Fluorescence Characteristics of Methoxycoumarins as Novel Fluorophores

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The fluorescence characteristics of various methoxycoumarin fluorophores for the development of fluorescence reagents were examined in relation to their structures. The fluorescence emission mechanisms were also considered from the viewpoint of the intramolecular charge-transfer (CT) between methoxyl substituents and the coumarin ring. The arrangement of 6-methoxyl and 3-acetyl group pairs on the coumarin ring significantly contributed to the fluorescence enhancement through the intramolecular CT. We found that the structural features of methoxycoumarins required for intense fluorescence are to hold both diether bonds at the 6- and 7-positions and an electron-withdrawing group at the 3-position, as shown in 3-acetyl-6,7-dimethoxycoumarin with 0.52 in quantum yield.

Keywords Methoxycoumarin, fluorophore, fluorescence reagent, fluorescence emission mechanism, intramolecular charge-transfer

A number of fluorogenic compounds have been widely used as reagents for microanalysis because they are both highly sensitive and selective. In general, most of the analytical reagents so far developed have been prepared by introducing some reactive groups coupled with functional groups in analytical samples into appropriate fluorophores. The reagent design, based upon detailed information concerning primary fluorophores, including their emission mechanisms, should permit a more efficient development of a variety of analytical reagents. However, only a few studies on the characteristics of the fluorophores under consideration for reagent design have been performed.^{1,2} Of many fluorophores, coumarins having variable fluorescence properties arising from the ring structure differs from those of rigid aromatic rings, such as anthracene or pyrene, have led to much interest not only concerning their application to analytical reagents, but also to an understanding of their emission mechanisms. We have reported on various coumarins as fluorescence derivatization reagents for high-performance liquid chromatographic analysis³⁻⁷, fluorescence probes for drug-protein binding studies⁸ and fluorescence ionophores.⁹ This paper describes the fluorescence characteristics of a series of methoxycoumarin fluorophores in relation to their structural features for reagent design.

Experimental

All chemicals were of reagent grade, unless noted

otherwise. The solvents (Luminasol) used for the fluorescence and absorption spectral measurements were purchased from Dojindo Laboratories (Kumamoto, Japan). Coumarins (I, II, and VII)¹⁰ and acetylcoumarins (III – VI, VIII and IX)¹¹ were synthesized by means of the Pechmann¹² and Knoevenagel¹³ type reactions from the corresponding phenols or hydroxybenzaldehydes¹⁴, respectively. The structures of the products were confirmed based on the known melting point, proton nuclear magnetic resonance (¹H-NMR) and mass spectral data.

The physical and spectral data of V, VI and IX were as follows:

[V] mp 243 – 244°C, ¹H-NMR (CDCl₃) δ: 2.34 (3H, s, C₇-CH₃), 2.73 (3H, s, C₃-COCH₃), 3.89 (3H, s, C₆-OCH₃), 6.91 (1H, s, C₈-H), 7.12 (1H, s, C₅-H), 8.49 (1H, s, C₄-H). MS *m/z*: 232 (M⁺).

[VI] mp 199 – 200°C, ¹H-NMR (CDCl₃) δ: 2.22 (3H, s, C₆-CH₃), 2.71 (3H, s, C₃-COCH₃), 3.94 (3H, s, C₇-OCH₃), 6.78 (1H, s, C₈-H), 7.36 (1H, s, C₅-H), 8.48 (1H, s, C₄-H). MS *m/z*: 232 (M⁺).

[IX] mp 162 – 163°C, ¹H-NMR (CDCl₃) δ: 2.33 (3H, s, C₆-CH₃), 2.39 (3H, s, C₇-CH₃), 2.72 (3H, s, C₃-COCH₃), 7.16 (1H, s, C₈-H), 7.37 (1H, s, C₅-H), and 8.46 (1H, s, C₄-H). MS *m/z*: 216 (M⁺).

Apparatus

The melting points were measured with a Yanagimoto micro-melting point apparatus, and were uncorrected. The absorption and fluorescence spectra were taken with a Hitachi U-3210 spectrophotometer and a Hitachi F-

4000 fluorescence spectrophotometer, respectively. The fluorescence quantum yields were determined according to the method of Paker and Rees¹⁵, the value (0.55) for quinine sulfate in 0.5 M H₂SO₄ was used as the standard. The time-resolved fluorescence spectra were taken with a Horiba NAES-700F spectrofluorometer. A nanosecond-flash lamp filled with hydrogen was used as an excitation light source. A sequence of light-pulses from this lamp was passed through a Hoya U 360 glass filter and a solution filter (CuSO₄·5H₂O, 250 g/l), then focused upon a rectangular sample cell made of quartz. The fluorescence lifetime measurements were based on an analysis of the fluorescence decay curves. ¹H-NMR were obtained with a JEOL JNM-GSX 500FT-NMR spectrometer using tetramethylsilane as an internal standard. The following abbreviations are used: s, singlet; d, doublet; t, triplet; m, multiplet. The mass spectra (MS) were taken with a JEOL JMS-DX303 spectrometer.

Results and Discussion

Although coumarin by itself is non-fluorescent, its derivatives with electron-donating groups at the 7-position develop intense fluorescence. This fluorescence emission mechanism has been interpreted based on the intramolecular charge-transfer (CT) arising from a push-pull electron relay, namely, electron-transfer from a substituent (donor) at the 7-position to a lactone carbonyl group (acceptor) on the coumarin ring.¹ Also, the substituent effects in 7-substituted coumarins were explained based on the Hammett rule by approximating

them to the disubstituted benzene (DSB)-model. According to this model, the relationship between substituents at the 7-position and the lactone carbonyl group corresponds to that of *para*-disubstituted benzenes for an electronic effect. Therefore, the substituents at the 6-position corresponding to the *meta*-relation of the lactone carbonyl group can be realized under these conditions.

The absorption and fluorescence spectral data of methoxycoumarins (I – VIII) prepared here are summarized in Table 1. The absorption maxima (λ_{UVS}) and the fluorescence emission maxima (λ_{FS}) of I – VIII shifted, on the whole, to a longer wavelength accompanying an enhancement of the absorbance and fluorescence intensities with increasing the solvent polarity. Their fluorescence quantum yields increased much more in methanol than in other solvents. This spectral behavior of I and III – VIII is essentially similar to that of II, as pointed out by Hinohara *et al.*¹ The lower quantum yields in acetonitrile than in methanol seem to be due to a difference in the solvation of the fluorophores with both solvents in the excited state. This may permit the fluorescence characteristics of these coumarins to be understood based on the viewpoint of their DSB-model treatment and intramolecular CT. Further characterization, including the emission mechanism, was performed based on the data obtained in methanol, because of the high quantum yield.

Monomethoxycoumarins

The λ_{UVS} and λ_{FS} of monomethoxycoumarins in

Table 1 Absorption and fluorescence spectral data of methoxycoumarins in various solvents

Compound No.	R ₃	R ₆	R ₇		Acetonitrile	Methanol	Dichloromethane	Benzene
I	H	OCH ₃	H	UV λ_{max}/nm	344	347	346	349
				F λ_{max}/nm	441 (0.01>)	453 (0.02)	438 (0.01>)	437 (0.01>)
II	H	H	OCH ₃	UV λ_{max}/nm	323	323	326	327
				F λ_{max}/nm	391 (0.01>)	390 (0.05)	388 (0.01)	389 (0.01>)
III	COCH ₃	OCH ₃	H	UV λ_{max}/nm	375	375	378	381
				F λ_{max}/nm	491 (0.02)	506 (0.06)	485 (0.01)	480 (0.01>)
IV	COCH ₃	H	OCH ₃	UV λ_{max}/nm	355	358	360	360
				F λ_{max}/nm	399 (0.01>)	428 (0.01>)	423 (0.01>)	433 (0.01>)
V	COCH ₃	OCH ₃	CH ₃	UV λ_{max}/nm	376	378	382	381
				F λ_{max}/nm	483 (0.12)	501 (0.25)	476 (0.09)	467 (0.02)
VI	COCH ₃	CH ₃	OCH ₃	UV λ_{max}/nm	368	369	372	370
				F λ_{max}/nm	428 (0.01>)	436 (0.01)	429 (0.01>)	n.d. ^a
VII	H	OCH ₃	OCH ₃	UV λ_{max}/nm	342	344	346	347
				F λ_{max}/nm	430 (0.07)	432 (0.25)	423 (0.09)	421 (0.02)
VIII	COCH ₃	OCH ₃	OCH ₃	UV λ_{max}/nm	383	385	382	389
				F λ_{max}/nm	466 (0.29)	482 (0.52)	406 (0.18)	456 (0.05)
IX	COCH ₃	CH ₃	CH ₃	UV λ_{max}/nm	350	351	359	359
				F λ_{max}/nm	n.d. ^a	n.d. ^a	n.d. ^a	n.d. ^a

a. Not detected. Parentheses stands for the quantum yield.

methanol were at 347 and 453 nm for **I** and 323 and 390 nm for **II**. Although the 6-methoxyl group in **I** corresponded to the *meta*-position of the lactone carbonyl group according to the DSB-model, its maximal wavelengths were longer than those of **II**, in which the 7-methoxyl group corresponds to the *para*-position. Although the reasons for these results are not fully understood, an electronic effect *via* the lactone ring oxygen atom from the 6-methoxyl group is probable in the coumarin ring. Yoshida *et al.*¹⁶ have described in a rule concerning the relationship between the chemical structures and fluorescence properties that coumarin fluoresces when electron-donating groups are introduced into electron-deficient positions (4-, 5- and 7-positions) and when electron-withdrawing groups are introduced into electron-sufficient positions (3-, 6- and 8-positions). Actually, 6- and 8-hydroxycoumarins are non-fluorescent.¹⁶ On the other hand, **I**, methylether of 6-hydroxycoumarin, apparently fluoresced with a quantum yield of 0.02, though it was smaller than that of **II**, corresponding to the *para*-relation in the DSB-model. This is considered to be based upon the difference in the solvation effects between the hydroxyl and methoxyl groups in the excited state.

Effects of an acetyl group

Compounds **III** and **IV**, which have an acetyl group at the 3-position, have another electron-withdrawing group in addition to that of the lactone carbonyl. Although it is slightly difficult to designate the electron-transfer route between the methoxyl and acetyl groups in these coumarins, the 6-methoxyl group in **III** from the electronic effect of the 3-acetyl group within the lactone ring may be considered to correspond to the *para*-relation of the acetyl group, while the 7-methoxyl group in **IV** corresponds to the *meta*-relation. Therefore, another electron-transfer route from the 6-methoxyl to the acetyl groups, namely a *para*-typed push-pull system, is presumed to be formed on the same ring.

The absorption and fluorescence bands of **III** and **IV** were shifted to longer wavelength regions by introducing acetyl groups into **I** and **II**, as shown in Table 1. This indicated the promotion of an electron-transfer character from the methoxyl substituents to the coumarin ring in both compounds. Compound **III** had a quantum yield of 0.06, increased by the acetyl group, while that for **IV** decreased to less than 0.01. Hammett's substituent constants (σ -values) take different signs according to the positions of substituents in substituted benzenes. For example, the methoxyl group acts as an electron-donating group at the *para*-position, but as an electron-withdrawing group at the *meta*-position. The acetyl carbonyl group also has a stronger electron-withdrawing effect than does the lactone carbonyl group, as reflected by the σ -values.

The results suggested that the arrangement of the methoxyl group at the 6-position and the acetyl group at the 3-position significantly contributed to the increase in the quantum yield of **III** through a push-pull system

between these groups. On the other hand, the lowered quantum yield of **IV** was presumed to have been due to the decreased contribution to the push-pull system of the 7-methoxyl group, which corresponds to the *meta*-position of the 3-acetyl group.

These results indicated that **III** is superior to **IV** in sensitivity when they are applied to analytical reagents. Fluorophores such as **IV** have already been used as good fluorescence derivatization reagents, for example, 7-methoxycoumarin-3-carbonyl chloride¹⁸ or 7-methoxycoumarin-3-carbonyl azide⁴ for alcohols and 3-bromoacetyl-7-methoxycoumarin⁶ for carboxylic acids. Actually, when the derivatizations of lauric acid with 3-bromoacetyl-6- (type **III**)¹⁹ and 7-methoxycoumarins (type **IV**)⁶ as an example were attempted for a comparison, it was found that their fluorescence quantum yields for derivatized products in methanol were 0.25²⁰ for the former and 0.02⁶ for the latter, respectively. This result is in accord with predictions based upon the above considerations.

Effect of a methyl group

A 3-acetyl group was found to appreciably contribute to the fluorescence enhancement of **I**, as shown in the quantum yield of **III**. The 6-methoxyl group in **III** corresponded to the *meta*-position, according to the DSB-model, for the electronic effect of the lactone carbonyl group. In the Hammett rule¹⁷ for substituted benzenes, a methyl group always acts as an electron-donating group at both the *meta*- and *para*-positions, unlike the action of the methoxyl group. Compounds **V** and **VI** were prepared in order to examine the electronic effects of the methyl group on the fluorescence characteristics of **III** and **IV**. Introducing a methyl group to the 7-position of **III** significantly enhanced the fluorescence from the observed quantum yield, to 0.25 for **V**. The quantum yield of **IV**, compared with that of **VI**, was also slightly restored by introducing a methyl group.

This effect of the methyl group supports the usefulness of the DSB-model for considering coumarin fluorophores. Furthermore, we estimated from these results that the structural requirements for good coumarin fluorophores in new reagent design are to hold an ether bond, an electron-donating alkyl group and an electron-withdrawing group, such as an acetyl group at the 6, 7 and 3 positions, respectively.

Dimethoxycoumarins

Compound **VII** afforded a high quantum yield of 0.25 compared with those of the corresponding monomethoxycoumarins, **I** and **II**. Although the reason for the increase in the fluorescence of **VII** remains obscure in considerations based on the electron-transfer effects between the substituents, the greater contribution of the 6-methoxyl group compared with that of the 7-methoxyl group to the fluorescence of **VII** was estimated based on a comparison of the λ_{UVS} and λ_{FS} of **I**, **II** and **VII**. More remarkable is the quite high quantum yield of 0.52 of

VIII, as shown in Table 1. This apparently indicates that introducing an acetyl group to the 3-position of **VII** caused an increase in the quantum yield. Figures 1 and 2 show the absorption and fluorescence spectra of **III**, **IV** and **VIII** for a comparison.

The absorption band of **VIII** is presumed to be made up of a superposition of bands **III** and **IV**. In contrast, an intense band was evident in its fluorescence spectrum. Compound **VIII** may have another electron-transfer route from 6-methoxyl to acetyl groups, such as a by-pass between the 7-methoxyl and lactone carbonyl groups, and have two push-pull systems through donor and acceptor groups. The substituents in each route are in *para*-relationships with each other. The mutual effects between two electron-transfer routes seem to contribute to the high quantum yield of **VIII**.

To elucidate the high quantum yield of **VIII**, time-resolved fluorescence spectra and the fluorescence lifetimes of **III**, **IV** and **VIII** were measured in methanol. Singly-exponential fluorescence decay with lifetime of 5.34 ns was observed for **VIII**, of which the emission seems to comprise of a single component. The fluorescence decays of **III** and **IV** were also singly-exponential with lifetimes of 4.44 and 0.3 ns, respectively. Since the lifetime of **VIII** was comparable to that of **III**, the main component of the emission of **VIII** may be of **III**.

It was suggested that the push-pull system between the 6-methoxyl and 3-acetyl groups, rather than between the 7-methoxyl and lactone carbonyl groups, effectively contributes to the CT-structure of **VIII** in the excited state to cause intense fluorescence. However, further studies, such as the role of the lactone carbonyl group for the fluorescence of **VIII**, are necessary to confirm the presence of two push-pull systems. We thus found from these results that the methoxyl substituents at the 6- and 7-positions on coumarin are at least required for strongly emitting fluorophores, and also that an additional substitution of an electron-withdrawing group, such as an acetyl group at the 3-position, is quite effective for fluorescence enhancement. In connection with this finding, **IX** having methyl groups at the 6- and 7-positions, exhibited no fluorescence, which may be due to a weaker electron-donating characteristic of the methyl group compared with that of the methoxyl group.

Such knowledge can be further extended to various coumarin reagents having 6,7-diether bonds and an electron-withdrawing group at the 3-position. This is also supported by the high quantum yields (above 0.8) of the coumarin derivatives obtained by the derivatization of fatty acids with 3-bromoacetyl-6,7-methylenedioxy-coumarin⁷ or 3-bromoacetyl-6,7-benzocrowned-coumarin²¹, as reported.

Thus, the construction of push-pull systems between substituents through π -electron media, such as intramolecular CT, may be a useful approach to the development of new analytical reagents.

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Fig. 1 Absorption spectra of **III**, **IV** and **VIII** in methanol.

Fig. 2 Fluorescence spectra of **III**, **IV** and **VIII** in methanol.

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 20. Mp 96–97°C. NMR (CDCl₃) δ : 0.88 (3H, t, laurate-CH₃), 1.26–2.49 (20H, m, laurate-CH₂), 3.87 (3H, s, C₆-OCH₃), 5.38 (2H, s, C₃-COCH₂-), 7.05 (1H, s, C₅-H), 7.27 (1H, s, C₇-H), 7.33 (1H, s, C₈-H), 8.59 (1H, s, C₄-H). MS m/z : 416 (M⁺).
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