Acridinium Ester Chemiluminescence: Methyl Substitution on the Acridine Moiety

Manabu Nakazono^{1*}, Shinkoh Nanbu², Takeyuki Akita¹, and Kenji Hamase¹

¹ Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, JAPAN

² Faculty of Science and Technology, Sophia University, 7-1 Kioi-Cho, Chiyoda-ku, Tokyo 102-8554, JAPAN

Abstract: Methyl groups were introduced on the acridine moiety in chemiluminescent acridinium esters that have electron-withdrawing groups (trifluoromethyl, cyano, nitro, ethoxycarbonyl) at the 4-position on the phenyl ester. The introduction of methyl groups at the 2-, 2,7-, and 2,3,6,7-positions on the acridine moiety shifted the optimal pH that gave relatively strong chemiluminescence intensity from neutral conditions to alkaline conditions. 4-(Ethoxycarbonyl)phenyl 2,3,6,7,10-pentamethyl-10 λ^4 -acridine-9-carboxylate, trifluoromethanesulfonate salt showed long-lasting chemiluminescence under alkaline conditions. Acridinium esters to determine hydrogen peroxide concentration at pH 7–10 were newly developed.

Key words: methyl substitution, chemiluminescence, acridinium ester, pH shift, emission spectrum

1 Introduction

Chemiluminescence (CL) is light emission produced by chemical reaction. Most of the CL is associated with the oxidation reaction. Chemiluminescent compounds have been used for detection, measurement, sensor, and imaging of various analytes. In CL studies, novel properties and high sensitivity have attracted much attention. The synthesis of new CL compounds is crucial to discovering novel properties and achieving high sensitivity. Compounds with extremely strong CL intensity that provide unprecedented CL conditions could be useful for highly selective CL assays^{1–7}. Acridinium ester and luminol derivatives are useful chemiluminescent compounds, and require the addition of a strong base and oxidant to produce strong CL intensity. Commonly, CL assays are not suitable as highly sensitive assays across a wide range of pH conditions, including neutral. In certain chemiluminescent compounds, therefore, it is useful to search for derivatizations that can shift the optimal pH on CL. In this investigation, we attempted to identify more facile substitution to give the optimal pH shift for the CL of acridinium ester derivatives.

Based on the possible CL mechanism of acridinium ester derivatives, acridinium ester derivatives react with hydrogen peroxide to produce 10-methyl-9(10H)-acridone (CL species) via a dioxetanone, with a maxima emission wavelength of approximately 430 nm (Scheme 1). Various acridinium ester derivatives were synthesized and their photo-

physical properties were investigated^{8–14}. Acridinium ester derivatives have been particularly useful compounds in CL enzyme immunoassays^{15–18}. We have recently clarified that acridinium ester derivatives having electron-withdrawing groups at the 4-position on the phenyl ester produced strong CL intensities at pH 7 and 8^{19,20}. The CL intensity at higher pH (alkaline conditions) apparently decreased. Upon further investigations, methoxycarbonyl-phenyl 10-methyl-10 λ^4 -2,7-disubstituted acridine-9-carboxylate derivatives were synthesized and their CL were measured, and the introduction of methyl groups at the 2,7-positions on the acridine moiety was effective in increasing the CL intensities at pH 9 and 10²¹.

For our present purposes, we considered that the hydrolysis of phenyl ester in acridinium ester derivatives under alkaline conditions may be related to this experimental result^{11, 22, 23}. Before the dioxetanone is produced, the hydrolysis of phenyl ester mainly proceeds and free carboxylic acid is formed (Scheme 1). Methyl substitutions on the acridine moiety could shift the optimal pH for the CL gradually from neutral conditions to alkaline conditions. Thus, the CL intensities of acridinium ester derivatives could be changed at different pH values by introducing different numbers of methyl groups on the acridine moiety.

Encouraged with these backgrounds, we introduced methyl groups at the 2-, 2,7- and 2,3,6,7-positions on the acridine moiety in acridinium ester derivatives with elec-

*Correspondence to: Manabu Nakazono, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, JAPAN

E-mail: nakazono@phar.kyushu-u.ac.jp

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Scheme 1 Possible chemiluminescence mechanism of acridinium ester derivatives via dioxetanone formation, and hydrolysis of phenyl ester.

Table 1 Chemical structures of **1a–3d**.

Compound	R	Yield [%]	Compound	R	Yield [%]	Compound	R	Yield [%]
1a	CF ₃	54	2a	CF ₃	39	3a	CF ₃	56
1b	CN	49	2b	CN	40	3b	CN	50
1c	NO ₂	65	2c	NO ₂	52	3c	NO ₂	49
1d	COOCH ₂ CH ₃	68	2d	COOCH ₂ CH ₃	40	3d	COOCH ₂ CH ₃	65

tron-withdrawing groups (trifluoromethyl, cyano, nitro, ethoxycarbonyl) at the 4-position on the phenyl ester (Table 1). CL intensities and spectra were evaluated at pH 7–10, and CL methods for measuring hydrogen peroxide at each pH value were developed.

2 Materials and Methods

2.1 Materials

General procedure for the synthesis of **1a–3d**

To a solution of 2-methylacridine-9-carbonyl chloride (70 mg, 0.27 mmol), 2,7-dimethylacridine-9-carbonyl chloride (110 mg, 0.41 mmol), or 2,3,6,7-tetramethylacridine-9-carbonyl chloride (70 mg, 0.24 mmol) in dry dichloromethane (5 mL) was added 4-dimethylaminopyridine (30 mg, 0.25 mmol), the corresponding phenols (0.54 mmol or 0.48 mmol), and triethylamine (0.4 mL). The mixture was stirred at ambient temperature overnight. Chloroform (80 mL) and water (60 mL) were added to the solution. The organic layer was dried with anhydrous magnesium sulfate.

The filtrate was evaporated *in vacuo*, and the residue was purified by column chromatography (silica gel 60N) to produce the desired acridine-9-carboxylates. These were *N*-methylated by methyl trifluoromethanesulfonate in dichloromethane, and the desired acridine-9-carboxylates as trifluoromethanesulfonate salts were purified by column chromatography (silica gel, chloroform-methanol). Compounds **1c**, **1d**, and **2a–2d** were obtained after washing the resulting precipitate with diethyl ether.

4-(Trifluoromethane)phenyl 2,10-dimethyl-10λ⁴-acridine-9-carboxylate, trifluoromethanesulfonate salt (**1a**).

80 mg (54% yield). Yellow solid. M.p. 248–250°C. ¹H NMR (500 MHz, DMSO-d₆): δ = 2.76 (s, 3H), 4.95 (s, 3H), 8.05 (s, 4H), 8.15 (t, *J* = 8 Hz, 1H), 8.37 (s, 1H), 8.42 (d, *J* = 10 Hz, 1H), 8.51 (t, *J* = 10 Hz, 1H), 8.64 (d, *J* = 8.5 Hz, 1H), 8.87 (d, *J* = 9 Hz, 1H), 8.92 (d, *J* = 9.5 Hz, 1H). ¹³C NMR (125.7 MHz, DMSO-d₆): δ = 21.4, 120.2, 120.3, 122.9, 123.1, 123.6, 125.4, 127.8, 127.9, 130.2, 139, 141.1, 141.5, 141.7, 142.3, 152.8, 163.4. HR-MS (FAB⁺) calcd for C₂₃H₁₇F₃NO₂⁺ 396.1211 (M⁺) found 396.1214. Anal calcd for C₂₄H₁₇F₆

NO₅S: C, 52.85; H, 3.14; N, 2.57 found: C, 52.34; H, 3.00; N, 2.53.

4-Cyanophenyl 2,10-dimethyl-10 λ^4 -acridine-9-carboxylate, trifluoromethanesulfonate salt (**1b**).

67 mg (49% yield). Yellow solid. M.p. 285–288°C. ¹H NMR (500 MHz, DMSO-d₆): δ = 2.75 (s, 3H), 4.95 (s, 3H), 8.04 (d, J = 9 Hz, 2H), 8.14 (m, 3H), 8.36 (s, 1H), 8.42 (dd, J = 1.5, 9.5 Hz, 1H), 8.5 (t, J = 9.5 Hz, 1H), 8.63 (d, J = 9 Hz, 1H), 8.87 (d, J = 9.5 Hz, 1H), 8.91 (d, J = 9 Hz, 1H). ¹³C NMR (125.7 MHz, DMSO-d₆): δ = 21.4, 110, 111.1, 118.5, 120.1, 120.3, 123, 123.1, 123.9, 125.4, 127.8, 130.2, 135, 139, 141.1, 141.5, 141.7, 142.3, 145.2, 153.2, 163.3. HR-MS (FAB⁺) calcd for C₂₃H₁₇N₂O₂⁺ 353.1290 (M⁺) found 353.1293. Anal calcd for C₂₄H₁₇F₃N₂O₅S: C, 57.37; H, 3.41; N, 5.58 found: C, 57.17; H, 3.26; N, 5.51.

4-Nitrophenyl 2,10-dimethyl-10 λ^4 -acridine-9-carboxylate, trifluoromethanesulfonate salt (**1c**).

92 mg (65% yield). Yellow solid. M.p. 294–297°C. ¹H NMR (500 MHz, DMSO-d₆): δ = 2.76 (s, 3H), 4.95 (s, 3H), 8.1 (m, 3H), 8.38 (s, 1H), 8.42 (d, J = 10 Hz, 1H), 8.5 (m, 3H), 8.65 (d, J = 8 Hz, 1H), 8.87 (d, J = 9.5 Hz, 1H), 8.92 (d, J = 9.5 Hz, 1H). ¹³C NMR (125.7 MHz, DMSO-d₆): δ = 21.4, 120.2, 120.3, 123, 123.2, 124, 125.4, 126.1, 127.8, 130.2, 139.1, 141.1, 141.5, 141.8, 142.4, 145.1, 146.8, 154.4, 163.2. HR-MS (FAB⁺) calcd for C₂₃H₁₇N₂O₄⁺ 373.1188 (M⁺) found 373.1178. Anal calcd for C₂₃H₁₇F₃N₂O₇S: C, 52.88; H, 3.28; N, 5.36 found: C, 52.83; H, 3.27; N, 5.26.

4-(Ethoxycarbonyl)phenyl 2,10-dimethyl-10 λ^4 -acridine-9-carboxylate, trifluoromethanesulfonate salt (**1d**).

87 mg (68% yield). Yellow solid. M.p. 268–270°C. ¹H NMR (500 MHz, DMSO-d₆): δ = 1.36 (t, J = 7.5 Hz, 3H), 2.76 (s, 3H), 4.37 (q, J = 7.5 Hz, 2H), 4.95 (s, 3H), 7.94 (d, J = 9 Hz, 2H), 8.15 (m, 3H), 8.35 (s, 1H), 8.42 (d, J = 9.5 Hz, 1H), 8.51 (t, J = 8.5 Hz, 1H), 8.61 (d, J = 8 Hz, 1H), 8.87 (d, J = 9 Hz, 1H), 8.92 (d, J = 9.5 Hz, 1H). ¹³C NMR (125.7 MHz, DMSO-d₆): δ = 14.6, 21.4, 61.6, 120.2, 120.3, 122.8, 123, 123.1, 125.3, 127.7, 129.7, 130.2, 131.6, 139, 141.1, 141.5, 141.8, 142.3, 145.5, 153.5, 163.4, 165.4. HR-MS (FAB⁺) calcd for C₂₅H₂₂NO₄⁺ 400.1549 (M⁺) found 400.1533. Anal calcd for C₂₆H₂₂F₃NO₇S: C, 56.83; H, 4.04; N, 2.55 found: C, 56.07; H, 3.84; N, 2.45.

4-(Trifluoromethyl)phenyl 2,7,10-trimethyl-10 λ^4 -acridine-9-carboxylate, trifluoromethanesulfonate salt (**2a**).

80 mg (39% yield). Yellow solid. M.p. 249–252°C. ¹H NMR (500 MHz, DMSO-d₆): δ = 2.73 (s, 6H), 4.91 (s, 3H), 8.03 (s, 4H), 8.29 (s, 2H), 8.35 (d, J = 9.5 Hz, 2H), 8.82 (d, J = 9.5 Hz, 2H). ¹³C NMR (125.7 MHz, DMSO-d₆): δ = 21.4, 120.1, 123.2, 123.6, 125.2, 127.9, 140.8, 140.9, 141.7, 144.1, 152.8, 163.4. HR-MS (FAB⁺) calcd for C₂₄H₁₉F₃NO₂⁺ 410.1368 (M⁺) found 410.1366. Anal calcd for C₂₅H₁₉F₆

NO₅S: C, 53.67; H, 3.42; N, 2.50 found: C, 51.97; H, 3.41; N, 2.46.

4-Cyanophenyl 2,7,10-trimethyl-10 λ^4 -acridine-9-carboxylate, trifluoromethanesulfonate salt (**2b**).

80 mg (40% yield). Yellow solid. M.p. 273–276°C. ¹H NMR (500 MHz, DMSO-d₆): δ = 2.72 (s, 6H), 4.91 (s, 3H), 8.02 (d, J = 9 Hz, 2H), 8.15 (d, J = 8.5 Hz, 2H), 8.29 (s, 2H), 8.35 (dd, J = 1.5, 9.5 Hz, 2H), 8.81 (d, J = 9.5 Hz, 2H). ¹³C NMR (125.7 MHz, DMSO-d₆): δ = 21.4, 111, 118.5, 120.1, 123.2, 123.9, 125.2, 135, 140.7, 141, 141.7, 143.9, 153.2, 163.3. HR-MS (FAB⁺) calcd for C₂₄H₁₉N₂O₂⁺ 367.1447 (M⁺) found 367.1447. Anal calcd for C₂₅H₁₉F₃N₂O₅S: C, 58.14; H, 3.71; N, 5.42 found: C, 57.72; H, 3.70; N, 5.65.

4-Nitrophenyl 2,7,10-trimethyl-10 λ^4 -acridine-9-carboxylate, trifluoromethanesulfonate salt (**2c**).

110 mg (52% yield). Yellow solid. M.p. 276–279°C. ¹H NMR (500 MHz, DMSO-d₆): δ = 2.73 (s, 6H), 4.92 (s, 3H), 8.08 (d, J = 8.5 Hz, 2H), 8.31 (s, 2H), 8.36 (dd, J = 1.5, 9.5 Hz, 2H), 8.48 (d, J = 9.5 Hz, 2H), 8.82 (d, J = 9.5 Hz, 2H). ¹³C NMR (125.7 MHz, DMSO-d₆): δ = 21.4, 120.1, 123.2, 124, 125.2, 126.1, 140.7, 141, 141.7, 143.8, 146.7, 154.4, 163.2. HR-MS (FAB⁺) calcd for C₂₃H₁₉N₂O₄⁺ 387.1345 (M⁺) found 387.1345. Anal calcd for C₂₄H₁₉F₃N₂O₇S: C, 53.73; H, 3.57; N, 5.22 found: C, 53.36; H, 3.61; N, 5.09.

4-(Ethoxycarbonyl)phenyl 2,7,10-trimethyl-10 λ^4 -acridine-9-carboxylate, trifluoromethanesulfonate salt (**2d**).

90 mg (40% yield). Yellow solid. M.p. 296–298°C. ¹H NMR (500 MHz, DMSO-d₆): δ = 1.34 (t, J = 7 Hz, 3H), 2.73 (s, 6H), 4.35 (q, J = 7 Hz, 2H), 4.91 (s, 3H), 7.91 (d, J = 8.5 Hz, 2H), 8.19 (d, J = 9 Hz, 2H), 8.27 (s, 2H), 8.35 (dd, J = 2, 10 Hz, 2H), 8.82 (d, J = 10 Hz, 2H). ¹³C NMR (125.7 MHz, DMSO-d₆): δ = 14.6, 21.4, 61.6, 120.1, 122.9, 123.1, 125.1, 129.5, 131.6, 140.7, 140.9, 141.6, 144.1, 153.4, 163.5, 165.4. HR-MS (FAB⁺) calcd for C₂₆H₂₄NO₄⁺ 414.1705 (M⁺) found 414.1705. Anal calcd for C₂₇H₂₄F₃NO₇S: C, 57.55; H, 4.29; N, 2.49 found: C, 56.90; H, 4.33; N, 2.83.

4-(Trifluoromethyl)phenyl 2,3,6,7,10-pentamethyl-10 λ^4 -acridine-9-carboxylate, trifluoromethanesulfonate salt (**3a**).

78 mg (56% yield). Yellow solid. M.p. 290–293°C. ¹H NMR (500 MHz, DMSO-d₆): δ = 2.63 (s, 6H), 2.73 (s, 6H), 4.83 (s, 3H), 8.01 (s, 4H), 8.22 (s, 2H), 8.67 (s, 2H). ¹³C NMR (125.7 MHz, acetone-d₆): δ = 19, 21.1, 39, 118.5, 121.5, 122.9, 124.3, 125.5, 127.4, 140.8, 140.9, 143.8, 152.5, 163.2. HR-MS (FAB⁺) calcd for C₂₆H₂₃F₃NO₂⁺ 438.1681 (M⁺) found 438.1681. Anal calcd for C₂₇H₂₃F₆NO₅S: C, 55.20; H, 3.95; N, 2.38 found: C, 54.83; H, 3.94; N, 2.39.

4-Cyanophenyl 2,3,6,7,10-pentamethyl-10 λ^4 -acridine-9-carboxylate, trifluoromethanesulfonate salt (**3b**).

64 mg (50% yield). Yellow solid. M.p. 266–269°C. ¹H

NMR (500 MHz, DMSO- d_6): δ = 2.62 (s, 6H), 2.72 (s, 6H), 4.83 (s, 3H), 8 (d, J = 8.5 Hz, 2H), 8.14 (d, J = 9 Hz, 2H), 8.21 (s, 2H), 8.67 (s, 2H). ^{13}C NMR (125.7 MHz, DMSO- d_6): δ = 19.8, 21.9, 110.9, 118.6, 119, 121.3, 124, 125.4, 135, 135.2, 140.6, 140.9, 142.8, 152.1, 153.1, 163.4. HR-MS (FAB $^+$) calcd for $\text{C}_{26}\text{H}_{23}\text{N}_2\text{O}_2^+$ 395.1760 (M^+) found 395.1757. Anal calcd for $\text{C}_{27}\text{H}_{23}\text{F}_3\text{N}_2\text{O}_5\text{S}$: C, 59.55; H, 4.26; N, 5.14 found: C, 59.04; H, 4.25; N, 5.13.

4-Nitrophenyl 2,3,6,7,10-pentamethyl-10 λ^4 -acridine-9-carboxylate, trifluoromethanesulfonate salt (**3c**).

65 mg (49% yield). Yellow solid. M.p. 267–270°C. ^1H NMR (500 MHz, DMSO- d_6): δ = 2.62 (s, 6H), 2.73 (s, 6H), 4.83 (s, 3H), 8.06 (d, J = 9.5 Hz, 2H), 8.23 (s, 2H), 8.47 (d, J = 9 Hz, 2H), 8.67 (s, 2H). ^{13}C NMR (125.7 MHz, DMSO- d_6): δ = 19.8, 21.9, 119, 121.3, 124, 125.4, 126, 126.1, 140.6, 140.9, 142.7, 146.6, 152.1, 154.4, 163.3. HR-MS (FAB $^+$) calcd for $\text{C}_{25}\text{H}_{23}\text{N}_2\text{O}_4^+$ 415.1658 (M^+) found 415.1660. Anal calcd for $\text{C}_{26}\text{H}_{23}\text{F}_3\text{N}_2\text{O}_7\text{S}$: C, 55.32; H, 4.11; N, 4.96 found: C, 54.60; H, 4.07; N, 4.87.

4-(Ethoxycarbonyl)phenyl 2,3,6,7,10-pentamethyl-10 λ^4 -acridine-9-carboxylate, trifluoromethanesulfonate salt (**3d**).

85 mg (65% yield). Yellow solid. M.p. 270–273°C. ^1H NMR (500 MHz, DMSO- d_6): δ = 1.34 (t, J = 7 Hz, 3H), 2.63 (s, 6H), 2.73 (s, 6H), 4.35 (q, J = 6.5 Hz, 2H), 4.83 (s, 3H), 7.89 (d, J = 9 Hz, 2H), 8.18 (d, J = 9 Hz, 2H), 8.2 (s, 2H), 8.67 (s, 2H). ^{13}C NMR (125.7 MHz, DMSO- d_6): δ = 14.6, 19.8, 21.9, 61.6, 119, 121.3, 122.9, 125.3, 129.4, 131.6, 140.7, 140.9, 143.1, 152.1, 153.4, 163.6, 165.3. HR-MS (FAB $^+$) calcd for $\text{C}_{28}\text{H}_{28}\text{NO}_4^+$ 442.2018 (M^+) found 442.2018. Anal calcd for $\text{C}_{29}\text{H}_{28}\text{F}_3\text{NO}_7\text{S}$: C, 58.88; H, 4.77; N, 2.37 found: C, 58.42; H, 4.78; N, 2.31.

2.2 Apparatus

The ^1H NMR (500 MHz) and ^{13}C NMR (125.7 MHz) spectra were obtained using an Inova-500 NMR (Agilent, Santa Clara, CA, USA) spectrometer. High resolution (HR) FAB-MS (positive) spectra were obtained using a JMS-HX110A (JEOL, Tokyo, Japan) mass spectrometer. Column chromatography was performed with silica gel 60 N (spherical, neutral 40–50 μm). Chemiluminescence was measured using a Lumat LB 9507 (Berthold, Bad Wildbad, Germany) luminometer, and CL spectra were recorded using a FP-6500 (JASCO, Tokyo, Japan) fluorometer.

2.3 Chemiluminescence measurement

To 50 μL of a 10 nM solution of **1a–3d** in dimethyl sulfoxide was added 100 μL of a buffer solution (100 mM Tris (hydroxymethyl)aminomethane hydrochloride (Tris-HCl) for pH 7 and pH 8, 100 mM glycine sodium hydroxide (Gly-NaOH) for pH 9 and pH 10). The mixture was allowed to stand for 20 s, and then the CL reaction was initiated by adding 100 μL of 1 mM aqueous hydrogen peroxide solu-

tion to the luminometer using an automatic injection system. The relative light unit integrated for 1 min was used to evaluate the CL intensity.

2.4 Chemiluminescence spectra measurement

To 0.5 mL of a 0.1 mM solution of **1a**, **2a**, and **3b** in dimethyl sulfoxide in a quartz cell (10 $\times$ 10 $\times$ 50 mm) was added 1 mL of a buffer solution (100 mM Tris-HCl for pH 7 and pH 8, 100 mM Gly-NaOH for pH 9 and pH 10). The mixture was allowed to stand for 20 s, and then the CL reaction was initiated by adding 1 mL of 100 mM aqueous hydrogen peroxide solution. After 1 min, the mixture was placed in a spectrometer (fluorescence bandwidth: 10 nm; response: 0.5 s; scanning speed: 500 nm/min) and the CL spectra was measured.

2.5 Determination of hydrogen peroxide concentration

To 50 μL of a 10 nM solution of **1a**, **3b**, and **3d** in dimethyl sulfoxide was added 100 μL of a buffer solution (100 mM Tris-HCl for pH 7 and pH 8; 100 mM Gly-NaOH for pH 9 and pH 10). The mixture was allowed to stand for 20 s, and then the CL reaction was initiated by adding 100 μL of 0.01–1 mM aqueous hydrogen peroxide solution to the luminometer using an automatic injection system. The relative light unit integrated for 1 min were used to evaluate the CL intensity.

3 Results and Discussion

Compounds **1a–3d** were new acridinium ester derivatives. Synthetic procedure of **1a–3d** was shown in Scheme 2. 4-Methyl-*N*-phenylaniline, di-*p*-tolylamine or bis(3,4-dimethylphenyl)amine was used as a starting material. These starting materials with oxalyl dichloride and aluminium chloride give isatin derivatives. The acridine ring was formed from an isatin structure in aqueous potassium hydroxide solution. The acridine derivatives were converted to acid chloride. Subsequently, esterification of phenols in the presence of 4-dimethylaminopyridine and triethylamine, and *N*-methylation of acridine using methyl trifluoromethanesulfonate proceeded in dichloromethane. The yields for **1a–3d** were 39%–68%.

In CL measurements, to the solution of **1a–3d** in dimethyl sulfoxide was added a buffer solution, and then aqueous hydrogen peroxide solution was added to the mixture. The CL emission was measured using luminometer. The time-courses for CL development of **1d** at pH 7–10 are shown in Fig. 1a. After the addition of hydrogen peroxide, the CL was flash-type and glow-type at pH 7 and pH 8, respectively. At pH 7, the intramolecular nucleophilic attack of OOH at the 9-position on the acridine moiety to the phenyl ester may rapidly occur (Scheme 1). CL intensities at pH 7 and 8 were stronger than those at pH 9 and 10. At pH 8, the

Fig. 1 (a) Time-course of the chemiluminescence development of **1d** at pH 7–10. (b) Time-course of the chemiluminescence development of **1d**, **2d**, and **3d** at pH 10. The concentration of **1d**, **2d**, and **3d** was 10 nM. The concentration of hydrogen peroxide was 1 mM.

time required to reach the maximum relative light units (RLU) was approximately 30 s after the addition of hydrogen peroxide. The time-courses of the CL development of **1a** and **1d** were similar at pH 7–10. **Fig. 1b** shows the time-courses of the CL development of **1d**, **2d**, and **3d** at pH 10. The descending order of CL intensities was as follows: **3d** > **2d** > **1d**. Thus, an increased number of methyl groups on the acridine moiety caused an increase of CL intensities under alkaline conditions. Interestingly, an acridinium ester that showed long-lasting CL was found, i.e., **3d**.

The CL intensities of **1a-3d** at pH 7–10 are shown in **Fig. 2**. Dramatic change of CL intensities was observed at different pH values. The optimal pH value was defined as pH value that gave maximum CL intensity. The optimal pH values for the CL of **1a-1d**, **2a-2d**, and **3a-3d** were pH 8, pH 8–9, and pH 9, respectively. The optimal pH value for CL shifted from neutral conditions to alkaline conditions. The introduction of methyl groups (weak electron-donating groups) at the 2-, 2,7- and 2,3,6,7-positions on the acridine moiety affected the optimal pH value for the CL of **1a-3d**. As shown in **Fig. 2**, the CL intensities of **1a-1d** decreased apparently at pH 9–10. For example, the CL intensity of **1d** at pH 8 was approximately 11-fold stronger than that at pH

9. Relative to the CL intensities of **1a-1d** at pH 7–10, the CL intensities of **2a-2d** were higher at pH 9–10 and lower at pH 7–8. In particular, the increase of CL intensity at pH 9 was noticeable. Similar results were obtained for the CL intensities of **3a-3d**, with the CL intensities of **3a-3d** at pH 10 being strong. The CL intensities of **3a-3d** at pH 10 were approximately 9-, 9-, 8-, and 15-fold stronger, respectively, than those of **1a-1d** at pH 10. Among several CL compounds, luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) has high CL intensities under alkaline conditions. When the concentrations of sodium hydroxide and hydrogen peroxide were 1 mM, the maximum CL intensity of 10 nM luminol was obtained. Compared to the CL intensities of **3d** (10 nM) and luminol (10 nM), those of **3d** at pH 9 and 10 were approximately 4-fold stronger than that of luminol.

In **1a-1d** at pH 9–10, after the hydroperoxide anion reacts with the carbon at the 9-position on the acridine moiety, the hydrolysis of phenyl ester may proceed (**Scheme 1**)^{11, 22, 23}. In contrast, in **2a-3d** at pH 9–10, the dioxetanone structure could mainly be formed as a chemiluminescent pathway, and the CL intensities increase. The introduction of methyl groups on the acridine moiety may suppress the hydrolysis of the phenyl ester in acridinium

Fig. 2 Chemiluminescence intensities of **1a–3d** at pH 7–10. The concentration of **1a–3d** and hydrogen peroxide was 10 nM and 1 mM, respectively. The relative light unit (RLU) integrated for 1 min was used to evaluate the chemiluminescence intensity.

Fig. 3 Chemiluminescence spectra of **3b** at pH 7–10. The concentration of **3b** and hydrogen peroxide was 0.1 mM and 100 mM, respectively.

ester derivatives.

Compounds **1a**, **2a**, and **3b** were selected for the measurement of CL spectra, because these three had the strongest CL intensities among **1a–1d**, **2a–2d**, and **3a–3d**, respectively. The CL spectra of **1a**, **2a**, and **3b** were measured at pH 7–10. The maxima wavelengths of CL emission of **3b** were approximately 440–450 nm (Fig. 3). The maximum wavelengths of CL emission of **1a** and **2a** was similar with that of **3b** (Fig. S1). There was no apparent change in the maxima wavelengths with increasing number of methyl groups.

Acridinium ester derivatives synthesized in this study were applied to determine hydrogen peroxide concentra-

tion at a wide range of pH conditions, including neutral. From the CL measurement of **1a–3d** at pH 7–10, the CL intensities of **1a** were maximal at pH 7 and 8. Compounds **3b** and **3d** gave maximum CL intensities at pH 9 and pH 10, respectively. Therefore, hydrogen peroxide was measured using **1a**, **3b** and **3d**. Standard curve of hydrogen peroxide using **3d** at pH 10 is shown in Fig. S2. The linear calibration ranges of hydrogen peroxide were 0.05–1 mM ($R = 0.994$) at pH 7 and 0.01–1 mM ($R = 0.999$) at pH 8 using **1a** (Fig. S2). The linear calibration ranges of hydrogen peroxide were 0.01–1 mM ($R = 0.999$) at pH 9 using **3b** (Fig. S2) and 0.01–1 mM ($R = 0.999$) at pH 10 using **3d**. A relatively long range of detectability and good linearities ($R > 0.994$) were obtained. The detection limit (defined as the mean value of blank + 5σ (standard deviation)) of hydrogen peroxide at pH 10 using **3d** was 4.5 mM. This CL method showed highly sensitive detection of hydrogen peroxide without an addition of catalyst. Compared to previous CL methods with an addition of catalyst for determining hydrogen peroxide using luminol hybrid Mg-Al- CO_3 layered double oxides²⁴, CL energy transfer by luminol-CdTe quantum dots²⁵, luminol- H_2O_2 CL system using nanoparticles (Co_3O_4 nanoparticle, $\alpha\text{-Fe}_2\text{O}_3$ nanorod, NiO nanoparticle)²⁶ or peroxalate CL (bis(2,4,6-trichlorophenyl) oxalate in the presence of rubrene and imidazole)²⁷, this CL method should achieve an approximately 20-fold increase in sensitivity.

4 Conclusion

Acridinium ester derivatives with electron-withdrawing groups at the 4-position in the phenyl ester were synthe-

sized, the optimal pH values shifted from neutral conditions to alkaline conditions by introducing methyl groups at the 2-, 2,7-, and 2,3,6,7-positions on the acridine moiety. These acridinium ester derivatives can determine hydrogen peroxide concentrations under optimal conditions. Facile methyl substitutions apparently changed the CL intensities at different pH values. Moreover, acridinium ester derivatives having long-lasting CL under alkaline conditions were developed. These experimental results could be crucial information for the molecular design of useful acridinium ester derivatives at pH 7–10.

Author Contributions

Manabu Nakazono: designed research, performed research, analyzed data, wrote the manuscript, funding acquisition. Shinkoh Nanbu: supervision. Takeyuki Akita: supervision. Kenji Hamase: supervision, funding acquisition.

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Conflicts of Interest

The authors declare no conflict of interest.

Supporting Information

This material is available free of charge via the Internet at doi: 10.5650/jos.ess21186. ¹H NMR and ¹³C NMR spectra of **1a–3d**, chemiluminescence spectra, and standard curves of hydrogen peroxide.

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