Chemiluminescence Characteristics of *N*-(4-Substituted Benzyl)isoluminol

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We synthesized *N*-(4-substituted benzyl)isoluminol which has 4-bromo-, 4-methyl-, 4-methoxy-, 4-nitro-groups. These compounds produced chemiluminescence by the reaction with the oxidizing agent, potassium hexacyanoferrate and hydrogen peroxide, in an alkaline medium. The chemiluminescence intensities of these compounds were 0.03—4.7 times that of isoluminol. We used Hamett substituent constants as a parameter for the electronic substituent effects. The relationship between the amino-H chemical shift value and the Hamett substituent constants showed a good linear correlation. The relationship between the chemiluminescence intensities and the Hamett substituent constants showed a good linear correlation. The relationship between the fluorescence intensities and the Hamett substituent constants also showed a good linear correlation. These results suggest that the change in the electron density around the amino group strongly influences the fluorescence intensities and corresponding chemiluminescence intensities of these derivatives.

Key words chemiluminescence; *N*-(4-substituted benzyl)isoluminol; luminol; isoluminol derivative

5-Amino-2,3-dihydro-1,4-phthalazine-1,4-dione (luminol) and 6-amino-2,3-dihydro-1,4-phthalazine-1,4-dione (isoluminol) are well-known chemiluminescence (CL) compounds, and many synthesized luminol and isoluminol derivatives have been widely used for various biomicroanalyses due to their high sensitivity. A practical labelling reagent having a luminol moiety has not been reported due to the difficulty of its synthesis and low CL intensity. On the other hand, various reagents having an isoluminol moiety, such as isoluminol-isothiocyanate (ILITC),[1] *N*-aminobutyl-*N*-ethylisoluminol (ABEI),[2,3] 4,5-diaminophthalhydrazide (DPH)[4] and 6-aminomethylphthalhydrazide (6-AMP)[5] have been used for the microanalyses of biological compounds. Most of these reagents react with the amino group or carboxylic acid of the analytes and a new CL labelling reagent which reacts with other functional groups has been required. The modification of the amino group of luminol with the electron donating group such as an alkyl group causes a decrease in the CL intensity. On the other hand, that of isoluminol with an electron donating group causes an increase in the intensity.[6,7] Thus we synthesized *N*-(4-substituted benzyl)isoluminols (**3a**—**e**) and the CL characteristic (relative CL intensity) of these isoluminol derivatives were studied. To clarify the relationship between the substituent groups and CL intensities, we used the Hamett substituent constants (σ) as a parameter for the electronic effects of the substituent groups.

Experimental

Reagents and Solutions Deionized H_2O purified with a Milli-Q II system (Japan Millipore, Tokyo, Japan) was used for the preparation of the aqueous solutions. Hydrogen peroxide (31%, v/v) was purchased from Mitsubishi Gas Kagaku (Tokyo, Japan). Luminol and isoluminol were purchased from Tokyo Kasei Industry (Tokyo, Japan). Luminol was purified as sodium luminol by 3 successive salting outs with 5% NaOH.[8] All other chemicals were of reagent grade and used without further purification. 4-Amino-*N*-methylphthalimide (**1**) was synthesized from 4-nitrophthalimide. The *N*-methylation of 4-nitrophthalimide was done using a method similar to that of Roswell *et al.*[9] *N*-methyl-4-nitrophthalimide was reduced to **1** by the method of Drew and Pearmann[10] Compound **1** was recrystallized from MeOH. Stock solutions of the CL compounds were prepared as follows: sodium luminol in 0.1 M sodium carbonate, and isoluminol and compounds **3a**—**e** in dimethyl sulfoxide (DMSO). These stock solutions were diluted to 1 mM or 10 nM solution with 0.1 M sodium carbonate.

Apparatus The CL reactions were carried out in a 75×12 mm round-bottom glass tube. The CL measurements were performed using a Lumat LB9507 luminometer (Berthold, Wildbad, Germany). Uncorrected fluorescence excitation and emission spectra were obtained using a Hitachi F2000 fluorescence spectrometer (Tokyo, Japan). FAB-MS were taken with a JEOL (Tokyo, Japan) JMS600 spectrometer. ^{1}H-NMR spectra were taken in chloroform-d_1 ($CDCl_3$) and DMSO-d_6 ($(CD_3)_2SO$) with a Varian UNITY plus spectrometer (U.S.A.) at 500 MHz.

Synthesis of 4-(*p*-Substituted Benzyl)amino-*N*-methylphthalimide (2a—e) Compound **1** (0.352 g, 2 mmol) and *p*-substituted benzyl bromide or chloride (2 mmol) were dissolved in *N*,*N*-dimethyl formamide (DMF) (5 ml). The mixture was refluxed with stirring for 8 h. The reaction mixture was purified by column chromatography on silica gel with $CHCl_3$–Me_2CO (15 : 1, v/v) as the eluent. Each fraction corresponding to **2a**—**e** was collected and evaporated to dryness under reduced pressure. The residue was recrystallized from MeOH–H_2O to give **2a**—**c**, **e** (yellow crystal), and **2d** (orange crystal).

2a: Yield 61%. mp=151—152 °C. ^{1}H-NMR 500 MHz ($CDCl_3$) δ: 3.09 (s, 3H, CH_3), 4.41 (d, 2H, CH_2, J=5.5 Hz), 4.76 (s, H, NH), 6.72 (q, H, ArH), 6.99 (d, H, ArH), 7.28—7.37 (m, 5H, ArH), 7.57 (d, H, ArH). FAB-MS (positive ion mode) m/z: 267.2 $[M+1]^+$.

2b: Yield 51%. mp=163—164 °C. ^{1}H-NMR 500 MHz ($CDCl_3$) δ: 2.33 (s, 3H, Ar-CH_3), 3.10 (s, 3H, CH_3), 4.36 (d, 2H, CH_2, J=5.5 Hz), 4.70 (s, H, NH), 6.71 (q, H, ArH), 6.98 (d, H, ArH), 7.15 (d, 2H, ArH, J=7.5 Hz), 7.20 (d, 2H, ArH, J=8 Hz), 7.56 (d, H, ArH) FAB-MS m/z: 281.2 $[M+1]^+$.

2c: Yield 41%. mp=177—178 °C. ^{1}H-NMR 500 MHz ($CDCl_3$) δ: 3.09 (s, 3H, CH_3), 3.79 (s, 3H, OCH_3), 4.33 (d, 2H, CH_2, J=5.5 Hz), 4.66 (s, H, NH), 6.71 (q, H, ArH), 6.87 (q, 2H, ArH), 6.98 (d, H, ArH, J=4 Hz), 7.26 (2H, ArH), 7.56 (d, H, ArH). FAB-MS m/z: 297.2 $[M+1]^+$.

2d: Yield 33%. mp=236—238 °C. ^{1}H-NMR 500 MHz ($CDCl_3$) δ: 3.09 (s, 3H, CH_3), 4.57 (d, 2H, CH_2, J=6 Hz), 4.91 (s, H, NH), 6.71 (q, H, ArH), 6.94 (d, H, ArH, J=2 Hz), 7.48 (d, 2H, ArH, J=8.5 Hz), 7.58 (d, H, ArH,

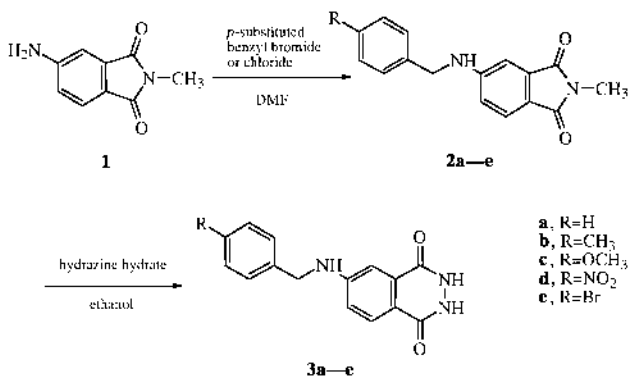

Fig. 1. Synthetic Route of **3a**—**e**

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J=8.5 Hz), 8.20 (d, 2H, ArH, J=9 Hz). FAB-MS m/z: 312.2 $[M+1]^+$.

2e: Yield 52%. mp=207—208 °C. ^{1}H-NMR 500 MHz ($CDCl_3$) δ: 3.09 (s, 3H, CH_3), 4.38 (d, 2H, CH_2, J=5.5 Hz), 4.76 (s, H, NH), 6.70 (q, H, ArH), 6.96 (d, H, ArH), 7.19 (d, 2H, ArH, J=8.5 Hz), 7.46 (d, 2H, ArH, J=8.5 Hz), 7.57 (d, H, ArH). FAB-MS m/z: 345.1 $[M+1]^+$.

Synthesis of *N*-(4-Substituted Benzyl)isoluminol (3a—e) Hydrazine hydrate (500 μl) was added to a suspension of **2a**—**e** (1 mmol) in EtOH (5 ml). The mixture was refluxed for 3 h. The mixture was then added to 1 M HCl (100 ml) and stirred at room temperature for 1 h. The resulting precipitate was collected and recrystallized from aqueous acetic acid to give compounds **3a**—**e** as white crystals **3a**, **b** and yellow crystals **3c**—**e**, respectively.

6-Benzylamino-2,3-dihydrophthalazine-1,4-dione (**3a**): Yield 23% from **2a**. mp=223—225 °C. ^{1}H-NMR 500 MHz (DMSO-d_6) δ: 4.39 (d, 2H, CH_2, J=5.5 Hz), 6.95 (s, H, ArH), 7.09 (dd, H, ArH, J=8.5, 2 Hz), 7.22 (t, H, ArNH, J=7 Hz), 7.28—7.37 (m, 5H, ArH), 7.75 (s, H, ArH). FAB-MS m/z: 268.2 $[M+1]^+$. *Anal*. Calcd for $C_{15}H_{13}N_3O_2 \cdot 1/2\ H_2O$: C, 65.20; H, 5.11; N, 15.21. Found: C, 65.35; H, 5.13; N, 15.04.

6-[(4-Methylphenyl)methyl]amino-2,3-dihydrophthalazine-1,4-dione (**3b**): Yield 86% from **2b**. mp=205 °C. ^{1}H-NMR 500 MHz (DMSO-d_6) δ: 2.26 (s, 3H, CH_3), 4.33 (d, 2H, CH_2, J=6 Hz), 6.93 (s, H, ArH), 7.07 (dd, H, ArH, J=8.5, 2 Hz), 7.12 (d, 2H, ArH, J=8 Hz), 7.23 (d, 2H, ArH, J=8 Hz), 7.24 (t, H, ArNH, J=5.5 Hz), 7.74 (s, H, ArH). FAB-MS m/z: 282.2 $[M+1]^+$. *Anal*. Calcd for $C_{16}H_{15}N_3O_2 \cdot H_2O$: C, 64.20; H, 5.72; N, 14.04. Found: C, 64.24; H, 5.59; N, 13.96.

6-[(4-Methoxyphenyl)methyl]amino-2,3-dihydrophthalazine-1,4-dione (**3c**): Yield 69% from **2c**. mp=224—225 °C. ^{1}H-NMR 500 MHz (DMSO-d_6) δ: 3.71 (s, 3H, OCH_3), 4.30 (d, 2H, CH_2, J=5.5 Hz), 6.88 (d, 2H, ArH, J=8.5 Hz), 6.94 (s, H, ArH), 7.07 (dd, H, ArH, J=8.5, 2.5 Hz), 7.21 (t, H, ArNH, J=6 Hz), 7.27 (d, 2H, ArH, J=8.5 Hz), 7.73 (s, H, ArH). FAB-MS m/z: 298.2 $[M+1]^+$. *Anal*. Calcd for $C_{16}H_{15}N_3O_3 \cdot 1/2\ H_2O$: C, 62.73; H, 5.26; N, 13.71. Found: C, 62.62; H, 5.35; N, 13.63.

6-[(4-Nitrophenyl)methyl]amino-2,3-dihydrophthalazine-1,4-dione (**3d**): Yield 76% from **2d**. mp>300 °C. ^{1}H-NMR 500 MHz (DMSO-d_6) δ: 4.57 (d, 2H, CH_2, J=6 Hz), 6.92 (s, H, ArH), 7.09 (dd, H, ArH, J=9, 2.5 Hz), 7.44 (t, H, ArNH, J=6 Hz), 7.61 (d, 2H, ArH, J=8.5 Hz), 7.76 (d, H, ArH, J=9 Hz), 8.19 (d, 2H, ArH, J=9 Hz). FAB-MS m/z: 313.2 $[M+1]^+$. *Anal*. Calcd for $C_{15}H_{12}N_4O_4 \cdot CH_3COOH$: C, 54.84; H, 4.33; N, 15.05. Found: C, 54.88; H, 4.42; N,14.94.

6-[(4-Bromophenyl)methyl]amino-2,3-dihydrophthalazine-1,4-dione (**3e**): Yield 58% from **2e**. mp>300 °C. ^{1}H-NMR 500 MHz (DMSO-d_6) δ: 4.37 (d, 2H, CH_2, J=5.5 Hz), 6.91 (s, H, ArH), 7.07 (dd, H, ArH, J=9, 2.5 Hz), 7.30 (d, 2H, ArH, J=8.5 Hz), 7.33 (t, H, ArNH), 7.51 (d, 2H, ArH, J=8 Hz), 7.74 (d, H, ArH, J=9 Hz). FAB-MS m/z: 346.0, 348.0 $[M+1]^+$. *Anal*. Calcd for $C_{15}H_{12}BrN_3O_2 \cdot 1/2\ H_2O$: C, 50.72; H, 3.69; N, 11.83. Found: C, 50.67; H, 5.74; N, 11.77.

CL Property To 200 μl of a 10 nM stock solution, 50 μl of 40 mM potassium hexacyanoferrate(III) in 0.1 M Na_2CO_3 and 50 μl of 20 mM hydrogen peroxide were added by automatic injection. The CL intensities were measured for 1 min and the integral photon counts were used.

Fluorescence of the CL Reaction Product To 400 μl of the 1 mM stock solution, 100 μl of 40 mM potassium hexacyanoferrate(III) in 0.1 M Na_2CO_3 and 100 μl of 20 mM hydrogen peroxide were added and allowed to stand at room temperature for 1 h. The fluorescence excitation, emission maxima and relative intensities of the resulting mixtures were measured by a fluorescence spectrometer.

Hammett Substituent Constants The Hamett substituent constants (σ_p) used in this study were cited from the review written by Hansch *et al.*[11] as follows; **3a** (H, 0.00), **3b** (Me, −0.17), **3c** (OMe, −0.27), **3d** (NO_2, 0.78), **3e** (Br, 0.23).

Results and Discussion

We synthesized isoluminol derivatives which were modified with a 4-substituted benzyl group through the amino group of isoluminol. By this modification, a change in the CL intensities was expected. Compound **3a**—**e** produce CL by the reaction with hydrogen peroxide and potassium hexacyanoferrate(III) in the presence of alkaline medium. The CL intensity reached a maximum in a few seconds after the addition of hydrogen peroxide and potassium hexacyanoferrate(III) and then almost disappeared within one minute.

The effects of the concentrations of hydrogen peroxide and potassium hexacyanoferrate(III) on the CL intensity were examined (Fig. 2). A 20 mM hydrogen peroxide and 40 mM potassium hexacyanoferrate(III) produced almost the maximum intensity. Under these reaction conditions, the CL intensities obtained from the seven derivatives were determined (Table 1). The CL intensities of **3a**—**e** were 0.03—4.7 times of isoluminol, and the intensities corresponded to 1/1000—9/50 of that obtained with luminol. In the CL of luminol, the 3-aminophthalate ion produced during the oxidizing reaction, has been shown to be the light emitter. Therefore, in the case of **3a**—**e**, the corresponding dicarboxylate ions should be emitting species. The fluorescence properties (excitation and emission maximum wavelengths of the fluorescence and relative intensities) of the species were measured after the CL reactions were completed (Table 2). The fluorescence intensities of **3a**—**e** were 0.02—1.9 times larger than that of luminol. The fluorescence excitation and emission maximum

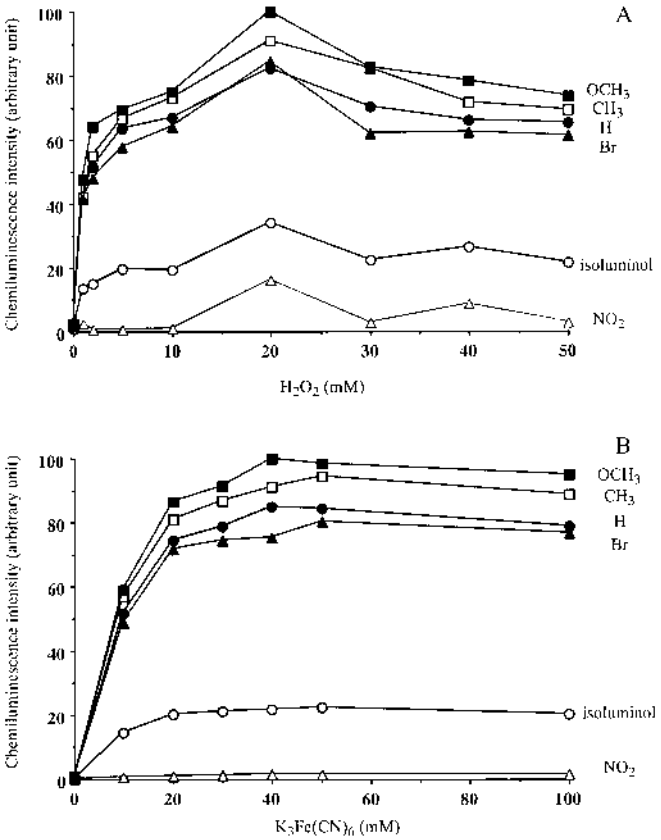

Fig. 2. Effects of Concentrations of (A) Hydrogen Peroxide and (B) Potassium Hexacyanoferrate(III) on the CL Intensities

Table 1. Relative CL Intensities (RCI) of *N*-(4-Substituted benzyl)isoluminols

Compound	
Sodium luminol	100.0[a]
Isoluminol	3.7
3a (R=H)	15.2
3b (R=CH_3)	17.0
3c (R=OCH_3)	17.6
3d (R=NO_2)	0.1
3e (R=Br)	14.6

a) Integrated CL intensity of luminol was taken as 100.

Table 2. Fluorescence Excitation (Ex), Emission (Em) Maxima and Relative Intensities (RFI) of *N*-(4-Substituted benzyl)isoluminols after the CL Reaction

Compound (CL reactant)	Ex (nm)	Em (nm)	RFI[a)]
Sodium luminol	307	465	100.0
Isoluminol	274	467	106.3
3a (R=H)	278	467	146.2
3b (R=CH_3)	279	466	168.8
3c (R=OCH_3)	278	465	190.7
3d (R=NO_2)	270	466	2.1
3e (R=Br)	285	466	78.6

a) Fluorescence intenstiy of luminol was taken as 100.

wavelengths of **3a**—**e** were almost the same as that of isoluminol.

The relationship between the amino-H chemical shift value and σ_p is shown in Fig. 3A. The electron donating substituent groups such as the methyl and methoxy groups cause upfield shifts of δ for the amine proton. On the other hand, the electron accepting groups such as the bromo and nitro groups cause downfield shifts of δ for the amine proton. Figure 3A showed a good linear correlation between the Hamett substituent constants and the amino-H chemical shift values. This result suggests that the change in the substituent group influences the electron density around the amino group. It is known that *N*-alkylated isoluminols have higher CL intensities than that of isoluminol. To explain this result, we examined the relationship between the CL intensities and the values of σ_p (Fig. 3B). Introduction of the electron donating groups onto the amino nitrogen caused an increase in the CL intensity. On the other hand, the electron accepting group caused a decrease in the intensity. The intensities are proportional to the fluorescence intensities of the final products of the CL reaction. Thus, we examined the relationship between the fluorescence intensities and the values of σ_p (Fig. 3C). Introduction of electron donating groups onto the amino nitrogen caused an increase in the fluorescence intensity, while the substitution of an electron accepting group onto the nitrogen caused a decrease in the intensity. These results suggest that the change in the electron density around the amino group strongly influences the fluorescence intensities and corresponding CL intensities of these derivatives. Thus, introduction of the electron donating groups onto the *p*-position of the benzyl group of the *N*-benzyl isoluminol enables us to develop luminol-type labelling reagents without any decrease in the CL intensity of the isoluminol. *N*-benzyl isoluminol can be easily synthesized and it can have various reactive substituent groups on the *p*-position of the benzyl group. The labelling reagent which has an electron donating group on the *p*-position of the benzyl group after the labelling reaction should have sufficient CL intensities and be useful for CL detection of biomicroanalyses. The synthesis of such labelling reagents for HPLC–CL analyses and their application are now in progress.

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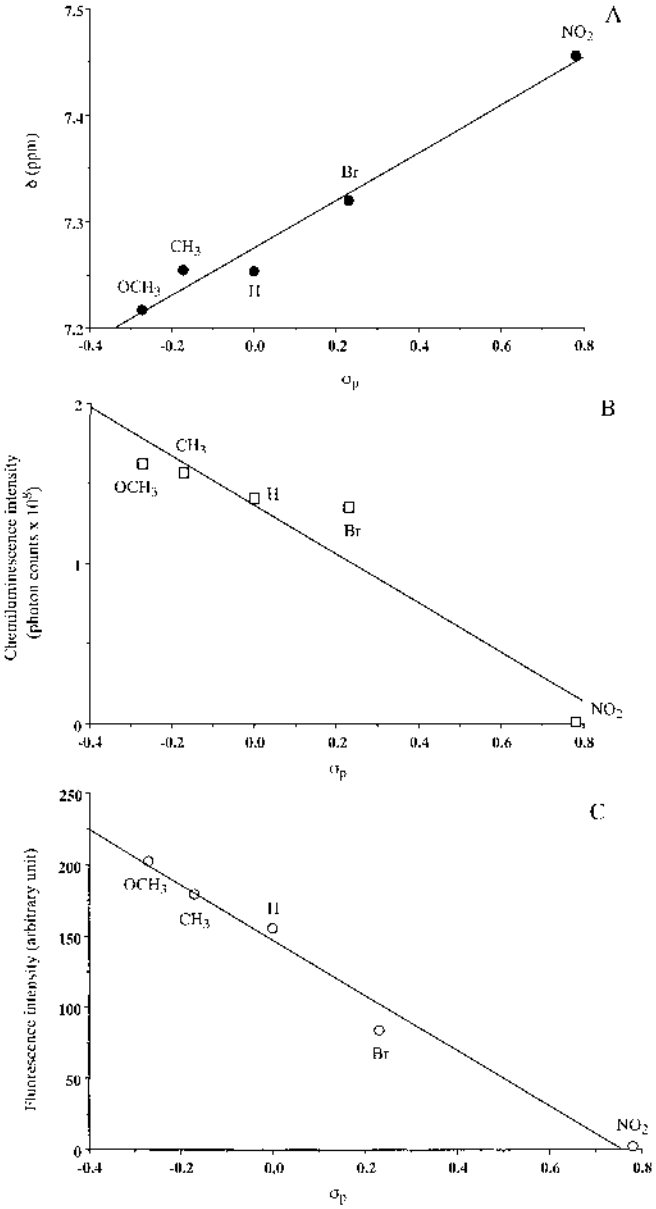

Fig. 3. The Relationship between the Hamett Substituent Constant and the Amine-H Chemical Shift Values (A), the Relative Chemiluminescence Intensities (B), and the Relative Fluorescence Intensities (C)

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