J Fluoresc (2010) 20:615–624
DOI 10.1007/s10895-009-0596-2

ORIGINAL PAPER

6,7-Difluoro-1,4-dihydro-1-methyl-4-oxo-3-quinolinecarboxylic Acid, a Newly Designed Fluorescence Enhancement-Type Derivatizing Reagent for Amino Compounds

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Received: 31 March 2009 / Accepted: 30 December 2009 / Published online: 28 January 2010

Abstract A novel fluorescence enhancement-type derivatizing reagent for amino compounds, 6,7-difluoro-1,4-dihydro-1-methyl-4-oxo-3-quinolinecarboxylic acid (FMQC), was developed. FMQC reacts with aliphatic primary amino compounds to afford strong fluorescent derivatives having high photo-and thermo-stabilities. The FMQC derivatives of amino compounds showed 12–159 times higher fluorescence quantum efficiencies than those of FMQC in aqueous and polar organic media. Additionally, the absorption and fluorescence emission wavelength of the derivatives are red-shifted from those of FMQC. These differences in the fluorescence properties between FMQC and the fluorescent derivative enabled the simple and highly sensitive determination of amino compounds without removing any excess unreacted FMQC by using a simple spectrofluorometer as well as HPLC.

Keywords Derivatizing reagent · Fluorescence · 4-Quinolone · Amino compound · HPLC

Introduction

Fluorescent derivatizing reagents enable the establishment of highly sensitive analytical methods of non-fluorescent compounds, and they are widely utilized in various research fields including biochemistry, clinical chemistry, and environmental chemistry. Up to now, a significant number of fluorescent derivatizing reagents have been reported [1–3]. Most of them are called fluorescent labeling reagents, showing the same fluorescence characteristics after reacting with the target molecules. When using a labeling type reagent, it is necessary to separate a fluorescent derivative of a target compound from the unreacted reagent. On the other hand, the fluorescent derivatizing reagent, which shows a drastic enhancement of the fluorescence intensity after reacting with a target molecule, has a significant value. By using the fluorescence enhancement-type derivatizing reagent, a target compound could be sensitively determined without separating the unreacted reagent. Therefore, in order to establish simple and highly sensitive analytical methods for various target compounds, such as physiologically active substances, pollutants, and drugs, significant efforts have been devoted to designing fluorescence enhancement-type derivatizing reagents.

In a previous study, we reported that 4-quinolone (4QO) analogs are useful photo-and thermo-stable fluorophores with high fluorescence quantum efficiencies (Φ) in aqueous media, and developed various fluorescent reagents utilizing the 4QO structure [4–8]. Among these reagents, [(6-methoxy-4-oxo-1,4-dihydroquinolin-3-yl)methyl]amine (6MOQ-NH_2) was a useful fluorescent reagent with a high stability, and enabled the sensitive determination of carboxylic acids [8]. However, 6MOQ-NH_2 is a labeling-type reagent, so its application is limited to separation analyses, such as HPLC. During the course of our investigation focusing on the relationship between the structure and fluorescence characteristics of 4QO derivatives in aqueous media, we found that the fluorescence properties could be modified by introducing electron withdrawing/donating groups to the 4QO moiety. Especially, the Φ of 4QO derivatives in H_2O drastically changed, for example, Φ is 0.001 for 6-nitro-4-quinolone, 0.017 for 4QO, and 0.498 for 6-methoxy-4-quinolone. The significant difference in the fluorescence characteristics of the 4QO derivatives led us to develop a highly sensitive and stable fluorescence enhancement-type derivatizing reagent utilizing the 4QO structure.

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In the present study, we focused on the development of a fluorescence enhancement-type derivatizing reagent having the 4QO structure which enables the highly sensitive determination of amino compounds. Amino compounds including amines and amino acids are present in various matrices as physiologically active substances and environmental substances, and therefore, numerous reagents for fluorescence determination have been reported. However, most of these reagents are the labeling-type ones, for example, 5-dimethylaminonaphthalene-1-sulfonylchloride (DNS-Cl) [9], 2-(5′,6′-dimethoxybenzothiazolyl)-benzenesulfonyl chloride (BHBT-SOCl) [10], 9-fluorenylmethyl chloroformate (Fmoc-Cl) [11], 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl chloride (DMEQ-COCl) [12], fluorescein isothiocyanate (FITC) [13], *N*-succinimidyl-1-naphthylcarbamate (SINC) [14], and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) [15]. On the other hand, the fluorescence enhancement-type reagents, such as *o*-phthalaldehyde (OPA) [16], fluorescamine [17], and 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) [18] are still limited. These reagents enable the rapid and sensitive determination of amines, and have been widely utilized in various analytical methods. However, these reagents are not always satisfactory with respect to the stability of the reagents and derivatives, showing decomposition by heating and light irradiation [19, 20].

In the present paper, we report a novel option for the stable fluorescence enhancement-type derivatizing reagent, 6,7-difluoro-1,4-dihydro-1-methyl-4-oxo-3-quinolinecarboxylic acid (FMQC), which is suitable for the simple and sensitive determination of amino compounds. By reacting with amino compounds, FMQC showed a drastic enhancement of the fluorescence intensity and wavelength shift, and therefore, enabled the highly sensitive determination of amino compounds without separating from the unreacted reagent. Furthermore, the synthesized FMQC derivative of the amino compound was stable and showed a strong fluorescence in water as well as organic solvents, such as MeOH and MeCN. Herein, we now report the design and synthesis of FMQC. The spectroscopic properties of FMQC and the FMQC derivatives of amino compounds were also indicated. As an application, the determination of amino compounds was demonstrated using a simple spectrofluorometer, and using a reversed-phase HPLC (RP-HPLC).

Experimental

General procedures

Commercially available materials were used without any additional purification. The solvents used for the spectral studies were of spectroscopic grade for dimethylsulfoxide (DMSO), and of HPLC grade for MeOH and MeCN. All the other solvents were of guaranteed reagent grade. The products were characterized by ^{1}H NMR, FAB-MS and elemental analyses. The ^{1}H NMR spectra were recorded using a Varian Unity-400 spectrometer (400 MHz) in dimethylsulfoxide-d_6 (DMSO-d_6). The mass spectra were obtained using a JEOL JMS-SX102A mass spectrometer.

Syntheses

7-Fluoro-4-quinolone (1). Compound **1** was synthesized as a colorless needle (yield 3.9%) in four steps from *m*-fluoroaniline according to previously reported methods [21–23]. Mp 254–256 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ ppm 6.02 (1H, d, J=7.5 Hz, H-3), 7.15 (1H, td, J=2.5, 8.8 Hz, H-6), 7.27 (1H, dd, J=2.4, 10.2 Hz, H-8), 7.89 (1H, d, J=7.3 Hz, H-2), 8.12 (1H, dd, J=6.4, 8.9 Hz, H-5), 11.74 (1H, br s, H-1). FABMS *m/z* = 164 (M+H). Anal. Calcd for C_9H_6NOF: C, 66.26; H, 3.71; N, 8.59. Found: C, 65.99; H, 3.71; N, 8.60.

7-Fluoro-1-methyl-4-quinolone (2). A mixture of **1** (1.5 g, 9.2 mmol) and anhydrous K_2CO_3 (3.18 g, 23 mmol) in dry DMF (15 mL) was heated at 50 °C for 2 h. MeI (2.86 mL, 46 mmol) was added, and the mixture was heated at 50 °C for 12 h. The reaction mixture was diluted with H_2O, and extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$, filtered, and evaporated. The crude compound was purified by column chromatography over silica gel with $CHCl_3$/MeOH (5:1) and with AcOEt/MeOH (4:1) to afford a colorless powder (yield 59.7%). Mp 155–156 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 3.76 (3H, s, Me), 6.03 (1H, d, J=7.6 Hz, H-3), 7.24 (1H, td, J=2.3, 8.7 Hz, H-6), 7.50 (1H, dd, J=2.5, 11.3 Hz, H-8), 7.95 (1H, d, J=7.6 Hz, H-2), 8.20 (1H, dd, J=6.7, 8.9 Hz, H-5). FABMS *m/z* = 178 (M+H). Anal. Calcd for $C_{10}H_8NOF$•0.5H_2O: C, 64.51; H, 4.87; N, 7.52. Found: C, 64.49; H, 4.91; N, 7.52.

6,7-Difluoro-1-methyl-4-quinolone (3). 6,7-Difluoro-4-quinolone was prepared using 3,4-difluoroaniline as described for **1**. The methylation of 6,7-difluoro-4-quinolone was carried out as described for **2** to afford a colorless powder (yield 22.7%). Mp 183–184 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 3.78 (3H, s, Me), 6.05 (1H, d, J=7.6 Hz, H-3), 7.83 (1H, dd, J=6.7, 12.5 Hz, H-5 or H-8), 7.98 (1H, d, J=7.6 Hz, H-2), 8.00 (1H, dd, J=9.2, 11.0 Hz, H-5 or H-8). FABMS *m/z* = 196 (M+H). Anal. Calcd for $C_{10}H_7NOF_2$•0.3H_2O: C, 59.88; H, 3.82; N, 6.98. Found: C, 60.02; H, 3.79; N, 7.02.

7-Fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid (4). The colorless powder (yield 71.9%) of **4** was obtained as the synthetic precursor of **1** according to previously reported

methods [21–23]. Mp 297–298 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 7.48 (1H, td, *J*=2.4, 8.9 Hz, H-6), 7.57 (1H, dd, *J*=2.4, 9.8 Hz, H-8), 8.36 (1H, dd, *J*=6.1, 8.9 Hz, H-5), 8.93 (1H, s, H-2), 13.36 (1H, br s, H-1), 15.11 (1H, s, CO_2H). FABMS *m/z* = 206 (M–H).

7-Fluoro-1,4-dihydro-1-methyl-4-oxo-3-quinolinecarboxylic acid (5). Compound **5** was synthesized in one step, starting from 7-fluoro-1,4-dihydro-4-oxo-3-quinoline carboxylic acid ethyl ester obtained in the following manner. A mixture of *m*-fluoroaniline (4.44 g, 40 mmol) and diethylethoxymethylenemalonate (DEEM)(8.65 g, 40 mmol) was heated at 120 °C for 100 min under N_2 gas flow. The obtained liquid was added to diphenyl ether (200 mL), and the mixture was refluxed for 1 h. After petroleum ether (200 mL) was added to the reaction mixture, the resulting precipitate was collected by filtration, and washed with acetone to afford 7-fluoro-1,4-dihydro-4-oxo-3-quinoline carboxylic acid ethyl ester as a colorless powder (yield 85.0%).

A mixture of 7-fluoro-1,4-dihydro-4-oxo-3-quinoline carboxylic acid ethyl ester (2.0 g, 8.5 mmol) and anhydrous K_2CO_3 (2.94 g, 21.3 mmol) in dry DMF (40 mL) was heated at 50 °C for 2 h. MeI (3.18 ml, 51 mmol) was then added, and the mixture was heated at 50 °C for 20 h. The reaction mixture was diluted with H_2O, and extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$, filtered, and evaporated. The residue was then dissolved in hot H_2O-MeOH and filtered. The resulting colorless powder was collected by filtration. To a suspension of the powder in H_2O (20 mL), H_2SO_4 (2 mL) and AcOH (12 mL) was added, and the mixture was refluxed for 30 min. The resulting precipitate was collected by filtration, then washed with H_2O to afford a colorless powder (yield 71.5%). Mp >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 4.06 (3H, s, Me), 7.56 (1H, td, *J*=2.4, 8.7 Hz, H-6), 7.86 (1H, dd, *J*= 2.4, 11.0 Hz, H-8), 8.43 (1H, dd, *J*=6.4, 8.9 Hz, H-5), 9.04 (1H, s, H-2), 15.05 (1H, s, CO_2H). FABMS *m/z* = 222 (M+ H). Anal. Calcd for $C_{11}H_8NO_3F$: C, 59.73; H, 3.65; N, 6.33. Found: C, 59.72; H, 3.66; N, 6.30.

6,7-Difluoro-1,4-dihydro-1-methyl-4-oxo-3-quinolinecarboxylic acid (6, FMQC). FMQC was synthesized in one step, starting from 6,7-difluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid ethyl ester obtained in the following manner. A mixture of 3,4-difluoroaniline (5.16 g, 40 mmol) and DEEM (8.65 g, 40 mmol) was heated at 120 °C for 100 min under N_2 gas flow. The obtained product was added to diphenyl ether (200 mL), and the mixture was refluxed for 1 h. After petroleum ether (200 mL) was added to the reaction mixture, the resulting precipitate was collected by filtration, and washed with acetone to afford 6,7-difluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid ethyl ester as a colorless powder (yield 71.8%).

A colorless powder of FMQC (yield 76.7%) was synthesized as described for **5** using 6,7-difluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid ethyl ester. Mp 296.5–297.5 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 4.07 (3H, s, Me), 8.19 (1H, dd, *J*=6.7, 12.2 Hz, H-5 or H-8), 8.26 (1H, dd, *J*=8.7, 10.5 Hz, H-5 or H-8), 9.05 (1H, s, H-2), 14.85 (1H, s, CO_2H). FABMS *m/z* = 238 (M–H). Anal. Calcd for $C_{11}H_7NO_3F_2$: C, 55.24; H, 2.95; N, 5.86. Found: C, 55.23; H, 2.96; N, 5.83.

6-Fluoro-1,4-dihydro-1-methyl-7-*n*-propylamino-4-oxo-3-quinolinecarboxylic acid (7). A mixture of FMQC (100 mg, 0.42 mmol) and *n*-propylamine (275 μL, 3.34 mmol) in MeCN (10 mL) was heated at 65 °C for 5 h. The resulting precipitate was collected by filtration, then washed with MeCN and H_2O to afford a colorless block (yield 60.2%). Mp 243–244 °C. ^{1}H NMR (400 MHz, DMSO-d_6, 50 °C): δ ppm 0.96 (3H, t, *J*=7.3 Hz, Me), 1.68 (2H, qt, J=7.0, 7.3 Hz, CH_2), 3.25–3.30 (2H, m, CH_2), 4.00 (3H, s, Me), 6.72 (1H, d, *J*=7.3 Hz, H-8), 6.87 (1H, br t, NH), 7.78 (1H, d, *J*=11.9 Hz, H-5), 8.79 (1H, s, H-2), 15.69 (1H, s, CO_2H). FABMS *m/z* = 277 (M–H). Anal. Calcd for $C_{14}H_{15}N_2O_3F$: C, 60.42; H, 5.43; N, 10.07. Found: C, 59.78; H, 5.49; N, 9.95.

7-*N*,*N*-Diethylamino-6-fluoro-1,4-dihydro-1-methyl-4-oxo-3-quinolinecarboxylic acid (8). A colorless block (yield 34.4%) of **8** was synthesized as described for **7** using diethylamine. Mp 210–211 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 1.19 (6H, t, *J*=7.0 Hz, Me), 3.50 (4H, q, J=7.0 Hz, CH_2), 4.02 (3H, s, Me), 6.87 (1H, d, *J* = 7.9 Hz, H–8), 7.83 (1H, d, *J* = 14.7 Hz, H–5), 8.86 (1H, s, H–2), 15.54 (1H, s, CO_2H). FABMS *m/z* = 291 (M–H). Anal. Calcd for $C_{15}H_{17}N_2O_3F$: C, 61.63; H, 5.86; N, 9.58. Found: C, 61.64; H, 5.86; N, 9.57.

Absorption and fluorescence studies

The absorption and corrected fluorescence emission spectra were obtained using a JASCO V-530 UV/Vis spectrophotometer and a JASCO F-6500 spectrofluorometer. The compounds were dissolved in DMSO at 10 mM concentrations for **1**–**6**, and at 1 mM concentrations for **7** and **8**. These solutions were diluted with various solvents to the appropriate concentration for the measurements. The relative fluorescence quantum efficiencies were obtained using quinine sulfate in aqueous 0.05 M H_2SO_4, which has a quantum efficiency of 0.55.

Reactivity of 1–6 for *n*-propylamine

Using each organic solvent (DMSO, DMF, MeCN), compounds **1**–**6** were prepared at a 10 mM concentration, while triethylamine (Et_3N) and *n*-propylamine were prepared at a 400 mM concentration. To a siliconized tube,

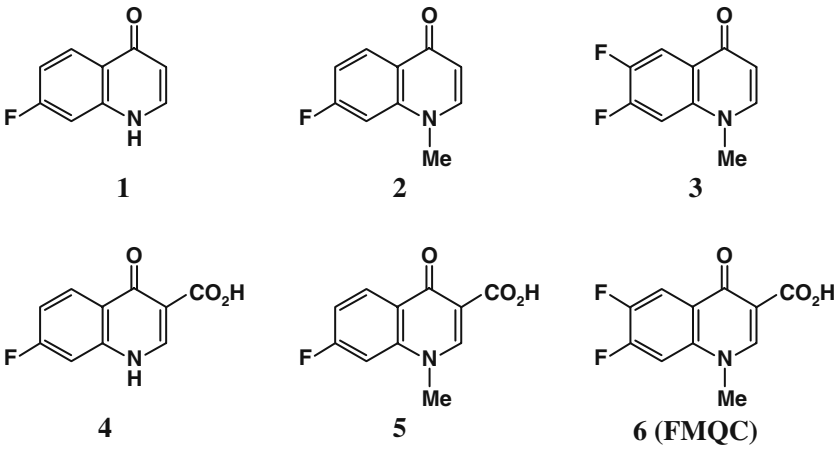

Fig. 1 Structures of designed candidates **1**–**6** as fluorescence enhancement-type derivatizing reagents for amino compounds

each **1**–**6** solution (100 μL), triethylamine (Et_3N) solution (5 μL), and *n*-propylamine solution (5 μL) were added. The tube was capped and then heated at 65 °C for 30 min. An aliquot (20 μL) of the reaction mixture was diluted with 50 mM phosphate buffer (pH 10) or aqueous 10 mM HCl to the appropriate concentration for the absorption and fluorescence measurements.

Fluorimetric analysis of *n*-propylamine with FMQC

To a siliconized tube, 5 mM FMQC in DMSO (100 μL), 5 mM Et_3N in DMSO (100 μL), *n*-propylamine (9, 45, 90, 450, 900 μM) in DMSO (100 μL) were added. The tube was capped and heated at 100 °C for 5 h. The reaction mixture was diluted with aqueous 10 mM HCl (2.7 mL), the emission intensity at 445 nm was measured with the excitation wavelength at 350 nm.

RP-HPLC of amines with FMQC

To a siliconized tube, 5 mM FMQC in DMSO (100 μL), 5 mM Et_3N in DMSO (100 μL), mixed amines (200 nM each of *n*-propylamine, *n*-butylamine, *n*-pentylamine, and *n*-hexylamine) in DMSO (100 μL) were added. The tube was capped and heated at 100 °C for 5 h. An aliquot (30 μL) of the reaction mixture was diluted with 10 mM aqueous HCl (970 μL), and 5 μL of the solution was subjected to the HPLC system. The HPLC system (Shiseido NANOSPACE

Table 1 Absorbance and fluorescence properties of 1-6 in various solvents at 20 °C

Compund	Solvent	Absorption max (nm)	Emission max (nm)	Molar absorptivity ($\times 10^4$ M^{-1} cm^{-1})	Φ_F
1	10 mM aqueous HCl	311	346	0.910	0.062
	H_2O	311	343	1.289	0.007
	50 mM Na-phosphate buffer (pH 10)	312	387	1.145	0.094
2	10 mM aqueous HCl	318	350	0.989	0.038
	H_2O	319	349	1.324	0.015
	50 mM Na-phosphate buffer (pH 10)	319	350	1.327	0.018
3	10 mM aqueous HCl	321	357	1.030	0.022
	H_2O	322	356	1.288	0.021
	50 mM Na-phosphate buffer (pH 10)	322	357	1.283	0.022
4	10 mM aqueous HCl	304	336	1.021	0.050
	H_2O	304	337	1.114	0.045
	50 mM Na-phosphate buffer (pH 10)	313	379	0.911	0.028
5	10 mM aqueous HCl	310	344	1.223	0.046
	H_2O	310	344	1.176	0.023
	50 mM Na-phosphate buffer (pH 10)	320	358	1.138	0.001
6	10 mM aqueous HCl	312	347	1.193	0.036
	H_2O	312	347	1.146	0.029
	50 mM Na-phosphate buffer (pH 10)	323	355	1.049	0.001

Φ_F: Fluorescence quantum efficiency

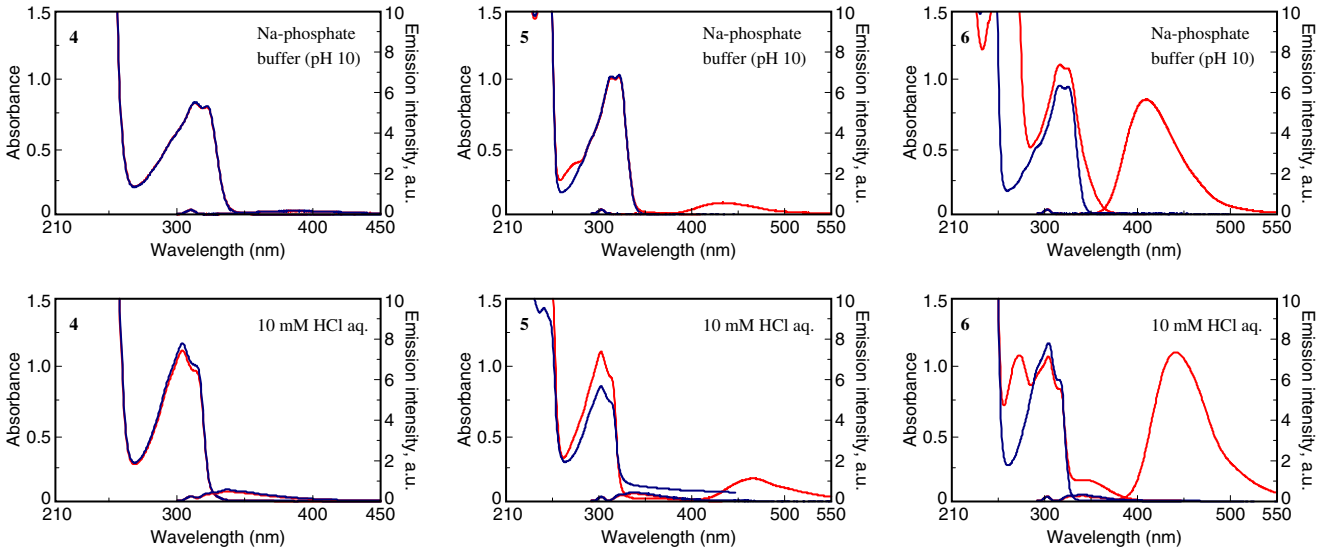

Fig. 2 Absorption and fluorescence emission spectra (excitation at 280 nm) of the reaction mixtures of **4**–**6** in the presence (red line) and absence of *n*-propylamine (blue line) in 50 mM phosphate buffer (pH 10) and 10 mM aqueous HCl

SI-2 series) consisted of a type 3101 pump, a 3007 injector, a 3014 column oven, and a 3013 fluorescence detector. A data processing program, Shiseido EZChrom Elite, was used to monitor the detector response. The analytical column used was a CAPCELL PAK C18 MGII S3 (1.0 mm i.d. x 150 mm, Shiseido) maintained at 40 °C. The mobile phase was MeCN-TFA-water (35:0.05:65, v/v/v), and the flow rate was 60 μL/min. The fluorescence detection was carried out at 445 nm with the excitation wavelength at 350 nm.

Results and discussion

Design and synthesis of 7-fluoro-4-quinolone derivatives as fluorescence enhancement-type derivatizing reagents for amino compounds

A number of 4QO derivatives has been synthesized as antibacterial agents. Among these derivatives, several 7-fluoro-4-quinolone derivatives are reported to undergo nucleophilic attack by amines, and form 7-amino-4-quinolone derivatives [24–27]. In our laboratory, we have also synthesized 6-and/or 7-substituted 4QO derivatives, and found that the derivatives having electron-withdrawing groups, such as NO_2, AcO, and Cl, show low Φ values, while the derivatives having electron-donating groups, such as MeO, EtO, and NMe_2, show high Φ values. These fundamental data strongly suggested that the 7-fluoro-4-quinolone derivatives show weak fluorescence, and the 7-amino-4-quinolone derivatives show strong fluorescence. Therefore, to develop fluorescence enhancement-type derivatizing reagents for amino compounds, we designed the six candidate compounds based on the 7-fluoro-4-quinolone structure. Figure 1 summarizes the designed compounds **1**–**6**. Compound **1** is 7-fluoro-4-quinolone. Compounds **2** and **3** have a Me group at the 1 position of the 4QO moiety. Compounds **4**–**6** are designed to have a COOH group at the 3 positions of **1**–**3**, respectively, to increase the reactivity for the amino compounds. Com-

Fig. 3 Syntheses of **7** and **8**

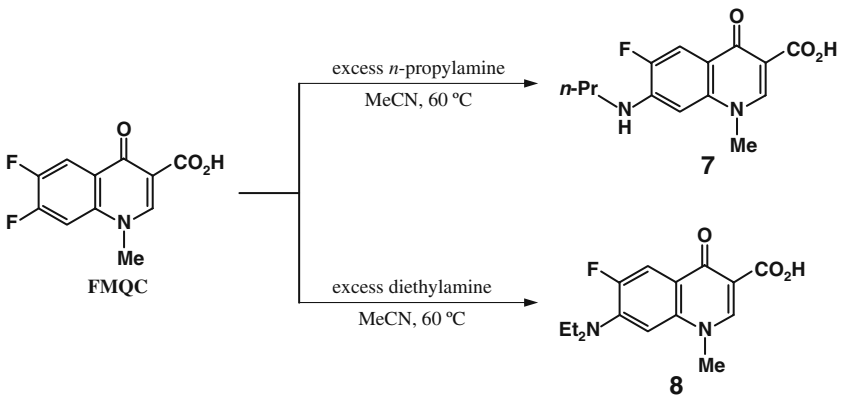

Table 2 Absorbance and fluorescence properties of FMQC, **7**, and **8** in various solvents at 20 ºC

Compound	Solvent	Absorption max (nm)	Emission max (nm)	Molar absorptivity ($\times 10^4$ M^{-1} cm^{-1})	Φ_F
FMQC	10 mM aqueous HCl	312	347	1.193	0.036
	H_2O	312	347	1.146	0.029
	50 mM Na-phosphate buffer (pH 10)	323	355	1.049	0.001
	MeOH	314	349	1.253	0.018
	MeCN	315	350	1.207	0.020
7	10 mM aqueous HCl	349	442	1.011	0.451
	H_2O	348	439	1.020	0.599
	50 mM Na-phosphate buffer (pH 10)	324	411	1.379	0.159
	MeOH	343	432	1.024	0.527
	MeCN	338	416	1.004	0.243
8	10 mM aqueous HCl	363	365,444	0.577	0.009
	H_2O	362	468	0.867	0.001
	50 mM Na-phosphate buffer (pH 10)	328	436	1.332	0.014
	MeOH	354	439	1.078	0.001
	MeCN	349	479	1.052	0.004

Φ_F: Fluorescence quantum efficiency

pounds **1**–**6** were synthesized in high yield following Gould-Jacobs reaction and literature methods [21–23].

Spectroscopic properties of synthesized analogs 1–6 and reactivities to amino compounds

The absorption and fluorescence properties of **1**–**6** were evaluated in 10 mM aqueous HCl, H_2O, and 50 mM phosphate buffer (pH 10) (Table 1). All the compounds exhibited similar absorption spectra with the maximum wavelengths of ca. 300–320 nm, and the molar absorptivities were on the order of $10^4 M^{-1} cm^{-1}$. In the fluorescence emission spectra, **1**–**6** showed very weak fluorescences as expected; the maximum Φ of 0.094 was observed for **1** in 50 mM phosphate buffer (pH 10).

We next investigated the reactivities of **1**–**6** to amino compounds. The mixture of each derivative and *n*-propylamine, which was selected as an amino compound, was heated in the presence of the basic catalyst Et_3N, and then diluted with 50 mM phosphate buffer (pH 10) or 10 mM aqueous HCl for the spectral measurement. Figure 2 represents the absorption and fluorescence emission spectra of the reaction mixtures of **4**–**6** in the presence (red line) and absence (blue line) of *n*-propylamine. Compounds **1**–**4** exhibited no change in the absorption and fluorescence emission spectra obtained in both alkaline and acidic solutions. On the other hand, for **5** and **6,** drastic spectral changes were observed, and the fluorescence enhancements (red line) were observed in the visible region of 400–500 nm in both alkaline and acidic solutions, indicating these two compounds were promising candidates for fluorescence enhancement-type derivatizing reagents for amino compounds. Concerning **5**, due to the poor solubility in H_2O, a precipitate was observed in the 10 mM aqueous HCl solution. Furthermore, the fluorescence enhancement was significantly greater for **6** than **5** (Fig. 2). Therefore, compound **6** (6,7-difluoro-1,4-dihydro-1-methyl-4-oxo-3-quinolinecarboxylic acid, FMQC) was employed in a subsequent study.

Spectroscopic properties of the FMQC derivative of *n*-propylamine

The derivative **7** described in Fig. 3 was obtained by reacting FMQC and *n*-propylamine on a synthetic scale in high yield, and the absorption and fluorescence properties were evaluated. Table 2 summarizes the spectroscopic properties of FMQC and **7** in five solvents. FMQC showed

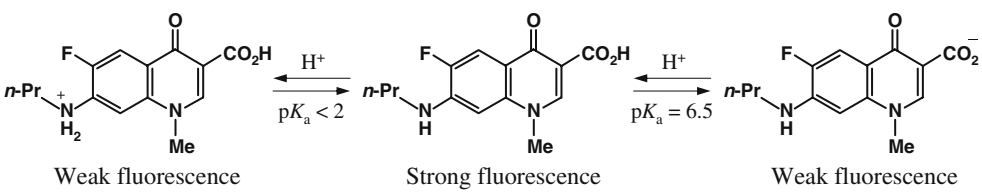

Fig. 4 Acid-base equilibriums of **7**

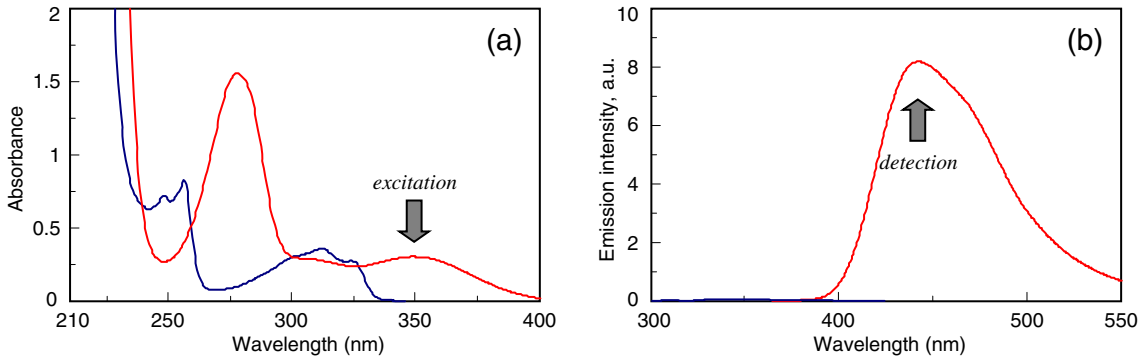

Fig. 5 **a** Absorption spectra of FMQC (*blue line*, 30 μM, 0.3% DMSO) and **7** (*red line*, 30 μM, 3% DMSO) in 10 mM HCl aq.; **b** fluorescence emission spectra (excitation at 350 nm) of FMQC (*blue line*, 3 μM, 0.03% DMSO) and **7** (*red line*, 3 μM, 0.3 % DMSO) in 10 mM aqueous HCl

a weak fluorescence even in organic solvents. On the other hand, compound **7** showed a strong fluorescence in all the solvents; the obtained Φ values were 0.159–0.599, which were 12–159 times higher than those of FMQC. The absorption maxima of **7** were 324–349 nm, ca. 30 nm red-shifted in comparison with FMQC except when using 50 mM phosphate buffer (pH 10). The fluorescence emission wavelengths of **7** were 411–442 nm, which were 60–90 nm red-shifted from those of FMQC. To understand the detailed spectroscopic properties, the acid-base equilibriums of both compounds were investigated. FMQC exhibited a blue-shift of the absorption spectrum and the decrease of the fluorescence intensity with a lowering of the pH, and the pK_a of COOH at the 3 position was estimated to be 5.9. These results indicate that FMQC is mainly the weakly fluorescent molecular form at pHs lower than 5.9, and is the non-fluorescent carboxylate ionic form over pH 5.9. Figure 4 shows the acid-base equilibrium of **7**. Based on the blue-shift of the absorption spectrum similar to that of FMQC, the pK_a of COOH at the 3 position of **7** was determined to be 6.5. In the fluorescence emission spectrum, the Φ decreased over pH 6, which was caused by the deprotonation of the COOH group at the 3 position. Furthermore, Φ also decreased at a pH lower than pH 4, which was thought to be caused by the protonation of the NH group at the 7 position, indicating that the pK_a of NH_2^+ is lower than ca. 2. These results suggested that **7** shows a strong fluorescence as its molecular form around pH 4.

The differences in the spectroscopic properties between FMQC and **7** indicated that the use of the appropriate excitation and emission wavelengths enables the highly sensitive determinations of amino compounds without separating any excess unreacted reagent, FMQC. Figure 5 represents the absorption and fluorescence emission spectra of FMQC and **7** in 10 mM aqueous HCl. When using an aqueous acidic solvent, the weak fluorescence of FMQC is quite negligible by employing the excitation wavelength of 350 nm and the emission wavelength of 445 nm, because the wavelengths of FMQC are 312 nm for the absorption and 347 nm for the emission. Concerning the stability, compound **7** exhibited no decomposition for more than a month during storage in a clear glass vial at room temperature. With respect to its stability, FMQC was superior to OPA, fluorescamine, and NBD-F reported as the fluorescence enhancement-type derivatizing reagents for amino compounds.

Reaction selectivity for amino compounds

The reaction selectivity of FMQC for amino compounds was investigated using aliphatic primary amino compounds (*n*-butylamine, *n*-pentylamine, *n*-hexylamine), aliphatic secondary amino compounds (diethylamine, di-*n*-propylamine), and aromatic amino compounds (aniline, *p*-methoxyaniline, *p*-chloroaniline). The mixture of FMQC and each amino compound was heated in the presence of Et_3N, and diluted with 10 mM aqueous HCl for the spectral measurements. For all the aliphatic primary amino compounds, the absorbance enhancements at 350 nm were observed similar to that of *n*-propylamine, and ca. one-sixth of the absorbance enhance-

Table 3 Emission intensities of reaction mixtures for various amino compounds derivatized with FMQC

Amine	Emission intensity, a.u.[a]
n-Propylamine	100
n-Butylamine	126
n-Propylamine	132
n-Hexylamine	150
Diethylamine	3.5
Di-*n*-propylamine	0.60
Aniline	0.67
p-Methoxyaniline	0.20
p-Chloroaniline	0.20
Blank	0.56

Each amine (450 μM) was derivatized with FMQC and Et3N at 100 ºC for 1 hr. a Emission intensity at 445 nm (excitation at 350 nm)

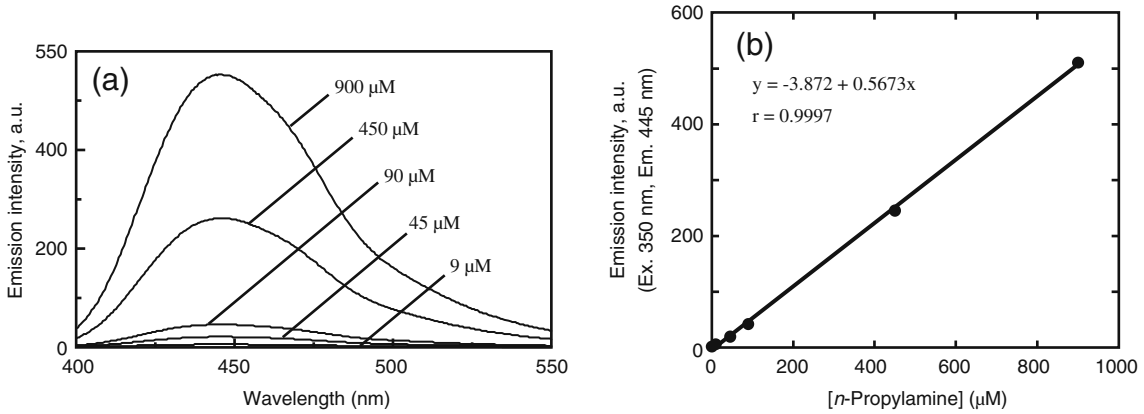

Fig. 6 **a** Fluorescence emission spectra (excitation at 350 nm) of the FMQC-derivatives for various amounts of *n*-propylamine in 10 mM aqueous HCl; **b** calibration line of derivatized *n*-propylamine

ment for the primary amino compound was obtained for diethylamine. Whereas, for all other amino compounds, no spectral change was obtained, suggesting that the derivatization reaction hardly proceeded for these compounds. Table 3 shows the fluorescence emission intensities at 445 nm detected with excitation at 350 nm. The reaction mixtures for all the aliphatic primary amino compounds exhibited a strong fluorescence similar to that of *n*-propylamine, however, the intensity for diethylamine was very weak.

The reaction mixture for diethylamine showed a slight fluorescence despite the change of the absorption spectrum. To clarify this phenomenon, we synthesized the derivative **8** by reacting FMQC and diethylamine as described in Fig. 3. The absorption and fluorescence properties of **8** were evaluated, and it was revealed that **8** exhibited a much weaker fluorescence than **7**, its Φ value was 0.009 in 10 mM aqueous HCl (Table 2). These obtained results indicated that FMQC is the fluorescence enhancement-type derivatizing reagent suitable for the analysis of aliphatic primary amino compounds.

Determination of aliphatic primary amino compounds derivatized with FMQC

After the optimizations of the basic catalyst and reaction conditions (temperature and time), the procedure indicated in the Experimental section was selected. With the optimized reaction conditions, various amounts of *n*-propylamine were derivatized with FMQC, and the fluorimetric determination was performed using the spectrofluorometer. Figure 6 shows the fluorescence emission spectra with the excitation wavelength at 350 nm. The fluorescence emission intensity quantitatively increased in accordance with the amount of *n*-propylamine added, and the calibration line for the derivatized *n*-propylamine was linear over the range from 9 μM to 900 μM (r=0.9997). We also demonstrated the separation analysis of four aliphatic primary amino compounds (*n*-propylamine, *n*-butylamine, *n*-pentylamine, *n*-hexylamine) using FMQC. These four amino compounds were derivatized with FMQC under the optimized conditions, and subjected to the RP-HPLC separation without removing the excess FMQC. Figure 7 shows the chromatogram of the derivatives for the four amino compounds, and the derivatives could be well separated within 45 min using the isocratic mobile phase of MeCN-TFA-water (35:0.05:65, v/v/v). Concerning the sensitivity, the signal-to-noise ratio (S/N) for each amino compound (10 fmol per injection) were 133, 140, 87, and 58, respectively, indicating that their lower limits of detection (S/N=3) were subfmol per injection, which are equivalent to that of the derivatives of NBD-F (fmol-subfmol) [28] and more sensitive than those of the derivatives for OPA (pmol) [29] and fluorescamine (pmol) [30]. Considering the high photo-and thermo-stabilities of the FMQC derivatives, FMQC could be a novel and useful option for the fluorescence enhancement-type derivatizing reagent for amino compounds.

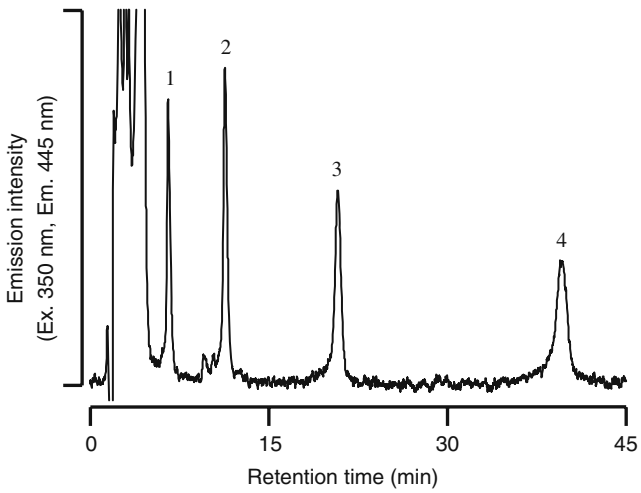

Fig. 7 Chromatogram of the derivatives for four aliphatic primary amino compounds (10 fmol): (1) *n*-propylamine, (2) *n*-butylamine, (3) *n*-pentylamine, (4) *n*-hexylamine

Conclusions

In the present study, we designed and synthesized six possible candidates as fluorescence enhancement-type derivatizing reagents for amino compounds. Among these compounds, FMQC exhibited a reactivity to amino compounds with a drastic fluorescence enhancement. The FMQC derivative of *n*-propylamine showed not only a high Φ, but also high photo-and thermo-stabilities. These properties enabled a simple and highly sensitive analysis using a spectrofluorometer as well as using an RP-HPLC, and the expanded applications of FMQC to various matrices are expected.

Acknowledgements This work was partially supported by the Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists (J.H.).

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