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Short communication

Novel stable fluorophore, 6-methoxy-4-quinolone, with strong fluorescence in wide pH range of aqueous media, and its application as a fluorescent labeling reagent

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Abstract

6-Methoxy-4-quinolone (6-MOQ, **1**), an oxidation product derived from 5-methoxyindole-3-acetic acid, is a novel fluorophore, which has several useful characteristics for biomedical analysis. Compound **1** has strong fluorescence with a large Stokes' shift in aqueous media, and the maximum fluorescence excitation and emission wavelengths are 243 nm and 374 nm, respectively. The molar absorptivity at the maximum excitation wavelength and fluorescence quantum yield in aqueous 10% (v/v) methanol are 32 600 L mol^{-1} cm^{-1} and 0.38, respectively. The fluorescence intensity of **1** is scarcely affected by changing the medium pH, showing strong fluorescence from pH 2.0 to 11.0. In addition, **1** is highly stable against light and heat, and no degradation was observed at 60 °C for 3 days with exposure to daylight. As a fluorescent labeling reagent, [(6-methoxy-4-oxo-1,4-dihydroquinolin-3-yl)methyl]amine (6-MOQ-NH_2, **2**) was synthesized, and determination of carboxylic acids was demonstrated; 50 pmol of standard propionic acid and isobutyric acid were derivatized, and the obtained S/N ratios for 10 fmol (injection amount) of these two acids were 206 and 164, respectively.

Keywords: 6-Methoxy-4-quinolone; Fluorescence; Carboxylic acids

1. Introduction

Fluorescence analyses are widely used in various fields of research including analytical chemistry, biochemistry, environmental chemistry and clinical chemistry. They enable highly sensitive and selective determinations of a large number of compounds (physiologically active substances, pollutants, drugs, etc.). When the analytes exhibit weak or no fluorescence, fluorescence derivatization methods that introduce fluorophores to the analytes are important. A great number of fluorescent labeling reagents have been developed [1–3]. Although these reagents could perform highly sensitive analyses of fmol level under specified conditions, they often have some defects mainly caused by the characteristics of the fluorophores such as decomposition by the exposure to light [4], low sensitivity in aqueous media [5,6] or changing the sensitivity by the variation of the pH [7–9]. Because a fluorescent labeling reagent having high applicability to a reversed-phase (RP)-HPLC or intracellular research is often required for biomedical analyses, a stable fluorophore having strong fluorescence in aqueous media at various pHs is needed.

In the previous papers, we have reported that melatonin (*N*-acetyl-5-methoxytryptamine) is converted to a fluorescent compound, *N*-[(6-methoxy-4-oxo-1,4-dihydroquinolin-3-yl)methyl]acetamide (6-MOQMA, **3**) by oxidation with hydrogen peroxide under alkaline conditions [10]. Compound **3** has strong fluorescence with a large Stokes' shift, and using this oxidation product, melatonin could be detected at amol level by the RP-HPLC system with a conventional fluorescence detector [10–13]. In addition to the fact that the sensitivity obtained with **3** is about 10 times higher than that reported with other fluorescent labeling reagents [1–3], **3** has good stability and strong fluorescence in aqueous media.

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The core structure showing such useful characteristics should be clarified. Thus, we focused on the oxidation product of 5-methoxyindole-3-acetic acid (MIAA); the fluorescence of the hitherto unknown oxidation product is similar to that of **3** [10,11], suggesting that these compounds have the same fluorophore. Considering that MIAA has similar but slightly different structure from melatonin, the structural analysis of this fluorescent oxidation product derived from MIAA should be helpful for the identification of the useful new fluorophore.

In the present paper, we determined the structure of the oxidation product of MIAA and found that the product is 6-methoxy-4-quinolone (6-MOQ, **1**). Because 6-methoxyquinoline and 4-quinolone have weaker fluorescence than **1**, and the fluorescence spectra were different from those of **1** and **3**, **1** (having both the methoxy group at the 6-position and the carbonyl group at the 4-position of quinoline moiety) is revealed to be a core structure having strong fluorescence. To our knowledge, although various quinoline and quinolone compounds have been synthesized and utilized as the key structure of antibiotics and some of them have been determined by their fluorescence [14–17], the 6-MOQ (**1**) moiety has never been reported to have strong fluorescence nor been utilized as the fluorophore. In this paper, the detailed fluorescence characteristics of **1** and the related compounds having substituents at the 3-position of the quinolone moiety including **3** were examined. As an application of this novel fluorophore, a new labeling reagent, [(6-methoxy-4-oxo-1,4-dihydroquinolin-3-yl)methyl]amine (6-MOQ-NH_2, **2**) was synthesized, and the determination of carboxylic acids was demonstrated by an RP-HPLC.

2. Experimental

2.1. Materials

Melatonin, 5-methoxyindole-3-acetic acid (MIAA), 5-methoxytryptamine, propionic anhydride, *n*-butyric anhydride, isobutyric anhydride, pivalic anhydride, propionic acid and isobutyric acid were purchased from Tokyo Kasei (Tokyo, Japan). Methanol (MeOH) and acetonitrile (MeCN) of HPLC grade, trifluoroacetic acid (TFA), sodium carbonate and aqueous hydrogen peroxide (31%, v/v) were the products of Wako (Osaka, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) of peptide synthesis grade was from Nacalai Tesque (Kyoto, Japan), and pyridine of HPLC grade was from Sigma-Aldrich Inc. (St. Louis, MO, USA). Water was purified using a Milli-Q gradient A10 system (Millipore, Bedford, MA, USA). All other reagents were of guaranteed reagent grade and used without further purification.

2.2. Apparatus

^{1}H and ^{13}C NMR spectra were obtained using a Unity-500 spectrometer (Varian, Palo Alto, CA, USA). Fast atom bombardment (FAB) mass and IR spectra were measured by a JMS 600 mass spectrometer (JEOL, Tokyo, Japan) and FT/IR-410 (JASCO, Tokyo, Japan). X-ray diffraction analysis was performed using a RAXIS-RAPID diffractometer (Rigaku, Tokyo, Japan).

2.3. Synthesis of the fluorescent oxidation product derived from MIAA

MIAA (100 mg) was dissolved in 1 mL of MeOH, and aqueous 100 mM sodium carbonate solution (20 mL) was added. The mixture was stirred at 80 °C, and 200 μL of aqueous 3 M H_2O_2 was added every 5 min for 4 h. After the reaction mixture was washed with 10 mL of ethyl acetate, the product was extracted with 10 mL of ethyl acetate four times and collected. The ethyl acetate layer was evaporated under reduced pressure, and the residue was recrystallized from H_2O to obtain colorless needles. For the X-ray diffraction analysis, the compound was slowly recrystallized from H_2O/MeOH = 10/1 (v/v).

Fluorescent oxidation product, 6-methoxy-4-quinolone (6-MOQ, **1**): m.p. 248 °C (decomp.). Yield, 6.02 mg (7.0%). ^{1}H NMR (500 MHz, DMSO-$^2H_6$): δ ppm 3.82 (3H, s, –OCH_3), 5.98 (1H, dd, J = 1.1, 7.3 Hz, H-3), 7.27 (1H, dd, J = 2.8, 9.0 Hz, H-7), 7.48–7.50 (2H, m, H-5, 8), 7.82 (1H, dd, J = 6.2, 7.3 Hz, H-2), 11.68 (1H, br s, H-1). ^{13}C NMR (125 MHz, $C^2H_3O^2H$): δ ppm 56.1 (–OCH_3), 104.8–158.3 (4-quinolone, C-2, 3, 5, 6, 7, 8, 9, 10,), 180.0 (4-quinolone, C-4). IR (KBr) 3155 (m), 3090 (m), 2993 (m), 1596 (s), 1561 (s), 1523 (s), 1492 (m), 1388 (m), 1232 (m), 816 (m) cm^{-1}. FAB-MS m/z = 176.1 (M + H). Anal. Calcd. for $C_{10}H_9NO_2$: C, 68.56; H, 5.18; N, 8.00. Found: C, 68.42; H, 5.15; N, 8.04.

2.4. Syntheses of four analogs of **3** having different alkyl groups instead of the methyl group of acetamide

These compounds were synthesized by the oxidation of the corresponding melatonin analogs that were synthesized in our laboratory by the reaction using 5-methoxytryptamine and the corresponding acid anhydrides. Melatonin analog (150 mg), 1 mL of MeOH and aqueous 100 mM sodium carbonate solution (15 mL) were mixed and stirred at 80 °C; then 400 μL of aqueous 3 M H_2O_2 was added every 10 min for 15–18 h (the progress of the reaction was monitored by TLC). After the reaction mixtures were cooled, the resulting colorless precipitates were obtained. These precipitates were recrystallized from H_2O for the ethyl analog, or from MeOH for other analogs, and the colorless crystals were obtained.

Ethyl analog, *N*-[(6-methoxy-4-oxo-1,4-dihydroquinolin-3-yl)methyl]propanamide (**4**): m.p. 262–264 °C. Yield, 40.66 mg (25.6%). ^{1}H NMR (500 MHz, DMSO-$^2H_6$): δ ppm 0.99 (3H, t, J = 7.6 Hz, –CH_3), 2.10 (2H, q, J = 7.6 Hz, –$COCH_2$–), 3.82 (3H, s, –OCH_3), 4.10 (2H, d, J = 5.5 Hz, –CH_2–), 7.27 (1H, dd, J = 3.0, 8.9 Hz, H-7), 7.49 (1H, d, J = 9.2 Hz, H-8), 7.51 (1H, d, J = 3.0 Hz, H-5), 7.77 (1H, s, H-2), 7.92 (1H, br t, J = 5.3 Hz, –NHCO–), 11.67 (1H, s, H-1).

FAB-MS m/z = 261.2 (M + H). Anal. Calcd. for $C_{14}H_{16}N_2O_3$: C, 64.60; H, 6.20; N, 10.76. Found: C, 64.41; H, 6.20; N, 10.70.

n-Propyl analog, *N*-[(6-methoxy-4-oxo-1,4-dihydroquinolin-3-yl)methyl]butanamide: m.p. 271–272 °C. Yield, 35.59 mg (22.5%). ^{1}H NMR (500 MHz, DMSO-$^2H_6$): δ ppm 0.84 (3H, t, J = 7.3 Hz, –CH_3), 1.51 (2H, m, –CH_2–), 2.07 (2H, t, J = 7.3 Hz, –$COCH_2$–), 3.82 (3H, s, –OCH_3), 4.10 (2H, d, J = 5.7 Hz, –CH_2–), 7.27 (1H, dd, J = 2.9, 9.0 Hz, H-7), 7.48 (1H, d, J = 8.9 Hz, H-8), 7.51 (1H, d, J = 3.0 Hz, H-5), 7.76 (1H, s, H-2), 7.95 (1H, br t, J = 5.2 Hz, –NHCO–), 11.67 (1H, s, H-1). FAB-MS m/z = 275.3 (M + H). Anal. Calcd. for $C_{15}H_{18}N_2O_3$: C, 65.68; H, 6.61; N, 10.21. Found: C, 65.48; H, 6.60; N, 10.12.

Isopropyl analog, *N*-[(6-methoxy-4-oxo-1,4-dihydroquinolin-3-yl)methyl]-2-methylpropanamide: m.p. 285 °C (decomp.). Yield, 51.99 mg (32.9%). ^{1}H NMR (500 MHz, DMSO-$^2H_6$): δ ppm 1.00 (6H, d, J = 6.9 Hz, (–CH_3)$_2$), 2.40 (1H, sept, J = 6.9 Hz, –CH–), 3.82 (3H, s, –OCH_3), 4.10 (2H, d, J = 5.5 Hz, –CH_2–), 7.27 (1H, dd, J = 2.9, 9.0 Hz, H-7), 7.48 (1H, d, J = 8.9 Hz, H-8), 7.51 (1H, d, J = 3.0 Hz, H-5), 7.73 (1H, s, H-2), 7.91 (1H, br t, J = 5.4 Hz, –NHCO–), 11.67 (1H, s, H-1). FAB-MS m/z = 275.2 (M + H). Anal. Calcd. for $C_{15}H_{18}N_2O_3$: C, 65.68; H, 6.61; N, 10.21. Found: C, 65.41; H, 6.49; N, 10.13.

t-Butyl analog, *N*-[(6-methoxy-4-oxo-1,4-dihydroquinolin-3-yl)methyl]-2,2-dimethylpropanamide: m.p. 272–273 °C. Yield, 58.58 mg (37.2%). ^{1}H NMR (500 MHz, DMSO-$^2H_6$): δ ppm 1.11 (9H, s, (–CH_3)$_3$), 3.83 (3H, s, –OCH_3), 4.13 (2H, d, J = 5.5 Hz, –CH_2–), 7.27 (1H, dd, J = 3.0, 8.9 Hz, H-7), 7.48 (1H, d, J = 8.9 Hz, H-8), 7.51 (1H, d, J = 3.0 Hz, H-5), 7.66 (1H, s, H-2), 7.74 (1H, br t, J = 5.5 Hz, –NHCO–), 11.67 (1H, s, H-1). FAB-MS m/z = 289.3 (M + H). Anal. Calcd. for $C_{16}H_{20}N_2O_3$: C, 66.65; H, 6.99; N, 9.72. Found: C, 66.50; H, 6.98; N, 9.65.

2.5. Synthesis of [(6-methoxy-4-oxo-1,4-dihydroquinolin-3-yl)methyl]amine (6-MOQ-NH_2, **2**)

The *n*-propyl analog of **3** (30 mg) was added to aqueous 0.1 M HCl (10 mL) and refluxed for 8 h, and the reaction mixture was chromatographed by the RP-HPLC as described below. The fraction corresponding to **2** was then collected, freeze-dried and recrystallized from MeOH/ethyl acetate to obtain colorless crystals as the HCl salts.

The HPLC system consisted of a DG-980-50 degasser (JASCO), a PU-2080 plus pump (JASCO), a 7725i injector (Rheodyne, Cotati, CA, USA), a CO-960 column oven (JASCO), a UV-970 UV detector (JASCO) and an 807-IT integrator (JASCO). As the analytical column, Super-ODS (10 mm i.d. × 100 mm, Tosoh, Tokyo, Japan) was used at 40 °C. The mobile phase was HCl–MeOH–water (0.05:5:95, w/v/v), and the flow rate was 5 mL min^{-1}. The absorbance at 260 nm was used for the detection.

6-MOQ-NH_2·HCl (2, HCl salt): m.p. 223–224 °C. Yield, 15.42 mg (58.8%). ^{1}H NMR (500 MHz, DMSO-$^2H_6$): δ ppm 3.84 (3H, s, –OCH_3), 3.89 (2H, s, –CH_2–), 7.35 (1H, dd, J = 3.0, 9.2 Hz, H-7), 7.53 (1H, d, J = 2.7 Hz, H-5), 7.59 (1H, d, J = 9.2 Hz, H-8), 7.95 (3H, br s, NH_3^+), 8.12 (1H, s, H-2), 12.31 (1H, br s, H-1). FAB-MS m/z = 205.2 (M + H). Anal. Calcd. for $C_{11}H_{13}N_2O_2Cl$: C, 54.89; H, 5.44; N, 11.64. Found: C, 54.57; H, 5.44; N, 11.60.

2.6. Determination of molar absorptivity and fluorescence quantum yield

The absorbances at maximum excitation wavelengths of **1**, four analogs of **3** and **2** were measured using 10 μM solution in aqueous 10% (v/v) MeOH with a Model 150-20 spectrophotometer (Hitachi, Tokyo, Japan). The absorbance of 50 μM quinine sulfate was also measured at 366 nm in aqueous 0.1 M sulfuric acid, and the molar absorptivities of these compounds were calculated from the obtained absorbances.

The corrected emission spectra of these compounds (1 μM solution in aqueous 10% (v/v) MeOH) were measured using a JASCO FP-750 fluorescence spectrophotometer, and the fluorescence quantum yields of these compounds were calculated using quinine sulfate (ϕ = 0.55, λ_{ex} = 366 nm in aqueous 0.1 M sulfuric acid) as a standard.

2.7. Precolumn derivatization of carboxylic acids with **2** and RP-HPLC of the fluorescent derivatives

To the aqueous solution (100 μL) of 10 mM **2** (HCl salt), aqueous 5% (w/w) NaOH (100 μL) was added, and the free base of **2** was extracted twice with 200 μL of MeCN. The MeCN layer was dried with $MgSO_4$, and the resulting **2** solution was used for the derivatization.

The MeCN solution of carboxylic acids (containing 5 μM propionic acid and 5 μM isobutyric acid, 10 μL) was placed into a microtube. To this solution, 20 μL of **2** solution and 10 μL of MeCN solution containing 180 mM EDC and 30% (v/v) pyridine were added and allowed to stand for 60 min at room temperature. The reaction mixture was then diluted with aqueous 0.1% (w/v) HCl to 4 mL and subjected to the solid-phase extraction cartridge (RP-select B, Merck, Darmstadt, Germany). After washing the cartridge with 5 mL of aqueous 0.1% (w/v) HCl containing 7.5% (v/v) MeCN, the derivatives were eluted with 1 mL of MeCN. The obtained eluate was diluted 10 times with H_2O, and 2 μL of this solution was injected into the HPLC system. For the determination of the derivatives, a micro-HPLC system, NANOSPACE SI-1 and SI-2 series (Shiseido, Tokyo, Japan), was used. The system consisted of a degasser (Type 2009), a pump (Type 2001), an injector (Type 2006), a column oven (Type 2004) and a fluorescence detector (Type 3013). The data processing program S-MicroChrom, Version 4.1 was used for the data treatment. The analytical column used was a Capcell Pak C_{18} ACR (1.0 mm i.d. × 150 mm, Shiseido) maintained at 40 °C. The mobile phase was TFA–MeCN–water (0.05:15:85, v/v/v),

and the flow rate was 50 μL min^{-1}. The fluorescence detection was carried out at 400 nm with the excitation at 245 nm.

3. Results and discussion

3.1. Preparation and structural determination of the highly fluorescent oxidation product of MIAA

As we reported in our previous papers [10,11], the production of a highly fluorescent compound was observed in the oxidation of MIAA by an RP-HPLC. However, the structure has not yet been clarified. In the present investigation, this fluorescent oxidation product of MIAA was authentically synthesized by adding hydrogen peroxide to MIAA under alkaline conditions. After purification of the product, colorless needles were obtained, and the structure of this fluorescent product was determined by instrumental analyses. In the ^{1}H NMR spectrum, a signal for methoxy protons and the peaks for six aromatic protons were observed. While, in the ^{13}C NMR spectra, a signal for a quaternary carbon, which was not observed in the spectra of MIAA, appeared at 180 ppm in addition to the peaks for methoxy carbon and eight aromatic carbons, and no other peaks were observed. The results of the X-ray single-crystal diffraction analysis showed that the fluorescent product derived from MIAA is **1**. The pseudomolecular ion peak of this product was observed at 176.1 [$M+H$] on FAB mass spectrum (the molecular weight of **1** is 175.2), and the elemental analysis data were also consistent with the calculated values as follows: calcd. for $C_{10}H_9NO_2$, 68.56% C, 5.18% H, 8.00% N; found, 68.42% C, 5.15% H, 8.04% N. These results indicate that the structure of this fluorescent oxidation product of MIAA is genuine **1**. Fig. 1 shows the fluorescence excitation and emission spectra of MIAA and **1** dissolved in aqueous 10% (v/v) MeOH. Compared to the spectra of MIAA (Ex_{max} 279 nm, Em_{max} 351 nm), **1** has a large Stokes' shift, and the maximum fluorescence excitation and emission wavelengths are 243 nm and 374 nm, respectively. The fluorescence intensity of **1** is 7.7 times higher than that of MIAA. The molar absorptivity (ε) at the maximum excitation wavelength (243 nm) and the fluorescence quantum yield (ϕ) were also determined, and the obtained values were 32 600 (ε) L mol^{-1} cm^{-1} and 0.38 (ϕ), respectively.

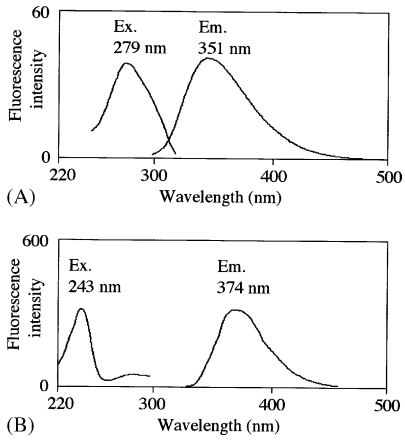


Fig. 1. Fluorescence excitation and emission spectra of (A) 1 μM MIAA and (B) 1 μM 6-MOQ (**1**) in aqueous 10% (v/v) MeOH.

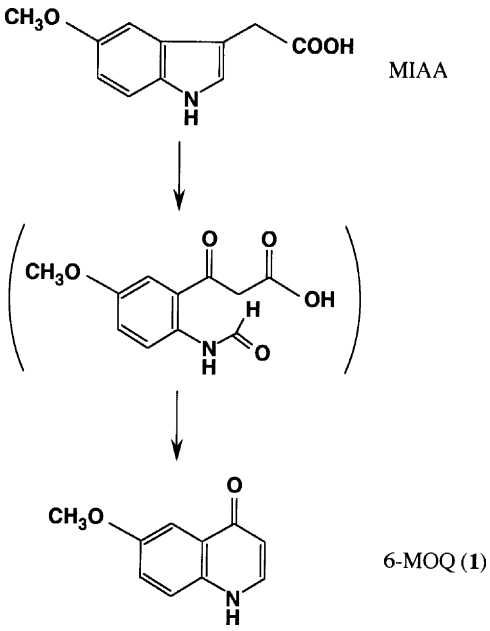


Fig. 2. Oxidative conversion of MIAA to 6-MOQ (**1**).

The reaction scheme of the oxidation of MIAA is thought to be as follows: in the first step of the reaction, the double bond between the 2- and 3-position of indole carbons is oxidized to form an intermediate, 3-[2-(formylamino)-5-methoxyphenyl]-3-oxopropanoic acid. Then, a 4-quinolone moiety was produced by the decarboxylation of a β-diketone and intramolecular condensation (Fig. 2). Although numerous pathways have been reported for the oxidation of indoles [18–21], similar oxidation reactions to that of MIAA are reported in several compounds [22–24]. *N*-Methoxy-3-substituted indoles are reported to form the oxidation products having *N*-formyl anthraniloyl group by ozonolysis, and these compounds are converted into *N*-methoxy quinolone derivatives by aldol condensation [22]. *N*-Methyltetrahydroharman is also known to form the compound, which has a 4-quinolone moiety, via an intermediate having a nine-membered lactam ring, by ozonolysis and intramolecular condensation [23]. Concerning the decarboxylation of β-diketone, it is generally known to proceed rapidly under alkaline conditions. Moreover, we previously demonstrated that melatonin is oxidized to **3** [10] under the same oxidative conditions described in the present investigation. These reports strongly support the expected reaction scheme presented in this paper for the oxidation of MIAA to **1**.

The fluorescence and absorbance properties of **1** are similar to those of 3, an oxidation product of melatonin identified in our previous investigation [10]. Compound **3** also has

strong fluorescence, and the maximum excitation and emission wavelengths are 245 nm and 378 nm, respectively. Considering that **3** has a 6-MOQ (**1**) moiety and the side chain at the 3-position, **1** is thought to be a core structure with strong fluorescence, and the side chain at the 3-position of **3** has a small effect on the fluorescence and absorbance properties. In addition, the fluorescence intensities of 6-methoxyquinoline (Ex_{max} 233 nm, Em_{max} 437 nm, RFI = 0.25 (the fluorescence intensity of **1** is taken as 1.0.)) and 4-quinolone (Ex_{max} 231 nm, Em_{max} 347 nm, RFI = 0.02) are much weaker than those of **1** and **3**. These results indicate that the presence of both the methoxy group at the 6-position and the carbonyl group at the 4-position strongly contributes to the fluorescence development of **1**. Quinolone compounds are mainly synthesized as antibiotic agents, and some of them could be detected by the fluorescence HPLC methods [14–16]. The structure of **1** has not been reported as a fluorophore; however, the present results showing that **1** has strong fluorescence are useful for the development of novel fluorescent reagents.

3.2. Fluorescence characteristics and stabilities of *1* and *3*

To examine the fluorescence characteristics of **1** and **3**, the effects of solvents and pH on their fluorescence were investigated. The maxima of their excitation and emission wavelengths slightly changed by the alteration of the solvents. However, the fluorescence intensities drastically changed, showing weak or no fluorescence in solvents of high organic solvent concentrations, while exhibiting strong fluorescence in water or aqueous 10% (v/v) organic solvents. Concerning the medium pH, the wavelengths of their emission maxima shifted about 20 nm longer at pH 2.0 than those observed at pH 7.0, while at other pHs the wavelengths of emission maxima were almost the same as those observed at pH 7.0. The wavelengths of excitation maxima and fluorescence intensities did not change by the alteration of pH. In addition, **1** and **3** have high stabilities against light and heat, and neither compound was degraded for at least 3 days at 60 °C under daylight.

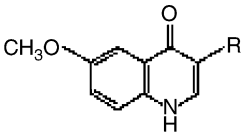
Until now, many fluorescent compounds such as NBD-derivatives, dansyl-derivatives and fluorescein derivatives have been reported and used for labeling reagents [1–7,25,26], pH indicators [27,28] and imaging probes [29,30]. The characteristics of these compounds almost depend on the fluorophores; however, these fluorophores often have some disadvantages, such as the quenching of fluorescence in aqueous media or decomposition by exposure to light. Concerning the 6-MOQ (**1**) moiety, which is discovered in the present study, the ε is over 30 000 L mol^{-1} cm^{-1}, and the ϕ is about 0.3 in aqueous 10% (v/v) MeOH, showing strong fluorescence in aqueous media under various pH conditions. Using this fluorescent compound, we have already demonstrated that the amol-level determination of melatonin (injection amount, 1.2–3.0 fmol of melatonin was derivatized and a portion of the reaction mixture was analyzed) could be accomplished after oxidative conversion of melatonin to **3** [10–13]. These results have been obtained using an HPLC system with a conventional fluorescence detector; the detection limit of **3** (200 amol, on column) is much lower than those of the dansyl-derivatives, NBD-derivatives and fluorescein derivatives.

The fluorescence characteristics of quinolones and quinolines have been studied in detail [31–34]. In general, *N*-hetero-cyclics, for example quinoline and acridine, are known to show weak fluorescence in the low polar solvents such as cyclohexane and benzene. On the other hand, they show strong fluorescence in high polar solvents such as alcohol and water, and the blue shifts in their emission spectra are observed with increasing pH. These reports are consistent with the characteristics of **1** and **3** demonstrated in the present investigation. Concerning **1**, despite its simple structure, there is no report showing the fluorescence characteristic of **1**, and labeling reagents using 6-MOQ (**1**) moiety have never been reported. Considering that **1** has strong fluorescence in aqueous media with a large Stokes' shift, and also has high stability against light and heat, **1** might be a useful novel fluorophore for the sensitive determination of biological substances.

3.3. Fluorescence characteristics of the related compounds of **1** and **3** having substituents at the 3-position of quinolone moiety

As described above, **1** and **3** exhibited almost the same fluorescence characteristics, suggesting that the introduction of some substituents to the 3-position of **1** via the methylene group scarcely affects the fluorescence characteristic of **1**. This is an advantage in utilizing the 6-MOQ (**1**) moiety for fluorescent labeling reagents after introducing reactive groups into the 3-position of the quinolone moiety via the methylene group. Therefore, to confirm this hypothesis, we synthesized several related compounds of **1** and **3** and investigated their fluorescence and absorbance properties. As the related compounds, we prepared **2**, which has methylamine at the 3-position of **1**, and four analogs of **3** having different alkyl groups instead of the methyl group of acetamide (**4**). Table 1 summarizes the fluorescence and absorbance properties of **1**, **3** and the related compounds observed in aqueous 10% (v/v) MeOH. All of these compounds have strong fluorescence, and their maximum excitation and emission wavelengths are about 245 nm and 375 nm, respectively. The molar absorptivities (ε) at the maximum excitation wavelengths and fluorescence quantum yields (ϕ) of all compounds are greater than 30 000 L mol^{-1} cm^{-1} and 0.3, respectively. The fluorescence properties of these compounds are quite similar to that of **1**, showing strong fluorescence in the aqueous solutions, and their fluorescence intensities were not affected by the medium pH. These results indicate that the introduction of some substituents to the 3-position of **1** via the methylene group does not affect the fluorescence characteristic of **1**, which is generally a suitable characteristic for the development of labeling reagents.

Table 1
Absorbance and fluorescence properties of 6-MOQ (**1**), 6-MOQMA (**3**) and related compounds



Compound	R	Absorbance		Fluorescence			
		(nm)	ε	Ex_{max} (nm)	Em_{max} (nm)	Intensity	ϕ
1	H	243	32 600	243	374	301.4	0.38
2	CH_2NH_2	244	41 500	244	375	315.0	0.31
3	$CH_2NHCOCH_3$	245	46 300	245	378	442.3	0.31
4[a]	$CH_2NHCOCH_2CH_3$	245	46 700	245	376	442.1	0.31

Fluorescence intensities were measured using 1 μM aqueous 10% (v/v) MeOH solutions; ε: molar absorptivity, ϕ: fluorescence quantum yield.
[a] *n*-Propyl, isopropyl and *t*-butyl analogs have almost the same absorbance and fluorescence properties as that of ethyl analog.

3.4. Application of the **1** *analog as a fluorescent labeling reagent*

To demonstrate the usefulness of **1** analogs, **2** was applied to the fluorescent labeling reagent for carboxylic acids. Derivatization is based on the reaction between the amino group of **2** and the carboxylic group (RCOOH) under alkaline conditions in the presence of EDC, a coupling reagent, to produce the corresponding amide compounds. Propionic acid and isobutyric acid were used as carboxylic acids. The reaction conditions were investigated by changing the concentrations of EDC and pyridine with various reaction times, and satisfactory results were obtained using the conditions described in the Section 2. After the derivatization reaction, a solid-phase extraction cartridge was used to remove excess reagents. Fig. 3(A) shows the chromatogram analyzing 10 fmol of propionic acid and isobutyric acid (injection amount), following the derivatization reaction of 50 pmol of these carboxylic acids with **2**. The derivatives of both carboxylic acids could be well separated within 20 min, and their signal-to-noise ratios were 206 and 164.

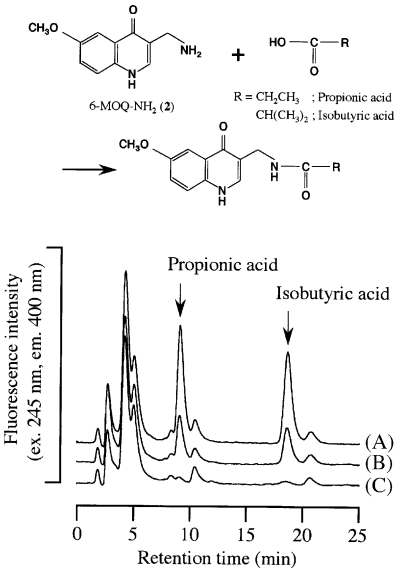


Fig. 3. Chromatograms of the derivatives for 10 fmol propionic acid and isobutyric acid (A) 50 pmol of the carboxylic acids were derivatized, 4 fmol, (B) 20 pmol of the carboxylic acids were derivatized, and the blank reaction mixture which the two carboxylic acids were omitted (C).

To date, various fluorescent labeling reagents have been developed and widely used in HPLC and HPCE [1–3]. Some of the reagents could provide the highly sensitive determination; however, most of the reagents have some limitations. For example, the fluorescence intensities of the dansyl-derivatives decrease under acidic conditions [35], and sensitive determination could not be performed under low pH conditions. Fluorescamine derivatives are hydrolyzed to nonfluorescent compounds and decrease their fluorescence immediately in acidic solutions [36]. NBD-derivatives, which are widely used due to their high sensitivities, are decomposed by light and heat [4].

As described in this paper, **1** has strong fluorescence in aqueous solutions, which are widely used as mobile phase of RP-HPLC, and its fluorescence does not depend on the change in pH. In addition, the 6-MOQ (**1**) moiety is stable against light and heat. These results indicate that the **1** is a useful moiety for labeling reagents.

4. Conclusions

In the present investigation, the oxidation product derived from MIAA has been synthesized and identified as **1**. This oxidation compound, **1**, is highly stable against light and heat and is revealed to be the core structure having strong fluorescence in aqueous media at various pHs with large Stokes' shifts. The applicability of this novel fluorophore has also been demonstrated using **2**, and RP-HPLC determination of carboxylic acids was performed. These results indicate that the 6-MOQ (**1**) moiety is a useful fluorophore, which could provide various powerful tools for biomedical analysis.

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