3-(7-Methoxycoumarin-3-carbonyl)- and 3-(7-Dimethylaminocoumarin-3-carbonyl)-2-oxazolones as New Fluorescent Labeling Reagents for High-Performance Liquid Chromatography

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3-(7-Methoxycoumarin-3-carbonyl)- and 3-(7-dimethylaminocoumarin-3-carbonyl)-2-oxazolones (IIa and IIb) were synthesized as fluorescent labeling reagents for high-performance liquid chromatography (HPLC). Treatment of 7-methoxy- and 7-dimethylaminocoumarin-3-carboxylic acids (Ia and Ib) with diphenyl 2-oxo-3-oxazolinylphosphonate in the presence of triethylamine in dichloromethane gave IIa and IIb in 45% and 26% yields, respectively. Compounds IIa and IIb reacted with primary and secondary amines at room temperature in chloroform to give the corresponding fluorescent 7-methoxy- and 7-dimethylamino-3-carboxamides (IIIa—i and IVa—i). A mixture of primary amines was labeled with IIa and chromatographed on a reversed-phase HPLC column (mobile phase: methanol–water) with a fluorescence detector. The detection limit of a test compound, benzylamine, was 19 fmol/100 μl by the use of IIa.

Keywords 3-(7-methoxycoumarin-3-carbonyl)-2-oxazolone; 3-(7-dimethylaminocoumarin-3-carbonyl)-2-oxazolone; labeling reagent; coumarin carboxamide; fluorescence-labeled amine; fluorescence quantum yield; synthesis; HPLC fluorescence detection

3-Acyl-2-oxazolones react as acylating reagents with amines, alcohols and thiols in the absence or presence of catalysts under mild conditions, giving amides and esters in high yields.[1] These compounds were utilized for peptide synthesis and selective protection of the amino group.[2]

In order to obtain highly reactive and selective labeling reagents for high-performance liquid chromatographic (HPLC) analysis of amines, alcohols or thiols, 3-(7-methoxycoumarin-3-carbonyl)- and 3-(7-dimethylaminocoumarin-3-carbonyl)-2-oxazolones (IIa and IIb) were synthesized (Chart 1) and their properties as labeling reagents were examined.

Materials and Methods

7-Dimethylaminocoumarin-3-carboxylic Acid (Ib) *m*-Dimethylaminophenol (20 g) was added to a solution of Vilsmeier reagent prepared from dimethylformamide (50 ml) and phosphoryl chloride (13.4 ml) at a temperature below 40 °C. The mixture was then heated at 90 °C for 2 h. After cooling, the reaction mixture was poured into ice-water, adjusted to pH 6.0—8.0 by the addition of sodium acetate, and let stand overnight in refrigerator. The resulting precipitates were collected by filtration and recrystallized from ethanol to give 6.9 g of *p*-dimethylaminosalicylaldehyde, mp 78—79 °C. This compound (7.5 g), according to the method of Rangaswami *et al.*,[3] was treated with ethyl malonate (25 ml) and piperidine (15 ml) in anhydrous ethanol (150 ml) to give ethyl 7-dimethylaminocoumarin-3-carboxylate (8.9 g) as yellow needles (ethanol). The coumarincarboxylate (8.0 g) was heated at 100 °C for 1 h in 6 N HCl aqueous solution (160 ml). After cooling, the resulting precipitates were collected by filtration and recrystallized from ethanol to give 4.3 g of Ib as yellow needles. mp 280—283 °C. *Anal.* Calcd for $C_{12}H_{11}NO_4$: C, 61.80; H, 4.75; N, 6.01. Found: C, 61.64; H, 4.77; N, 5.84. IR ν_{max}^{KBr} cm^{-1}: 1730. ^{1}H-NMR dimethyl sulfoxide-d_6 (DMSO-d_6) δ: 3.10 (6H, s, $N(CH_3)_2$), 6.58 (1H, d, C_8-H), 6.81 (1H, q, C_6-H), 7.65 (1H, d, C_5-H), 8.56 (1H, s, C_4-H).

3-(7-Methoxycoumarin-3-carbonyl)-2-oxazolone(IIa) 7-Methoxycoumarin-3-carboxylic acid (Ia, 1.0 g)[3] and diphenyl 2-oxazolinylphosphonate (DPPOx, 1.44 g)[4] were dissolved in dry dichloromethane (50 ml), and then triethylamine (2 ml) in dichloromethane (10 ml) was added dropwise at 0 °C. After stirring at 50 °C for 12 h, the reaction mixture was concentrated under reduced pressure until precipitates separated out, and then poured into water. The resulting precipitates were collected by filtration and recrystallized from acetonitrile to give 0.44 g of IIa and colorless prisms. mp 229—232 °C. *Anal.* Calcd for $C_{14}H_9NO_6$: C, 58.54; H, 3.16; N, 4.88. Found: C, 58.45; H, 3.35; N, 4.60. IR ν_{max}^{KBr} cm^{-1}: 1786, 1738, 1705. ^{1}H-NMR (DMSO-d_6) δ: 3.93 (3H, s, C_7-OCH_3), 6.92—7.22 (2H, m, C_6- and C_8-H), 7.50 (1H, d, oxazolone-H), 7.64 (1H, d, oxazolone-H), 7.80 (1H, d, C_5-H), 8.44 (1H, s, C_4-H).

3-(7-Dimethylaminocoumarin-3-carbonyl)-2-oxazolone(IIb) 7-Dimethylaminocoumarin-3-carboxylic acid (Ib, 1.0 g) and DPPOx (1.6 g) were treated with triethylamine in the same manner as above, and the mixture was stirred at *ca.* 60 °C for 12 h. The precipitates obtained were placed on the silica gel column and eluted with benzene–ethyl acetate (1 : 1, v/v). The crude products were recrystallized from benzene to give 0.34 g of IIb as yellow prisms. mp 223—225 °C. *Anal.* Calcd for $C_{15}H_{12}N_2O_5$: C, 60.00; H, 4.03; N, 9.33. Found: C, 60.05; H, 4.09; N, 9.19. IR ν_{max}^{KBr} cm^{-1}: 1794, 1720, 1694. ^{1}H-NMR (DMSO-d_6) δ: 3.12 (6H, s, C_7-$N(CH_3)_2$), 6.68 (1H, q, C_6-H), 6.90 (1H, d, C_8-H), 7.46 (1H, d, oxazolone-H), 7.52—7.76 (2H, m, C_5-H and oxazolone-H), 8.31 (1H, s, C_4-H).

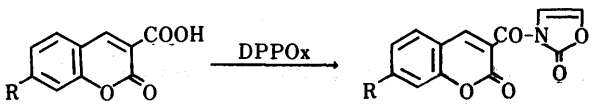
Labeling of Amines with IIa Labeling for Identifying Optimum Reaction Conditions: Stock solutions of benzylamine (428 ng/ml) and IIa (112 μg/ml) were prepared in each test solvent. A mixture of benzylamine (100 μl) and IIa (100 μl) solutions was stirred at room temperature and then an aliquot (10 μl) of the mixture was injected into the liquid chromatograph.

Labeling for Obtaining the Working Curve: Standard solutions of benzylamine (1.0—0.2 ng/ml), IIa (670 ng/ml) and internal standard (30 ng/ml) were prepared in chloroform. A mixture of benzylamine (50 μl), IIa (20 μl) and internal standard (30 μl) was stirred at room temperature for 90 min, then an aliquot (10 μl) of the reaction mixture was injected directly into the chromatograph.

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 Hitachi R-600, employing tetramethylsilane as an internal standard. The abbreviations used are as follows: s, singlet; d, doublet; q, quartet; m, multiplet. Fluorescence spectra were measured with a Hitachi 650-60 fluorescence spectrophotometer. Fluorescence quantum yields were determined according to the method of Parker and Rees[5] using quinine sulfate in 1 N H_2SO_4 as the standard. HPLC was carried out at room temperature on a Hitachi 655 equipped with a stainless steel column (250 × 4.6 mm i.d., Unisil Pack 5C18-250A) packed with Unisil NQ-8 silica gel (5 μm). MeOH–H_2O (10 : 3, v/v) was used as the mobile phase at flow rate of 0.8 ml/min. The fluorescence was measured at 405 nm with excitation at 350 nm for IIIa—f on a Hitachi 650-10S fluorescence spectrophotometer equipped with a flow cell of 18 μl volume.

Results and Discussion

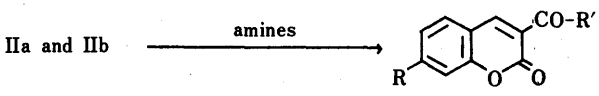
Synthesis and Properties of IIa and IIb Compound Ia was prepared by the acid hydrolysis of ethyl 7-methoxycoumarin-3-carboxylate obtained by the method of Rangaswami *et al.*[3] Compound Ib was prepared by condensing ethyl malonate with *p*-dimethylaminosalicylaldehyde obtained by Vilsmeier formylation of *m*-dimethylaminophenol, followed by hydrolysis with 6 N hydro-



Ia : R=OCH_3
Ib : R=$N(CH_3)_2$

IIa : R=OCH_3
IIb : R=$N(CH_3)_2$

Chart 1



IIIa—i: R=OCH_3, IVa—i: R=$N(CH_3)_2$
IIIa, IVa: R'=$NHCH_2CH_2CH_3$
IIIb, IVb: R'=$NHCH(C_6H_5)CH_3$
IIIc, IVc: R'=$NHCH_2CH_2C_6H_5$
IIId, IVd: R'=$NHCH_2C_6H_5$
IIIe, IVe: R'=NHC_6H_{11} (cyclo)
IIIf, IVf: R'=$NHC(CH_3)_3$
IIIg, IVg: R'=$N(C_2H_5)_2$
IIIh, IVh: R'=$N(CH_3)CH_2C_6H_5$
IIIi, IVi: R'=NC_5H_{10} (piperidyl)

Chart 2

chloric acid. Compounds Ia and Ib reacted with DPPOx[4] to give the desired 3-(7-methoxycoumarin-3-carbonyl)- and 3-(7-dimethylaminocoumarin-3-carbonyl)-2-oxazolones (IIa and IIb) in 45% and 26% yields, respectively. The structures of IIa and IIb shown in Chart 1 were confirmed as follows. Compound IIa showed an IR absorption band due to the carbonyl group in the oxazolone ring at 1786 cm^{-1}. The ^{1}H-NMR spectrum of IIa showed signals due to a coumarin ring [δ 3.93 (C_7-OCH_3), 6.92—7.22 ($C_{6,8}$-H), 7.80 (C_5-H) and 8.44 (C_4-H)], and an oxazolone ring (δ 7.50 and 7.64). These spectral and elemental analysis (see Materials and Methods) data were consistent with the expected structure of IIa. The structure of IIb was also confirmed in the same way. Compounds IIa and IIb were stable for a few months in a desiccator shielded from light.

As shown in Chart 2, IIa and IIb were found to react with aliphatic primary and secondary amines in the absence of a catalyst, by stirring at room temperature in chloroform, giving the corresponding coumarin carboxamides (IIIa—i and IVa—i). On the other hand, the reaction with aromatic amines such as anilines gave no products. The reaction products (IIIa—i and IVa—i) were recrystallized from ethanol after isolation by preparative thin layer chromatography (solvent system, benzene : ethyl acetate = 1 : 1 or 4 : 1, v/v) and stored for use as standard samples in HPLC. The structures of IIIa—i and IVa—i were confirmed by elemental analysis, IR and ^{1}H-NMR spectral data (Tables I and II). Compounds IIa and IIb did not react with benzyl alcohol, while with thiobenzyl alcohol they reacted in the presence of triethylamine, giving the corresponding benzylthio-esters 45% and 62% yields, respectively. The limited data obtained here suggest their utility as thiol reagents, and hence further investigations on the labeling of thiols are required.

Fluorescence Properties The fluorescence quantum yields of IIa and IIb themselves in methanol–water (10 : 3, v/v) were 0.210 and 0.098, respectively, which are not large enough to have a serious influence on the fluorescence detection.

TABLE I. Preparation and Physical Properties of 3-Substituted Carbamoyl-7-methoxy(and 7-dimethylamino)coumarins

Compd. No.	Yield[a] (%)	mp (°C)	Formula	Analysis (%) Calcd (Found)		
				C	H	N
IIIa	33	143—144	$C_{14}H_{15}NO_4$	64.36	5.79	5.36
				(64.34	5.57	5.36)
IIIb	44	159—162	$C_{19}H_{17}NO_4$	70.57	5.30	4.33
				(70.77	5.41	4.31)
IIIc	62	172—176	$C_{19}H_{17}NO_4$	70.57	5.30	4.33
				(70.68	5.14	4.33)
IIId	37	182—185	$C_{18}H_{15}NO_4$	69.89	4.89	4.53
				(69.63	5.15	4.65)
IIIe	68	200—205	$C_{17}H_{19}NO_4$	67.76	6.36	4.65
				(68.20	6.27	4.59)
IIIf	59	233—235	$C_{15}H_{17}NO_4$	65.44	6.22	5.09
				(65.50	6.21	5.04)
IIIg	24	125—126	$C_{15}H_{17}NO_4$	65.44	6.22	5.09
				(65.53	6.19	5.00)
IIIh	47	58—59	$C_{19}H_{17}NO_4$	70.57	5.30	4.33
				(70.49	5.25	4.05)
IIIi	33	159—160	$C_{16}H_{17}NO_4$	66.88	5.96	4.88
				(67.03	5.78	4.89)
IVa	44	160—163	$C_{15}H_{18}N_2O_3$	65.67	6.61	10.21
				(65.67	6.45	10.05)
IVb	60	181—182	$C_{20}H_{20}N_2O_3$	71.41	5.99	8.33
				(71.45	5.86	8.17)
IVc	67	197—200	$C_{20}H_{20}N_2O_3$	71.41	5.99	8.33
				(71.67	6.17	8.40)
IVd	71	203—205	$C_{19}H_{18}N_2O_3$	70.79	5.63	8.69
				(70.47	5.35	8.51)
IVe	45	190—193	$C_{18}H_{22}N_2O_3$	68.77	7.05	8.91
				(68.47	6.89	8.84)
IVf	66	225—230	$C_{16}H_{20}N_2O_3$	66.64	6.99	9.72
				(66.70	6.68	9.21)
IVg	56	175—178	$C_{16}H_{20}N_2O_3$	66.64	6.99	9.72
				(66.98	6.94	9.64)
IVh	62	190—193	$C_{20}H_{20}N_2O_3$	71.41	5.99	8.33
				(71.36	6.05	8.23)
IVi	37	184—185	$C_{17}H_{20}N_2O_3$	67.72	6.71	9.33
				(67.98	6.63	9.22)

a) Isolated yields.

The fluorescence spectral data of 7-methoxy- and 7-dimethylaminocoumarin-3-carboxamides (IIIa—i and IVa—i) in various solvents are summarized in Table III. The fluorescence maxima of IIIa—i were observed at around 400 nm, with excitation at 350 nm. Of these compounds, IIIa—f having secondary amide groups at the C_3 position of the coumarin ring gave large fluorescence quantum yields (0.312—0.569) in polar solvents such as ethanol, acetonitrile and methanol–water, while IIIg—i having tertiary amide groups showed practically no or very weak fluorescence in the solvents used. The above results suggested that the amide hydrogen at the C_3 position is significant for the fluorescence of these compounds. On the other hand, IVa—i showed fluorescence maxima in longer wavelength regions (428—469 nm) than those of IIIa—i when were excited at 400 nm. The fluorescence quantum yields (0.304—0.633) of IVa—f were quite large in less polar solvents such as benzene and cyclohexane, in contrast to those of IIIa—f, and were fairly large even in polar solvents. Compounds IVg—i, moreover, showed the tendency for the fluorescence quantum yields to increase with

TABLE II. IR and ^{1}H-NMR Spectral Data for 3-Substituted Carbamoyl-7-methoxy(and 7-dimethylamino)coumarins

Compd. No.	IR ν_{max}^{KBr} cm^{-1} (NH)	^{1}H-NMR ($CDCl_3$) δ						
		C_4-H	C_5-H	C_6-H	C_8-H	C_7-OCH$_3$	C_7-N(CH$_3$)$_2$	Substituent
IIIa	3324	8.85	7.52	6.95	6.88	3.92		1.00 (CH_3), 1.3—1.9 (CH_2), 3.2—3.7 (NCH_2), 8.6—9.0 (NH)
IIIb	3332	8.83	7.56	6.93	6.85	3.91		1.58 (CH_3), 5.0—5.5 (CH), 7.45 (C_6H_5), 8.9—9.3 (NH)
IIIc	3352	8.84	7.60	6.97	6.88	3.91		2.95 (CH_2), 3.5—4.2 (NCH_2), 7.30 (C_6H_5), 8.6—9.2 (NH)
IIId	3356	8.88	7.51	6.95	6.88	3.91		4.66 (CH_2), 7.35 (C_6H_5), 8.9—9.2 (NH)
IIIe	3340	8.84	7.52	6.95	6.88	3.92		1.0—2.3 (C_6H_{11}), 8.5—9.0 (NH)
IIIf	3328	8.81	7.49	7.01	6.86	3.91		1.48 ($CH_3 \times 3$), 8.6—8.9 (NH)
IIIg		7.77	7.35	6.90	6.82	3.89		1.0—1.5 ($CH_3 \times 2$), 3.0—3.8 ($CH_2 \times 2$)
IIIh		7.92	7.45	6.93	6.88	3.89		3.01 (CH_3), 4.69 (CH_2), 7.40 (C_6H_5)
IIIi		7.84	7.46	6.90	6.82	3.89		1.2—1.9, 3.2—3.9 (C_5H_{10})
IVa	3344	8.61	7.37	6.46	6.68		3.00	0.83 (CH_3), 1.1—1.8 (CH_2), 3.1—3.6 (NCH_2), 8.5—8.9 (NH)
IVb	3300	8.71	7.40	6.56	6.74		3.09	1.58 (CH_3), 5.1—5.6 (CH), 7.35 (C_6H_5), 9.0—9.3 (NH)
IVc	3332	8.54	7.30	6.37	6.61		2.96	2.80 (CH_2), 3.3—3.8 (NCH_2), 7.13 (C_6H_5), 8.5—8.9 (NH)
IVd	3360	8.76	7.44	6.56	6.75		3.10	4.67 (CH_2), 7.33 (C_6H_5), 9.0—9.3 (NH)
IVe	3320	8.72	7.38	6.56	6.76		3.11	1.0—2.0 (C_6H_{11}), 9.0—9.3 (NH)
IVf	3300	8.69	7.36	6.56	6.74		3.11	1.50 ($CH_3 \times 3$), 8.6—8.8 (NH)
IVg		7.71	7.24	6.55	6.74		3.09	1.20 ($CH_3 \times 2$), 3.1—3.8 ($CH_2 \times 2$)
IVh		7.85	7.35	6.51	6.78		3.07	2.95 (CH_3), 4.70 (CH_2), 7.35 (C_6H_5)
IVi		7.79	7.25	6.50	6.70		3.08	1.4—1.8, 3.2—3.8 (C_5H_{10})

TABLE III. Fluorescence Spectral Data for 3-Substituted Carbamoyl-7-methoxy(and 7-dimethylamino)coumarins in Various Solvents

Compd. No.	F λ_{max} nm (Quantum yield)[a]				
	Ethanol	Aceto-nitrile	Benzene	Cyclo-hexane	Methanol–water[b] (10:3, v/v)
IIIa	403 (0.506)	402 (0.424)	399 (0.262)	391 (0.130)	407 (0.634)
IIIb	405 (0.501)	402 (0.423)	401 (0.199)	392 (0.065)	407 (0.579)
IIIc	403 (0.569)	403 (0.480)	401 (0.318)	391 (0.173)	407 (0.635)
IIId	405 (0.565)	404 (0.513)	400 (0.280)	391 (0.116)	406 (0.580)
IIIe	403 (0.488)	403 (0.312)	400 (0.165)	392 (0.065)	405 (0.544)
IIIf	403 (0.489)	402 (0.425)	400 (0.271)	392 (0.104)	405 (0.558)
IIIg	405 (0.012)	400 (<0.01)	401 (<0.01)	399 (<0.01)	404 (0.018)
IIIh	404 (0.025)	401 (<0.01)	402 (<0.01)	398 (<0.01)	406 (0.028)
IIIi	400 (<0.01)	389 (<0.01)	392 (<0.01)	387 (<0.01)	399 (<0.01)
IVa	468 (0.178)	466 (0.277)	439 (0.625)	430 (0.419)	
IVb	468 (0.166)	466 (0.250)	441 (0.633)	429 (0.512)	
IVc	468 (0.159)	466 (0.273)	440 (0.636)	429 (0.304)	
IVd	469 (0.175)	467 (0.240)	439 (0.621)	439 (0.473)	
IVe	467 (0.040)	466 (0.268)	438 (0.566)	428 (0.318)	
IVf	467 (0.208)	465 (0.296)	440 (0.598)	427 (0.321)	
IVg	467 (0.410)	461 (0.578)	438 (0.311)	430 (0.058)	
IVh	469 (0.345)	461 (0.563)	438 (0.462)	431 (0.129)	
IVi	467 (0.372)	460 (0.586)	437 (0.210)	429 (0.024)	

a) Excitation at 350 nm for IIIa—i and at 400 nm for IVa—i. *b*) Mobile phase for HPLC.

increasing polarity of solvents.

Fluorescence characteristics for 7-substituted coumarins have been discussed in detail from the standpoint of the relative positions of $\pi\pi^*$ and $n\pi^*$ electronic energy levels in excited states by Hinohara *et al.*[6] The differences of characteristics between 7-methoxy- and 7-dimethylamino-coumarins shown in Table III are agreement with those described by them.

Thus, it was expected from the reactivity of IIa and the fluorescence characteristics of IIIa—i that IIa may be applicable to the labeling of primary amines in HPLC.

Fluorescence Labeling of Primary Amines with IIa The labeling of benzylamine with IIa was carried out at room temperature in various solvents. The labeling yields of 3-(*N*-benzylcarbamoyl)-7-methoxycoumarin(IIId) were estimated by comparison of the fluorescence intensities at 405 nm with that of a standard sample at regular time intervals (Fig. 1). It was found that the labeling was effectively achieved in chloroform and gave the highest and constant value after 90 min. Furthermore, the effect of the concentration of IIa in the labeling of benzylamine was examined by monitoring the fluorescence intensities of IIId on HPLC, indicating that about 100-fold molar excess of reagent was suitable for the labeling. Thus, the labeling of primary amines was carried out by stirring them with about 100-fold molar excess of IIa in chloroform at room temperature for 90 min.

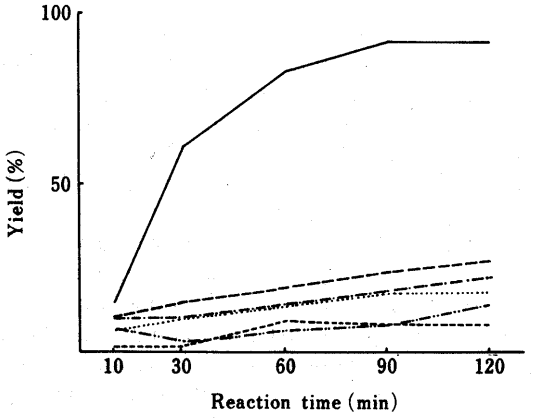


Fig. 1. Time Course of the Reaction of Benzylamine with IIa at Room Temperature in Various Solvents

Solvents: chloroform (——), dichloromethane (– – – –), ethanol (— · —), acetonitrile (·····), acetone (------), tetrahydrofuran (——— · ———).

HPLC Figure 2 shows the chromatogram of five amines labeled with IIa. Although most of the amines examined were clearly separated under these conditions, the separation between the labeled α-and β-phenylethylamines (IIIb and IIIc) was difficult even with the use of other mobile phases such as an acetonitrile–water system. Therefore, IIIc was excluded from the experiments for Fig. 2. Benzylamine was also determined by measuring the peak

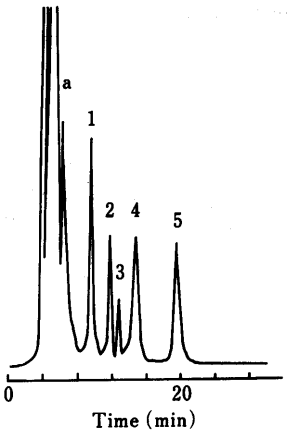


Fig. 2. Chromatogram of Reaction Mixture of Amines after Labeling with IIa

A mixture of *n*-propylamine, α-phenylethylamine, benzylamine (100 pmol each), cyclohexylamine and *tert*-butylamine (200 pmol each) was reacted with IIa (100 nmol) in chloroform (200 μl). An aliquot (10 μl) of the final reaction mixture was injected into the chromatograph. Column: Unisil Pack 5C18-250A. Mobile phase: methanol–water (10 : 3, v/v). Flow rate: 0.8 ml/min. a, unreacted reagent and its decomposition product; 1, *n*-propylamine; 2, benzylamine; 3, *tert*-butylamine; 4, α-phenylethylamine; 5, cyclohexylamine.

height ratios of benzylamine with IIa to internal standard, cyclohexyl 7-methoxycoumarin-3-carbamate.[7] A linear relationship was obtained in the range from 93 to 467 fmol in reaction solutions (100 μl), and the detection limit of benzylamine was 19 fmol/100 μl ($S/N=3$).

In the present study, 3-(7-methoxycoumarin-3-carbonyl)- and 3-(7-dimethylaminocoumarin-3-carbonyl)-2-oxazolones (IIa and IIb) could be easily synthesized from the corresponding coumarin carboxylic acids. Compounds IIa and IIb reacted with primary and secondary amines to yield fluorescent 3-substituted carbamoylcoumarins. In particular, the products obtained by the reactions of IIa with primary amines showed remarkably intense fluorescence, in contrast to the products from secondary amines. The detection sensitivity in the fluorescence was comparable with those of other reagents for amines.[8] These reagents are sparingly soluble in water, and therefore, in the case of analysis of the species present in biological fluids, the sampling should be done with chemical means prior to the labeling.

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References and Notes

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