

Fluorescent α,β -Unsaturated Carbonyl Compounds and 2-Methylpyridines. Their Application to a Quantitative Analysis of Carnitine

Ken-ichi Nakaya, Kazumasa Funabiki, Katsuyoshi Shibata,
Hiroshige Muramatsu, and Masaki Matsui*

Department of Chemistry, Faculty of Engineering, Gifu University, Yanagido, Gifu 501-11

(Received May 27, 1996)

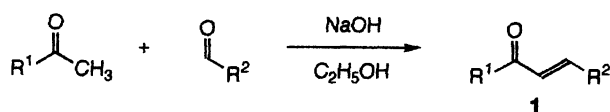
α,β -Unsaturated carbonyl compounds and 2-methylpyridines containing a 4-(dialkylamino)phenyl substituent can be used as fluorescent labeling reagents for the quantitative analysis of carnitine. 6-(4-Aminophenyl)-3-cyano-4-[4-(diethylamino)phenyl]-2-methylpyridine showed the best properties among the synthesized reagents.

Carnitine acts as a carrier of long-chain fatty acids into a mitochondrial matrix.¹⁾ To analyze carnitine, radiometric,²⁾ enzyme colorimetric,³⁾ and fluorescent HPLC⁴⁾ methods have been proposed. The HPLC method is the most facile among them. Though 9-anthryldiazomethane (ADAM, λ_{ex} : 365 nm, λ_{em} : 450 nm) has generally been used as a fluorescent labeling reagent of carnitine,⁵⁾ this compound is not stable to store for a long time. There are many compounds, which show absorption and emission bands in UV region (< 300 nm) and around 325—400 nm, respectively in the components of serum. Therefore, it is required that the fluorescent labeling reagents are stable enough to store and have bathochromic excitation (> 300 nm) and emission (> 450 nm) bands. In this report, strongly fluorescent α,β -unsaturated carbonyl compounds and 2-methylpyridines with bathochromic excitation and emission bands have been synthesized. Their application to the quantitative analysis of carnitine have also been examined.

Results and Discussion

α,β -Unsaturated carbonyl compounds **1** were prepared by an aldol condensation reaction, as shown in Scheme 1. Methyl ketones reacted with aldehydes in the presence of sodium hydroxide to give α,β -unsaturated carbonyl compounds **1** in moderate to good yields. 3-[4-(Dimethylamino)phenyl]-1-(4-hydroxyphenyl)prop-2-en-1-one (**1c**) was obtained by the demethylation of 3-[4-(dimethylamino)phenyl]-1-(4-methoxyphenyl)prop-2-en-1-one (**1c'**).⁶⁾

Table 1 summarizes the fluorescence spectra of prop-2-en-1-one derivatives **1**. All of the derivatives showed suitable

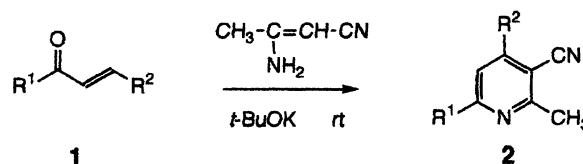


Scheme 1. Synthesis of α,β -unsaturated carbonyl compounds **1**.

excitation and emission bands (λ_{ex} : > 350 nm, λ_{em} : > 500 nm) as the labeling reagents of carnitine. 1-(4-Aminophenyl) derivative **1a** showed more intense fluorescence than did 1-(3-aminophenyl) and -(4-hydroxyphenyl) derivatives **1b** and **1c**. Furyl, thienyl, anthryl, and pyrenyl derivatives **1i—l** were less intense fluorescent compounds than **1a**. Interestingly, 1-[4-(dimethylamino)phenyl] derivative **1a** showed much more stronger fluorescence than did 3-[4-(dimethylamino)phenyl] derivative **1d**. 3-[4-(Dialkylamino)phenyl] derivatives **1a—c, e, f** were found to be strong fluorescent compounds.

The fluorescence intensity increases by extending a π -conjugated planar system.⁷⁾ To obtain more intense fluorescent compounds, 2-methylpyridines were prepared from strongly fluorescent α,β -unsaturated carbonyl compounds, as shown in Scheme 2. The reaction of α,β -unsaturated carbonyl compounds **1** with β -aminocrotononitrile in the presence of potassium *t*-butoxide at room temperature gave the 3-cyano-2-methylpyridines **2** in moderate to good yields.

A plausible reaction mechanism for the formation of **2** is shown in Scheme 3. β -Aminocrotononitrile exists as *Z* and *E* isomers, which are in equilibrium with amino and imino tautomers in solution. A Michael addition of a carbanion derived from the imino tautomer to α,β -unsaturated carbonyl compounds **1** can form an enolic adduct which is in equilibrium with the keto-enamine form, followed by an intramolecular condensation and oxidation by oxygen in the atmosphere to give a final products **2**.

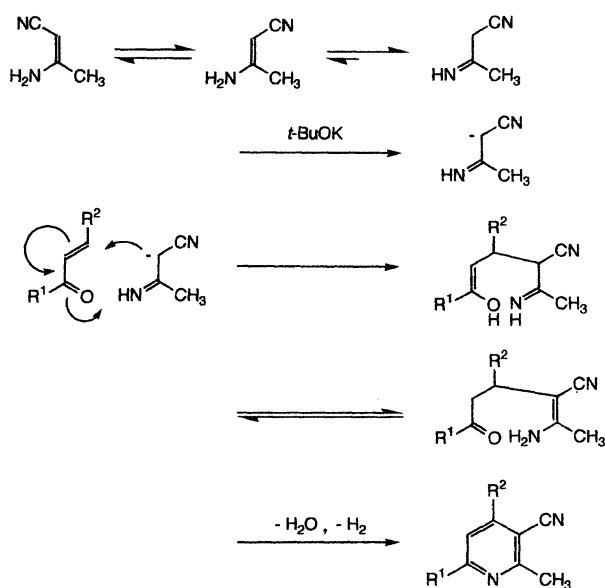


Scheme 2. Synthesis of 3-cyano-2-methylpyridines **2**.

Table 1. Fluorescence Spectra and Relative Sensitivity of α,β -Unsaturated Carbonyl Compounds 1

$\text{R}^1-\text{C}(=\text{O})-\text{CH}=\text{CH}-\text{R}^2$						
Compd	R ¹	R ²	$\lambda_{\text{ex}}^{\text{a)}}$ /nm	$\lambda_{\text{em}}^{\text{a)}}$ /nm	RFI ^{b)}	Rel sens ^{c)}
1a			399 422 ^{d)}	510 540 ^{d)}	100 5 ^{d)}	100
1b			406	512	70	75
1c			399	519	60	60
1d			392	522	1	—
1e			419	510	130	132
1f			429	538	62	17
1g			352	520	< 1	—
1h			360	523	1	—
1i			357	520	< 1	—
1j			357	531	< 1	—
1k			346	496	< 1	—
1l			389	522	1	—

a) Measured in CH₃CN. b) Relative fluorescence intensity measured in CH₃CN (1×10^{-5} mol dm⁻³, 25 °C, **1a** = 100). c) Relative sensitivity (**1a** = 100). d) Measured in an acetonitrile–potassium dihydrogenphosphate (50 mM) mixed solution (6 : 4) containing trifluoroacetic acid (TFA, 10 mM).

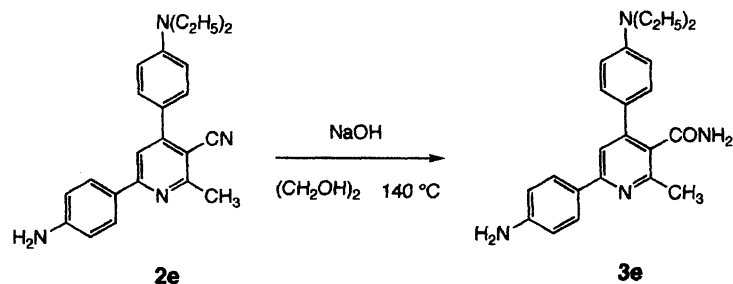
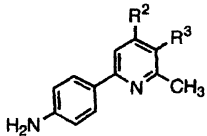


Scheme 3. Plausible mechanism for the formation of 3-cyano-2-methylpyridines **2**.

3-Cyano-2-methylpyridine **2e** was hydrolyzed to give the 3-carbamoyl derivative **3e** (Scheme 4).

The fluorescence spectra of 2-methylpyridine derivatives **2** and **3e** are indicated in Table 2. These compounds also showed suitable fluorescence bands (λ_{ex} : > 325 nm, λ_{em} : > 500 nm). As expected, the relative fluorescence intensity (RFI) of compounds **2a** and **2e** were larger than those of **1a** and **1e**, respectively. Unexpectedly, the juloridino derivative **2f** showed less fluorescence than did **2e**. 3-Cyano derivative **2e** showed more intense fluorescence than the 3-carbamoyl derivative **3e**. Both the excitation and emission maxima of **1a** and **2e** showed positive bathochromic shifts (**1a**: 23 and 30 nm, respectively, **2e**: 10 and 26 nm, respectively) in an acetonitrile–potassium dihydrogenphosphate mixed solution used as a mobile phase in HPLC analysis. Unfortunately, the fluorescence intensity of both **1a** and **2e** remarkably decreased in the mobile phase used in the HPLC analysis. The fluorescence intensity of **2e** was found to be the highest, about 8-times that of **1a** in the mobile phase.

The reactivity of **2e** with carnitine was examined by an HPLC analysis. The result is given in Table 3. Carnitine did not react with **2e** in the absence of 1-ethyl-3-[3-(dimeth-

Scheme 4. Synthesis of 3-carbamoyl-2-methylpyridine **3e**.Table 2. Fluorescence Spectra and Relative Sensitivity of 2-Methylpyridines **2** and **3e**


Compd	R ²	R ³	$\lambda_{\text{ex}}^{\text{a)}}$ /nm	$\lambda_{\text{em}}^{\text{a)}}$ /nm	RFI ^{b)}	Rel sens ^{c)}
2a		CN	353	510	153	192
2e		CN	353 463 ^{d)}	508 533 ^{d)}	205 38 ^{d)}	804
2f		CN	344	549	40	73
3e		CONH ₂	326	499	58	—

a) Measured in CH₃CN. b) Relative fluorescence intensity measured in CH₃CN (1×10^{-5} mol dm⁻³, 25 °C. **1a** = 100). c) Relative sensitivity (**1a** = 100). d) Measured in an acetonitrile–potassium dihydrogenphosphate (50 mM) mixed solution (6 : 4) containing trifluoroacetic acid (TFA, 10 mM).

Table 3. Reactivity of Carnitine with **2e**

Run	Additive	Reaction time	Reaction temp	Relative reactivity ^{a)}
		h	°C	
1	None	6	60	0
2	DCC	6	60	1
3	EDC	6	60	100
4	EDC	6	20	100
5	EDC	0.5	20	96
6	EDC	2	20	100

a) Reactivity was calculated on the basis of fluorescence intensity by HPLC analysis.

ylamino)propyl]carbodiimide hydrochloride (EDC). In the presence of EDC, the reaction proceeded smoothly under mild conditions (20 °C) in a short time (2 h).

The results of the relative sensitivity for the reaction of carnitine with selected α,β -unsaturated carbonyl compounds **1** and 2-methylpyridines **2**, measured by an HPLC analysis, are also summarized in Tables 1 and 2. 3-Cyano-2-methylpyridines **2** were more sensitive than α,β -unsaturated carbonyl compounds **1**. This can be attributed to the more intense fluorescence of **2** than **1** in the mobile phase.

A typical chromatogram in the analysis of carnitine by **2e** is shown in Fig. 1. The component at a retention time of 3.4 min showed its MH⁺ ion peak at m/z 500 by an LC-MS analysis.

In conclusion, 6-(4-aminophenyl)-3-cyano-4-[4-(diethylamino)phenyl]-2-methylpyridine (**2e**) was found to be the most suitable fluorescent labeling reagent for a quantitative analysis of carnitine among the synthesized reagents. The detection limit of **2e** was 60 fmol. This detection limit was about one tenth compared with that of ADAM.

Experimental

Instruments: The melting points were measured with a Yanagimoto MP-S2 micro-melting-point apparatus. NMR spectra were taken on JEOL 270-GX and α -400 spectrometers. Mass spectra were recorded on Shimadzu QP-1000 (for EIMS) and LC/MS-QP1100EX (for LC/MS) instruments. The fluorescence spectra were measured with a Hitachi F-4500 spectrometer. Liquid chromatography was performed with a JEOL Trirotor-V instrument.

Materials: 9-Formyljulolidine was prepared by Vilsmeier formylation of julolidine. Methyl ketones, aldehydes, potassium *t*-butoxide, and β -aminocrotononitrile were purchased from Tokyo Kasei Co., Ltd.

Synthesis of α,β -Unsaturated Carbonyl Compounds (1). To an ethanol solution (20 ml) of methyl ketone (10 mmol) and aldehyde (10 mmol) was added sodium hydroxide (0.5 g), and stirred overnight. After the reaction was complete, the mixture was concentrated and poured into water. The resulting precipitate was filtered, dried, purified by column chromatography (SiO₂, CH₂Cl₂), and recrystallized from ethanol. 3-[4-(Dimethylamino)phenyl]-1-(4-hydroxyphenyl)prop-2-en-1-one (**1c**) was obtained by demethylation of 3-[4-(dimethylamino)phenyl]-1-(4-methoxyphenyl)prop-2-en-1-one (**1c'**) using sodium ethanethiolate.⁶⁾ The physical and spectral data are given below.

1-(4-Aminophenyl)-3-[4-(dimethylamino)phenyl]prop-2-en-1-one (1a): Yield 49%; mp 187–188 °C; ¹H NMR (DMSO-*d*₆) δ = 2.99 (s, 6H), 6.04 (s, 2H), 6.62 (d, J = 8.8 Hz, 2H), 6.73 (d, J = 8.8 Hz, 2H), 7.58 (s, 2H), 7.65 (d, J = 8.8 Hz, 2H), 7.89 (d, J = 8.8 Hz, 2H); EIMS (70 eV) m/z (rel intensity) 266 (M⁺; 100), 265 (34), 121 (36), 120 (37), 119 (21), 92 (22), 65 (28); UV (CH₃CN) 399 (30600) nm.

1-(3-Aminophenyl)-3-[4-(dimethylamino)phenyl]prop-2-en-1-one (1b): Yield 50%; mp 157–158 °C; ¹H NMR (DMSO-*d*₆) δ = 3.04 (s, 6H), 3.80 (br s, 2H), 6.69 (d, J = 8.8 Hz, 2H), 6.86 (dd, J = 7.8 and 3.0 Hz, 1H), 7.28–7.31 (m, 2H), 7.29 (d, J = 15.6 Hz, 1H), 7.36–7.39 (m, 1H), 7.75 (d, J = 8.8 Hz, 2H), 7.77 (d, J = 15.6 Hz, 1H); EIMS (70 eV) m/z (rel intensity) 266 (M⁺; 100), 174 (38), 173 (33), 146 (19), 121 (37), 120 (32), 92 (32), 91 (34), 65 (19); UV (CH₃CN) 406 (28500) nm.

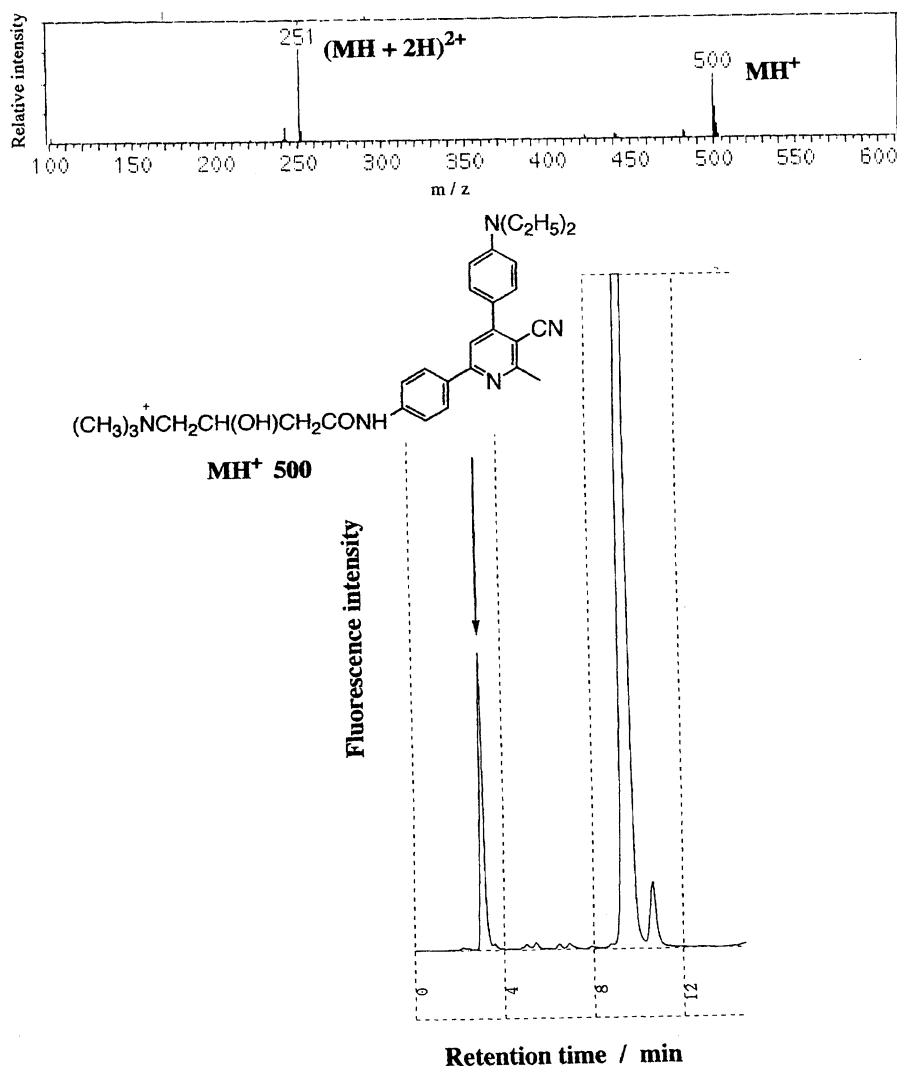


Fig. 1. HPLC analysis of carnitine.

3-[4-(Dimethylamino)phenyl]-1-(4-hydroxyphenyl)prop-2-en-1-one (1c): Yield 66%; mp 229–231 °C; $^1\text{H NMR}$ (DMSO- d_6) δ = 3.01 (s, 6H), 6.73 (d, J = 8.5 Hz, 2H), 6.87 (d, J = 8.5 Hz, 2H), 7.59 (d, J = 7.1 Hz, 2H), 7.64 (d, J = 7.7 Hz, 2H), 8.00 (d, J = 7.7 Hz, 2H), 10.22 (s, 1H); EIMS (70 eV) m/z (rel intensity) 267 (M^+ ; 100), 174 (24), 121 (38); UV (CH₃CN) 399 (25400) nm.

3-[4-(Dimethylamino)phenyl]-1-(4-methoxyphenyl)prop-2-en-1-one (1c'): Yield 57%; mp 129–130 °C; $^1\text{H NMR}$ (DMSO- d_6) δ = 3.04 (s, 6H), 3.89 (s, 3H), 6.70 (d, J = 9.0 Hz, 2H), 6.97 (d, J = 8.7 Hz, 2H), 7.36 (d, J = 15.6 Hz, 1H), 7.55 (d, J = 9.0 Hz, 2H), 7.79 (d, J = 15.6 Hz, 1H), 8.03 (d, J = 8.7 Hz, 2H); EIMS (70 eV) m/z (rel intensity) 281 (M^+ ; 100), 266 (28), 238 (26), 174 (31), 146 (32), 144 (40), 135 (42), 121 (47), 92 (51), 77 (74).

3-(4-Aminophenyl)-3-[(4-dimethylamino)phenyl]prop-2-en-1-one (1d): Yield 11%; mp 261–263 °C; $^1\text{H NMR}$ (DMSO- d_6) δ = 3.01 (s, 6H), 5.77 (s, 2H), 6.59 (d, J = 8.5 Hz, 2H), 6.75 (d, J = 8.5 Hz, 2H), 7.52 (d, J = 8.5 Hz, 2H), 7.53 (s, 2H), 7.98 (d, J = 8.5 Hz, 2H); EIMS (70 eV) m/z (rel intensity) 266 (M^+ ; 100), 238 (26); UV (CH₃CN) 392 (37000) nm.

1-(4-Aminophenyl)-3-[4-(diethylamino)phenyl]prop-2-en-1-one (1e): Yield 23%; mp 138–140 °C; $^1\text{H NMR}$ (DMSO- d_6) δ = 1.12 (t, J = 7.1 Hz, 6H), 3.40 (q, J = 7.1 Hz, 4H), 6.02, (s, 2H),

6.60 (d, J = 8.8 Hz, 2H), 6.68 (d, J = 9.0 Hz, 2H), 7.53 (s, 2H), 7.61 (d, J = 9.0 Hz, 2H), 7.87 (d, J = 8.8 Hz, 2H); EIMS (70 eV) m/z (rel intensity) 294 (M^+ ; 52), 280 (91), 279 (100), 120 (26); UV (CH₃CN) 419 (42700) nm.

1-(4-Aminophenyl)-3-(julolidine-9-yl)prop-2-en-1-one (1f): Yield 26%; mp 212–214 °C; $^1\text{H NMR}$ (CDCl₃) δ = 1.97 (quintet, J = 6.1 Hz, 4H), 2.67 (t, J = 6.1 Hz, 4H), 3.16 (t, J = 6.1 Hz, 4H), 4.06 (br s, 2H), 6.69 (d, J = 8.3 Hz, 2H), 7.10 (s, 2H), 7.38 (d, J = 16.3 Hz, 1H), 7.69 (d, J = 16.3 Hz, 1H), 7.92 (d, J = 8.3 Hz, 2H); EIMS (70 eV) m/z (rel intensity) 318 (M^+ ; 100), 317 (37), 145 (27), 145 (27), 144 (31), 120 (37); UV (CH₃CN) 429 (29600) nm.

1-(4-Aminophenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (1g): Yield 28%; mp 114–115 °C; $^1\text{H NMR}$ (DMSO- d_6) δ = 3.82 (s, 3H), 6.14 (s, 2H), 6.66 (d, J = 8.5 Hz, 2H), 7.01 (d, J = 8.3 Hz, 2H), 7.62 (d, J = 15.5 Hz, 1H), 7.75 (d, J = 15.5 Hz, 1H), 7.80 (d, J = 8.3 Hz, 2H), 7.95 (d, J = 8.5 Hz, 2H); EIMS (70 eV) m/z (rel intensity) 253 (M^+ ; 100), 252 (23), 238 (30), 120 (28); UV (CH₃CN) 352 (32000) nm.

1-(4-Aminophenyl)-3-(4-cyanophenyl)prop-2-en-1-one (1h): Yield 47%; mp 228–230 °C; $^1\text{H NMR}$ (DMSO- d_6) δ = 6.23 (s, 2H), 6.63 (d, J = 8.7 Hz, 2H), 7.64 (d, J = 15.4 Hz, 1H), 7.90 (d, J = 8.3 Hz, 2H), 7.95 (d, J = 8.7 Hz, 2H), 8.03 (d, J = 15.4 Hz,

1H), 8.05 (d, $J = 8.3$ Hz, 2H); EIMS (70 eV) m/z (rel intensity) 248 (M^+ ; 67), 220 (45), 120 (100), 92 (43), 65 (55); UV (CH_3CN) 360 (26300) nm.

1-(4-Aminophenyl)-3-(2-furyl)prop-2-en-1-one (1i): Yield 40%; mp 118–119 °C; 1H NMR ($DMSO-d_6$) $\delta = 6.19$ (s, 2H), 6.63 (d, $J = 7.7$ Hz, 2H), 6.66 (dd, $J = 3.4$ and 1.7 Hz, 1H), 7.01 (d, $J = 3.4$ Hz, 1H), 7.46 (d, $J = 15.4$ Hz, 1H), 7.53 (d, $J = 15.4$ Hz, 1H), 7.85 (d, $J = 7.7$ Hz, 2H), 7.86 (d, $J = 1.7$ Hz, 1H); EIMS (70 eV) m/z (rel intensity) 213 (M^+ ; 100), 185 (23), 159 (30), 156 (23), 120 (44); UV (CH_3CN) 357 (30400) nm.

1-(4-Aminophenyl)-3-(2-thienyl)prop-2-en-1-one (1j): Yield 11%; mp 135–136 °C; 1H NMR ($DMSO-d_6$) $\delta = 6.18$ (s, 2H), 6.63 (d, $J = 8.8$ Hz, 2H), 7.17 (dd, $J = 4.9$ and 3.7 Hz, 1H), 7.51 (d, $J = 15.1$ Hz, 1H), 7.61 (d, $J = 3.7$ Hz, 1H), 7.72 (d, $J = 4.9$ Hz, 1H), 7.79 (d, $J = 15.1$ Hz, 1H), 7.88 (d, $J = 8.8$ Hz, 2H); EIMS (70 eV) m/z (rel intensity) 229 (M^+ ; 100), 201 (39), 200 (24), 120 (44); UV (CH_3CN) 357 (27600) nm.

1-(4-Aminophenyl)-3-(9-anthryl)prop-2-en-1-one (1k): Yield 76%; mp 196–198 °C; 1H NMR ($CDCl_3$) $\delta = 3.96$ (br s, 2H), 6.37 (d, $J = 8.4$ Hz, 2H), 7.40 (d, $J = 12.2$ Hz, 1H), 7.41–7.43 (m, 4H), 7.59 (d, $J = 8.4$ Hz, 2H), 7.76 (d, $J = 12.2$ Hz, 1H), 7.93–7.96 (m, 2H), 8.11–8.13 (m, 2H), 8.34 (s, 1H); EIMS (70 eV) m/z (rel intensity) 323 (M^+ ; 15), 202 (18), 201 (11), 120 (100), 119 (73), 92 (14); UV (CH_3CN) 346 (24600) nm.

1-(4-Aminophenyl)-3-(1-pyrenyl)prop-2-en-1-one (1l): Yield 42%; mp 229–231 °C; 1H NMR ($CDCl_3$) $\delta = 6.21$ (br s, 2H), 6.67 (d, $J = 8.5$ Hz, 2H), 8.04 (d, $J = 8.5$ Hz, 2H), 8.12–8.15 (m, 1H), 8.18 (d, $J = 15.4$ Hz, 1H), 8.24–8.38 (m, 6H), 8.61 (d, $J = 8.6$ Hz, 1H), 8.77 (d, $J = 15.4$ Hz, 1H), 8.79 (d, $J = 8.6$ Hz, 1H); EIMS (70 eV) m/z (rel intensity) 347 (M^+ ; 60), 346 (20), 266 (30), 202 (15), 160 (14), 159 (20), 120 (100), 92 (26), 65 (23); UV (CH_3CN) 389 (25600) nm.

Synthesis of 4,6-Disubstituted 3-Cyano-2-methylpyridines (2). To an acetonitrile suspension (50 ml) of α,β -unsaturated carbonyl compound **1** (2 mmol) and β -aminocrotononitrile (2.4 mmol) was added potassium *t*-butoxide (10 mmol) and stirred overnight at room temperature. After the reaction was complete, the mixture was concentrated and poured into water. The resulting precipitate was filtered, dried, purified by column chromatography, and recrystallized from ethanol. The physical and spectral data are given below.

6-(4-Aminophenyl)-3-cyano-4-[4-(dimethylamino)phenyl]-2-methylpyridine (2a): Yield 60%; mp 257–260 °C; 1H NMR ($CDCl_3$) $\delta = 2.85$ (s, 3H), 3.05 (s, 6H), 3.93 (br s, 2H), 6.71 (d, $J = 8.8$ Hz, 2H), 6.82 (d, $J = 8.8$ Hz, 2H), 7.54 (s, 1H), 7.59 (d, $J = 8.8$ Hz, 2H), 7.93 (d, $J = 8.8$ Hz, 2H); EIMS (70 eV) m/z (rel intensity) 328 (M^+ ; 100), 327 (49), 164 (12), 163 (26); UV (CH_3CN) 353 (30600) nm.

6-(4-Aminophenyl)-3-cyano-4-[4-(diethylamino)phenyl]-2-methylpyridine (2e): Yield 58%; mp 151–152 °C; 1H NMR ($CDCl_3$) $\delta = 1.22$ (t, $J = 7.1$ Hz, 6H), 2.84 (s, 3H), 3.43 (q, $J = 7.1$ Hz, 4H), 3.93 (br s, 2H), 6.75 (d, $J = 9.0$ Hz, 2H), 6.77 (d, $J = 8.8$ Hz, 2H), 7.53 (s, 1H), 7.57 (d, $J = 9.0$ Hz, 2H), 7.92 (d, $J = 8.8$ Hz, 2H); EIMS (70 eV) m/z (rel intensity) 356 (M^+ ; 49), 342 (25), 341 (100), 313 (15), 157 (11), 156 (11); UV (CH_3CN) 353 (37000) nm. Found: C, 77.58; H, 6.72; N, 15.57%. Calcd for $C_{23}H_{24}N_4$: C, 77.50; H, 6.79; N, 15.72%.

6-(4-Aminophenyl)-3-cyano-4-(julolidine-9-yl)-2-methylpyridine (2f): Yield 18%; mp 251–254 °C; 1H NMR ($CDCl_3$) $\delta = 2.00$ (quintet, $J = 6.0$ Hz, 4H), 2.82 (s, 3H), 2.82 (t, $J = 6.0$ Hz, 4H), 3.23 (t, $J = 6.0$ Hz, 4H), 3.91 (br s, 2H), 6.75 (d, $J = 8.5$ Hz, 2H), 7.14 (s, 2H), 7.49 (s, 1H), 7.91 (d, $J = 8.5$ Hz, 2H); EIMS (70

eV) m/z (rel intensity) 380 (M^+ ; 100), 379 (39), 190 (15), 189 (10); UV (CH_3CN) 344 (28600) nm.

Synthesis of 6-(4-Aminophenyl)-3-carbamoyl-4-[4-(diethylamino)phenyl]-2-methylpyridine (3e). To an ethylene glycol solution (20 ml) of 6-(4-aminophenyl)-3-cyano-4-[4-(diethylamino)phenyl]-2-methylpyridine (**2e**) (0.48 g, 1.35 mmol) were added sodium hydroxide (2 g, 50 mmol) and water (1 ml) and heated at 140 °C for 5 h. After the reaction was complete, to the mixture was added brine and then extracted with dichloromethane. The product was washed with brine, dried, purified by column chromatography, and recrystallized from benzene. The physical and spectral data are given below. Yield 51%; mp 222–223 °C; 1H NMR ($CDCl_3$) $\delta = 1.20$ (t, $J = 7.1$ Hz, 6H), 2.70 (s, 3H), 3.40 (q, $J = 7.1$ Hz, 4H), 3.82 (br s, 2H), 5.55 (br s, 2H), 6.71 (d, $J = 9.0$ Hz, 2H), 6.75 (d, $J = 8.8$ Hz, 2H), 7.43 (d, $J = 9.0$ Hz, 2H), 7.44 (s, 1H), 7.86 (d, $J = 8.8$ Hz, 2H); EIMS (70 eV) m/z (rel intensity) 374 (M^+ ; 54), 359 (100); UV (CH_3CN) 326 (26400) nm.

Measurement of Fluorescence Spectra. After completely dissolving the samples in a solvent, the concentration of the solution was adjusted to 1×10^{-5} mol dm $^{-3}$. The fluorescence spectra were measured at 25 °C. The emission spectra were measured by irradiating the wavelength of the excitation maximum of each compound (shown in Tables 1 and 2). The relative fluorescence intensity was calculated by measuring the intensity (number) in the detector.

Measurement of Relative Sensitivity. To carnitine (8 μ g, 0.05 μ mol) was added an acetonitrile–pyridine mixed solution (8:2, 1 ml) of **1** or **2** (2 μ mol) and EDC (3 μ mol). The mixture was allowed to stand for 2 h at 20 °C. The solution was analyzed by HPLC (column: Develosil ODS-HG-5 (4.6 $\times$ 150 mm), mobile phase: acetonitrile–potassium dihydrogenphosphate (50 mM) mixed solution (6:4) (1 M = 1 mol dm $^{-3}$) containing trifluoroacetic acid (TFA, 10 mM), 0.8 ml min $^{-1}$). After measuring the excitation wavelength of each compound, the relative fluorescence intensity in each emission wavelength was measured.

HPLC Analysis of Carnitine. To carnitine (8 μ g, 0.05 μ mol) was added an acetonitrile–pyridine mixed solution (8:2, 1 ml) of **2e** (1 μ mol) and EDC (3 μ mol). The mixture was allowed to stand for 2 h at 20 °C. The solution was analyzed by HPLC (column: Develosil ODS-HG-5 (4.6 $\times$ 150 mm), mobile phase: acetonitrile–potassium dihydrogenphosphate (50 mM) mixed solution (6:4) containing trifluoroacetic acid (TFA, 10 mM), 0.8 ml min $^{-1}$, excitation wavelength: 363 nm, emission wavelength: 533 nm). The detection limit of carnitine was defined by S/N ratio to be larger than 3.

The authors are grateful to Miss Harumi Kudo of Shimadzu Co. for LC/MS measurement.

References

- 1) P. R. Borum, *Ann. Rev. Nutr.*, **3**, 233 (1983).
- 2) H. U. Bergmeyer, "Methods of Enzymatic Analysis," VCH, Weinheim (1985), p. 474.
- 3) H. U. Bergmeyer, "Methods of Enzymatic Analysis," VCH, Weinheim (1985), p. 481.
- 4) P. E. Minkler, E. P. Brass, W. R. Hiatt, S. T. Ingalls, and C. L. Hoppel, *Anal. Biochem.*, **231**, 315 (1995).
- 5) P. E. Minkler and C. L. Hoppel, *J. Chromatogr.*, **613**, 203 (1993).
- 6) G. I. Feutrell and R. N. Mirrington, *Aust. J. Chem.*, **25**, 1719 (1972).

- 7) J. Griffith, V. Millar, and Z. Defong, "The Third International Symposium on Functional Dyes Program and Abstracts," 1995, Abstr., p. 95.
-