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N-Aryl-1,8-naphthalimides as highly sensitive fluorescent labeling reagents for carnitine

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Abstract

4-(2-Aminoethylamino)-*N*-(4-methoxyphenyl)-1,8-naphthalimide can be used as a fluorescent labeling reagent for carnitine. The excitation and emission maxima of this reagent were observed at 436 and 524 nm in acetonitrile, respectively and the reagent reacted smoothly with carnitine to afford the labeled product under mild conditions. The detection limit of carnitine by using this reagent was ca. 30 fmol. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Carnitine; *N*-Aryl-1,8-naphthalimides; Fluorescent labeling reagents

1. Introduction

Carnitine plays an important role as a carrier of long-chain fatty acids into mitochondrial matrix [1]. Naphthalene, anthracene, pyrene, coumarin, xanthene, and pyrromethene derivatives are known as fluorescent labeling reagents for carboxylic acids in HPLC analysis [2,3]. 9-Anthryldiazomethane (ADAM, λ_{ex}: 365 nm, λ_{em}: 412 nm in CH_3CN-CH_3OH) has been used as a reagent for carboxylic acids [4]. In a previous paper, 6-(4-aminophenyl)-3-cyano-4-[4-(diethylamino)phenyl]-2-methylpyridine (λ_{ex}: 353 nm, λ_{em}: 508 nm in CH_3CN) has been reported to show good properties as a fluorescent labeling reagent for carnitine [5]. Intense bathochromic and bathofluoric compounds having a large Stokes shift are necessary properties for the reagents. Since the analysis is performed by reverse phase chromatography, it is also desirable that the fluorescence intensity is not decreased by the addition of water. 4-Amino-1,8-naphthalimides are known as intense fluorophores and have been used as fluorescent whitening dyes for cotton, wool, silk, synthetic fibers, and plastics [6,7]. A series of Lucifer dyes (4-amino-1,8-naphthalimidedisulfonates, Molecular Probes Co. Inc.) have been used as fluorescent probes [8–12]. Thus, 1,8-naphthalimides are interesting fluorescent compounds and we report here the application of *N*-aryl-1,8-naphthalimides as fluorescent labeling reagents for carnitine.

2. Results and discussion

The synthesis of 4-alkylamino-*N*-aryl-1,8-naphthalimides **5** is shown in Scheme 1.

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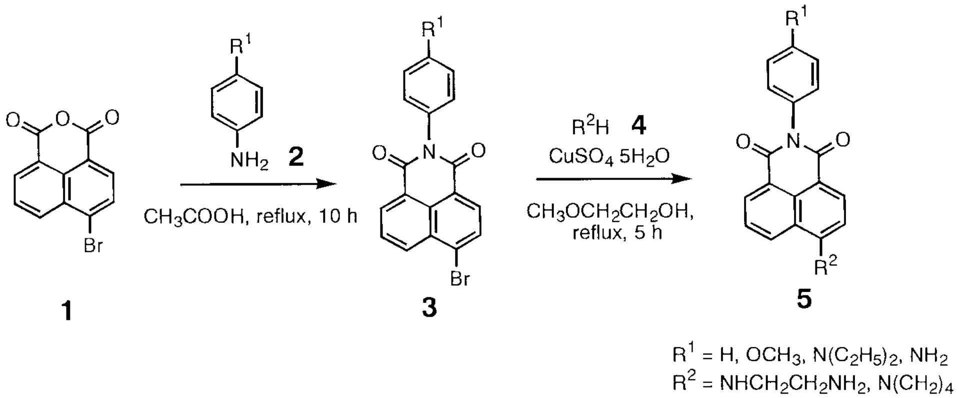

Scheme 1. Synthesis of *N*-aryl-1,8-naphthalimides **5**.

4-Bromo-1,8-naphthalic anhydride (**1**) reacted with arylamines **2** to provide *N*-aryl-1,8-naphthalimides **3** in moderate to good yields; further reaction with amines **4** afforded the 4-alkylamino derivatives **5** in moderate to good yields.

The fluorescence spectra of **5** in acetonitrile are summarized in Table 1. The excitation (λ_{ex}) and emission maxima (λ_{em}) of **5** were in the region around 436 and 524 nm, respectively. The relative fluorescence intensity (RFI) of the 4-methoxyphenyl derivative **5b** was the highest of the 4-alkylamino-*N*-aryl-1,8-naphthalimides **5**. The RFI of **5b** in acetonitrile was lower than that of 6-(4-aminophenyl)-3-cyano-4-[4-(diethylamino)phenyl]-2-methylpyridine.

HPLC analysis of amino acids is normally performed by reverse phase chromatography. Therefore, the effect of water on the RFI of **5b** was examined. The result is shown in Fig. 1. The RFI of 6-(4-aminophenyl)-3- cyano-4-[4-(diethylamino)phenyl] - 2-methylpyridine was significantly decreased by addition of water into acetonitrile, whilst that of **5b** did not decrease with up to 80% of water. Thus, the RFI of **5b** in an acetonitrile-water mixed solvent was higher than that of 6-(4-aminophenyl)-3-cyano-4-[4-(diethylamino)phenyl]-2-methylpyridine. A series of 4-amino-1,8-naphthalimide-3,6-disulfonates are known as Lucifer dyes. The RFI of the commercially available 1,8-naphthalimide derivative, disodium 4-amino-*N*-(2-aminoethyl)-1,8-naphthalimide-3,6-disulfonate [Lucifer Yellow Ethylenediamine, λ_{ex}: 434 nm, λ_{em}: 529 nm in an acetonitrile:water (10:90) mixed solution], was measured to be 51. Compound **5b** was sufficiently soluble to be used as a fluorescent labeling reagent in an acetonitrile-water mixed solvent. These results suggest that 4-(2-aminoethylamino)-*N*-(4-methoxyphenyl)-1,8-naphthalimide (**5b**) has good properties for the quantitative HPLC analysis of carnitine.

Table 1
Fluorescence spectra of *N*-aryl-1,8-naphthalimides **5**

Compund	R[1]	R[2]	λ_{ex}[a]/nm	λ_{em}[a]/nm	RFI[b]
5a	H	$NHCH_2CH_2NH_2$	436	523	77
5b	OCH_3	$NHCH_2CH_2NH_2$	436	524	79
5c	$N(C_2H_5)_2$	$NHCH_2CH_2NH_2$	435	523	1
5d	NH_2	$N(CH_2)_4$	446	524	16

[a] Measured in CH_3CN.

[b] Relative fluorescence intensity measured in CH_3CN (1×10^{-5} mol dm^{-3}, 25°C, 6-(4-aminophenyl)-3-cyano-4-[4-(diethylamino)phenyl]-2-methylpyridine ($\lambda_{ex}=353$ nm, λ_{em}:508 nm in CH_3CN) = 100).

The reactivity of **5b** with carnitine is summarized in Table 2. The reactivity in the presence of dicyclohexylcarbodiimide (DCC) was low. However, compound **5b** reacted smoothly with carnitine in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbo diimide hydrochloride (EDC) under mild conditions.

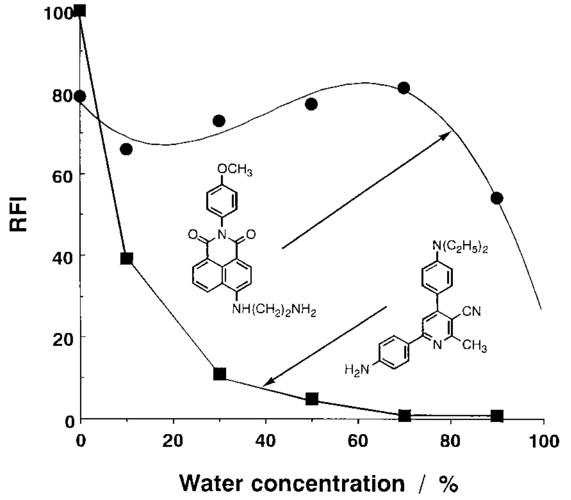

Fig. 1. Effect of added water on relative fluorescence intensity. The relative fluorescence intensities of 1×10^{-5} mol dm^{-3} of substrates (**5b**: $\lambda_{ex}=436$ nm: $\lambda_{em}=524$ nm, 6-(4-aminophenyl)-3-cyano-4-[4-(diethylamino)-phenyl]-2-methylpyridine: $\lambda_{ex}=353$ nm; $\lambda_{em}=508$ nm) were measured at 25°C.

Table 2
Reactivity of **5b** with carnitine

Run	Additive	Reaction time	Reaction tempeature	Relative reactivity[c]
		h	°C	
1	none	6	60	0
2	DCC[a]	6	60	1
3	EDC[b]	6	60	100
4	EDC[b]	6	20	100
5	EDC[b]	0.5	20	60
6	EDC[b]	6	20	100

[a] Dicyclohexylcarbodiimide.

[b] 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride.

[c] Determined on the basis of fluorescence intensity by HPLC analysis.

A typical chromatogram in the analysis of carnitine by using **5b** is shown in Fig. 2. The components with retention times at 5.025 and 6.379 min were **5b** and labeled carnitine, respectively. The detection limit (defined by the S/N ratio being higher than 3) of carnitine using **5b** was calculated to be ca. 30 fmol.

In conclusion, a series of *N*-aryl-1,8-naphthalimides have been prepared as fluorescent labeling reagents for carnitine. The RFI of **5b** did not decrease by the addition of water. The detection limit of carnitine by using **5b** was calculated to be about 30 fmol, being twice that of 6-(4-aminophenyl)- 3-cyano- 4-[4-(diethylamino)phenyl]-2-methylpyridine.

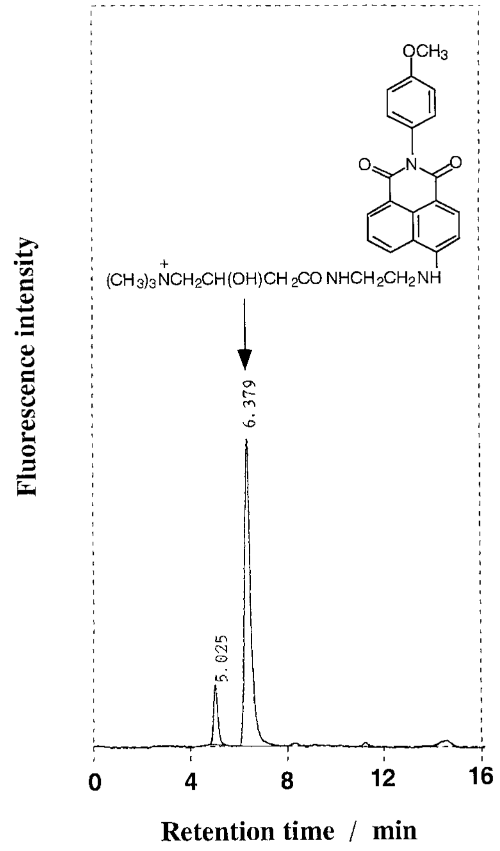

Fig. 2. Chromatogram of labeled carnitine with **5b**. Column: TSK gel ODS 80 TM (4.6×150 mm); mobile phase: CH_3CN-50 mM KH_2PO_4 (1:3), flow rate: 0.8 ml min^{-1}; excitation: 436 nm, detection: 524 nm.

3. Experimental

3.1. Instruments

Melting points were measured with a Yanagimoto MP-S2 micro-melting-point apparatus. ^{1}H-NMR spectra were taken on a Jeol α-400 spectrometer; EIMS spectra were recorded on a Shimadzu QP-1000 spectrometer; Fluorescence spectra were measured with a Jasco FP-777 spectrofluorometer and liquid chromatography was performed with a Jasco Triroter-V instrument.

3.2. Materials

4-Bromo-1,8-naphthalic anhydride (**1**), aniline (**2a**), 4-methoxyaniline (**2b**), *N,N*-diethyl-*p*-phenylenediamine (**2c**), *p*-phenylenediamine (**2d**), ethylenediamine (**4e**), and pyrrolidine (**4f**) were purchased from Tokyo Kasei Co., Ltd. 6-(4-Aminophenyl)-3-cyano-4-[4-(diethylamino)phenyl]-2-methylpyridine was prepared as described in our previous paper [5].

*3.3. Synthesis of N-Aryl-4-bromo-1,8-naphthalimides **3***

To an acetic acid solution (12 ml) of 4-bromo-1,8-naphthalic anhydrides **1** (1 g, 3.6 mmol) was added the appropriate aniline **2** (4.4 mmol). The mixture was refluxed for 10 h; after reaction was complete, the mixture was poured into ice-water. The resulting precipitate was filtered and purified by column chromatography (SiO_2, CH_2Cl_2). Characterisation data are given below.

*3.3.1. 4-Bromo-N-phenyl-1,8-naphthalimide (**3a**)*

Yield 69%; M.p. 239–240°C; ^1H NMR ($CDCl_3$) $\delta = 7.31$–7.33 (m, 2H), 7.48–7.58 (m, 3H), 7.90 (t, $J = 7.8$ Hz, 1H), 8.09 (d, $J = 7.8$ Hz, 1H), 8.47 (d, $J = 7.8$ Hz, 1H), 8.64 (d, $J = 7.8$ Hz, 1H), 8.71 (d, $J = 7.8$ Hz, 1H); MS (EI, 70 eV) *m/z* 353 (99) [$M^+ + 2$], 351 (100) [M^+], 350 (87).

*3.3.2. 4-Bromo-N-(4-methoxyphenyl)-1,8-naphthalimide (**3b**)*

Yield 80%; M.p. 237–238°C; ^1H NMR ($CDCl_3$) $\delta = 3.88$ (s, 3H), 7.06 (d, $J = 8.9$ Hz, 2H), 7.22 (d, $J = 8.9$ Hz, 2H), 7.89 (dd, $J = 8.5$ and 7.3 Hz, 1H), 8.08 (d, $J = 7.9$ Hz, 1H), 8.46 (d, $J = 7.9$ Hz, 1H), 8.64 (dd, $J = 8.5$ and 1.2 Hz, 1H), 8.70 (dd, $J = 7.3$ and 1.2 Hz, 1H); MS (EI, 70 eV) *m/z* 383 (99) [$M^+ + 2$], 381 (100) [M^+], 260 (72), 258 (75).

*3.3.3. 4-Bromo-N-[4-(diethylamino)phenyl]-1,8-naphthalimide (**3c**)*

Yield 72%; M.p. 274–275°C; ^1H NMR ($CDCl_3$) $\delta = 1.20$ (t, $J = 7.1$ Hz, 6H), 3.40 (q, $J = 7.1$ Hz, 4H), 6.77 (d, $J = 9.0$ Hz, 2H), 7.09 (d, $J = 9.0$ Hz, 2H), 7.87 (dd, $J = 8.5$ and 7.3 Hz, 1H), 8.06 (d, $J = 7.8$ Hz, 1H), 8.45 (d, $J = 7.8$ Hz, 1H), 8.61 (dd, $J = 8.5$ and 1.2 Hz, 1H), 8.70 (dd, $J = 7.3$ and 1.2 Hz, 1H); MS (EI, 70 eV) *m/z* 424 (60) [$M^+ + 2$], 422 (59) [M^+], 409 (100), 407 (100), 260 (12), 258 (12).

*3.3.4. 4-Bromo-*N*-(4-Aminophenyl)-1,8-naphthalimide (**3d**)*

Yield 50%; M.p. >300°C; ^1H NMR (DMSO-d_6) $\delta = 5.28$ (s, 2H), 6.64 (d, $J = 8.8$ Hz, 2H), 6.95 (d, $J = 8.8$ Hz, 2H), 8.03 (t, $J = 7.9$ Hz, 1H), 8.25 (d, $J = 7.9$ Hz, 1H), 8.34 (d, $J = 7.9$ Hz, 1H), 8.59 (d, $J = 7.9$ Hz, 1H), 8.60 (d, $J = 7.9$ Hz, 1H); MS (EI, 70 eV) *m/z* 368 (96) [$M^+ + 2$], 366 (100) [M^+], 260 (63), 258 (63).

*3.4. Synthesis of 4-Alkylamino-N-aryl-1,8-naphthalimides **5***

To a 2-methoxyethanol solution (10 ml) of the 4-bromo-*N*-arylnaphthalimide **3** (292 mg, 0.84 mmol) were added copper(II) sulfate pentahydrate (100 mg) and an amine **4** (11.8 mmol). The mixture was refluxed for 5 h and after reaction was complete, the mixture was poured into ice-water. The resulting precipitate was filtered, purified by column chromatography (SiO_2, CH_2Cl_2), and recrystallised (**5a** and **5d**: ClC_6H_5; **5b** and **5c**: $CH_3C_6H_5$). Characterisation data are given below.

*3.4.1. 4-(2-Aminoethylamino)-N-phenyl-1,8-naphthalimide (**5a**)*

Yield 66%; M.p. 232–234°C; ^1H NMR ($CDCl_3$) $\delta = 3.20$ (t, $J = 5.6$ Hz, 2H), 3.45 (q, $J = 5.6$ Hz, 2H), 6.23 (br s, 1H), 6.75 (d, $J = 8.5$ Hz, 1H), 7.26–7.32 (m, 2H), 7.43–7.55 (m, 3H), 7.67 (t, $J = 8.5$ Hz, 1H), 8.24 (d, $J = 8.5$ Hz, 1H), 8.50 (d, $J = 8.5$ Hz, 1H), 8.63 (d, $J = 8.5$ Hz, 1H); MS (EI, 70 eV) *m/z* 331 (60) [M^+], 302 (53), 301 (100), 182 (56).

*3.4.2. 4-(2-Aminoethylamino)-N-(4-methoxyphenyl)-1,8-naphthalimide (**5b**)*

Yield 85%; M.p. 280°C (dec); ^1H NMR ($CDCl_3$) $\delta = 2.89$ (t, $J = 6.3$ Hz, 2H), 3.40 (t, $J = 6.3$ Hz, 2H), 3.08 (s, 3H), 6.84 (d, $J = 8.5$ Hz, 1H), 7.03 (d, $J = 9.0$ Hz, 2H), 7.20 (d, $J = 9.0$ Hz, 2H), 7.71 (t, $J = 8.3$ Hz, 1H), 8.25 (d, $J = 8.5$ Hz, 1H), 8.43 (d, $J = 8.3$ Hz, 1H), 8.75 (d, $J = 8.3$ Hz, 1H); MS (EI, 70 eV) *m/z* 361 (60) [M^+], 332 (52), 331 (94), 182 (72), 181 (59), 112 (100), 77 (77). Found:

C, 70.04; H, 5.57; N, 11.34%. Calcd for $C_{21}H_{19}N_3O_3$: C, 69.79; H, 5.30; N, 11.63%.

3.4.3. *4-(2-Aminoethylamino)-N-[4-(diethylamino) phenyl]-1,8-naphthalimide (**5c**)*

Yield 68%; M.p. 205–207°C; 1H NMR ($CDCl_3$) $\delta = 1.19$ (t, $J = 7.1$ Hz, 6H), 3.19 (t, $J = 5.9$ Hz, 2H), 3.39 (q, $J = 7.1$ Hz, 4H), 3.44 (q, $J = 5.9$ Hz, 2H), 6.18 (br, 1H), 6.73 (d, $J = 8.5$ Hz, 1H), 6.76 (d, $J = 9.2$ Hz, 2H), 7.10 (d, $J = 9.2$ Hz, 2H), 7.64 (t, $J = 8.1$ Hz, 1H), 8.21 (d, $J = 8.1$ Hz, 1H), 8.50 (d, $J = 8.5$ Hz, 1H), 8.62 (d, $J = 8.1$ Hz, 1H); MS (EI, 70 eV) *m/z* 402 (16) [M^+], 387 (32), 359 (41), 345 (26), 344 (100), 316 (18), 195 (17), 194 (17).

3.4.4. N-*(4-Aminophenyl)-4-(1-pyrrolidinyl)-1,8-naphthalimide (**5d**)*

Yield 45%; M.p. >300°C; ^1H NMR ($CDCl_3$) $\delta = 2.10$–2.14 (m, 4H), 3.77–3.81 (m, 4H), 6.81 (d, $J = 8.7$ Hz, 2H), 6.84 (d, $J = 8.7$ Hz, 1H), 7.08 (d, $J = 8.7$ Hz, 2H), 7.55 (dd, $J = 8.5$ and 7.2 Hz, 1H), 8.45 (d, $J = 8.7$ Hz, 1H), 8.59 (dd, $J = 7.2$ and 1.0 Hz, 1H), 8.62 (dd, $J = 8.5$ and 1.0 Hz, 1H); MS (EI, 70 eV) *m/z* 357 (100) [M^+], 249 (34).

3.5. *Fluorescence spectra*

After completely dissolving a sample (1×10^{-5} mol dm^{-3}) in a solvent, the fluorescence spectra were measured at 25°C. The emission spectrum was measured by irradiating at the wavelength of the excitation maximum of each compound. The relative fluorescence intensity was calculated by measuring the fluorescence intensity (intensity number in the detector).

3.6. *Relative reactivity of **5b** with carnitine*

An acetonitrile-pyridine mixed solution (8:2, 1 ml) of **5b** (1 μmol) was added to carnitine (10 μg, 0.06 μmol). To the mixture was added an additive (DCC or EDC: 60 μmol). The mixture was then analyzed by HPLC (column: TSK gel ODS 80 TM (4.6×150 mm); mobile phase: CH_3CN-50 mM KH_2PO_4 (1:3), flow rate: 0.8 ml min^{-1}; excitation: 436 nm, detection: 524 nm). The reactivity was calculated on the basis of the fluorescence intensity (intensity number in the detector) of the product.

3.7. *HPLC Analysis*

An acetonitrile-pyridine solution (8:2, 1 ml) of **5b** (12 μmol) and EDC (60 μmol) was added to carnitine (1 μg, 6.2 nmol). The mixture was stirred for 2 h at 20°C. The solution was then analyzed by HPLC (column: TSK gel ODS 80 TM (4.6 ×150 mm); mobile phase: CH_3CN-50 mM KH_2PO_4 (1:3), flow rate: 0.8 ml min^{-1}; excitation: 436 nm, detection: 524 nm).

References

[1] Borum PR. Ann Rev Nutr 1983;3:233.
[2] Schwedt G, Ichinose N, Schnepel F-M. Fluorescent analytical chemistry. Baihukan, Tokyo, 1987 [p. 154].
[3] Blau K, Halket J. Handbook of derivatives for chromatography. John Wiley and Sons, London, 1993.
[4] Yshida T, Aetake A, Yamaguchi H, Nimura N, Kinoshita T. J Chromatogr 1988;445:175.
[5] Nakaya K, Funabiki K, Shibata K, Muramatsu H, Matsui M. Bull Chem Soc Jpn 1996;69:2961.
[6] Peters AT, Bide MJ. Dyes and Pigm 1985;6:349.
[7] Konstantinova TN, Meallier P, Grabchev I. Dyes and Pigm 1993;22:191.
[8] Lee JA, Fortes PAG. Biochem 1985;24:322.
[9] Maranto AR. Science 1982;217:953.
[10] Lewis JA, Baird RA, Leverenz EL, Koyama H. Science 1982;215:1641.
[11] Michaels RL, Sheridan JD. Science 1981;214:801.
[12] Miller JP, Selverston AI. Science 1979;206:702.