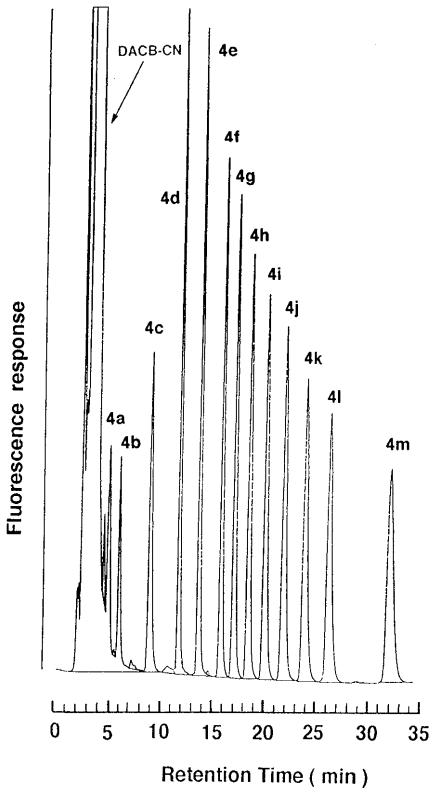

Fig. 1. Chromatogram of the DACB-Esters of Saturated Alcohols (**4a**—**m**) after Treatment with (Trimethylsilyl)diazomethane

Each peak area corresponds to 5 pmol alcohol.

the amount of cetyl alcohol was observed over the range 5 fmol—100 pmol/injection volume (10 μl) of the alcohol (linear correlation coefficient: 0.999), and the detection limit in this case was 1—2 fmol/10 μl ($S/N=3$).

Next, the simultaneous determination of saturated alcohols (C_5—C_{22}) was examined. To minimize the tailing by carboxylic acid (**1**) generated by the hydrolysis of DACB-CN during the derivatization reaction, **1** was converted into the methyl ester (**5**) by treatment of the reactant with (trimethylsilyl)diazomethane. The peaks of the DACB-derivatives of a series of saturated alcohols (C_5—C_{22}) (**4a**—**m**) were completely separated by gradient elution with methanol-water as the mobile phase within 35 min. (Fig. 1).

The analytical precision was established by repeated determination ($n=10$) using a mixture of eleven alcohols (5 pmol each per 10 μl). The yield of the fluorescent derivatives under these conditions was 72% (**4c** for octyl alcohol), 92% (**4d** for decyl alcohol), and 95—100% for long-chain alcohols (**4e**, **4g**—**m**; C_{12}—C_{22}). Further, the coefficient of variation was 3.6% for octyl alcohol, 1.5% for decyl alcohol, and did not exceed 0.6% for long-chain alcohols (C_{12}—C_{22}). Since the derivatization reaction was performed in an open vial, the yield and reproducibility for short-chain alcohols (C_5, C_6) were poor. However, this derivatization method is useful for the determination of high-boiling alcohols.

Based on the study described above, we have further investigated the derivatization of monohydroxysteroids. The determinations were performed by measuring the peak height ratios of steroids derivatized with DACB-CN to an internal standard, the DACB-ester of cholestanol (**4n**). (3β)-7-Dehydrocholesterol and dihydrocholesterol were derivatized in good yield [72% for **4o** and 91% for **4p**, and a coefficient of variation ($n=5$, 5 pmol/10 μl) of 4.0% and 1.4%, respectively] and separated within 38 min by reversed-phase HPLC using methanol as a mobile phase.

In conclusion, the reaction of DACB-CN with primary and secondary alcohols is complete within a short time to give highly fluorescent DACB-esters (**4**) in good yield. Further, DACB-esters have the advantage that they exhibit small differences in the fluorescence maxima (F. λ_{max}) and fluorescence intensities for the solvents used as mobile phases for reversed-phase HPLC. DACB-CN as a fluorescent derivatization reagent will be useful for the trace analysis by HPLC of certain series of alcohols, such as hydroxysteroids, with satisfactory accuracy and reliability at a femtomol level.

Experimental

Apparatus All melting points were determined on a Yamato melting point apparatus (Yanaco MP-J3) and are uncorrected. Infrared (IR) spectra were recorded on JASCO FT/IR-300 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on JEOL JNM-LA-300 and JEOL JNM-EX-400 spectrometers. Mass spectra (MS) were determined with 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 equipped with an R928F photomultiplier (200—900 nm), respectively. 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 × 4.6 mm i.d.; particle size, 5 μm; Gasukuro Kogyo, Tokyo).

Ethoxycarbonyl 4-[7-(Diethylamino)-2-oxo-2*H*-1-benzopyran-3-yl]-benzoate (2) Ethyl chloroformate (1.2 ml, 12 mmol) and triethylamine (1.7 ml, 12 mmol) were added to a solution of carboxylic acid (**1**,[1b] 1.17 g, 3.5 mmol) in THF (50 ml) at room temperature, and stirred overnight at the same temperature. THF was evaporated *in vacuo* and the residue was chromatographed on silica-gel using AcOEt–hexane (1 : 6, v/v) as the eluent to give **2** (1.21 g, 85%). Yellow prisms (from AcOEt–hexane), mp 126—127 °C. IR (Nujol): 1785, 1740, 1710 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.24 (6H, t, $J=7$ Hz, $N(CH_2\underline{CH}_3)_2$), 1.43 (3H, t, $J=7$ Hz, $OCH_2\underline{CH}_3$), 3.45 (4H, q, $J=7$ Hz, $N(\underline{CH}_2CH_3)_2$), 4.42 (2H, q, $J=7$ Hz, $O\underline{CH}_2CH_3$), 6.54 (1H, d, $J=2$ Hz, ArH), 6.62 (1H, dd, $J=9$, 2 Hz, ArH), 7.35 (1H, d, $J=9$ Hz, ArH), 7.81 (1H, s, ArH), 7.86 (2H, d, $J=9$ Hz, ArH), 8.10 (2H, d, $J=9$ Hz, ArH). MS m/z: 409 (M^+). *Anal.* Calcd for $C_{23}H_{23}NO_6$: C, 67.46; H, 5.66; N, 3.42. Found: C, 67.51; H, 5.77; N, 3.34.

4-[7-(Diethylamino)-2-oxo-2*H*-1-benzopyran-3-yl]benzoyl Cyanide (DACB-CN, 3) A mixture of mixed anhydride (**2**, 1.21 g, 3.0 mmol), trimethylsilyl cyanide (1.4 ml, 10.9 mmol) and zinc iodide (40 mg, 0.1 mmol) in $CHCl_3$ (30 ml) was refluxed for 24 h. The resulting precipitates were collected by filtration and recrystallized from AcOEt to give **3** (0.89 g, 86%). Reddish needles, mp 257—260 °C. IR (Nujol): 2220, 1710 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.25 (6H, t, $J=7$ Hz, $N(CH_2\underline{CH}_3)_2$), 3.46 (4H, q, $J=7$ Hz, $N(\underline{CH}_2CH_3)_2$), 6.53 (1H, d, $J=2$ Hz, ArH), 6.64 (1H, dd, $J=9$, 2 Hz, ArH), 7.37 (1H, d, $J=9$ Hz, ArH), 7.87 (1H, s, ArH), 8.00 (2H, d, $J=8$ Hz, ArH), 8.16 (2H, d, $J=8$ Hz, ArH). MS m/z: 346 (M^+). *Anal.* Calcd for $C_{21}H_{18}N_2O_3$: C, 72.82; H, 5.24; N, 8.09. Found: C, 72.84; H, 5.38; N, 8.10.

Synthesis of DACB-Esters (4a—p): General Procedure A mixture of DACB-CN (**3**, 104 mg, 0.3 mmol), an alcohol (C_5—C_{22}, cholesterol, 7-dehydrocholesterol, or dihydrocholesterol) (0.3 mmol), and DMAP (147 mg, 1.2 mmol) in acetonitrile (9 ml) was refluxed for 1.5 h. The solvent was evaporated *in vacuo* and the residue was chromatographed on silica-gel using $CHCl_3$ as the eluent to give the corresponding DACB-ester (**4a**—**p**). The yield, physical properties and spectral data for the DACB-esters are listed in Table 1.

Derivatization Procedure and HPLC Conditions Stock solutions of alcohol (1.0 mM), DACB-CN (**3**, 2.0 mM), DMAP (10 mM) and internal standard (**4f**, 1.0 mM) were prepared in THF. To 10 μl of a test solution

of alcohol were sequentially added 100 μl DACB-CN and 10 μl each of **4f** and DMAP solutions. The mixture in a reaction vial was heated without sealing to 85 °C for 30 min. After cooling, the reaction mixture dissolved in MeOH (2 ml) was treated with (trimethylsilyl)diazomethane (50 μl) at room temperature for 30 min. The reaction mixture was diluted with acetonitrile to 5 ml, and then an aliquot (10 μl) was injected into the liquid chromatograph. The eluent from the column was monitored with a fluorophotometric detector at an excitation wavelength of 413 nm and an emission wavelength of 489 nm. The eluent flow-rate was 1.0 ml/min.

Calibration Curve for Formation of 4h Stock solutions of cetyl alcohol (0.25 μM—5.0 mM), DACB-CN (**3**, 4.0 mM), internal standard (**4f**, 1.0 μM—1.0 mM), and DMAP (50 mM) were prepared in THF. The derivatization reaction and detection were carried out according to the standard procedure described above.

Simultaneous Separation of Saturated Alcohols Stock solutions of twelve alcohols (C_5—C_{22}) (0.25 mM each), DACB-CN (**3**, 5.0 mM), internal standard (**4f**, 0.25 mM), and DMAP (2.5 mM) were prepared in THF. The derivatization reaction and detection were carried out according to the standard procedure described above. Simultaneous separation was attained by gradient elution with MeOH/H_2O (MeOH concentration in the mobile phase: 92%, 0—6 min; 100%, 6—40 min).

Separation of Monohydroxysteroids Stock solutions of 7-dehydrocholesterol and dihydrocholesterol (0.25 mM each), DACB-CN (**3**, 5.0 mM), internal standard [DACB-ester of cholestanol (**4n**), 0.25 mM)], and DMAP (2.5 mM) were prepared in THF. The derivatization reaction and detection were carried out according to the standard procedure described above. Simultaneous separation was attained by elution with MeOH.

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References and Notes

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