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

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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(1H)-quinoxalinone,^{9,10} 2-(4-hydrazinocarbonylphenyl)-4,5-diphenylimidazole,^{11,12} N-[4-(2benzimidazolyl)phenyl]maleimide,13 pyrene-1-carbonyl cyanide,14 and 6-(4-aminophenyl)-3-cyano-4-[4-(dimethylamino)phenyl]-2-methylpyridine¹⁵ 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. 7H-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 7H-benz[de]benzimidazo[2,1-a]isoquinoline-7-ones can be formed. Fluorescent 7H-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-(2aminoethylamino)-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 (SiO₂, C₆H₆). 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 ¹H and ¹³C 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 ¹³C NMR spectra were attributed to C-7 and C-13a, respectively. Then, the significant ³*J* 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-



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

No.	$\delta_{ m H}$	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 ¹H–¹H 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





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

Compound	$\lambda_{max}{}^{a)}$	$\boldsymbol{\varepsilon}^{\mathrm{a})}$	$\lambda_{ex}{}^{a)}$	$\lambda_{em}{}^{a)}$	RFI ^{b)}
	nm		nm	nm	
5	451	24200	454	508	207
5'	484	12400	487	589	2

a) Measured in acetonitrile. b) Relative fluorescence intensity measured in acetonitrile $(1 \times 10^{-5} \text{ mol dm}^{-3}, 25 \text{ °C}, 6\text{-}(4\text{-aminophen$ yl)-3-cyano-4-[4-(diethylamino)phenyl]-2-methylpyridine: λ_{ex} = 365 nm, $\lambda_{em} = 514$ 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-(4aminophenyl)-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



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

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	Reaction temp.	Reactivity ^{a)}
		h	°C	
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.



Retention time / min

Fig. 3. HPLC analysis of labeled carnitine. Column: TSK gel ODS 80 TM (4.6 mm \times 150 mm); mobile phase: CH₃CN-50 mM KH₂PO₄ (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-7one were more bathochromic than those of ADAM (λ_{ex} : 365 nm, λ_{em} : 412 nm in CH₃CN–CH₃OH), 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 (SiO₂, C₆H₆) 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 (SiO₂, C₆H₆), 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; ¹H NMR (CDCl₃) \delta 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; ¹H NMR (CDCl₃) \delta 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 \times 2), purified by column chromatography (SiO₂, CHCl₃ then CH₃OH), 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; ¹H NMR (CDCl₃) \delta 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 C₂₀H₁₆N₄O: C, 73.15; H, 4.91; N, 17.06%.**

HPLC Analysis of Carnitine. A methanol solution of carnitine $(1 \ \mu g \ mL^{-1})$ 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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