Electronic Supplementary Information

A Novel Luciferin-Based Bright Chemiluminescent Probe for the Detection of Reactive Oxygen Species

Maki Sekiya, Keitaro Umezawa, Akemi Sato , Daniel Citterio and Koji Suzuki*

Abbreviations

CLA : 2-methyl-6-phenylimidazo[l,2-*a*]pyrazin-3(7*H*)-one[1, 2]
MCLA : 2-methyl-6-(4-methoxyphenyl)imidazo[l,2-*a*]pyrazin-3(7*H*)-one[1, 3]
FCLA : 2-methyl-6-[*p*-[2-[sodium3-carboxylato-4-(6-hydroxy-3-xanthenon-9-yl)phenylthioureylene]-ethyleneoxy]phenyl]-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one, consisting of the moieties of MCLA and fluorescein[1, 4]
Red-CLA : consisting of the moieties of MCLA and sulforhodamine 101[1, 5]

Chemicals and Instruments

All chemical reagents and solvents for synthesis were purchased from commercial suppliers (Wako Pure Chemical, Tokyo Kasei Industry, Invitrogen and Aldrich Chemical) and were used without further purification. The composition of mixed solvents is given as the volume ratio (v/v). ^{1}H-NMR spectra were recorded on a JEOL JNM-LA 300 (JEOL Ltd., Tokyo, Japan) spectrometer at room temperature. The measurements were performed at 300 MHz. All chemical shifts are relative to an internal standard of tetramethylsilane (δ= 0.0 ppm), and coupling constants (*J*) are given in Hz. Flash chromatography separation was undertaken using a YFLC-Al-560 chromatograph (Yamazen Co., Osaka, Japan). High-resolution MS spectra (HR-MS) were recorded on a JEOL JMS-T100LCS (JEOL Ltd., Tokyo, Japan) with MeOH as the eluent.

Synthesis of KBI

1,3,5,7-Tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene: Under a nitrogen atmosphere, 2,4-dimethylpyrrole (5.19 g, 54.6 mmol) was dissolved in dichloromethane (100 ml) and stirred at room temperature. Triethoxymethane (4.5 ml, 27.1 mmol) and trifluoroacetic acid (3.0 ml, 40.4 mmol) were added into the mixture, and stirring continued for 2 h. The organic phase was washed with water and brine, dried over Na_2SO_4 and evaporated. Under an argon atmosphere, the resulting residue (5.66 g, 28.3 mmol) and triethylamine (10.4 ml, 74.4 mmol) were dissolved in toluene (200 ml) and stirred at room temperature. Boron trifluoride-ethyl ether complex (15.6 ml, 124 mmol) was added dropwise to the mixture, and stirring continued for 0.5 h. The reaction mixture was poured into saturated aqueous $NaHCO_3$ solution to neutralize. The organic phase was washed with water and brine, dried over Na_2SO_4 and evaporated. The resulting residue was purified by chromatography (silica gel, eluent: *n*-hexane/toluene = 1/3) to obtain 1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene as a red solid (3.85 g, 56.9 %). ^{1}H-NMR ($CDCl_3$): δ = 7.04 (s, 1H), 6.04 (s, 2H), 2.53 (s, 6H), 2.24 (s, 6H).

2-Bromo-1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene: Under a nitrogen atmosphere, 1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (206 mg, 0.83 mmol) was dissolved in dichloromethane (500 ml) and stirred at room temperature. *N*-bromosuccinimide (147 mg, 0.83 mmol) was added to the mixture portionwise over 3 h, and stirring continued for 20 h. The solvent was evaporated and the resulting residue was purified by chromatography (silica gel, eluent: *n*-hexane/toluene = 1/3) to obtain 2-bromo-1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene as an orange solid (225 mg, 82.8 %). ^{1}H-NMR ($CDCl_3$): δ = 7.05 (s, 1H), 6.10 (s, 1H), 2.55 (s, 6H), 2.26 (s, 3H), 2.22 (s, 3H).

1,3,5,7-Tetramethyl-2-[4,4,5,5-tetramethyl-1,3,2-dioxaborolanyl]-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene: Under an argon atmosphere, 2-bromo-1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (604 mg, 1.85 mmol), bis(pinacolato)diboron (939 mg, 3.70 mmol), and potassium acetate (216 mg, 2.77 mmol) were dissolved in dioxane (11 ml) and degassed *in vacuo*. A catalytic amount of [1,1'-bis(diphenylphosphino)ferrocene]palladium(II) dichloride was added to the mixture and heated at 90 °C for 21 h. After cooling, the organic phase was washed with water and brine, dried over Na_2SO_4 and evaporated. The resulting residue was purified by flash chromatography (silica gel, eluent: *n*-hexane/chloroform = 3/7) to obtain 1,3,5,7-tetramethyl-2-[4,4,5,5-tetramethyl-1,3,2-dioxaborolanyl]-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene as an orange solid (173mg, 25.0 %). ^{1}H-NMR ($CDCl_3$): δ = 7.10 (s, 1H), 6.07 (s, 1H), 2.72 (s, 3H), 2.53 (s, 3H), 2.41 (s, 3H), 2.25 (s, 3H), 1.31 (s, 12H). HR-MS: found *m/z* 397.2055 [M+Na^+], calculated for [$C_{19}H_{26}B_2F_2N_2O_2$+Na^+] : 397.2046.

5-Bromo-2-aminopyrazine: Under an argon atmosphere, 2-aminopyrazine (542 mg, 5.70 mmol) was dissolved in dichloromethane (60 ml), degassed *in vacuo*, and stirred at 0 ^{o}C. *N*-bromosuccinimide (1020 mg, 5.73 mmol) was added to the mixture portionwise over 30 min, and stirring was continued for 1 h. The reaction mixture was poured into saturated aqueous $NaHCO_3$ solution. The organic phase was washed with water and brine, dried over Na_2SO_4 and evaporated. The resulting residue was purified by flash chromatography (silica gel, eluent: *n*-hexane/ethyl acetate = 1/1) to obtain 5-bromo-2-aminopyrazine as a creamy white solid (0.994 g, 51.0 %). ^{1}H-NMR ($CDCl_3$): δ = 8.09 (d, *J* = 1.2 Hz, 1H), 7.77 (d, *J* = 1.2 Hz, 1H), 4.63 (s, 2H).

2-[5-(2-Aminopyrazin)-yl]-1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene: Under an argon atmosphere, 1,3,5,7-tetramethyl-2-[4,4,5,5-tetramethyl-1,3,2-dioxaborolanyl]-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (148 mg, 0.39 mmol) and 5-bromo-2-aminopyrazine (57.4 mg, 0.33 mmol) were dissolved in a mixture of toluene (24 ml), methanol (6 ml), and 2M $NaCO_3$ aqueous solution (400 µl), and degassed *in vacuo*. A catalytic amount of tetrakis(triphenylphosphine)palladium(0) was added into the mixture and heated at 75 ^{o}C for 6 h. After cooling, the organic phase was washed with water and brine, dried over Na_2SO_4 and evaporated. The resulting residue was purified by chromatography (silica gel, eluent: chloroform /methanol = 50/1) to obtain 2-[5-(2-Aminopyrazin)-yl]-1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene as a red solid (56.0 mg, 50.0 %). ^{1}H-NMR ($CDCl_3$): δ =8.10 (d, *J* = 1.7 Hz, 1H), 8.02 (d, *J* = 1.5 Hz, 1H), 7.13 (s, 1H), 6.09 (s, 1H), 4.63 (s, 2H), 2.62 (s, 3H), 2.56 (s, 3H), 2.31 (s, 3H), 2.28 (s, 3H). HR-MS: found *m/z* 364.1499 [M+Na^+], calculated for [$C_{17}H_{18}BF_2N_5$+Na^+] : 364.1521.

2-[6-(2-Methylimidazo[1,2-*a*]pyrazin-3(7*H*)-one)-yl]-1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (KBI): Under an argon atmosphere, 2-[5-(2-aminopyrazin)-yl]-1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (56.1 mg, 0.16 mmol) and pyruvaldehyde solution (62.0 mg, 0.25 mmol) were dissolved in a mixture of 10% HCl aq (200 µl), water (200 µl), and dioxane (1.2 ml), stirred and heated at 70 ^{o}C for 2 h. After cooling, the organic phase was washed with water and brine, dried over Na_2SO_4 and evaporated. The resulting residue was purified by flash chromatography (silica gel, eluent: chloroform /methanol = 10/1) to obtain 2-[6-(2-methylimidazo[1,2-*a*]pyrazin-3(7*H*)-one)-yl]-1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene as a red solid (18.7 mg, 28.9 %). ^{1}H-NMR ($CDCl_3$): δ =7.91 (s, 1H), 7.59 (s, 1H), 7.40 (s, 1H), 6.26 (s, 1H), 2.53 (s, 3H), 2.51 (s, 3H), 2.45 (s, 3H), 2.34 (s, 6H). HR-MS: found *m/z* 418.1645 [M+Na^+], calculated for [$C_{20}H_{20}BF_2N_5O$+Na^+] : 418.1627.

2-[5-(*N*-pyrazin-2-yl-acetylamin)-yl]-1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (ring-opened KBI): Under an argon atmosphere, 2-[5-(2-Aminopyrazin)-yl]-1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (23.0 mg, 0.067 mmol)) was dissolved in dichloromethane (800 µl) and stirred at 0 ^{o}C. Pyridine (seven drops) and acetyl chloride (two drops) were added into the mixture, and stirring continued for 1.0 h. The reaction mixture was poured into saturated aqueous $NaHCO_3$ solution. The organic phase was washed with water and brine, dried over Na_2SO_4 and evaporated. The resulting residue was purified by chromatography (silica gel, eluent: acetic acid/toluene acetate = 3/2) to obtain 2-[5-(*N*-pyrazin-2-yl-acetylamin)-yl]-1,3,5,7-tetramethyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene as a red solid (16.4 mg, 63.6 %). ^{1}H-NMR ($CDCl_3$): δ = 9.58 (s, 1H), 8.25 (d, *J* = 1.5 Hz, 1H), 7.94 (s, 1H), 7.17 (s, 1H), 6.12 (s, 1H), 2.66 (s, 3H), 2.58 (s, 3H), 2.36 (s, 3H), 2.29 (s, 3H), 2.29 (s, 3H). HR-MS: found *m/z* 406.1661 [M+Na^+], calculated for [$C_{19}H_{20}BF_2N_5O$+Na^+] : 406.1627.

Chemiluminescence spectra

MOPS buffer (pH 7.2) prepared from 0.02 M MOPS, 0.2 M KCl, and 0.01 M KOH was used for all the experiments. An XOD solution (3.2 U/ml, 20µl) was added to a quartz glass cell and it was set in the AB-1850 LumiFL-Spectocapture (ATTO corporation). The chemiluminescence was accumulated for 175 second after injecting a mixture of each chemiluminescence probe solution (2.5 µl, 200 µl) and HPX solution (1 mM, 40 µl), and the spectra were recorded. KBI was dissolved in MOPS buffer (containing 5 % MeOH), while the other reagents were dissolved in pure MOPS buffer.

The mechanism of chemiluminescence and fluorescence, and fluorescence spectra

Fig. S-1 schematically illustrates the chemiluminescence mechanism of KBI oxidized by ROS and the fluorescence mechanism of the ring-opened KBI (assumed singlet-excited). According to the reported mechanism of the imidazopyrazine chemiluminescence,[1] the decomposition of the dioxetanone oxidized by ROS results in a singlet-excited amide, which then relaxes to the ground state by emitting light. Fig. S-2 shows

the fluorescence spectra of ring-opened KBI and of BODIPY. An obvious difference between ring-opened KBI and BODIPY in terms of emission wavelength was confirmed. In this experiment, all reagents were dissolved in chloroform and fluorescence emision spectra were recorded on a F-4500 fluorophotometer (Hitachi corporation).

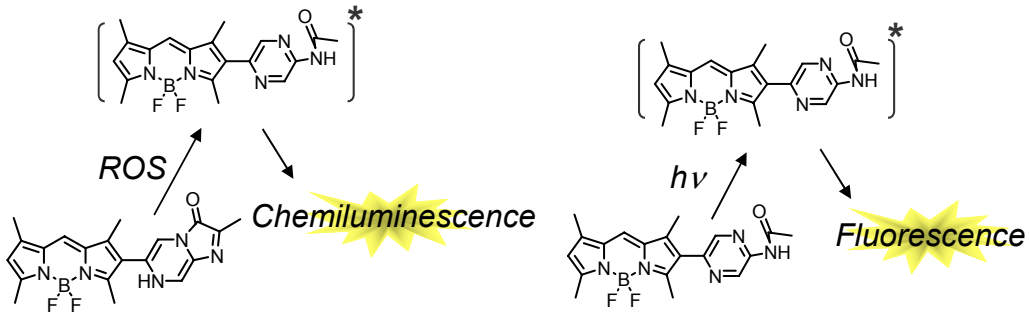

Fig. S-1 The mechanism of chemiluminescence and fluorescence

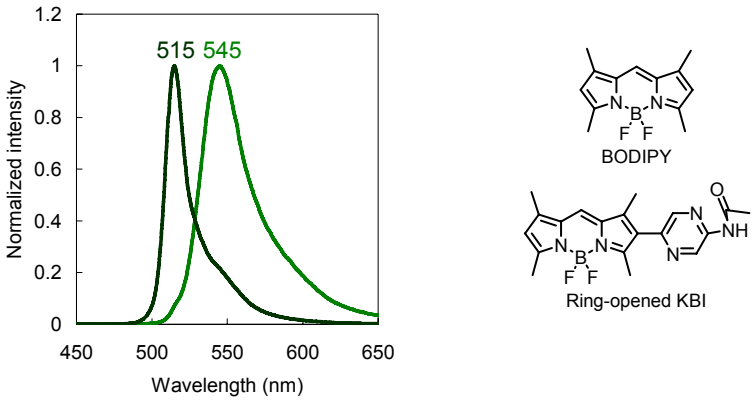

Fig. S-2 Fluorescence spectra of BODIPY and ring-opened KBI (in chloroform, Ex: 515 nm)

Chemiluminescence colors

The chemiluminescence probes (KBI and MCLA) and horseradish peroxidase (HRP) were diluted with MeOH and stirred at room temperature. The chemiluminescence was accumulated for a few minutes with a digital camera after injecting an excess of H_2O_2. In order to be able to collect sufficient chemiluminescence with a standard digital camera, high concentrations of probes were used. Due to the limites solubility of KBI in 100% aqueous environment, MeOH was used as the solvent for this experiment.

The relative chemiluminescence intensity (RCI) of KBI

The following list describes the detailed experimental conditions used to determine the RCI of KBI in the presence of various ROS (O_2^-, H_2O_2, ClO^-, $ONOO^-$, $^{\cdot}OH$, $^1O_2$) and antioxidants (SOD and catalase). KBI was dissolved in MOPS buffer (containing 5 % MeOH), while the other reagents were dissolved in pure MOPS buffer.

Blank	: KBI solution (5.2 µM, 200 µl)
O_2^-	: HPX (1 mM, 50 µl) and XOD (1.6 U/ml, 20 µl)
H_2O_2	: Hydrogen peroxide (10 mM, 5 µl) and horseradish peroxidase (40 U/ml, 50 µl)
ClO^-	: Sodium hypochlorite (1 mM, 50 µl)
$ONOO^-$	: Peroxynitrite in solution (4.5 mM, 50 µl)
$^{\cdot}OH$	: Hydrogen peroxide (0.1 M, 5 µl) and iron perchlorate (1 mM, 50 µl)
$^1O_2$	: Hydrogen peroxide (0.1 M, 5 µl) and sodium hypochlorite (1 mM, 50 µl)
O_2^- + SOD	: HPX (1 mM, 50 µl), XOD (1.6 U/ml, 20 µl), and SOD (400 U/ml, 200 µl)
H_2O_2 + catalase	: Hydrogen peroxide (0.1 M, 5 µl), horseradish peroxidase (40 U/ml, 50 µl), and catalase (400 U/ml, 200 µl)

The detection of ROS generated from HL-60 cells

KBI was dissolved in HBSS buffer containing 1 % DMSO, while MCLA was dissolved in pure HBSS buffer. The reason for using MCLA instead of FCLA was that the chemiluminescence intensity of MCLA was higher than that of FCLA (Fig. 1). HL-60 cells (10^6 cells) from human acute promyelotic leukemia had been cultured with RPMI-1640 containing 10 % fetal bovine serum (FBS). In this experiment, HL-60 cells were washed with HBSS buffer twice and incubated with the solution of each chemiluminescent probe (1 µM, 200 µl) at 5 % CO_2 and 37 ^oC for 40 min in 1.5 ml plastic tubes. Then, each suspension containing probe-loaded cells was transferred to a black 96-well microplate. The microplate was set in the AB-2350 PHELIOS, and the background chemiluminescence was accumulated. Ten minutes later, the microplate was ejected and phorbol 12-myristate 13-acetate (PMA: HBSS buffer, pH 7.4 contaoning 0.1 % DMSO: 1 µM, 20 µl) was manually pipetted as stimulating agent to each well. Then chemiluminescence was accumulated again for 40 minutes. ROS were generated when HL-60 cells were stimulated with PMA, which was detected with each chemiluminescent reagent. The chemiluminescence at each well was collected every seven seconds for KBI, and every ten seconds for MCLA, respectively.

Fig. S-3 shows the raw data. Higher chemiluminescence intensities were observed with KBI for stimulated cells (green line), compared to non-stimulated cells (light green line). This indicates that KBI responds to ROS generated from HL-60 cells. On the other hand, with MCLA (blue lines) this detection was hardly possible for stimulated cells. The difference of the chemiluminescence intensities between stimulated cells and non-stimulated cells (light blue line) was too small to allow reliable ROS detection, indicating a significantly lower sensitivity of MCLA compared to KBI. While the chemiluminescence of KBI generated from stimulated cells was much larger than that of MCLA, the background was relatively high, too. This is assumed to be due to autooxidation of KBI and oxidation by ROS generated from HL-60 cells without stimulation.

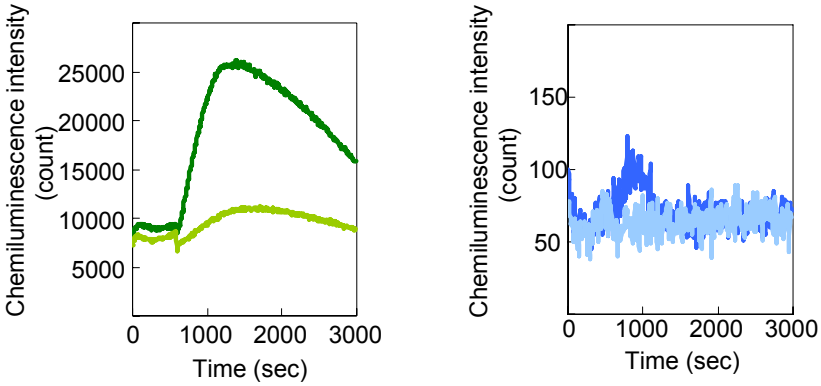

Fig. S-3 Chemiluminescence intensity of KBI and MCLA (1.0 µM), oxidized by ROS generated from HL-60 cells.(green line: KBI for stimulated cells, light green line: KBI for non- stimulated cells, blue line: MCLA for stimulated cells, light blue line: MCLA for non- stimulated cells)

Reference

1 K. Teranishi, *Bioorg. Chem.*, 2007, **35**, 82
2 T. Goto and T. Takagi, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 833; T. Kobayashi, K. Saga, S. Shimizu and T. Goto, *Agric. Biol. Chem.*, 1981, **45**, 1403
3 A. Nishida, H. Kimura, M. Nakano and T. Goto, *Clinica. Chimica. Acta.*, 1989, **179**, 177
4 N. Suzuki, K. Suetsuna, S. Mashiko, B. Yoda, T. Nomoto, Y. Toya, H. Inaba and T. Goto, *Agric. Biol. Chem.*, 1991, **55**, 157
5 K. Teranishi, *Luminescence,* 2007, **22**, 147