Improvement of Fluorescence Characteristics of Coumarins: Syntheses and Fluorescence Properties of 6-Methoxycoumarin and Benzocoumarin Derivatives as Novel Fluorophores Emitting in the Longer Wavelength Region and Their Application to Analytical Reagents

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To improve the fluorescence characteristics, especially emission wavelength, of coumarins, various 3-substituted-6-methoxycoumarin derivatives were synthesized, and then benzocoumarin derivatives were also synthesized in expectation of the shift to the longer wavelength region by the extension of the conjugated system. Their fluorescence properties were investigated spectrophotometrically in acetonitrile and evaluated from the viewpoint of the intramolecular charge transfer (ICT) between push- and pull-substituents in the ground and the excited states. Among them, benzocoumarin derivatives especially fluoresced in the longer wavelength around 540 nm with remarkably large Stokes shifts beyond 10000 cm^{-1}. Using such fluorophores, some novel fluorescence derivatization reagents for carboxylic acids, alcohols, phenols, and amines were preliminarily prepared as an example, and their derivatized products were also found to fluoresce in the longer wavelength region with large Stokes shifts.

Key words fluorescence characteristic; stokes shift; methoxycoumarin; benzocoumarin; intramolecular charge transfer; fluorescence derivatization

Fluorometric analysis is one of the most sensitive methods for detecting organic and/or inorganic compounds and therefore it has been widely used in many scientific fields with improving analytical instruments such as high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), fluorescence confocal microscopy, *etc*. For these purposes, a great deal of effort has gone into the development of fluorescent reagents using various fluorophores, which can be utilized for derivatizing biologically active small molecules, labeling macromolecules such as proteins and DNAs, and probing ions such as biologically relevant metal cations and inorganic or organic anions.[1—4] In the development of such fluorescent reagents, it should first be ensured that the fluorophore used has a high fluorescence quantum yield because of the reliably high sensitivity in the detection. Fluorescein is one of the most widely used fluorophore for biological experiments, since it has a high fluorescence quantum yield of 0.90 or grater in aqueous solution and its excitation and emission wavelengths are in the visible region. Recently, Nagano and coworkers have succeeded in developing fluorescent reagents for the specific detection of nitric oxide (NO),[5—8] singlet oxygen ($^1O_2$),[9,10] and others[11,12] using fluorescein as the fluorophore. 4,4-Difluoro-4-bora-3a,4a-diaza-*s*-indacenes (BODIPYs) are also well-known fluorophores that have very sharp and narrow fluorescence bandwidths in addition to their high fluorescence quantum yield in aqueous solution.[4,13,14] Therefore they have been used as mother fluorophores for many analytical purposes.[15—18] However, the difference between the excitation and emission wavelengths of these derivatives are very small, less than approximately 30 nm for fluoresceins and 15 nm for BODIPYs, and it is necessary to correct their spectra from interferences such as Rayleigh or Raman scattering light.

On the other hand, coumarin (2*H*-benzopyran-2-one), is also another interesting fluorophore, since its fluorescence changes drastically with substituents and their introduced positions.[19—21] In previous papers, we reported the fluorescence characteristics of methoxycoumarins and discussed the structural features of strongly fluorescing methoxycoumarins from the viewpoint of intramolecular charge transfer (ICT) between push- and pull-substituents in the ground and the excited states.[22,23] Based on the above findings, we succeeded in developing novel fluorescent reagents with both high sensitivity and functions such as self-catalytic reactivity.[24—26] In the course of those studies, we suspected that most coumarin derivatives showed large Stokes shifts in their fluorescence spectra as compared with other fluorophores. These large Stokes shifts appear to be advantageous for escaping autofluorescence from biological molecules and reducing the self-absorption of chromophores. Further, if the emission bands of coumarins could be shifted to longer wavelengths with large Stokes shifts, analytical detection limits would markedly increase because of the clearing of interferences off and higher signal-to–noise (S/N) ratio. With this in mind, we began to design novel coumarin fluorophores that can fluoresce in the longer wavelength region while considering the relationship between the chemical structure of coumarins and their fluorescence properties. Here, we report the synthesis and the fluorescence properties of 6-methoxycoumarin and benzocoumarin (naphtopyranone) derivatives as candidate novel fluorophores emitting in the longer wavelength region, and the preliminary investigation of their application to fluorescence derivatization reagents using synthesized fluorophores as an example.

Results and Discussion

Design of Coumarin Fluorophores Although coumarin by itself is nonfluorescent, its derivatives with both electron-

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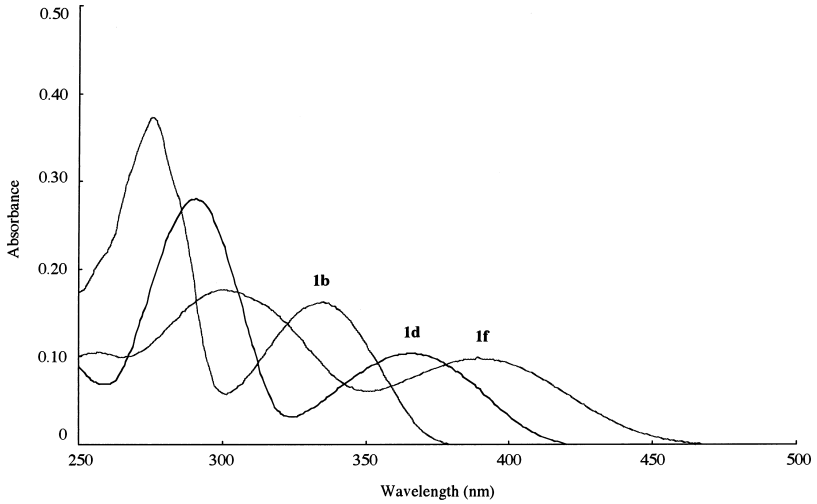


Fig. 1. Absorption Spectra of 3-Substituted-6-methoxycumarins **1b**, **1d**, and **1f** in Acetonitrile at 25 °C
Concentration of 3-substituted-6-methoxycumarins $=2.0\times10^{-5}$ M.

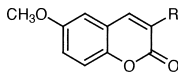
donating groups at the 6- and 7-positions and an electron-withdrawing group at the 3-position develop intense fluorescence,[19—21] as shown in 3-acetyl-6,7-dimethoxycoumarin with a quantum yield of 0.52 and a large Stokes shift of 5200 cm^{-1} in methanol.[22] In previous studied, we examined in detail the fluorescence characteristics of coumarins from the viewpoint of the substituent effect in connection with two ICT in the coumarin skeleton and postulated that one ICT from an electron-donating group at the 6-position to an electron-withdrawing group at the 3-position contributed to the fluorescence wavelength and the other ICT from an electron-donating group at the 7-position to an electron-withdrawing lactone carbonyl group at the 2-position contributed to the fluorescence intensity.[22,23] Based upon the above hypotheses, only the former ICT may be allowed to shift the emission wavelength of coumarin to the longer wavelength region. Thus 6-methoxycoumarin derivatives with various substituents at the 3-position were synthesized and their fundamental fluorescence behaviors were investigated spectrophotometrically.

It is well known that seminaphthofluoresceins (SNAFLs) and naphthofluoresceins, which are recognized as annellated derivatives of fluorescein by one or two aromatic ring, have longer emission wavelengths at 623 nm and 663 nm, respectively, compared with fluorescein (516 nm).[4,27—29] We thought that the introduction of such conjugated systems into the coumarin skeleton would make it possible to emit fluorescence in the longer wavelength region. However, it should be noted that the direction of annellation possibly affects the fluorescence properties of coumarin, as observed in studies of SNAFLs and naphthofluoresceins. Therefore a series of benzo-annellated coumarins, that is, 5,6-benzocoumarin (3*H*-naphto[2,1-b]pyran-3-one), 6,7-benzocoumarin (2*H*-naphto[2,3-b]pyran-2-one), and 7,8-benzocoumarin (2*H*-naphto[1,2-b]pyran-2-one) derivatives were first synthesized, and their fundamental fluorescence properties were investigated for comparison.

Syntheses 6-Methoxycoumarin **1a** was synthesized according to the method described in the literature.[30] Other derivatives **1b**—**f**, with various substituents at the 3-position, were synthesized from 5-methoxysalicylaldehyde[31] with active methylene compounds such as ethyl acetoacetate, diethyl malonate, ethyl cyanoacetate, and ethyl nitroacetate by means of Knoevenagel condensation[32] using piperidine as a catalyst in dry ethanol, and with phosphoranes such as carbethoxymethyl triphenylphosphorane and carbethoxyethylidene triphenylphosphorane in diethylaniline, respectively. The benzocoumarin derivatives **4a** and **b**, **5a** and **b**, and **6a**—**d** and **f** were also obtained from the appropriate hydroxynaphthaldehyde in a similar manner to that for 6-methoxycoumarin derivatives. 3-Formyl-6,7-benzocoumarin **6e** was obtained by the oxidation of **6d** with selenium dioxide in toluene.[33]

Effects of Substituents on Spectroscopic Properties of 6-Methoxycoumarins The typical absorption spectra of 3-substituted-6-methoxycoumarins (**1b**, **1d**, **1f**) in acetonitrile are shown in Fig. 1. Their spectroscopic properties, absorption maxima (λ_{max}), molar absorptivities (ε), fluorescence excitation maxima (λ_{Ex}), fluorescence emission maxima (λ_{Em}), Stokes shifts ($\Delta\nu$), and relative fluorescence intensities (RFIs) are listed in Table 1, together with the Hammett substituent constants (σ_p-values).[34,35] The RFIs are represented as values against the fluorescence intensity (100) of the standard compound **1a**. Although the Hammett substituent constants are commonly used for the estimation of reactivity and there is no theoretical connection between σ_p constants and fluorescence, these parameters appeared to be suitable and easily available to represent the total electronic effects in the ground state because of the fluorescence of coumarins based upon the typical ICT between push- and pull-subtituents in a molecule. Gottlieb and coworkers reported that the ^{13}C-chemical shifts of 6- and 7-substituted coumarins correlated to the Hammett constants.[36] Furthermore, Sherman and Robins pointed out the close correlation of the fluorescence of substituted 7-hydroxycoumarins with the Hammett constants.[37] Recently, Imai and coworkers have also succeeded in predicting the fluorescence characteristics of benzofurazan compounds using the Hammett substituent constants.[38] Therefore it can be expected that the ICT character of coumarin derivatives in the ground state primarily reflects their emissive ICT in the excited state. As shown in Fig. 1, the absorption spectra of 6-methoxycoumarins were influ-

Table 1. Spectral Properties of 3-Substituted-6-methoxycoumarins in Acetonitrile



Compound No.	R (σ_p)	Absorption		Fluorescence			
		λ_{max}/nm	ε	λ_{Ex}/nm	λ_{Em}/nm	$\Delta\nu$/cm^{-1}	RFI
1a	H (0)	344	4000	344	441	6400	100
1b	CH_3 (−0.17)	334	8000	335	437	7000	233
1c	$COOC_2H_5$ (0.45)	366	5200	367	478	6300	1871
1d	$COCH_3$ (0.50)	367	5200	375	501	6700	1573
1e	CN (0.66)	374	4800	374	485	6100	1067
1f	NO_2 (0.78)	393	4900	393	553	7400	223

enced by a substituent at the 3-position. The λ_{max} values of 3-substituted compounds (**1a**—**f**) shifted to longer wavelengths with an increase in electron-withdrawing ability (see σ_p) of substituents in the order of **1f**>**1e**>**1d**>**1c**>**1a**>**1b**, whereas their molar absorptivities (ε) were similar except for that of **1b** (Table 1). In contrast to the absorption spectra, their fluorescence spectra differed markedly. In the RFI, a drastic increase was observed for **1c**—**e** substituted with electron-withdrawing groups, *i.e.*, ethoxycarbonyl (ester) to cyano groups, as compared with that of **1a** and **b**. The RFI value of **1f**, with the strongest electron-withdrawing nitro group, was comparable with that of **1b**, with an electron-donating methyl group. This prominent decrease in **1f** may be attributed to the nonemissive twisted ICT (TICT) excited state,[39—43] where push-pull π-electron systems between a methoxyl group at the 6-position and a nitro group at the 3-position in a molecule must be fully separated in polar solvent such as acetonitrile. Rettig and Klock reported that the very weak fluorescence of 6-aminocoumarin with a stronger electron-donating amino group than the methoxyl group was attributed to this TICT between the amino group and coumarin ring.[44] In spite of 6-methoxycoumarins **1a** and **1c**—**f** having similar molar absorptivities (ε), the reason for the distinct differences in their fluorescence intensities is not yet obvious. It is likely that the ICT characters such as dipole moments of 6-methoxycoumarins in the excited state are more amplified than those in the ground state. In future, the study of computer calculations of substituent effects in the excited state may enable the fluorescence characteristics to be predicted more precisely. On the other hand, the λ_{Em} values of these compounds were also shifted to longer wavelength regions, with an increase in the electron-withdrawing ability of substituents at the 3-position. Furthermore, the wavelengths were also about 20 nm longer than that of 6,7-dimethoxycoumarins and resulted in the Stokes shifts ($\Delta\nu$) beyond 6000 cm^{-1}. The results were in accordance with our hypothesis that an ICT from a methoxyl group at the 6-position to a substituent such as ethoxycarbonyl and acetyl groups at the 3-position greatly contributes to the fluorescence wavelength. Although the fluorescence quantum yields of 6-methoxycoumarins were lower than those of fluoresceins and BODIPYs, as shown in **1d** with a quantum yield of 0.02 in acetonitrile,[22] their large $\Delta\nu$ values appear to be useful for fluorometric analysis. To confirm this hypothesis, we tried to synthesize novel fluorescence derivatizing reagents based on the 6-methoxycoumarin skeleton and investigated the fluorescence properties of the compounds derivatized by such reagents.

Fluorescence Derivatization by 6-Methoxycoumarin Reagents In general, the fluorescence derivatization reagents consist of two parts, a mother fluorophore and a group reactive to target molecules. We have already reported the effects of the reactive groups of 6,7-dimethoxycoumarin reagents, such as the bromoacetyl group for carboxylic acids, hydrazinocarbonyl group for ketones or aldehydes, and chlorocarbonyl group for hydroxyl and amino groups, on the fluorescence characteristics of derivatized compounds and found that the compounds derivatized by both bromoacetyl- and chlorocarbonyl-reactive groups showed excellent fluorescence quantum yields in acetonitrile and aqueous acetonitrile.[45] In this study, we also chose bromoacetyl, carboxylic, and chlorocarbonyl groups as reactive groups of derivatizing reagents and introduced them into the 3-position of the 6-methoxycoumarin skeleton. The synthetic route for the above derivatization reagents and their derivatization reactions are shown in Chart 1. One reagent, **2a** with a bromoacetyl-reactive group, was synthesized from **1d** as previously reported,[46,47] and the derivatization reaction of carboxylic acids with **2a** was carried out using acetic acid, lauric acid, and benzoic acid as model carboxylic acids in the presence of catalysts such as $KHCO_3$ and 18-crown-6 to yield the derivatized compounds **2b**—**d**, respectively. Another reagent, **3a** with a carboxylic group as a reactive group, was synthesized by the acid hydrolysis of **1c**, and the derivatized compounds esters **3b** and **c** and amides **3d**—**f** were obtained using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC HCl) and 4-*N*,*N*-dimethylaminopyridine (DMAP) as usual catalysts in dichloromethane. The fluorescence properties of these derivatized compounds in methanol and acetonitrile are listed in Table 2. The RFIs in this table are represented as the relative values against the fluorescence intensity (100) of reference compound **1d**. These results showed that the difference of excitation and emission wavelengths ($\Delta\lambda$) and Stokes shifts ($\Delta\nu$) of all derivatized compounds were greater than 100 nm and 6000 cm^{-1}, respectively, except for **3f**. In particular, those of **2b**—**d** obtained using reagent **2a** reached more than 120 nm and 6900 cm^{-1}, respectively. These large shifts in the fluorescence spectra in the fluorescence spectra may be due to the strong electron-withdrawing ability of a newly introduced functional group, which has two carbonyl groups at the 3-position of the coumarin skeleton due to the derivatization reaction. Al-

Table 2. Fluorescence Spectral Data of Derivatized Products with 6-Methoxycoumarin Reagents in Acetonitrile and Methanol

Compound No.	Acetonitrile					Methanol				
	λ_{Ex}/nm	λ_{Em}/nm	($\Delta\lambda^{a)}$/nm)	$\Delta\nu$/cm^{-1}	RFI	λ_{Ex}/nm	λ_{Em}/nm	($\Delta\lambda^{a)}$/nm)	$\Delta\nu$/cm^{-1}	RFI
1d	367	478	(111)	6300	76	369	480	(111)	6300	100
2b	380	506	(126)	6600	114	380	519	(139)	7000	134
2c	380	506	(126)	6600	126	380	514	(134)	6900	149
2d	384	485	(101)	5400	186	383	515	(132)	6700	200
3b	368	479	(111)	6300	90	370	480	(110)	6200	119
3c	372	482	(110)	6100	137	373	484	(111)	6100	153
3d	363	467	(104)	6100	51	367	471	(104)	6000	76
3e	350	464	(114)	7000	19	352	469	(117)	7100	36
3f	358	n.d.$^{b)}$	—	—	—	358	n.d.$^{b)}$	—	—	—

a) $\Delta\lambda=\lambda_{Em}-\lambda_{Ex}$, *b*) not detected.

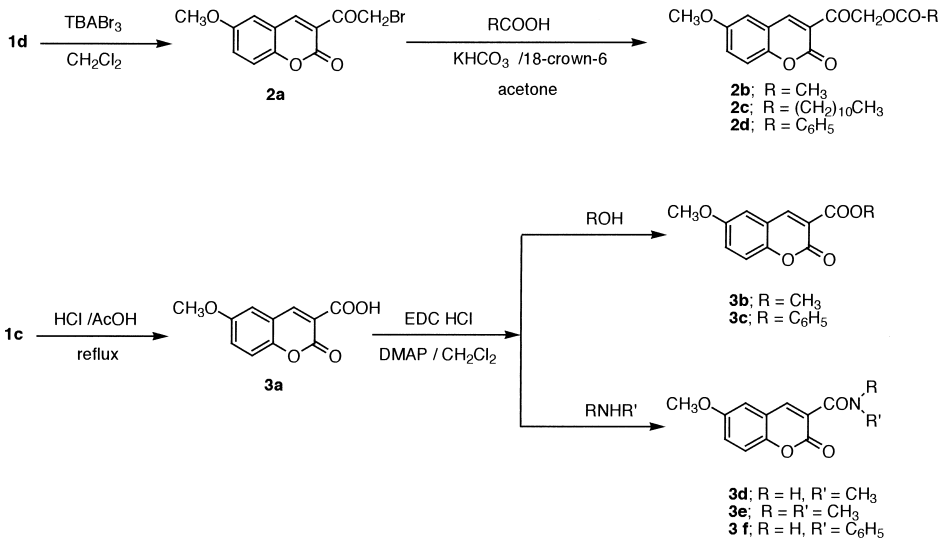


Chart 1

though a fluorescence quantum yield for **2c** in methanol has already been reported to be 0.25 (RFI=149 in Table 2),[22] those of **2b** and **3b** and **c** appear to be high as well. On the other hand, the RFIs of **3d**—**f** obtained using reagent **3a** were slightly low as compared with the above compounds, because thir electron-withdrawing ability at the 3-position were reduced by an amide group. However, these Stokes shifts were still greater than 6000 cm^{-1}. This trend in fluorescence intensity was also observed in the study of 6,7-dimethoxycoumarin reagents.[22,23] Therefore these fluorescence derivatization reagents based on the 6-methoxycoumarin skeleton are expected to have fluorescence properties superior to that of the 6,7-dimethoxycoumarin skeleton from the viewpoint of the highly sensitive fluorescence detection of analytes in the longer wavelength region.

Effects of Annellation on the Spectroscopic Properties of Benzocoumarins The absorption and fluorescence spectra of three different benzocoumarins, 3-acetyl-5,6-benzocoumarin **4b**, 3-acetyl-7,8-benzocoumarin **5b**, and 3-acetyl-6,7-benzocoumarin **6b**, in acetonitrile are shown in Figs. 2 and 3, respectively, and the results are summarized in Table 3. As predicted, the direction of annellation affected both the absorption and fluorescence properties of benzocoumarin derivatives. The shape of the absorption band of **6b** from around 300 to 450 nm is markedly different from that of **4b** and **5b**. Linear compound **6b** had a λ_{max} at 337 nm with a small shoulder band at around 370—450 nm, whereas angulated compounds **4b** and **5b** definitely had two splitting absorption bands at 337 and 382 nm for **4b** and 329 and 390 nm for **5b**, respectively, as shown in Fig. 2. Although such annellation effects on the absorption spectra were frequently observed in condensed aromatic compounds, for example, a linear compound such as anthracene generally has a λ_{max} ascribed to Platt's ^1L_b or Clar's *p* bands in the longer wavelength region than that of an angulated phenanthrene. The λ_{max} values of benzocoumarin derivatives shifted to longer wavelengths in the order of **5b**>**4b**>**6b** with decreasing absorbance. The same tendency was also observed in the 3-carboethoxy compounds (**5a**>**4a**>**6a**), as shown in Table 3. These differences in the absorption spectra apparently were attributed to the substituent effect at the 3-position of the benzocoumarin skeleton. The changes in transition moment in a molecule may be reflected in their absorption spectra as the critical difference between linear compound and angulated compounds. That is, this may be due to the increase in the CT character between the benzocoumarin skeleton and a substituent at the 3-position. However, the fluorescence intensity increased in the order of **5b**>>**4b**>**6b**, as shown in Fig. 3. Konishi and coworkers also observed the same behaviors in the fluorescence intensity of angulated and linear benzocoumarins in ethanol and benzene.[48] Among these benzocoumarins, **4a** of the ester-type and **5b** of the acetyl-type

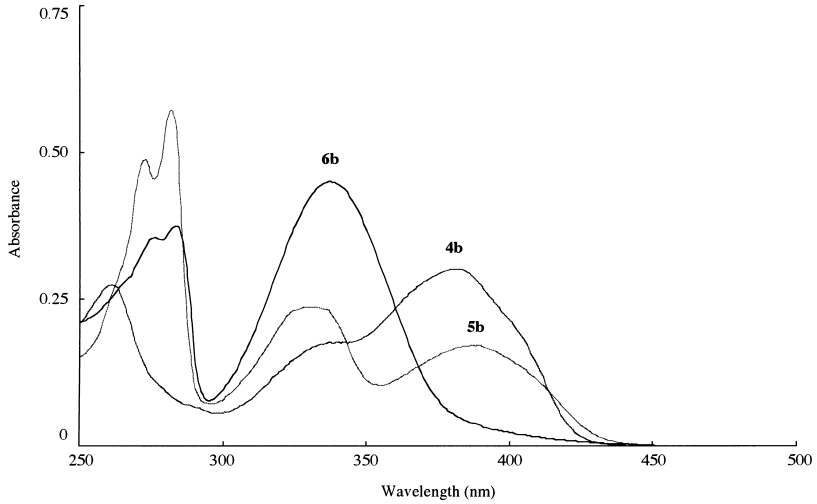


Fig. 2. Absorption Spectra of Benzocoumarins **4b**, **5b**, and **6b** in Acetonitrile at 25 °C
Concentration of benzocoumarins = 2.0×10^{-5} M.

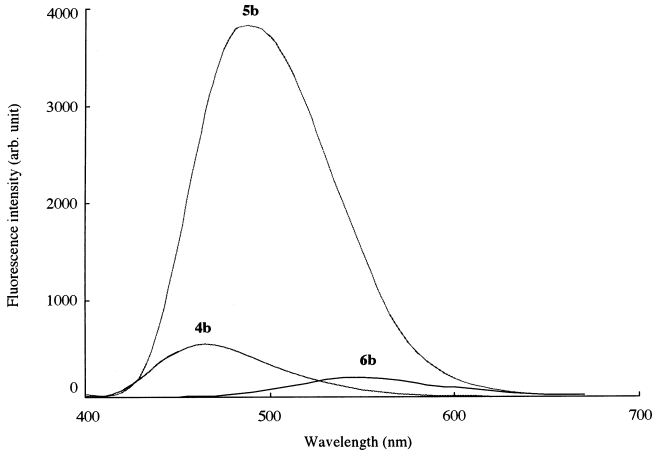
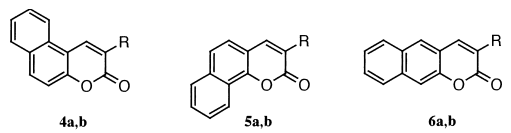


Fig. 3. Fluorescence Spectra of Benzocoumarins **4b**, **5b**, and **6b** in Acetonitrile at 25 °C
Concentration of benzocoumarins = 1.0×10^{-6} M.

Table 3. Spectral Properties of Benzocoumarins in Acetonitrile

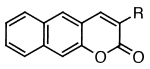
Compound No.	Absorption		Fluorescence			
	λ_{max}/nm	ε	λ_{Ex}/nm	λ_{Em}/nm	$\Delta\nu$/cm^{-1}	RFI
4a	371	14200	378	445	4000	4050
4b	382	15100	384	465	4500	90
5a	379	7900	380	470	5000	1570
5b	390	8600	389	491	5300	770
6a	331	24200	336	535	11100	100
6b	337	22500	341	549	11100	40



(**a**; R = $COOC_2H_5$, **b**; R = $COCH_3$)

compounds showed the highest fluorescence intensity (Table 3). These results apparently indicate that the fluorescence intensity of the benzocoumarins is influenced not only by the direction of annellation, but also by the electronic effect of substituents at the 3-position. In contrast to these behaviors in the fluorescence intensity, the λ_{Em} values of the benzocoumarins shifted to the longer wavelength region in the order of **6**>**5**>**4**. In particular, that of linear compound **6b** reached the longest wavelength of 549 nm, in spite of λ_{Ex} in shorter wavelength (341 nm) and lower RFI. As a result, the linear compounds **6a** and **b** showed remarkably large Stokes shifts ($\Delta\nu$) of greater than 11000 cm^{-1}, values two-fold greater than those of the angulated compounds. Although such fluorescence characteristics of the linear compounds **6a** and **b** are not clear, their emissive ICT characters in the excited state may be stabilized by the solvation of polar solvents such as acetonitrile, resulting in shifts of their λ_{Em} values to the longer wavelength region and the lowering of their fluorescence intensities.[43,49] Thus the 6,7-benzocoumarin skeleton was chosen as a novel fluorophore emitting in the longer wavelength region, and its derivatives with various substituents at the 3-position were synthesized to understand the detailed fluorescence properties. Table 4 shows the fluorescence spectral data of 3-substituted 6,7-benzocoumarins **6a**—**f**. All the benzocoumarin compounds with electron-withdrawing groups such as ester (**6a**), acetyl (**6b**), and formyl (**6e**) groups at the 3-position developed intense fluorescence. Langmuir and coworkers reported that the fluorescence quantum yields of linear benzocoumarin compounds were very weak compared with those of angulated compounds.[49] For example, a quantum yield of methyl 7-methoxy-2*H*-naphto[2,3,b]pyran-2-oxo-3-calboxylate, which is a family compound of **6a**, was found to be 0.13 in ethanol, but its $\Delta\lambda$ value was 167 nm, resulting in a large $\Delta\nu$ value (8500 cm^{-1}). It was observed in this study that the λ_{Em} values of the compounds with electron-withdrawing groups shifted to the longer wavelength region with larger $\Delta\nu$ values than those of nonsubstituted derivative **6f** and derivative **6d** with an electron-donating methyl group at the 3-position. In contrast, the RFI of **6c** with a strong electron-withdrawing cyano group apparently decreased. The reduction in the fluorescence intensity of **6c** may be also ascribed to the nonemissive TICT excited state due to the strong electron-withdrawing ability of the cyano group, as well as in the case of 6-methoxy-3-nitrocoumarin **1f**. In this investigation, we con-

Table 4. Spectral Properties of 3-Substituted-6,7-benzocoumarins in Acetonitrile



Compound No.	R (σ_p)	Absorption		Fluorescence			
		λ_{max}/nm	ε	λ_{Ex}/nm	λ_{Em}/nm	$\Delta\nu$/cm^{-1}	RFI
6a	$COOC_2H_5$ (0.45)	331	24200	331	534	11500	345
6b	$COCH_3$ (0.50)	337	22500	340	540	10900	375
6c	CN (0.66)	335	24000	329	533	11600	67
6d	CH_3 (−0.17)	322	19400	322	465	9600	82
6e	CHO (0.44)	345	25100	321	547	12900	126
6f	H (0)	321	19000	320	429	7900	100

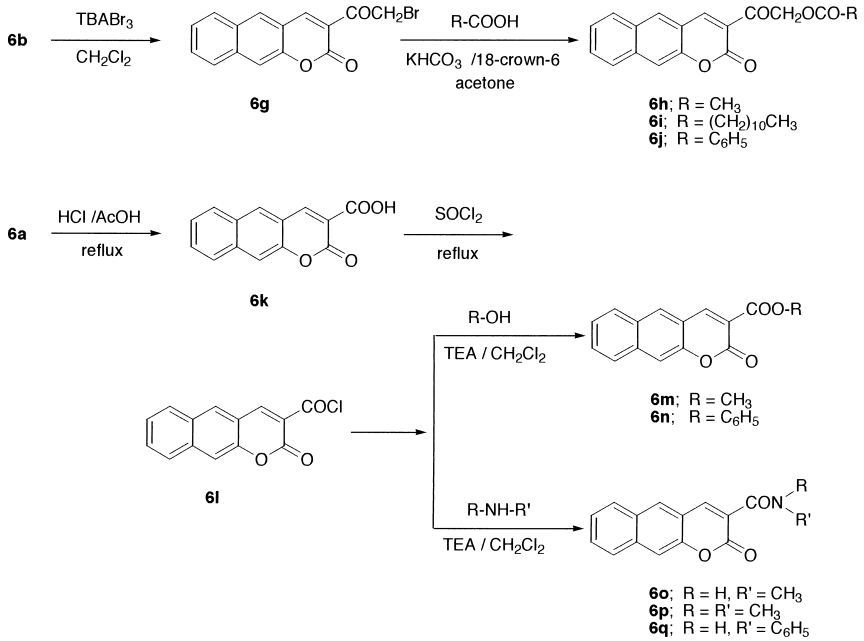


Chart 2

firmed that the linear benzocoumarin compounds with electron-withdrawing groups at the 3-position had remarkably large Stokes shifts of greater than 10000 cm^{-1} in spite of low fluorescence intensity, and therefore that the introduction of such conjugated systems into the coumarin skeleton was effective for the development of longer wavelength emissive fluorophores. Next, we tried to synthesize novel fluorescence derivatizing reagents based on the 6,7-benzocoumarin skeleton and investigated fluorescence properties of the derivatives prepared by such reagents.

Fluorescence Derivatization by Benzocoumarin Reagents The synthetic routes of novel fluorescence derivatization reagents based on the 6,7-benzocoumarin skeleton and their derivatization reactions are shown in Chart 2. A bromoacetyl group was also selected as a reactive group for carboxylic acids, and the derivatization reaction with reagent **6g** was carried out in a similar manner to that with **2a**. On the other hand, a more reactive chlorocarbonyl group[45] than the carboxylic group in 6-methoxycoumarin reagent **3a** was introduced into the benzocoumarin skeleton to give reagent **6l**, and the derivatization reactions of alcohols, phenols, and amines with reagent **6l** proceeded smoothly at room temperature as compared with **3a**. The fluorescence spectral data of the derivatives **6h**—**j** and **6m**—**q** obtained with these reagents in acetonitrile and methanol, which are frequently used as solvents in HPLC and CE, are summarized in Table 5, with reference compound **6b**. The λ_{Em} values of these derivatives were shifted to the longer wavelength region with an increase in the electron-donating ability of the new functional group (**6h**—**j**>**6m**, **n**>**6o**, **p**) introduced by the derivatization reaction, and these λ_{Em} values reached more than 520 nm. As a result, their $\Delta\nu$ values were surprisingly large, almost greater than 10000 cm^{-1}. The actual fluorescence of these products in methanol appeared greenish yellow with excitation at 365 nm on the transilluminator apparatus. A further examination of Table 5 shows that the tendency of these derivatives in terms of fluorescence intensity was apparently different from those of reagents **2a** and **3a** based on the 6-methoxycoumarin skeleton. Among the derivatives, the fluorescence intensities of **6h**—**j** obtained by a reagent **6g** were lower and those of **6o**—**q** obtained with reagent **6l** were higher, except for **6q** in methanol. Interestingly, this tendency was reversed in acetonitrile. A strong electron-withdrawing group with two carbonyl groups is present in derivatives **6h**—**j**, whereas a weak electron-withdrawing amide group is present in derivatives **6o**, **p**. Therefore the difference in fluo-

Table 5. Fluorescence Spectral Data of Derivatized Products with Benzocoumarin Reagents in Acetonitrile and Methanol

Compound No.	Acetonitrile					Methanol				
	λ_{Ex}/nm	λ_{Em}/nm	($\Delta\lambda^{a)}$/nm)	$\Delta\nu$/cm^{-1}	RFI	λ_{Ex}/nm	λ_{Em}/nm	($\Delta\lambda^{a)}$/nm)	$\Delta\nu$/cm^{-1}	RFI
6h	345	544	(199)	10700	131	346	550	(204)	10700	55
6i	345	544	(199)	10600	125	346	550	(204)	10700	58
6j	346	544	(198)	10500	136	347	550	(203)	10600	64
6b	331	534	(203)	11500	52	336	535	(199)	11100	100
6m	332	534	(202)	11400	60	336	536	(200)	11100	114
6n	337	539	(202)	11100	115	339	539	(200)	10900	138
6o	334	515	(181)	10500	270	336	525	(189)	10700	471
6p	326	510	(184)	11100	31	328	521	(193)	11300	124
6q	343	n.d.$^{b)}$	—	—	—	345	n.d.$^{b)}$	—	—	—

a) $\Delta\lambda=\lambda_{Em}-\lambda_{Ex}$, *b*) not detected.

rescence intensity could be attributed to the difference in electron-donating ability between the introduced benzene ring and methoxyl group. It is likely that the ICT characters involving solvation of these benzocoumarins in the excited state are different from those of 6-methoxycoumarin derivatives. In any case, these findings indicate that such derivatization reagents based on the benzocoumarin skeleton would be useful for selective and sensitive detection of specific molecules in complex biological systems, because of their large Stokes shifts of more than 10000 cm^{-1}. In the future, we will attempt to confirm the above results in further, more precise spectroscopic experiments and elucidate the theoretical relationships between spectroscopic behaviors such as absorption and fluorescence and chemical structures involving the substituent effects of coumarin derivatives.

In conclusion, we have found two candidates to be novel fluorophores, 6-methoxycoumarin and 6,7-benzocoumarin, which can fluoresce in the longer wavelength region with large Stokes shifts. The former was designed based on the emission mechanism of coumarins, while the latter was based on the introduction of conjugated systems due to annellation.

Using the fluorophores, we obtained fluorescence derivatization reagents **2a**, **3a**, **6g**, and **6l** for target molecules. Since the derivatized compounds can emit fluorescence in the longer wavelength region with large Stokes shifts, they should be useful for selective and sensitive detection of various biological molecules in analytical procedures such as HPLC and CE. In addition, the application of these reagents to multicolored imaging, which can distinguish many target molecules simultaneously owing to the varying bright fluorescence from white blue to greenish yellow even if excited at the same wavelength, would be possible.

Experimental

^{1}H-NMR spectra were obtained with a JEOL LNM-GSX 500FT-NMR spectrometer. Chemical shifts are expressed in δ ppm downfield from an internal tetramethylsilane (TMS) signal. The following abbreviations are used: s, singlet; d, doublet; t, triplet; and m, multiplet. The mass spectra (MS) were recorded with a JEOL JMS-DX303 spectrometer using the electron impact ionization (EI) mode at 70 eV. The fluorescence spectra were recorded with a Hitachi F-4500 fluorescence spectrophotometer. The slit width was 5.0 nm for both excitation and emission. RFI values were obtained by comparing the area under the recorded spectrum of coumarin derivatives with the standard one. All chemicals were of reagent grade and were used without further purification. The solvents (Luminasol) used for the fluorescence measurement were purchased from Dojindo Laboratories (Kumamoto, Japan). Silica gel chromatography was performed using Silica gel 60 (Merck).

Preparation of 1b This compound was prepared from 5-methoxysalicylaldehyde[31] and calbethoxyethylidene triphenylphosphorane in diethylaniline according to the method described in a literature.[30] Crude product was subjected to silica gel chromatography using chloroform as the eluent and recrystallized from hexane to give **1b** as pale yellow needles. ^{1}H-NMR ($CDCl_3$, ppm) δ: 2.22 (3H, s, C_3-CH_3), 3.84 (3H, s, C_6-OCH_3), 6.86 (1H, s, C_5-H), 7.04 (1H, d, C_7-H), 7.25 (1H, d, C_8-H), and C_8-H), 7.48 (1H, s, C_4-H). HR-MS (EI$^+$) *m/z*: 190.0628 (Calcd for $C_{11}H_{10}O_3$: 190.0630).

General Synthetic Method of 1c—f A mixture of 5-methoxysalicylaldehyde (10 mmol) and the appropriate ester (10 mmol) in 25 ml of absolute ethanol was refluxed in the presence of a few drops of piperidine for 30 min. After cooling, the resulting precipitates were recrystallized from ethanol to give 6-methoxycoumarins **1c**—**f** as pale yellow needles.

Ethyl 6-Methoxycoumarin-3-carboxylate (**1c**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 1.41 (3H, t, –CH_3), 3.87 (3H, s, C_6-OCH_3), 4.42 (2H, q, –CH_2–), 7.01 (1H, s, C_5-H), 7.22—7.31 (2H, m, C_7-, C_8-H), 8.48 (1H, s, C_4-H). HR-MS (EI$^+$) *m/z*: 248.0685 (Calcd for $C_{13}H_{12}O_5$: 248.0685).

3-Acetyl-6-methoxycoumarin (**1d**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 2.73 (3H, t, C_3-$COCH_3$), 3.87 (3H, s, C_6-OCH_3), 7.04 (1H, s, C_5-H), 7.24 (1H, d, C_7-H), 7.31 (1H, d, C_8-H), 8.47 (1H, s, C_4-H). HR-MS (EI$^+$) *m/z*: 218.0579 (Calcd for $C_{12}H_{10}O_4$: 218.0579).

3-Cyano-6-methoxycoumarin (**1e**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 3.88 (3H, s, C_6-OCH_3), 6.98 (1H, s, C_5-H), 7.26—7.35 (2H, m, C_7-, C_8-H), 8.21 (1H, s, C_4-H). HR-MS (EI$^+$) *m/z*: 201.0423 (Calcd for $C_{11}H_7O_3$: 201.0426).

3-Nitro-6-methoxycoumarin (**1f**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 3.90 (3H, s, C_6-OCH_3), 7.09 (1H, s, C_5-H), 7.36—7.39 (2H, m, C_7-, C_8-H), 8.70 (1H, s, C_4-H). HR-MS (EI$^+$) *m/z*: 221.0326 (Calcd for $C_{10}H_7O_5$: 221.0324).

3-Bromoacetyl-6-methoxycoumarin (**2a**): This compound was prepared by the bromination of acetyl compound **1c** using tetrabuthylammonium tribromide ($TBABr_3$) as previously reported[46,47] and recrystallized from ethanol to give yellow needles of **2a**. ^{1}H-NMR ($CDCl_3$, ppm) δ: 3.38 (3H, s, C_6-OCH_3), 4.77 (2H, s, $COCH_2Br$), 7.07 (1H, s, C_5-H), 7.28 (1H, d, C_7-H), 7.34 (1H, d, C_8-H), 8.60 (1H, s, C_4-H). HR-MS (EI$^+$) *m/z*: 295.9691 (Calcd for $C_{12}H_9O_4Br$: 295.9684).

General Synthetic Method of Derivatized Products 2b—d[46,47] A mixture of **2a** (1.1 mmol), the appropriate carboxylic acid (1.2 mmol), 18-crown-6 (1.0 mmol), and $KHCO_3$ (800 mg) in acetone (200 ml) was stirred at room temperature for 30 min. The solvent was evaporated to dryness under reduced pressure. The residue was purified by column chromatography on silica gel eluted with chloroform–ethyl acetate (9 : 1). Recrystallization from ethanol gave **2b**—**d** as pale yellow needles in 60—80% yields.

6-Methoxycoumarin-3-carbonylmethyl Acetate (**2b**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 2.22 (3H, s, –CH_3), 3.88 (3H, s, C_6-OCH_3), 5.39 (2H, s, –$COCH_2$–), 7.06 (1H, s, C_5-H), 7.29—7.35 (2H, m, C_7-, C_8-H), 8.60 (1H, s, C_4-H). HR-MS (EI$^+$) *m/z*: 276.0630 (Calcd for $C_{14}H_{12}O_6$: 276.0634).

6-Methoxycoumarin-3-carbonylmethyl Laurate (**2c**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 0.88 (3H, t, –CH_3), 1.27—1.38 (16H, m, –$(CH_2)_8$–), 1.70 (2H, m, –CH_2–), 2.47 (2H, t, –CH_2–), 3.87 (3H, s, C_6-OCH_3), 5.38 (2H, s, –$COCH_2$–), 7.05 (1H, s, C_5-H), 7.27—7.34 (2H, m, C_7-, C_8-H), 8.59 (1H, s, C_4-H). HR-MS (EI$^+$) *m/z*: 416.2216 (Calcd for $C_{24}H_{32}O_6$: 416.2199).

6-Methoxycoumarin-3-carbonylmethyl Benzoate (**2d**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 3.88 (3H, s, C_6-OCH_3), 5.64 (2H, s, –$COCH_2$–), 7.07 (1H, s, C_5-H), 7.29 (1H, d, C_7-H), 7.36 (1H, d, C_8-H), 7.48—8.14 (5H, m, Ar-H), 8.62 (1H, s, C_4-H). HR-MS (EI$^+$) *m/z*: 338.0781 (Calcd for $C_{19}H_{14}O_6$: 338.0790).

6-Methoxycoumarin-3-carboxylic Acid (**3a**): A suspension of **1c**

(5.0 mmol) in concentrated hydrochloric acid (10 ml) containing acetic acid (5 ml) was refluxed for 2 h. The mixture was poured into ice-water (100 ml), and then the resulting precipitate was collected, washed with water, and recrystallized from ethanol to give **3a** as yellow needles. ^{1}H-NMR (DMSO-d_6, ppm) δ: 3.82 (3H, s, C_6-OCH_3), 7.33 (1H, d), 7.40 (1H, d), 7.48 (1H, s), 8.70 (1H, s), 13.25 (1H, broads, C_3-COOH). HR-MS (EI$^+$) *m/z*: 220.0365 (Calcd for $C_{11}H_8O_5$: 220.0372).

General Synthetic Method of Derivatized Products 3b—f To an ice-cold suspension of **3a** (1.0 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC HCl) (1.1 mmol), *N*,*N*-dimethylaminopyridine (DMAP) (1.1 mmol) in dichloromethane (20 ml) was added dropwise to the appropriate alcohols, phenols, or amines with stirring. The reaction mixture was stirred for a further 1 h at room temperature, and then the mixture was washed with saturated aqueous $NaHCO_3$ and water. The solution was dried over anhydrous Na_2SO_4 and the solvent was evaporated. The residue was purified by column chromatography on silica gel eluted with ethyl acetate. Recrystallization from ethanol gave **3b**—**f** as pale yellow needles in 62—92% yield.

Methyl 6-Methoxycoumarin-3-carboxylate (**3b**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 3.87 (3H, s, C_6-OCH_3), 3.96 (3H, s, $COOCH_3$), 7.03 (1H, s, C_5-H), 7.24 (1H, d, C_7-H), 7.31 (1H, d, C_8-H), 8.53 (1H, s, C_4-H). HR-MS (EI$^+$) *m/z*: 234.0521 (Calcd for $C_{12}H_{10}O_5$: 234.0528).

Phenyl 6-Methoxycoumarin-3-carboxylate (**3c**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 3.88 (3H, s, C_6-OCH_3), 7.06 (1H, s, C_5-H), 7.24—7.30 (4H, m, C_7-H, Ar-H), 7.34 (1H, d, C_8-H), 7.44 (2H, t, Ar-H), 8.69 (1H, s, C_4-H). HR-MS (EI$^+$) *m/z*: 296.0690 (Calcd for $C_{17}H_{12}O_5$: 296.0685).

6-Methoxycoumarin-3-*N*-methylcarboxamide (**3d**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 3.03 (3H, d, $NHCH_3$), 3.88 (3H, s, C_6-OCH_3), 7.08 (1H, s, C_5-H), 7.23 (1H, d, C_7-H), 7.34 (1H, d, C_8-H), 8.88 (1H, s, C_4-H). HR-MS (EI$^+$) *m/z*: 233.0682 (Calcd for $C_{12}H_{11}NO_4$: 233.0688).

6-Methoxycoumarin-3-*N*,*N*-dimethylcarboxamide (**3e**): ^{1}H-NMR (DMSO-d_6, ppm) δ: 3.02 (3H, s, N-CH_3), 3.13 (3H, s, N-CH_3), 3.86 (3H, s, C_6-OCH_3), 6.94 (1H, s, C_5-H), 7.13 (1H, d, C_7-H), 7.30 (1H, d, C_8-H), 7.86 (1H, s, C_4-H). HR-MS (EI$^+$) *m/z*: 247.0843 (Calcd for $C_{13}H_{13}NO_4$: 247.0845).

6-Methoxycoumarin-3-*N*-phenylcarboxamide (**3f**): ^{1}H-NMR (DMSO-d_6, ppm) δ: 3.90 (3H, s, C_6-OCH_3), 7.12 (1H, s, C_5-H), 7.17 (1H, t, Ar-H), 7.28 (1H, m, C_7-H), 7.37—7.40 (3H, m, C_8-H, Ar-H), 7.75 (2H, d, Ar-H), 8.98 (1H, s, C_4-H), 10.90 (1H, s, NH). HR-MS (EI$^+$) *m/z*: 295.0848 (Calcd for $C_{17}H_{13}NO_4$: 295.0845).

General Synthetic Method of 4a and b, 5a and b, and 6a—c A mixture of the hydroxynaphtaldehyde, 2-hydroxy-1-naphtaldehyde for **4a** and **b**, 1-hydroxy-2-naphtaldehyde for **5a** and **b**, and 2-hydroxy-3-naphtaldehyde[50] for **6a**—**c** (10 mmol) and the appropriate ester (10 mmol) in 25 ml of absolute ethanol was refluxed in the presence of a few drops of piperidine for 30 min. After cooling, the resulting precipitates were recrystallized from ethanol to give benzocoumarins as yellow needles.

Ethyl 5,6-Benzocoumarin-3-carboxylate (**4a**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 1.46 (3H, t, –CH_3), 4.49 (2H, q, –CH_2–), 7.49 (1H, d), 7.63 (1H, t), 7.77 (1H, t), 7.95 (1H, d), 8.11 (1H, d), 8.35 (1H, d), 9.35 (1H, s). HR-MS (EI$^+$) *m/z*: 268.0739 (Calcd for $C_{16}H_{12}O_4$: 268.0736).

3-Acetyl-5,6-benzocoumarin (**4b**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 2.80 (3H, s, C_3-$COCH_3$), 7.50 (1H, d), 7.63 (1H, t), 7.77 (1H, t), 7.94 (1H, d), 8.12 (1H, d), 8.39 (1H, d), 9.35 (1H, s). HR-MS (EI$^+$) *m/z*: 238.0639 (Calcd for $C_{15}H_{10}O_3$: 238.0630).

Ethyl 7,8-Benzocoumarin-3-carboxylate (**5a**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 1.44 (3H, t, –CH_3), 4.45 (2H, q, –CH_2–), 7.54 (1H, d), 7.68—7.73 (3H, m), 7.77 (1H, t), 7.90 (1H, d), 8.60 (1H, d), 8.35 (1H, d), 8.68 (1H, s). HR-MS (EI$^+$) *m/z*: 268.0724 (Calcd for $C_{16}H_{12}O_4$: 268.0736).

3-Acetyl-7,8-benzocoumarin (**5b**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 2.79 (3H, s, C_3-$COCH_3$), 7.58 (1H, d), 7.70—7.74 (3H, m), 7.91 (1H, d), 8.60 (1H, d), 8.67 (1H, s). HR-MS (EI$^+$) *m/z*: 238.0620 (Calcd for $C_{15}H_{10}O_3$: 238.0630).

Ethyl 6,7-Benzocoumarin-3-carboxylate (**6a**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 1.44 (3H, t, –CH_3), 4.44 (2H, q, –CH_2–), 7.54 (1H, d), 7.64 (1H, t), 7.73 (1H, s), 7.90 (1H, d), 7.96 (1H, d), 8.17 (1H, s), 8.66 (1H, s). HR-MS (EI$^+$) *m/z*: 268.0746 (Calcd for $C_{16}H_{12}O_4$: 268.0736).

3-Acetyl-6,7-benzocoumarin (**6b**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 2.77 (3H, s, C_3-$COCH_3$), 7.55 (1H, d), 7.65 (1H, t), 7.75 (1H, s), 7.90 (1H, d), 7.97 (1H, d), 8.22 (1H, s), 8.65 (1H, s). HR-MS (EI$^+$) *m/z*: 238.0620 (Calcd for $C_{15}H_{10}O_3$: 238.0630).

3-Cyano-6,7-benzocoumarin (**6c**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 7.59 (1H, t), 7.70 (1H, s), 7.81 (1H, s), 7.93 (1H, d), 7.99 (1H, d), 8.16 (1H, s), 8.39 (1H, s). HR-MS (EI$^+$) *m/z*: 221.0479 (Calcd for $C_{14}H_7NO_2$: 221.0477).

3-Methyl-6,7-benzocoumarin (**6d**): This compound was synthesized according to the method of Ishii *et al.*[51]

3-Formyl-6,7-benzocoumarin (**6e**): A solution of **6d** (1.5 mmol) dissolved in toluene was refluxed with selenium dioxide (2.0 mmol) for 2 h. The mixture was filtered hot to remove black selenium, and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography on silica gel eluted with chloroform–ethyl acetate (7 : 3), and the desired fractions were concentrated to give **6e** as yellow crystals. ^{1}H-NMR ($CDCl_3$, ppm) δ: 7.56 (1H, t), 7.67 (1H, t), 7.77 (1H, s), 7.91 (1H, d), 7.98 (1H, d), 8.26 (1H, d), 8.56 (1H, s), 10.36 (1H, s, C_3-CHO). HR-MS (EI$^+$) *m/z*: 224.0475 (Calcd for $C_{14}H_8O_3$: 224.0474).

6,7-Benzocoumarin (**6f**): This compound was synthesized in a similar manner to that described for **1b** and purified by recrystallization from ethanol–chloroform to give the product as yellow needles.

3-Bromoacetyl-6,7-benzocoumarin (**6g**): This compound was synthesized from acetyl compound **6b** in a similar manner to that described for **2a** and purified by recrystallization from ethanol–chloroform to give the product as yellow needles. ^{1}H-NMR ($CDCl_3$, ppm) δ: 4.80 (2H, s, C_3-$COCH_2Br$), 7.56 (1H, t), 7.67 (1H, t), 7.77 (1H, s), 7.92 (1H, d), 7.99 (1H, d), 8.27(1H, s), 8.78 (1H, s). HR-MS (EI$^+$) *m/z*: 315.9734 (Calcd for $C_{15}H_9O_3Br$: 315.9735).

General Synthetic Method of Derivatized Products 6h—j These compounds were synthesized from **6g** in a similar manner to that described for **2b**—**d** and purified by column chromatography on silica gel eluted with chloroform–ethyl acetate (9 : 1). Recrystallization from ethanol–chloroform gave **6h**—**j** as pale yellow needles in 60—92% yields.

6,7-Benzocoumarin-3-carbonylmethyl acetate (**6h**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 2.24 (3H, s, –CH_3), 5.42 (2H, s, –$COCH_2$–), 7.54—8.25 (6H, m), 8.77 (1H, s). HR-MS (EI$^+$) *m/z*: 296.0694 (Calcd for $C_{17}H_{12}O_5$: 296.0685).

6,7-Benzocoumarin-3-carbonylmethyl Laurate (**6i**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 0.89 (3H, t, –CH_3), 1.25—1.39 (16H, m), 1.72 (2H, m), 2.49 (2H, t), 5.42 (2H, s, –$COCH_2$–), 7.56 (1H, t), 7.67 (2H, t), 7.77 (1H, s), 7.92 (1H, d), 7.98 (1H, d), 8.25 (1H, s), 8.77 (1H, s). HR-MS (EI$^+$) *m/z*: 436.2242 (Calcd for $C_{27}H_{32}O_5$: 436.2250).

6,7-Benzocoumarin-3-carbonylmethyl Benzoate (**6j**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 5.67 (2H, s, –$COCH_2$–), 7.49 (2H, t), 7.55—7.69 (3H, m), 7.78 (1H, s), 7.92 (1H, d), 7.98 (1H, d), 8.27 (1H, s), 8.15 (2H, d), 8.26 (1H, s), 8.79 (1H, s). HR-MS (EI$^+$) *m/z*: 358.0842 (Calcd for $C_{22}H_{14}O_5$: 358.0841).

6,7-Benzocoumarin-3-carbonylchloride (**6l**): A suspension of **6a** (10 mmol) in concentrated hydrochloric acid (50 ml) containing acetic acid (10 ml) was refluxed for 2 h. The mixture was poured into ice-water (500 ml), and then the resulting precipitate was collected, washed with water, and recrystallized from ethanol to give **6k** as yellow needles. ^{1}H-NMR (DMSO-d_6, ppm) δ: 7.58 (1H, t), 7.69 (1H, t), 7.92 (1H, s), 8.04 (1H, d), 8.08 (1H, d), 8.56 (1H, s), 8.84 (1H, s), 13.32 (1H, broads, C_3-COOH). HR-MS (EI$^+$) *m/z*: 240.0432 (Calcd for $C_{14}H_8O_4$: 240.0423). A suspension of **6k** (5.0 mmol) in thionyl chloride (15 ml) was refluxed for 12 h. After cooling, excess thionyl chloride was evaporated to afford a crude product. This was purified by recrystallization from benzene–petroleum ether to give pale yellow needles. ^{1}H-NMR ($CDCl_3$, ppm) δ: 7.59 (1H, t), 7.71 (1H, t), 7.76 (1H, s), 7.93 (1H, d), 8.01 (1H, d), 8.32 (1H, s), 9.00 (1H, s). HR-MS (EI$^+$) *m/z*: 258.0087 (Calcd for $C_{14}H_7O_3Cl$: 258.0084).

General Synthetic Method of Derivatized Products 6m—q To an ice-cold solution of the appropriate alcohol, phenol, or amine (1.0 mmol) and triethylamine (TEA) (1.1 mmol) in dichloromethane (20 ml) was added dropwise **6l** in dichloromethane (5 ml) with stirring. The reaction mixture was stirred for a further 1 h at room temperature, and then the mixture was washed with saturated aqueous $NaHCO_3$ and water. The solution was dried over anhydrous Na_2SO_4 and the solvent was evaporated. The residue was purified by recrystallization from ethanol to give **6m**—**q** as pale yellow needles in 60—70% yield.

Methyl 6,7-Benzocoumarin-3-carboxylate (**6m**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 3.99 (3H, s, –$COOCH_3$), 7.54 (1H, t), 7.64 (1H, t), 7.73 (1H, s), 7.90 (1H, d), 7.96 (1H, d), 8.17 (1H, s), 8.70 (1H, s). HR-MS (EI$^+$) *m/z*: 254.0589 (Calcd for $C_{15}H_{14}O_4$: 254.0579).

Phenyl 6,7-Benzocoumarin-3-carboxylate (**6n**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 7.28—7.32 (3H, m), 7.45 (2H, t), 7.56 (1H, t), 7.67 (1H, t), 7.78 (1H, s), 7.93 (1H, d), 7.99 (1H, d), 8.24 (1H, s), 8.87 (1H, s). HR-MS (EI$^+$) *m/z*: 316.0733 (Calcd for $C_{20}H_{12}O_4$: 316.0736).

6,7-Benzocoumarin-3-*N*-methylcarboxamide (**6o**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 3.05 (3H, d, –$NHCH_3$), 7.56 (1H, t), 7.65 (1H, t), 7.78 (1H, s), 7.92 (1H, d), 7.99 (1H, d), 8.25 (1H, s), 8.78 (1H, s, –NH–), 9.06 (1H, s). HR-MS (EI$^+$) *m/z*: 253.0740 (Calcd for $C_{11}H_{13}NO_3$: 253.0739).

6,7-Benzocoumarin-3-*N*,*N*-dimethylcarboxamide (**6p**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 3.05 (3H, s, N–CH_3), 3.15 (3H, s, N–CH_3), 7.53 (1H, t), 7.62 (1H, t), 7.75 (1H, s), 7.90 (1H, d), 7.95 (1H, d), 8.04 (1H, s), 8.07 (1H, s). HR-

MS (EI$^+$) *m/z*: 267.0899 (Calcd for $C_{16}H_{13}NO_3$: 267.0896).

6,7-Benzocoumarin-3-*N*-phenylcarboxamide (**6q**): ^{1}H-NMR ($CDCl_3$, ppm) δ: 7.18 (1H, t), 7.40 (2H, t), 7.57 (1H, t), 7.67 (1H, t), 7.77 (2H, d), 7.83 (1H, s), 7.94 (1H, d), 8.02 (1H, d), 8.30 (1H, s), 9.16 (1H, s), 10.86 (1H, s, –NH–). HR-MS (EI$^+$) *m/z*: 315.0909 (Calcd for $C_{20}H_{13}NO_3$: 315.0896).

References

1) Mason W. T. (ed.), "Fluorescent and Luminescent Probes for Biological Activity," Academic Press, London, 1999.
2) Kraayenhof R., Visser A. J. W. G., Gerritsen H. C. (eds.), "Fluorecence Spectroscopy, Imaging and Probes," Springer, Berlin, 2002.
3) Toyooka T. (ed.), "Modern Derivatization Methods for Separation Sciences," John Wiley, Chichester, 1999.
4) Haugland R. P., "Handbook of Fluorescent Probes and Research Chemicals," 6th ed., Molecular Probes, Inc., Eugene, OR, 1996, pp. 531—540.
5) Kojima H., Sakurai K., Kikuchi K., Kawahara S., Kirino Y., Nagoshi H., Hirata Y., Nagano T., *Chem. Pharm. Bull.*, **46**, 373—375 (1998).
6) Kojima H., Nakatsubo N., Kikuchi K., Kawahara S., Kirino Y., Nagoshi H., Hirata Y., Nagano T., *Anal. Chem.*, **70**, 2446—2453 (1998).
7) Nakatsubo N., Kojima H., Kikuchi K., Nagoshi H., Hirata Y., Maeda D., Imai Y., Irimura T., Nagano T., *FEBS Lett.*, **427**, 263—266 (1998).
8) Okamoto T., Akaike T., Nagano T., Miyajima S., Suga M., Ando M., Ichimori K., Nakazawa H., Maeda H., *Arch. Biochem. Biophys.*, **342**, 261—274 (1997).
9) Umezawa N., Tanaka K., Urano Y., Kikuchi K., Higuchi T., Nagano T., *Angew. Chem. Int. Ed.*, **38**, 2899—2901 (1999).
10) Tanaka K., Miura T., Umezawa N., Urano Y., Kikuchi K., Higuchi T., Nagano T., *J. Am. Chem. Soc.*, **123**, 2530—2536 (2001).
11) Hirano T., Kikuchi K., Urano Y., Higuchi T., Nagano T., *J. Am. Chem. Soc.*, **122**, 12399—12400 (2000).
12) Hirano T., Kikuchi K., Urano Y., Nagano T., *J. Am. Chem. Soc.*, **124**, 6555—6562 (2000).
13) Kalolin J., Johansson L. B. A., Strandberg L., Ny T., *J. Am. Chem. Soc.*, **116**, 7801—7806 (1994).
14) Kollmannsberger M., Gareis T., Heinl S., Breu J., Daub J., *Angew. Chem., Int. Ed.*, **36**, 1333—1335 (1997).
15) Kollmannsberger M., Rurack K., Resch-Genger U., Daub J., *J. Phys. Chem. A*, **102**, 10211—10220 (1998).
16) Rurack K., Kollmannsberger M., Resch-Genger U., Daub J., *J. Am. Chem. Soc.*, **122**, 968—969 (2000).
17) Gabe Y., Urano Y., Kikuchi K., Kojima H., Nagano T., *J. Am. Chem. Soc.*, **126**, 3357—3367 (2004).
18) Koutaku H., Kosuge J., Fukasaku N., Hirano T., Kikuchi K., Urano Y., Kojima H., Nagano T., *Chem. Pharm. Bull.*, **52**, 700—703 (2004).
19) Wheelock C. E., *J. Am. Chem. Soc.*, **81**, 1348—1352 (1959).
20) Sherman W. R., Robins E., *Anal. Chem.*, **40**. 803—805 (1968).
21) Atkins R. L., Bliss D. E., *J. Org. Chem.*, **43**, 1975—1980 (1978).
22) Takadate A., Masuda T., Murata C., Tanaka T., Irikura M., Goya S., *Anal. Sci.*, **11**, 97—101 (1995).
23) Takadate A., Masuda T., Murata C., Shibuya M., Isobe A., *Chem. Pharm. Bull.*, **48**, 256—260 (2000).
24) Takadate A., Masuda T., Murata C., Tanaka T., Miyahara H., Goya S., *Chem. Lett.*, **1993**, 811—814 (1993).
25) Takadate A., Masuda T., Murata C., Tanaka T., Goya S., *Bull. Chem. Soc. Jpn.*, **68**, 3105—3110 (1995).
26) Masuda T., Kaneko T., Tanaka T., Goya S., Murata C., Takadate A., *Bull. Chem. Soc. Jpn.*, **73**, 1213—1217 (2000).
27) Lee L. G., Berry G. M., Chen C.-H., *Cytometry*, **10**, 151—162 (1989).
28) Whitaker J. E., Haugland R. P., Prendergast F. G., *Anal. Biochem.*, **194**, 330—344 (1991).
29) Fabian W. M. M., Schuppler S., Wolfbes O., *J. Chem. Soc. Perkin Trans. 2*, **1996**, 853—856 (1996).
30) Gunther H., Prestien J., *Org. Magn. Resonance*, **7**, 339—344 (1979).
31) Baker L., Castro., *J. Chem. Soc. Perkin Trans. 1*, **1990**, 47—65 (1990).
32) Horning E. C., Horning M. G., *J. Am. Chem. Soc.*, **69**, 968—969 (1947).
33) Ito K., Nakajima K., *J. Heterocyclic Chem.*, **25**, 511—515 (1988).
34) Hammett L. O., *J. Am. Chem. Soc.*, **59**, 96—103 (1937).
35) Hansch C., Leo A., Taft R. W., *Chem. Rev.*, **91**, 165—195 (1991).
36) Gottlieb E. H., de Lima R. A., delle Monache F., *J. Chem. Soc. Perkin Trans. II*, **1979**, 435—437 (1979).
37) Sherman W. R., Robins E., *Anal. Chem.*, **40**, 803—805 (1968).
38) Uchiyama S., Santa T., Fukushima T., Homma H., Imai K., *J. Chem. Soc. Perkin Trans. 2*, **1998**, 2165—2173 (1998).
39) Jones G. II, Jackson W. R., Halpern M., *Chem. Phys. Lett.*, **72**, 391—395 (1980).
40) Jones G. II, Griffin S. F., Choi C.-Y., Bergmark W. R., *J. Org. Chem.*, **49**, 2705—2708 (1984).
41) Jones G. II, Jackson W. R., Choi C.-Y., *J. Phys. Chem.*, **89**, 294—300 (1985).
42) Jones G. II, Jackson W. R., Kanoktanaporn S., Bergmark W. R., *Photochem. Photobiol.*, **42**, 477—483 (1985).
43) Rettig W., *Angew. Chem. Int. Ed. Engl.*, **25**, 971—988 (1986).
44) Rettig W., Klock A., *Can. J. Chem.*, **63**, 1649—1653 (1985).
45) Takadate A., Masuda T., Murata C. Isobe A., Shinohara T., Irikura M., Goya S., *Anal. Sci.*, **13**, 753—756 (1997).
46) Takadate A., Masuda T., Tajima C., Murata C., Irikura M., Goya S., *Anal. Sci.*, **8**, 663—668 (1992).
47) Takadate A., Masuda T., Murata C., Haratake C., Isobe A., Irikura M., Goya S., *Anal. Sci.*, **8**, 695—697 (1992).
48) Umemoto H., Morii S., Kitao T., Konishi K., *Kogyo Kagaku Zasshi*, **74**, 2123—2126 (1971).
49) Langmuir M. E., Yang J.-R., Moussa A. M., Laura R., LeCompte K. A., *Tetrahedron Lett.*, **36**, 3989—3992 (1995).
50) Narasimhan N. S., Mali R. S., *Tetrahedron*, **31**, 1005—1009 (1975).
51) Ishii H., Kaneko Y., Miyazaki H., Harayama T., *Chem. Pharm. Bull.*, **38**, 3100—3102 (1991).