4-(2-Aminoethylamino)-7*H*-benz[*de*]benzimidazo[2,1-*a*]isoquinoline-7-one as a Highly Sensitive Fluorescent Labeling Reagent for Carnitine

Ken-ichi Nakaya, Toshiyuki Tanaka,† Yoshiaki Shirataki,†† Hisayoshi Shiozaki,†††
Kazumasa Funabiki, Katsuyoshi Shibata, and Masaki Matsui*

Department of Chemistry, Faculty of Engineering, Gifu University, Yanagido, Gifu 501-1193

† Gifu Prefectural Institute of Health and Environmental Sciences, 1-1 Naka Fudogaoka, Kakamigahara, Gifu 504-0838

†† Faculty of Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakato, Saitama 350-0295

††† Technology Research Institute of Osaka Prefecture, 2-7-1 Ayumino, Izumi, Osaka 594-1157

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4-(2-Aminoethylamino)-7*H*-benz[*de*]benzimidazo[2,1-*a*]isoquinoline-7-one, synthesized and identified by a HMBC study, can be used as a fluorescent labeling reagent for carnitine. The excitation and emission maxima in acetonitrile were observed at λ 454 and 508 nm, respectively. This reagent smoothly reacted with carnitine in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride to afford the corresponding amide under mild conditions. The detection limit (S/N > 3) of carnitine was ca. 12 fmol.

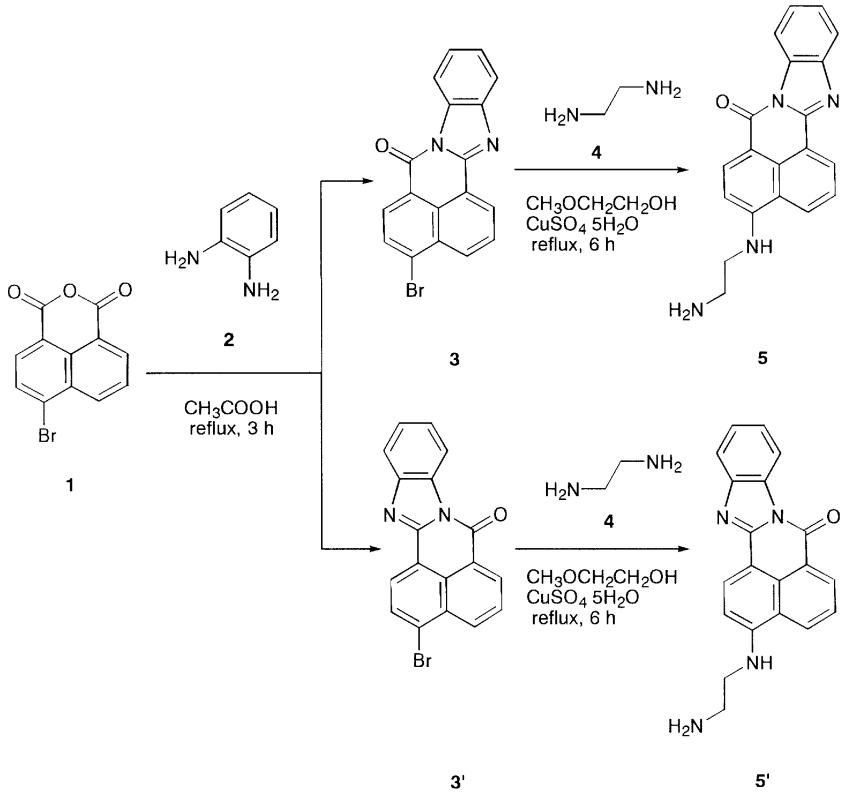
Carnitine, (4-(trimethylammonio)-3-hydroxybutanoate), which acts as a carrier of long-chain fatty acids into the mitochondrial matrix, has been analyzed by high-performance liquid chromatography (HPLC) using fluorescent labeling reagents. 9-Anthryldiazomethane (ADAM),[1,2] 4-bromophenacyl trifluoromethanesulfonate,[3] 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate,[3] *N*-(9-acridinyl)maleimide,[4] 9-fluorenylmethyl chloroformate,[5–7] 1-aminoanthracene,[8] 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone,[9,10] 2-(4-hydrazinocarbonylphenyl)-4,5-diphenylimidazole,[11,12] *N*-[4-(2-benzimidazolyl)phenyl]maleimide,[13] pyrene-1-carbonyl cyanide,[14] and 6-(4-aminophenyl)-3-cyano-4-[4-(dimethylamino)phenyl]-2-methylpyridine[15] have been reported as the fluorescent labeling reagents for carnitine. Fluorescent labeling reagents are required to have high sensitivity, appropriate excitation and emission maxima, and a functional group smoothly reacting with target compounds. It is of significance to obtain new fluorescent labeling reagents having a high detection limit and good fluorescent spectral features. 7*H*-Benz[*de*]benzimidazo[2,1-*a*]isoquinoline-7-ones have been prepared by the condensation of 1,8-naphthalenedicarboxylic anhydrides with *o*-phenylenediamines. This reaction can produce isomers. For example, when 4-substituted 1,8-naphthalenedicarboxylic anhydride reacts with *o*-phenyenediamine, 3- and 4-substituted 7*H*-benz[*de*]benzimidazo[2,1-*a*]isoquinoline-7-ones can be formed. Fluorescent 7*H*-benz[*de*]benzimidazo[2,1-*a*]isoquinoline-7-ones have been used as dyes and pigments for synthetic fibers and plastics.[16–24] Therefore, less attention has been paid to the separation and identification of these isomers. We report here the synthesis, identification, and application of novel 4-(2-aminoethylamino)-7*H*-benz[*de*]benzimidazo[2,1-*a*]isoquinoline-7-one as a fluorescent labeling reagent for carnitine.

Results and Discussion

The synthesis of the 4- and 3-(2-aminoethylamino)-7*H*-benz[*de*]benzimidazo[2,1-*a*]isoquinoline-7-ones **5** and **5′** is shown in Scheme 1. 4-Bromo-1,8-naphthalenedicarboxylic anhydride (**1**) reacted with *o*-phenylenediamine (**2**) to give the 4- and 3-bromo derivatives **3** and **3′**, which were carefully isolated by column chromatography (SiO_2, C_6H_6). The separated components, **3** and **3′**, reacted with ethylenediamine (**4**) to afford the corresponding substitution products, **5** and **5′**, respectively.

The structure of **5** was confirmed by elemental analysis, high-resolution MS, and NMR spectra. The ^{1}H and ^{13}C NMR spectral data and CH long-range correlations in the HMBC spectrum of **5** are shown in Table 1 and Fig. 1, respectively. Normally, carbonyl-carbon is deshielded more strongly than imino-carbon, due to a larger electron-withdrawing nature of the oxygen atom.[25] Therefore, the peaks at δ 159.7 and 149.3 in the ^{13}C NMR spectra were attributed to C-7 and C-13a, respectively. Then, the significant ^3J long-range correlations were observed between H-1′/C-4, H-6/C-4, and H-6/C-7, as indicated by the dotted allows in Fig. 1. Thus, compound **5** was identified as 4-(2-aminoethylamino)-7*H*-benz[*de*]benzimidazo[2,1-*a*]isoquinoline-7-one.

The absorption and fluorescence spectra of **5** and **5′** in acetonitrile are summarized in Table 2. Both the absorption (λ_{max}) and emission maxima (λ_{em}) of **5** were more hypsochromic than that of **5′**. The MO (INDO/S) calculation supported that the 4-



Scheme 1.

Table 1. ^{1}H and ^{13}C NMR spectral data of **5**[a)]

No.	δ_H	δ_C
1	8.65 dd (7.3, 0.9)	126.8
2	7.65 dd (8.2, 7.3)	126.2
3	8.57 dd (8.2, 0.9)	126.2
3a		120.4
4		152.0
5	6.79 d (8.6)	104.3
6	8.36 d (8.6)	134.9
6a		107.6
7		159.7
8a		131.5
9	8.42 m	115.3
10	7.40 m	124.1
11	7.42 m	124.8
12	7.79 m	119.3
12a		143.4
13a		149.3
13b		119.4
13c		128.7
1′	3.37 t (6.4)	36.5
2′	2.88 t (6.4)	40.0

a) Measured in DMSO-d_6 (500 and 125 MHz). All signals were assigned by ^{1}H–^{1}H COSY, HMQC, and HMBC spectra. The coupling constants (parentheses) are in Hz

(2-aminoethylamino) derivative **5** (LUMO: –1.11 eV, HOMO: –7.39 eV) was more hypsochromic than **5′** (LUMO: –1.13 eV, HOMO: –7.26 eV). The introduction of the electron-donating 2-aminoethylamino group at the 3-position can raise the energy

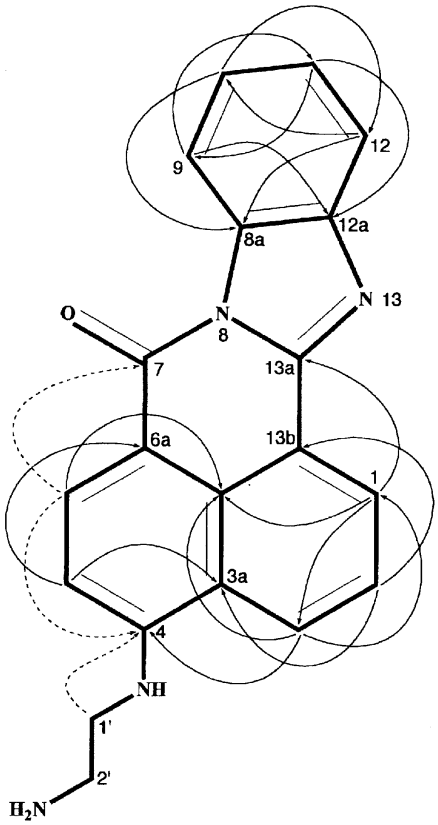


Fig. 1. CH long range correlation in HMBC spectrum of **5**. The important correlations are shown in dotted allows.

Table 2. Fluorescence spectra of 4- and 3-(2-aminoethylamino)-7*H*-benz[*de*]benzimidazo[2,1-*a*]isoquinoline-7-ones **5** and **5′**

Compound	λ_{max}[a)] nm	ε[a)]	λ_{ex}[a)] nm	λ_{em}[a)] nm	RFI[b)]
5	451	24200	454	508	207
5′	484	12400	487	589	2

a) Measured in acetonitrile. b) Relative fluorescence intensity measured in acetonitrile (1×10^{-5} mol dm^{-3}, 25 °C, 6-(4-aminophenyl)-3-cyano-4-[4-(diethylamino)phenyl]-2-methylpyridine: λ_{ex} = 365 nm, λ_{em} = 514 nm, RFI = 100; 7-(diethylamino)-4-methylcoumarin: λ_{ex} = 372 nm, λ_{em} = 434 nm, RFI = 333).

level of HOMO to produce a bathochromic shift in **5′**.

Interestingly, the relative fluorescence intensity (RFI) of the 4-isomer **5** was much greater than that of the 3-isomer **5′**. The RFI of **5** was about two-times more intense than that of 6-(4-aminophenyl)-3-cyano-4-[4-(diethylamino)phenyl]-2-methylpyridine, a previously reported fluorescent labeling reagent for carnitine.[15] The Stoke's shift of **5** and **5′** were observed to be 54 and 102 nm, respectively. This result suggests a more rigid conformation of **5** in the excited state than that of **5′**.

Since the HPLC analysis of amino acids, such as carnitine, is usually performed in a reverse phase, the effect of water on the RFI in **5** was examined. The result is shown in Fig. 2. Though the RFI of the 6-(4-aminophenyl)-3-cyano-4-[4-(diethylamino)phenyl]-2-methylpyridine decreased with the addition of water, that of **5** slightly increased up to a water concentration of 70%, and then decreased. Compound **5** was sufficiently soluble in aqueous acetonitrile used as the solvent in the HPLC analysis.

The reactivity of **5** with carnitine is summarized in Table 3. Compound **5** smoothly reacted with carnitine in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) under mild conditions.

The HPLC analysis of carnitine by **5** is shown in Fig. 3 Major peaks were observed at retention times of 6.774 and 9.247 min. The former component was the labeling reagent **5**. The high resolution MS spectrum of the latter component showed its MH^+ ion peak (relative intensity: 100%) at *m*/*z* 472.2352,

Table 3. Reactivity of **5** with carnitine

Run	Additive	Reaction time h	Reaction temp. °C	Reactivity[a)]
1	none	6	60	0
2	DCC[b)]	6	60	0
3	EDC[c)]	6	60	100
4	EDC[c)]	6	20	100
5	EDC[c)]	0.5	20	91
6	EDC[c)]	2	20	100

a) Reactivity was calculated on the basis of fluorescence intensity by HPLC analysis (column: Inert SIL (4.6 mm × 150 mm); mobile phase: hexane–choroform = 7 : 3; flow rate: 0.8 ml min^{-1}; detection: 254 nm). b) Dicyclohexylcarbodiimide. c) 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride.

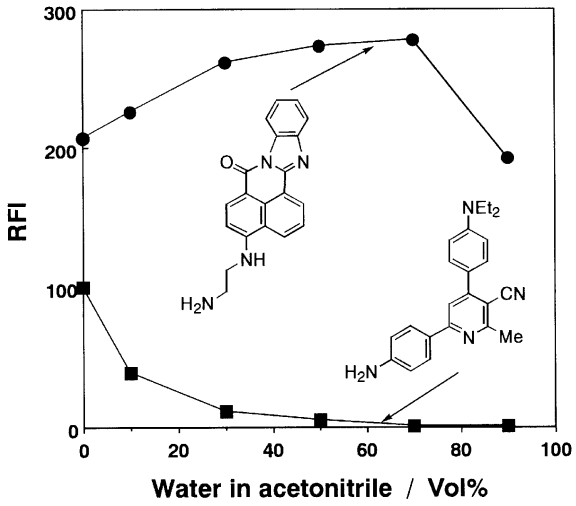


Fig. 2. Effect of added water on the relative fluorescence intensity of **5**. The relative fluorescence intensity was measured at 25 °C on 1×10^{-5} mol dm^{-3} of substrate (**5**: λ_{ex} = 454 nm, λ_{em} = 508 nm, 6-(4-aminophenyl)-3-cyano-4-[4-(diethylamino)phenyl]-2-methylpyridine: λ_{ex} = 365 nm, λ_{em} = 514 nm).

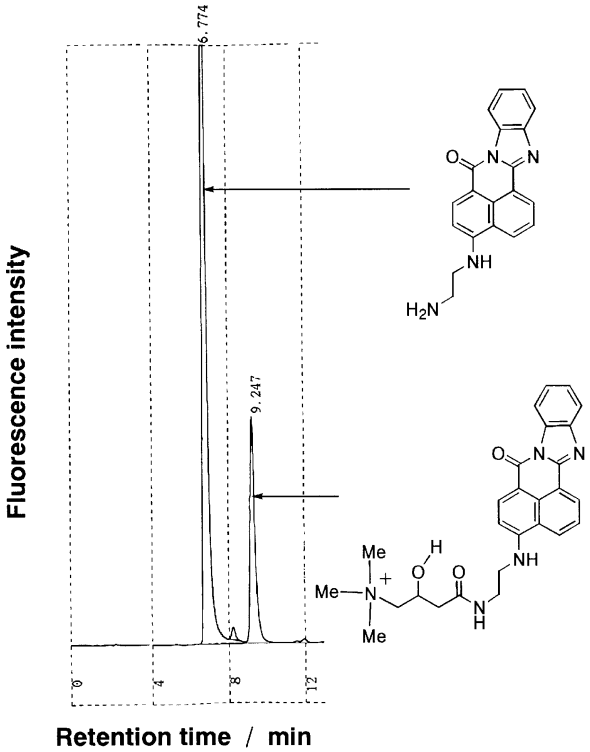


Fig. 3. HPLC analysis of labeled carnitine. Column: TSK gel ODS 80 TM (4.6 mm × 150 mm); mobile phase: CH_3CN–50 mM KH_2PO_4 (100 : 225) containing 10 mM of TFA, flow rate: 0.8 ml min^{-1}; excitation: 454 nm, detection: 508 nm.

showing labeled carnitine (calcd for $C_{27}H_{30}N_5O_3$: 472.2349).

Conclusion

We have synthesized 4-(2-aminoethylamino)-7*H*-benz[*de*]-benzimidazo[2,1-*a*]isoquinoline-7-one. This compound was identified by a HMBC study. The excitation and emission maxima in acetonitrile were observed at λ 454 and 508 nm, respectively. This reagent smoothly reacted with carnitine in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride to afford the labeled product under mild conditions. The detection limit (S/N > 3) of carnitine by this reagent was ca. 12 fmol, similar to that of ADAM. However, both the excitation and emission maxima of 4-(2-aminoethylamino)-7*H*-benz[*de*]benzimidazo[2,1-*a*]isoquinoline-7-one were more bathochromic than those of ADAM (λ_{ex}: 365 nm, λ_{em}: 412 nm in CH_3CN–CH_3OH), being the preferred properties for fluorescent labeling reagents.

Experimental

Melting points were measured with a Yanagimoto MP-S2 micro-melting-point apparatus. Fluorescence spectra were measured with a Jasco FP-777 spectrofluorometer. NMR spectra were taken on JEOL α-400 and α-500 spectrometers. MS spectra were recorded on Shimadzu QP-1000 and JEOL JMS-700 spectrometers. Liquid chromatography was performed with a Jasco Triroter-V instrument. DL-Carnitine hydrochloride was obtained from NAKALAI TESQUE, Inc. 4-Bromo-1,8-naphthalenedicarboxylic anhydride (**1**), *o*-phenylenediamine (**2**), and ethylenediamine (**4**) were purchased from Tokyo Kasei Co., Ltd.

Synthesis of Bromo-7*H*-benz[*de*]benzimidazo[2,1-*a*]isoquinoline-7-ones 3 and 3′. To an acetic acid solution (50 ml) of 4-bromo-1,8-naphthalenedicarboxylic anhydride **1** (5.0 g, 18 mmol) was added *o*-phenylenediamine **2** (2.4 g, 22 mmol), and the mixture was refluxed for 3 h. After the reaction was completed, the products were extracted with chloroform (100 ml × 2). The Rf value of **3′** in TLC (SiO_2, C_6H_6) was slightly larger (R_f = 0.36) than that of **3** (R_f = 0.34). Therefore, compounds **3** and **3′** were separated by column chromatography (SiO_2, C_6H_6), with checking the purity of the eluate by HPLC (Mightysil Si 60 150-4.6 (5 μm), hexane : chloroform = 8 : 2, 254 nm). These products were recrystallized from toluene.

4-Bromo-7*H*-benz[*de*]benzimidazo[2,1-*a*]isoquinoline-7-one (3): Yield 2.584 g (41%); mp 291–292 °C; ^{1}H NMR ($CDCl_3$) δ 7.48–7.52 (m, 2H), 7.82–7.93 (m, 2H), 8.13 (d, *J* = 7.6 Hz, 1H), 8.53–8.55 (m, 1H), 8.54 (d, *J* = 7.6 Hz, 1H), 8.61 (d, *J* = 7.6 Hz, 1H), 8.91 (d, *J* = 7.3 Hz, 1H); EIMS (70 eV) *m*/*z* (rel intensity) 350 (M^++2; 98), 348 (M^+; 100).

3-Bromo-7*H*-benz[*de*]benzimidazo[2,1-*a*]isoquinoline-7-one (3′): Yield 2.457 g (39%); mp 262–263 °C; ^{1}H NMR ($CDCl_3$) δ 7.48–7.52 (m, 2H), 7.82–7.88 (m, 1H), 7.93 (dd, *J* = 7.8 and 7.3 Hz, 1H), 8.10 (d, *J* = 7.8 Hz, 1H), 8.52–8.57 (m, 1H), 8.68 (d, *J* = 7.8 Hz, 1H), 8.69 (dd, *J* = 7.8 and 1.0 Hz, 1H), 8.86 (dd, *J* = 7.3 and 1.0 Hz, 1H); EIMS (70 eV) *m*/*z* (rel intensity) 350 (M^++2; 98), 348 (M^+; 100).

Synthesis of (2-Aminoethylamino)-7*H*-benz[*de*]benzimidazo[2,1-*a*]isoquinoline-7-ones 5 and 5′. To a 2-methoxyethanol solution (10 ml) of bromo-7*H*-benz[*de*]benzimidazo[2,1-*a*]isoquinoline-7-one **3** or **3′** (350 mg, 1 mmol) were added copper(II) sulfate pentahydrate (100 mg) and ethylenediamine **4** (840 mg, 14 mmol). The mixture was refluxed for 6 h. After the reaction was completed, the product was extracted with chloroform (100 ml × 2), purified by column chromatography (SiO_2, $CHCl_3$ then CH_3OH), and recrystallized from toluene. The physical and spectral data are given below.

4-(2-Aminoethylamino)-7*H*-benz[*de*]benzimidazo[2,1-*a*]isoquinoline-7-one (5): Yield 42.6 mg (13%); mp 242–244 °C; EIMS (70 eV) *m*/*z* (rel intensity) 328 (M^+; 45), 298 (100), 285 (30), 270 (20); HRMS Calcd for $C_{20}H_{16}N_4O$: 328.1323. Found: *m*/*z* 328.1322. Found: C, 72.98; H, 5.11; N, 17.18%. Calcd for $C_{20}H_{16}N_4O$: C, 73.15; H, 4.91; N, 17.06%.

3-(2-Aminoethylamino)-7*H*-benz[*de*]benzimidazo[2,1-*a*]isoquinoline-7-one (5′): Yield 32.8 mg (10%); mp 128–129 °C; ^{1}H NMR ($CDCl_3$) δ 3.20 (t, *J* = 5.7 Hz, 2H), 3.44 (q, *J* = 5.7 Hz, 2H), 6.08 (br s), 6.81 (d, *J* = 8.4 Hz, 1H), 7.37–7.47 (m, 2H), 7.74 (dd, *J* = 8.3 and 7.3 Hz, 1H), 7.81–7.83 (m, 1H), 8.32 (d, *J* = 8.3 Hz, 1H), 8.55–8.57 (m, 1H), 8.71 (d, *J* = 8.4 Hz, 1H), 8.82 (d, *J* = 7.3 Hz, 1H); EIMS (70 eV) *m*/*z* (rel intensity) 328 (M^+; 79), 298 (100), 285 (11), 270 (41). Found: C, 73.23; H, 5.03; N, 17.23%. Calcd for $C_{20}H_{16}N_4O$: C, 73.15; H, 4.91; N, 17.06%.

HPLC Analysis of Carnitine. A methanol solution of carnitine (1 μg mL^{-1}) was prepared. After evaporating one ml of this solution, an acetonitrile–pyridine mixed solution (8:2, 1 mL^{-1}) of **5** (12 nmol) and EDC (6.2 nmol) was added. The mixture was stirred for 2 h at 20 °C. The solution was then analyzed by HPLC. The analytical conditions in the HPLC are shown in Fig. 3.

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