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New Labeling Reagents for Alcohols in Fluorescence High-Performance Liquid Chromatography

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7-Methoxycoumarin-3 (and 4)-carbonyl azides (I and II) were synthesized as highly sensitive fluorescent labeling reagents for use in high-performance liquid chromatography (HPLC). Treatment of 7-methoxycoumarin-3 (and 4)-carbonyl chlorides with a suspension of activated sodium azide in dry acetone gave I and II in 56% and 54% yields, respectively. Compounds I and II reacted with alcohols in dichloromethane to give the corresponding highly fluorescent 7-methoxycoumarin-3 (and 4)-carbamic acid esters (IIIa—e and IVa—g). 4-Amino-7-methoxycoumarin (V), isolated as a by-product on labeling with II, was also obtained by heating II in tetrahydrofuran. Mixtures of primary and secondary alcohols were labeled with I or II and chromatographed on a reversed-phase HPLC column (mobile phase: methanol–water and methanol–chloroform) with a fluorescence detector. The detection limits of a test sample, cholesterol, were 50 fg and 10 ng/100 μl on labeling with I and II, respectively.

Keywords——7-methoxycoumarin-3-carbonyl azide; 7-methoxycoumarin-4-carbonyl azide; 7-methoxycoumarincarbamic acid ester; 4-amino-7-methoxycoumarin; fluorescence labeled alcohol; HPLC fluorescence detection; fluorescence quantum yield

Several labeling reagents for alcohols for use in high-performance liquid chromatography (HPLC) with fluorescence detection have recently been developed. Namely, Nambara *et al.*[1] reported the preparation of some naphthoyl- and anthroyl-nitriles and their applicability to the labeling of hydroxysteroids. We also reported the reactivity of 7-methoxycoumarins and a dansyl derivative prepared as labeling reagents for alcohols.[2] Although these reagents have fairly good properties as regards reactivity, sensitivity and selectivity, there remains scope for the further development of labeling reagents for alcohols from various sources.

Thus, in order to obtain additional highly sensitive labeling reagents for alcohols, 7-methoxycoumarin-3(and 4)-carbonyl azides (I and II) were synthesized and their properties as labeling reagents were examined.

Results and Discussion

Synthesis and Properties of I and II

Acyl azides in general can be synthesized from the corresponding acyl halides by treatment with sodium azide in aqueous-organic solvent mixtures such as water and acetone.[3] However, these methods gave poor yields in the present cases. On the other hand, the treatment of 7-methoxycoumarin-3 (and 4)-carbonyl chlorides[2a,4] with a suspension of activated sodium azide[5] in dry acetone gave the desired 7-methoxycoumarin-3 (and 4)-carbonyl azides (I and II) in 56% and 54% yields, respectively. The structures of I and II shown in Chart 1 were confirmed as follows. Compound I showed the infrared (IR)

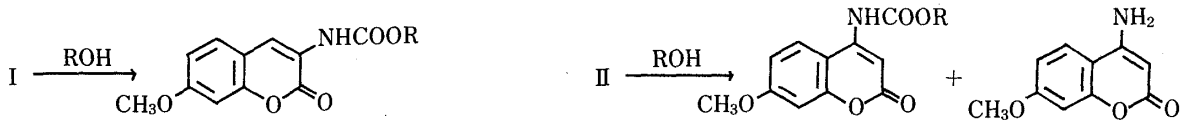
I II

Chart 1

absorption band due to the azido group at 2130 cm^{-1} and proton nuclear magnetic resonance (^{1}H-NMR) signals due to methoxy and aromatic ($C_{6,8}$-H, C_5-H and C_4-H) protons at 3.90, 6.80—7.10, 7.30 and 8.60 ppm, respectively. These spectral data and elemental analysis data were consistent with the expected structure of I. The structure of II was also confirmed in the same way. Compounds I and II were stable for a few months in a desiccator shielded from light. Compounds I and II themselves showed weak fluorescence, having no significant influence on the fluorescence detection.

Fluorescence Labeling of Alcohols with I and II

It is well-known that acyl azides can rearrange in inert solvents such as benzene and chloroform in the presence of alcohols, which react with the intermediate isocyanates to form carbamic acid esters.[6] As shown in Chart 2, I and II were found to react with alcohols under heating in tetrahydrofuran (THF), giving highly fluorescent coumarincarbamic acid esters (IIIa—e and IVa—g). On the other hand, the starting materials were recovered when the reaction was carried out at room temperature. THF was chosen as a solvent for the labeling because of the low solubility of the reagents and some alcohols in inert solvents on a preparative scale. The esters obtained were purified and stored for use as standard samples. The structures of IIIa—e and IVa—g were confirmed by elemental analysis, and the IR and ^{1}H-NMR spectral data shown in Tables I and II. The labeling of tertiary alcohols and phenols was also examined, but no reaction products could be isolated. Furthermore, trace amounts of V were obtained in the reaction with II as a fluorescent by-product (Chart 2). The structure



IIIa: $R=C_2H_5$
IIIb: $R=C_6H_{11}$
IIIc: $R=C_{27}H_{45}$ (cholesterol)
IIId: $R=C_{27}H_{47}$ (cholestanol)
IIIe: $R=CH_2C_6H_5$

IVa: $R=C_2H_5$
IVb: $R=C_6H_{11}$
IVc: $R=CH(CH_3)_2$
IVd: $R=C_{27}H_{45}$ (cholesterol)
IVe: $R=C_{27}H_{47}$ (cholestanol)
IVf: $R=C_{18}H_{23}O$ (estradiol)
IVg: $R=CH_2C_6H_5$

V

Chart 2

of V was easily determined to be 4-amino-7-methoxycoumarin from the presence of two absorption bands at 3350 and 3180 cm^{-1} (νNH_2) in the IR spectrum, and a broad singlet at 7.23 ppm (which disappeared on adding heavy water) and other peaks in the ^{1}H-NMR spectrum. In the absence of alcohols, V could be obtained by heating II in THF in 65% yield. The introduction of an amino group at the 4-position of coumarin is difficult, and the preparation of 4-aminocoumarin from 4-chlorocoumarin and liquid ammonia is the only known example.[7] Thus, the present method is useful for the preparation of 4-aminocoumarin derivatives.

In order to find the optimum labeling conditions for HPLC, the labeling of a test

TABLE I. Preparation and Physical Properties of 7-Methoxycoumarin-3 (and 4)-carbamic Acid Esters

Compound No.	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found)		
				C	H	N
IIIa	94	123—124	$C_{13}H_{13}NO_5$	59.31 (59.04)	4.98 (4.97)	5.23 (5.32)
IIIb	69	137—138	$C_{17}H_{19}NO_5$	64.34 (64.49)	6.04 (6.11)	4.41 (4.49)
IIIc	77	226—228	$C_{38}H_{53}NO_5$	75.58 (75.44)	8.85 (8.72)	2.32 (2.44)
IIId	79	179—180	$C_{38}H_{55}NO_5$	75.33 (74.99)	9.15 (8.99)	2.31 (2.50)
IIIe	96	167—168	$C_{18}H_{15}NO_5$	66.45 (66.31)	4.65 (4.70)	4.31 (4.46)
IVa	65	178—180	$C_{13}H_{13}NO_5$	59.31 (59.60)	4.98 (4.83)	5.32 (5.50)
IVb	76	198—199	$C_{17}H_{19}NO_5$	64.34 (64.51)	6.04 (5.89)	4.41 (4.65)
IVc	33	173—176	$C_{14}H_{15}NO_5$	60.64 (60.94)	5.45 (5.43)	5.05 (4.52)
IVd	90	270—272	$C_{38}H_{53}NO_5$	75.58 (75.58)	8.85 (8.78)	2.32 (2.31)
IVe	42	291—292	$C_{38}H_{55}NO_5$	75.33 (75.00)	9.15 (9.43)	2.31 (2.55)
IVf	35	255—257	$C_{29}H_{31}NO_6$	71.14 (71.16)	6.38 (6.53)	2.86 (2.54)
IVg	40	197—199	$C_{18}H_{15}NO_5$	66.45 (66.26)	4.65 (4.62)	4.31 (4.46)

TABLE II. IR and ^{1}H-NMR Spectral Data for 7-Methoxycoumarin-3 (and 4)-carbamic Acid Esters

Compound No.	IR ν_{max}^{KBr} cm^{-1} (NH)	^{1}H-NMR ($CDCl_3$) δ						
		C_3-H	C_4-H	C_5-H	C_6-H	C_8-H	C_7-OCH_3	Substituent
IIIa	3400		8.25	7.25	6.85	6.77	3.85	1.35, 4.25 (CH_2CH_3)
IIIb	3390		8.25	7.30	6.85	6.77	3.85	1.00—2.15 (C_6H_{11})
IIIc	3360		8.22	7.31	6.81	6.75	3.82	0.60—2.60 ($C_{27}H_{45}$)
IIId	3360		8.21	7.30	6.85	6.78	3.83	0.50—2.25 ($C_{27}H_{47}$)
IIIe	3290		8.25	7.28	6.90	6.80	3.86	5.25, 7.47 ($CH_2C_6H_5$)
IVa	3290	6.96		8.15	6.87	6.78	3.85	1.32, 4.22 (CH_2CH_3)
IVb	3290	7.03		7.43	6.89	6.78	3.83	0.80—2.30 (C_6H_{11})
IVc	3260	7.04		7.40	6.87	6.78	3.87	1.32, 4.48—5.30 (C_3H_7)
IVd	3300	7.01		7.35	6.88	6.79	3.85	0.50—2.60 ($C_{27}H_{45}$)
IVe	3290	7.02		7.38	6.90	6.83	3.87	0.30—2.30 ($C_{27}H_{47}$)
IVf	3280	—		—	—	—[a]	3.85	0.50—2.90 ($C_{12}H_{20}O$)
IVg	3300	6.94		8.12	6.86	6.82	3.85	5.37, 7.42 ($CH_2C_6H_5$)

a) Multiplet at 6.33—7.25 ppm.

compound, cholesterol, with I and II was examined in various solvents, dichloromethane, THF, acetonitrile and benzene. The labeling yields of cholesteryl coumarincarbamates (IIIc and IVd) in various solvents were estimated by comparison of the fluorescence intensities at 406 nm (IIIc) and 398 nm (IVd) with those of standard samples at regular time intervals. As can be seen in Fig. 1, the labeling in dichloromethane gave higher yields (81% for IIIc and 93% for IVd) than those in other solvents, indicating that the labeling of cholesterol with I and II proceeds quite effectively in dichloromethane. The labeling of some other alcohols with I or II gave similar results. The effect of the concentration of I and II on the labeling reactions was also examined by monitoring the fluorescence intensities of IIIc and IVd obtained under the conditions described above. As shown in Fig. 2, about 5-fold molar excess of reagents was suitable for labeling.

Thus, the labeling of all alcohols was carried out by heating them with about 5-fold molar excess of I or II in dichloromethane at 60 °C for 60 min.

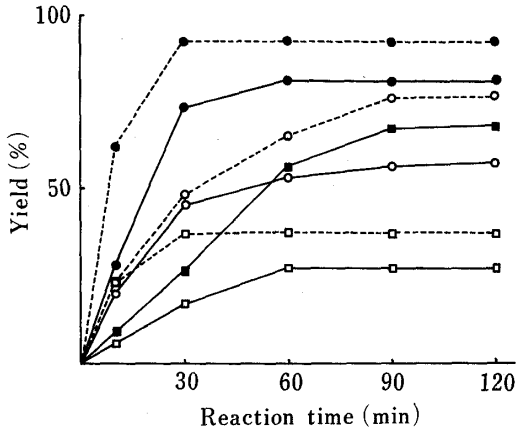


Fig. 1. Time Course of the Fluorescence Development of Cholesterol during the Reaction with I (——) or II (– – – –) in Various Solvents

●, dichloromethane; ○, benzene; ■, acetonitrile; □, tetrahydrofuran

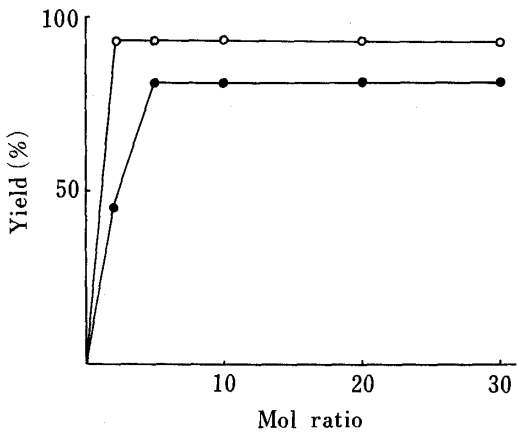


Fig. 2. Effects of 7-Methoxycoumarin-3 (and 4)-carbonyl Azide (I and II) Concentration on Fluorescent Derivative (IIIc and IVd) Formation

●, [I]/[cholesterol]; ○, [II]/[cholesterol]. Labeling of cholesterol (1.3 μg) was carried out under heating at 60 °C for 60 min with I or II (1.7—25 μg) in dichloromethane (100 μl).

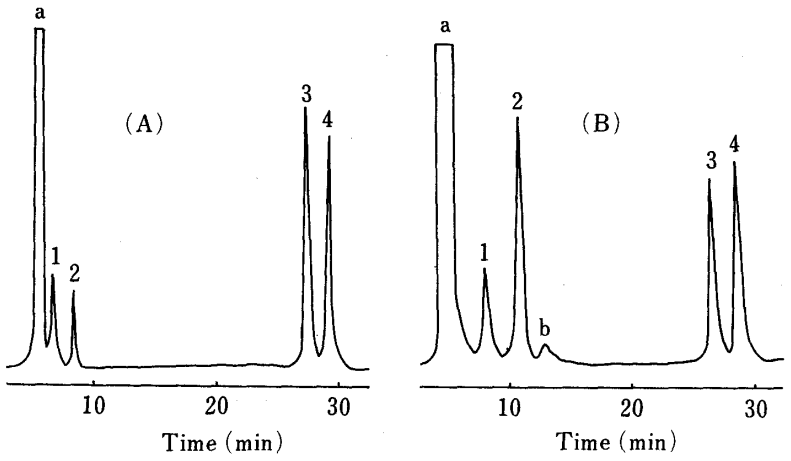


Fig. 3. Chromatograms of Reaction Mixtures after Labeling with I (A) or II (B)

A mixture of alcohols (A, 200 pmol; B, 10 nmol) was labeled with I (5 nmol) or II (250 nmol) in dichloromethane (100 μl), and an aliquot (10 μl) of the reaction mixture was injected directly.

Column: Unisil Pack 5C18-250A. Mobile phase: from 0 to 4 min, methanol–water (10 : 1, v/v) and from 4 to 40 min, methanol–chloroform (4 : 1 v/v) for (A) and (9 : 1 v/v) for (B). Flow rate: 0.8 ml/min.

(A): a, decomposition product of reagent; 1, benzyl alcohol; 2, cyclohexanol; 3, cholesterol; 4, cholestanol.

(B): a and b, decomposition products of reagent; 1, cyclohexanol; 2, estradiol; 3, cholesterol; 4, cholestanol.

Separation by HPLC

Two standard mixtures of alcohols were examined after prelabeling reactions with I or II. The following elution conditions were selected on the basis of preliminary experiments using the prepurified samples (IIIa—e and IVa—g). The elutions were carried out by using methanol–water (10 : 1, v/v) followed 4 min later by methanol–chloroform (4 : 1 for IIIa—e and 9 : 1 v/v for IVa—g) at a flow rate of 0.8 ml/min. Figure 3 shows the chromatograms of alcohols labeled with I or II. Although most of the alcohols examined were clearly separated under these conditions, IIIa, IVa, IVc and IVg were not detected because of interference by

TABLE III. Fluorescence Spectral Data for 7-Methoxycoumarin-3 (and 4)-carbamic Acid Esters in Various Solvents

Compound No.	F λ_{max} nm (Quantum yield)				
	Ethanol	Acetonitrile	Benzene	Cyclohexane	Methanol–chloroform (4:1)
IIIa	407 (0.506)	399 (0.552)	391 (0.487)	390 (0.458)	409 (0.530)
IIIb	407 (0.597)	399 (0.625)	390 (0.518)	388 (0.540)	409 (0.530)
IIIc	405 (0.583)	400 (0.608)	397 (0.507)	389 (0.527)	407 (0.602)
IIId	407 (0.601)	399 (0.619)	390 (0.502)	389 (0.534)	407 (0.548)
IIIe	407 (0.541)	400 (0.590)	392 (0.521)	389 (0.473)	409 (0.548)
IVa	389 (0.080)	383 (0.032)	368 (0.014)	362 (0.010)	389 (0.073)
IVb	387 (0.089)	383 (0.041)	363 (0.015)	361 (0.011)	387 (0.068)
IVc	385 (0.087)	385 (0.040)	367 (0.015)	359 (0.012)	389 (0.068)
IVd	382 (0.090)	382 (0.043)	363 (0.013)	363 (0.010)	387 (0.065)
IVe	388 (0.090)	383 (0.038)	367 (0.017)	362 (0.014)	386 (0.069)
IVf	385 (0.088)	384 (0.042)	367 (0.013)	361 (0.011)	387 (0.070)
IVg	388 (0.092)	385 (0.038)	366 (0.014)	359 (0.014)	386 (0.069)

Excitation wavelength: 335 nm for IIIa—e and 318 nm for IVa—g.

decomposed products derived from the reagents. The quantity of decomposed products from I was less than that from II as shown in Fig. 3. Furthermore, the determinations of cholesterol were done by measuring the peak height ratios of cholesterol labeled with I or II to internal standards, cholestanyl coumarincarbamates (IIId and IVe). Linear relationships were obtained in the range from 0.1 to 1.2 pg/100 μl by the use of I and 50 to 600 ng/100 μl by the use of II for cholesterol.

Fluorescence Properties of the Labeled Compounds

The fluorescence spectral data for the labeled compounds (IIIa—e and IVa—g) in various solvents are summarized in Table III. Compounds IIIa—e showed fluorescence maxima around 400 nm with large fluorescence quantum yields of 0.458—0.625. On the other hand, IVa—g showed fluorescence maxima in shorter wavelength regions (359—389 nm) with smaller quantum yields (0.010—0.092) than those of IIIa—e. From these observations, it appears that the substituents at the 3-position contribute significantly to the fluorescence of these derivatives.

In the present study, 7-methoxycoumarin-3 (and 4)-carbonyl azides (I and II) could be easily synthesized from the corresponding coumarincarbonyl chlorides, and were found to possess excellent storage properties. Compounds I and II react with primary and secondary alcohols under mild conditions to yield highly fluorescent 7-methoxycoumarin-3 (and 4)-carbamic acid esters. In particular, the esters obtained from I showed remarkably intense fluorescence in comparison with those from II. Thus, compound I is a useful, highly sensitive labeling reagent for alcohols.

Experimental

Synthesis——7-Methoxycoumarin-3-carbonyl Azide (I): 7-Methoxycoumarin-3-carbonyl chloride (1.0 g)[4] was dissolved in dry acetone (25 ml) and activated sodium azide (0.32 g)[5] was added at 0 °C. After being stirred at 0 °C for 2 h, the reaction mixture was poured into ice-water (200 ml). The resulting precipitates were collected by filtration and washed with cold water. The product was purified by precipitation from benzene solution with petroleum ether, followed by drying under reduced pressure to yield 0.59 g as pale yellow needles. mp 170—171 °C. *Anal.* Calcd for $C_{11}H_7N_3O_4$: C, 53.88; H, 2.88; N, 17.14. Found: C, 53.25; H, 2.95; N, 17.08. IR ν_{max}^{KBr} cm^{-1}: 2130, 1760. ^{1}H-NMR

($CDCl_3$) δ: 3.90 (3H, s, OCH_3), 6.80—7.10 (2H, m, C_6- and C_8-H), 7.30 (1H, d, C_5-H), 8.60 (1H, s, C_4-H).

7-Methoxycoumarin-4-carbonyl Azide (II): 7-Methoxycoumarin-4-carbonyl chloride (1.0 g)[2a] was treated in the same manner as above to yield 0.56 g of yellow needles. mp 120—121 °C. *Anal.* Calcd for $C_{11}H_7N_3O_4$: C, 53.88; H, 2.88; N, 17.14. Found: C, 53.48; H, 2.93; N, 17.05. IR ν_{max}^{KBr} cm^{-1}: 2130, 1760. ^{1}H-NMR ($CDCl_3$) δ: 3.96 (3H, s, OCH_3), 6.60 (1H, s, C_3-H), 6.85 (1H, s, C_8-H), 6.97 (1H, q, C_6-H), 7.13 (1H, d, C_5-H).

4-Amino-7-methoxycoumarin (V): II (0.1 g) in THF (10 ml) was refluxed for 1 h. After cooling, the resulting precipitates were collected by filtration and washed with acetone. Recrystallization from dimethylformamide gave 0.051 g of yellow prisms. mp >300 °C. *Anal.* Calcd for $C_{10}H_9NO_3$: C, 62.82; H, 4.75; N, 7.33. Found: C, 62.46; H, 4.53; N, 7.50. IR ν_{max}^{KBr} cm^{-1}: 3350, 3180. ^{1}H-NMR (DMSO-d_6) δ: 3.83 (3H, s, OCH_3), 5.09 (1H, s, C_3-H), 6.82 (1H, s, C_8-H), 6.91 (1H, q, C_6-H), 7.23 (2H, s, NH_2), 7.89 (1H, d, C_5-H). MS *m/e*: 191 (M^+).

Labeling of Alcohols with I and II——Labeling for Identifying Optimum Reaction Conditions: Cholesterol (1.3 μg) was treated with I or II (4.5 μg) in a test solvent (100 μl) under heating at the boiling points of the solvent used and then an aliquot (10 μl) of the mixture was injected into the liquid chromatograph.

Labeling for Obtaining the Working Curves: Standard solutions of cholesterol (2.5—30 pg/ml and 1.25—150 μg/ml for labeling with I and II, respectively), reagents (I, 190 pg/ml; II, 95 μg/ml) and internal standards (IIId, 47 pg/ml; IVe, 23.5 μg/ml) were prepared in dichloromethane. A mixture of cholesterol (40 μl), reagent (40 μl) and internal standard (20 μl) was heated at 60 °C for 60 min, then an aliquot (10 μl) of the reaction mixture was injected directly. Standard samples were prepared in a similar manner, except that they were refluxed in THF and recrystallized from EtOH after isolation by preparative thin layer chromatography (solvent system: benzene–ethyl acetate = 2 : 1 or 4 : 1 v/v).

Apparatus and Measurements——All melting points were measured with a Yanagimoto micro-melting point apparatus, and are uncorrected. IR spectra were taken with a JASCO IRA-1. ^{1}H-NMR spectra were taken with a JEOL JMN-PMX 60, employing tetramethylsilane as an internal standard. The abbreviations used are as follows: s, singlet; d, doublet; q, quartet; m, multiplet. Mass spectra were taken with a JEOL JMS-01 SG. Fluorescence spectra were measured with a Hitachi 650-60 fluorescence spectrophotometer. Fluorescence quantum yields were determined according to the method of Parker and Rees[8] using quinine sulfate in 1 N H_2SO_4 as the standard. HPLC was carried out on a Hitachi 655 equipped with a stainless steel column (250 × 4.6 mm i.d., Unisil Pack 5C18-250A) packed with Unisil NQ-18 silica gel (5 μm) at room temperature. MeOH–H_2O (10 : 1 v/v) and MeOH–chloroform (4 : 1 and 9 : 1 v/v) were used as the mobile phase at a flow rate of 0.8 ml/min. The fluorescence was measured at 406 nm with excitation at 335 nm for IIIa—e and at 398 nm with excitation at 318 nm for IVa—g on a Hitachi 650-10S fluorescence spectrophotometer equipped with a flow cell of 18 μl volume.

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