Electronic Supplementary Material (ESI) for ChemComm.

SUPPORTING INFORMATION

Highly Sensitive and Selective Bioluminescence Based Ozone Probes and Their Applications to Detect Ambient Ozone

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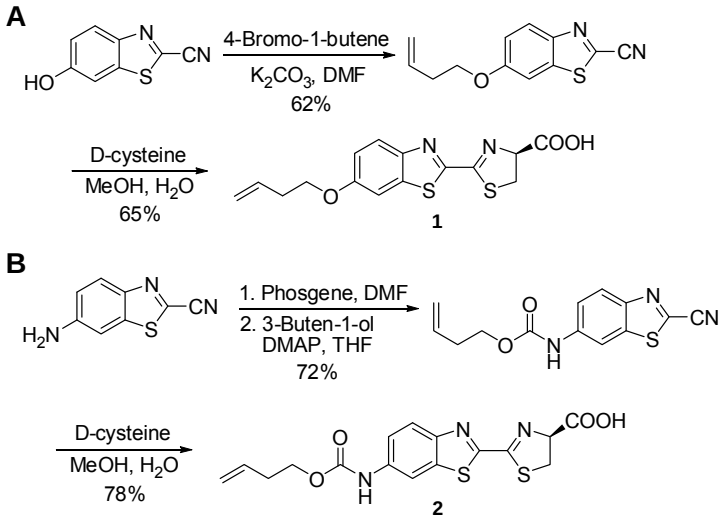
Supplementary Experimental Methods

General methods

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and Alfa Aesar (Ward Hill, MA). Analytical thin-layer chromatography was performed using silica gel 60 F254 glass plates (Merck, Darmstadt). Compound spots on TLC were visualized by UV light (254 nm) and/or by staining with 10 wt% phosphomolybdic acid in ethanol. Flash column chromatography was conducted on silica gel 60 (230-400 Mesh, Merck). NMR spectra were recorded with a Bruke Avance II-400 or DPX-250 instrument. Coupling constants were reported in Hertz (Hz). Analytical reverse-phased HPLC analysis was performed on Denali C-18 column (250 x 4.6 mm) at a flow rate of 1.0 mL/min with a Waters HPLC system. Electrospray ionization (ESI) mass spectra were recorded in a positive ion mode on a Waters 3100 LC/MS System.



Scheme S1. Synthesis of bioluminescent ozone probes (A) **1** and (B) **2**.

2-Cyano-6-(3-butenyloxy)benzothiazole. A stirring mixture of 2-cyano-6-hydroxybenzothiazole[1] (25 mg, 0.14 mmol), K_2CO_3 (58.9 mg, 0.43 mmol) and 4-bromo-1-butene (43.2 μL, 0.43 mmol) in 2 mL DMF was heated at 70 ^oC for 24 h. The reaction mixture was poured into water (25 mL) and extracted with EtOAc (30 mL x 3). The combined organic layers were washed with water and brine, dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo*. The residue was purified by flash column chromatography (hexane : EtOAc = 4:1) to afford a product as a white solid in 62% yield: ^1H NMR (400 MHz, $CDCl_3$) δ 8.08 (d, 1 H, J = 9.5 Hz), 7.35 (d, 1 H, J = 2.2 Hz), 7.24 (dd, 1 H, J = 10.3, 2.2 Hz), 5.86 - 5.97 (m, 1 H), 5.18 - 5.24 (m, 1 H), 5.15 (dd, 1 H, J = 10.3, 2.2 Hz), 4.12 (t, 2 H, J = 7.0 Hz), 2.58 - 2.65 (m, 2 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.9, 147.0, 137.5, 133.9, 133.4, 125.9, 118,9, 117,7, 113.3, 103.8, 68.2, 33.5; ESI-MS calcd for $C_{12}H_{11}N_2OS$ $[M + H]^+$ 231.05, found 231.27.

Synthesis of 1. D-Cysteine hydrochloride monohydrate (71.6 mg, 0.41 mmol) was dissolved in water (2 mL) degassed with argon and pH of the solution was adjusted to 8.0 with 0.5 M K_2CO_3. 2-Cyano-6-(3-butenyloxy)benzothiazole (39.1 mg, 0.17 mmol) dissolved in MeOH (4 mL) degassed with argon was added to the D-cysteine solution. The mixture was stirred at room temperature with light shielding for 1 h under argon atmosphere. The reaction mixture was adjusted to about pH 4 with addition of several drops of concentrated HCl. The organic solvent was removed under reduced pressure. The remaining aqueous solution was extracted with ethyl acetate three times. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography (CH_2Cl_2 : EtOAc : MeOH = 3:3:1) to provide a product as a yellow solid in 65% yield: ^{1}H NMR (400 MHz, CD_3OD) δ 7.94 (d, 1 H, *J* = 8.8 Hz), 7.53 (d, 1 H, *J* = 2.9 Hz), 7.15 (dd, 1 H, *J* = 9.2, 2.6 Hz), 5.91 - 6.02 (m, 1 H), 5.29 (t, 1 H, *J* = 9.2 Hz), 5.20 (dq, 1 H, *J* = 17.1, 1.6 Hz), 5.11 (dt, 1 H, *J*=10.3, 2.2 Hz), 4.12 (t, 2 H, *J*=6.2 Hz), 3.76 (d, 2 H, *J* = 9.5 Hz), 2.58 (qt, 2 H, *J* = 6.6, 1.5 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.4, 161.3, 158.8, 157.9, 147.0, 137.0, 134.7, 124.5, 117.1, 117.0, 105.3, 81.7, 67.4, 35.7, 32.9; ESI-MS calcd for $C_{15}H_{15}N_2O_3S_2$ $[M + H]^+$ 335.05, found 335.19.

6-Butenyloxycarbonylamino-2-cyanobenzothiazole. To a stirred solution of 6-amino-2-cyanobenzothiazole[2] (98 mg, 0.6 mmol) in dry THF (10 mL) was added phosgene (2.7 mL, 20% w/v in toluene, 5.5 mmol). The solution was heated at reflux for 4.5 h. After cooling to room temperature, the remaining phosgene was removed by purging with nitrogen into a KOH trap for 10 min. The solvent was removed under reduced pressure to give a white solid. The solid was dissolved into anhydrous toluene (10 mL) and then the solvent was removed again under reduced pressure. After re-dissolving the resulting residue in dry THF (5 mL) under N_2 atmosphere, 3-buten-1-ol (96 μL, 1.2 mmol) and DMAP (6.0 mg, 0.05 mmol) were added to the solution. After stirring overnight at room temperature, the volatile material was removed under reduced pressure. The residue was re-dissolved in EtOAc and the organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane : EtOAc = 5:1) to give a product as a pale yellow solid in 72% yield: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.41 (s, 1 H), 8.08 (d, 1 H, *J* = 9.5 Hz), 7.38 (dd, 1 H, *J* = 9.2, 1.8 Hz), 7.17 (s, 1 H), 5.74 - 5.90 (m, 1 H), 5.09 - 5.20 (m, 2 H), 4.28 (t, 2 H, *J* = 6.6 Hz), 2.43 - 2.50 (m, 2 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.1, 147.6, 140.2, 136.9, 134.3, 134.1, 124.5, 119.7, 116.3, 112.8, 109.1, 64.1, 33.1; ESI-MS calcd for $C_{13}H_{12}N_3O_2S$ $[M + H]^+$ 274.06, found 274.16.

Synthesis of 2. D-Cysteine hydrochloride monohydrate (77.2 mg, 0.44 mmol) was dissolved in water (2 mL) degassed with argon and pH of the solution was adjusted to 8.0 with 0.5 M K_2CO_3. 6-Butenyloxycarbonylamino-2-cyanobenzothiazole (50 mg, 0.18 mmol) dissolved in MeOH (4 mL) degassed with argon was added to the D-cysteine solution. The mixture was stirred at room temperature with light shielding for 1 h under argon atmosphere. The reaction mixture was adjusted to about pH 4 with addition of several drops of concentrated HCl. The organic solvent was removed under reduced pressure. The remaining aqueous solution was

extracted with ethyl acetate three times. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography (CH_2Cl_2 : EtOAc : MeOH = 3:3:1) to provide a product as a yellow solid in 78% yield: ^{1}H NMR (400 MHz, CD_3OD) δ 8.26 (s, 1 H), 7.95 (d, 1 H, *J* = 9.5 Hz), 7.52 (dd, 1 H, *J* = 9.2, 1.8 Hz), 5.82 - 5.97 (m, 1 H), 5.29 (t, 1 H, *J* = 9.2 Hz), 5.06 - 5.22 (m, 2 H), 4.18 - 4.28 (m, 2 H), 3.76 (d, 2 H, *J* = 9.5 Hz), 2.42 - 2.52 (m, 2 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.2, 161.7, 159.5, 153.7, 148.2, 138.6, 136.4, 134.7, 124.1, 118.8, 117.4, 110.0, 81.9, 63.7, 35.9, 33.0; ESI-MS calcd for $C_{16}H_{16}N_3O_4S_2$ $[M + H]^+$ 378.05, found 378.18.

Production of ozone and determination of its concentration. Ozone produced by using an ozone generator (Fischer, Meckenheim, Germany) was collected in potassium phosphate buffer (5 mM, pH 7.0). The concentration of ozone solutions was determined by iodometric titration and UV spectroscopy methods.

Measurements of ozone concentrations by iodometric titration. Ozone concentrations by iodometric titration were determined according to the method described previously.[3] Briefly, to a solution of ozone (5 mL) in potassium phosphate buffer (5 mM, pH 7.0) was added 5 mL of 0.2 M KI solution. The pH of solutions was adjusted to *ca.* 2.0 by adding drops of 1 M H_2SO_4. The solutions were titrated with 2 mM $Na_2S_2O_3$ solutions until the yellow solutions became coloreless.

Measurements of ozone concentrations by UV spectroscopy.[4] Ozone concentrations were determined by using a JASCO Corporation UV/VIS spectrophotometer ($\varepsilon_{254\,nm}$ (O_3) = 3150 $M^{-1}\cdot cm^{-1}$).

HPLC analysis of products obtained from reaction of ozone and probes. A solution (2 mM) of probe **1** or **2** in DMSO (50 μL) was diluted with 1 mL potassium phosphate buffer (5 mM, pH 7). Each probe (a final concentration: 100 μM) in potassium phosphate buffer (5 mM, pH 7.0) was reacted with ozone (a final concentration: 150 μM) in potassium phosphate buffer (5 mM, pH 7.0) for 1 h at 37 °C. The mixture was treated with 2 mM pyrrolidine for 5 min. The mixture treated or untreated with pyrrolidine was analyzed by analytical RP-HPLC on a C-18 column at a flow rate of 1 mL/min with a linear gradient of 5-100% CH_3CN (0.1% TFA) in water (0.1% TFA) over 45 min. The products were characterized by ESI-MS.

Fluorescent detection of a mixture of probe 1 or 2 with ozone. A solution of each probe (40 μM) in potassium phosphate buffer (5 mM, pH 7.0) containing 0.5% DMSO was treated with various concentrations of ozone (0–120 μM) in potassium phosphate buffer at room temperature for 30 min and then further treated with 2 mM pyrrolidine for 5 min. Fluorescent spectra were recorded on a Perkin Elmer LS 55 fluorometer. The samples were excited at 330 nm for probe **1** and 350 nm for probe **2**.

Luciferase activity test. To a reaction mixture (10 μL) of **1** or **2** with ozone in potassium phosphate buffer was added a solution (10 μL) of luciferase (100 μg/mL) in assay buffer (1 mM ATP, 60 mM DTT 0.6 mM CoA and 16 mM Mg^{2+} in 50 mM Tris buffer at pH 8.0) at 25 ^{o}C. Black, opaque 384-well plates were used for bioluminescent measurements. Bioluminescence intensity was measured by using a Victor X5 Multilabel Plate Reader. The experiment was performed in triplicate.

Bioluminescent detection of a mixture of probe 1 or 2 with ozone. A solution of each probe (1 mM) in potassium phosphate buffer (5 mM, pH 7.0) containing 5% DMSO was treated with various concentrations of ozone (0–500 μM) in potassium phosphate buffer at room temperature for 30 min and then further treated with 2 mM pyrrolidine. After 5 min, luciferase (100 μg/mL) in assay buffer was added to the mixture. After incubation for 1 min at 25 ^{o}C, bioluminescence spectra were recorded on a Perkin Elmer LS 55 fluorimeter (a bioluminescence measurement mode) at room temperature. Bioluminescence images were obtained by using a Xenogen IVIS Spectrum In Vivo Imaging System.

Time-dependent changes in bioluminescence intensity. A solution of each probe (2 μM) in potassium phosphate buffer (5 mM, pH 7.0) containing 0.5% DMSO was treated with ozone (10 μM) in potassium phosphate buffer at 25 ^{o}C. After incubation for the indicated time, luciferase (100 μg/mL) in assay buffer was added to the mixture and then bioluminescence intensity was measured by using a bioluminescent microplate reader.

Response of probes to ROS. A solution of each probe (2 μM) in potassium phosphate buffer (5 mM, pH 7.0) containing 0.5% DMSO were treated with 20 μM O_3, 1 mM $^1O_2$, 1 mM H_2O_2, 1 mM $^{\cdot}OH$, 1 mM $O_2^{-\cdot}$ or 1 mM OCl^- at 25 °C. After incubation for 1 h at 37 °C, luciferase (100 μg/mL) in assay buffer was added to the mixture, and bioluminescence intensity was measured by using a bioluminescent microplate reader.

Ozone: A solution of probe **1** or **2** (2 μM) in potassium phosphate buffer (5 mM, pH 7.0) containing 0.5% DMSO were treated with ozone (20 μM) in potassium phosphate buffer for 1 h at 37 °C. Luciferase activity was measured as described above.

Hydrogen peroxide (H_2O_2): A solution of probe **1** or **2** (2 μM) in potassium phosphate buffer (5 mM, pH 7.0) containing 0.5% DMSO were treated with 1 mM hydrogen peroxide in water for 1 h at 37 °C. Luciferase activity was measured as described above.

Hydroxyl radical ($^{\cdot}OH$): A solution of probe **1** or **2** (2 μM) in potassium phosphate buffer (5 mM, pH 7.0) containing 0.5% DMSO were treated with 1 mM hydroxyl radical generated from a mixture of $FeSO_4{\cdot}7H_2O$ and H_2O_2 in water for 1 h at 37 °C. Luciferase activity was measured as described above.

Superoxide ($O_2^{\cdot-}$): A solution of probe **1** or **2** (2 μM) in potassium phosphate buffer (5 mM, pH 7.0) containing 0.5% DMSO were treated with 1 mM superoxide generated from KO_2 in DMSO for 1 h at 37 °C. Luciferase activity was measured as described above.

Singlet oxygen ($^1O_2$): A solution of probe **1** or **2** (2 μM) in potassium phosphate buffer (5 mM, pH 7.0) containing 0.5% DMSO were treated with 1 mM singlet oxygen generated from NaOCl and H_2O_2 for 1 h at 37 °C. Luciferase activity was measured as described above.

Hyperchlorite ion (OCl^-): A solution of probe **1** or **2** (2 μM) in potassium phosphate buffer (5 mM, pH 7.0) containing 0.5% DMSO were treated with 1 mM hyperchlorite ion generated from NaOCl and NaOH for 1 h at 37 °C. Luciferase activity was measured as described above.

Myeloperoxidase (MPO): A solution of probe **1** or **2** (2 μM) in potassium phosphate buffer(5 mM, pH 7.0) containing 0.5% DMSO were treated with 40 nM myeloperoxidase and 200 μM H_2O_2 in 150 mM NaCl in water for 1 h at 37°C. Luciferase activity was measured as described above.

Bioluminescent detection of a mixture of probe 1, amino acid and 6-FP after UV light irradiation. A solution of probe **1** (20 μM), 6-FP (100 μM) and 2 mM of each amino acid (Cys, Met, Trp, His, Lys, Ala) was irradiated for 5 min at 3.2 mW/cm^2 by using a UVA irradiation apparatus (XX-15BLB; UVP). The mixture was treated with 2 mM pyrrolidine. After 5 min, luciferase (100 μg/mL) in assay buffer was added to the mixture. After incubation for 5 min, bioluminescence intensity was measured by using a bioluminescent microplate reader. Ozone (1 μM) was used as a positive control. For detection using indigo carmine, a solution of indigo carmine (60 μM), 6-FP (100 μM) and each amino acid (Cys, Met, Trp, His, Lys, Ala; 0.5-2 mM) was irradiated for 5 min at 3.2 mW/cm^2 by using a UVA irradiation apparatus. Decomposition of indigo carmine was monitored by measuring its absorbance at 610 nm.

Measurements of ozone concentrations in ambient samples. Open, wide mouth glass bottles containing potassium phosphate buffer (5 mM, pH 7.0) were placed for 8 h at four locations with different atmospheric conditions. During this period, the sample bottles were protected from direct sunlight. A blank used as a negative control indicates the potassium phosphate buffer (5 mM, pH 7.0) without exposing to ozone. The collected samples were treated with 10 μM **1** for 30 min and then with 2 mM pyrrolidine for 5 min. The luciferase activity of each sample was measured as described above. The experiments were carried out three times.

Supplementary References

1. D. C. McCutcheon, M. A. Paley, R. C. Steinhardt, J. A. Prescher. *J. Am. Chem. Soc*., 2012, **134**, 7604.
2. H. Takakura, R. Kojima, Y. Urano, T. Terai, K. Hanaoka, T. Nagano, *Chem. Asian. J.*, 2011, **6**, 1800.
3. H. Gallard, U. von Gunten, *Water Res.,* 2002, **36**, 65-74.
4. A. L. Garner, C. M. St Croix, B. R. Pitt, G. D. Leikauf, S. Ando, K. Koide, *Nat Chem.,* 2009, **1**, 316.

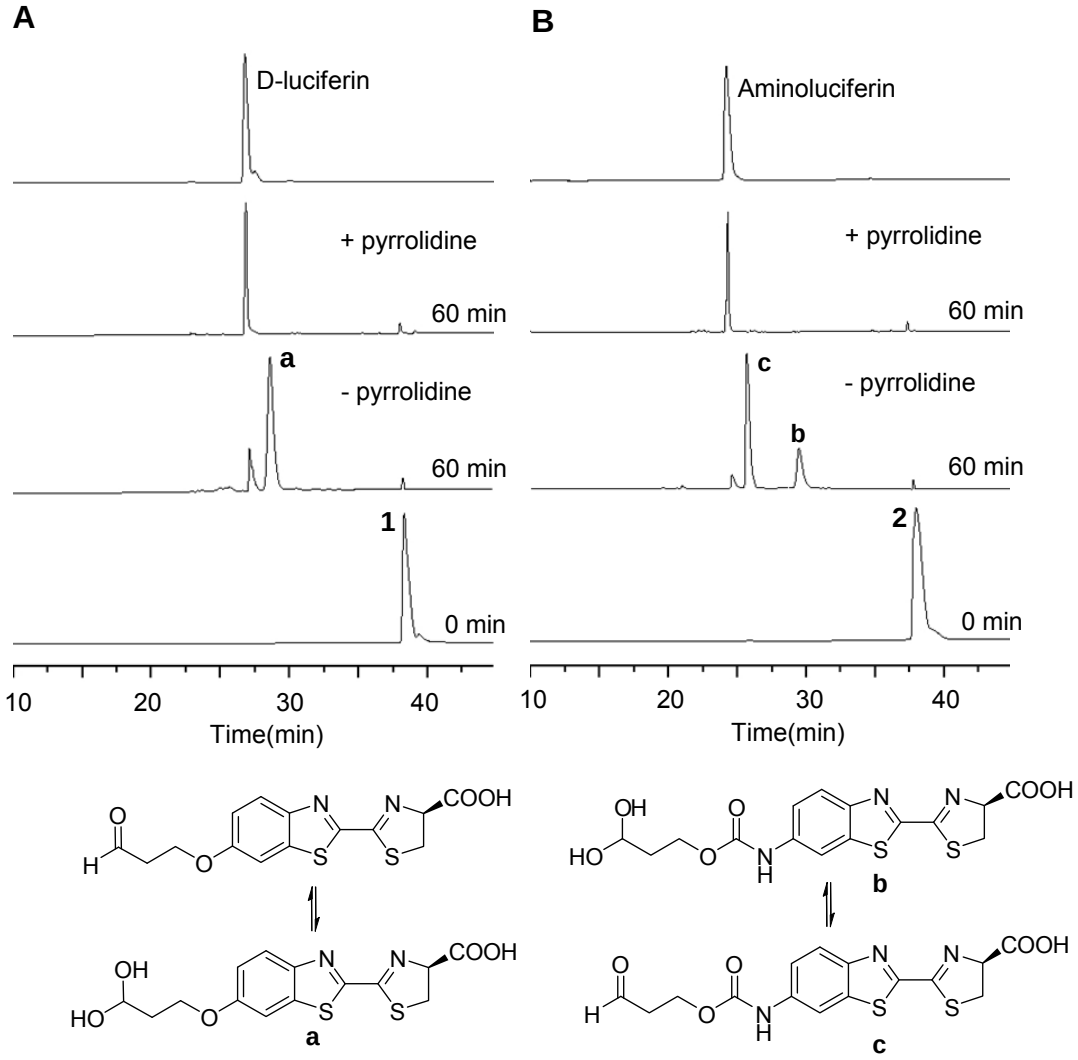


Fig. S1. HPLC profiles of products of reaction of probe (A) **1** and (B) **2** with ozone. A solution of each probe (100 μM) in potassium phosphate buffer (5 mM, pH 7.0) containing 0.5% DMSO was treated with 150 μM ozone in potassium phosphate buffer in the absence or presence of pyrrolidine (2 mM) for 1 h at 37 °C.

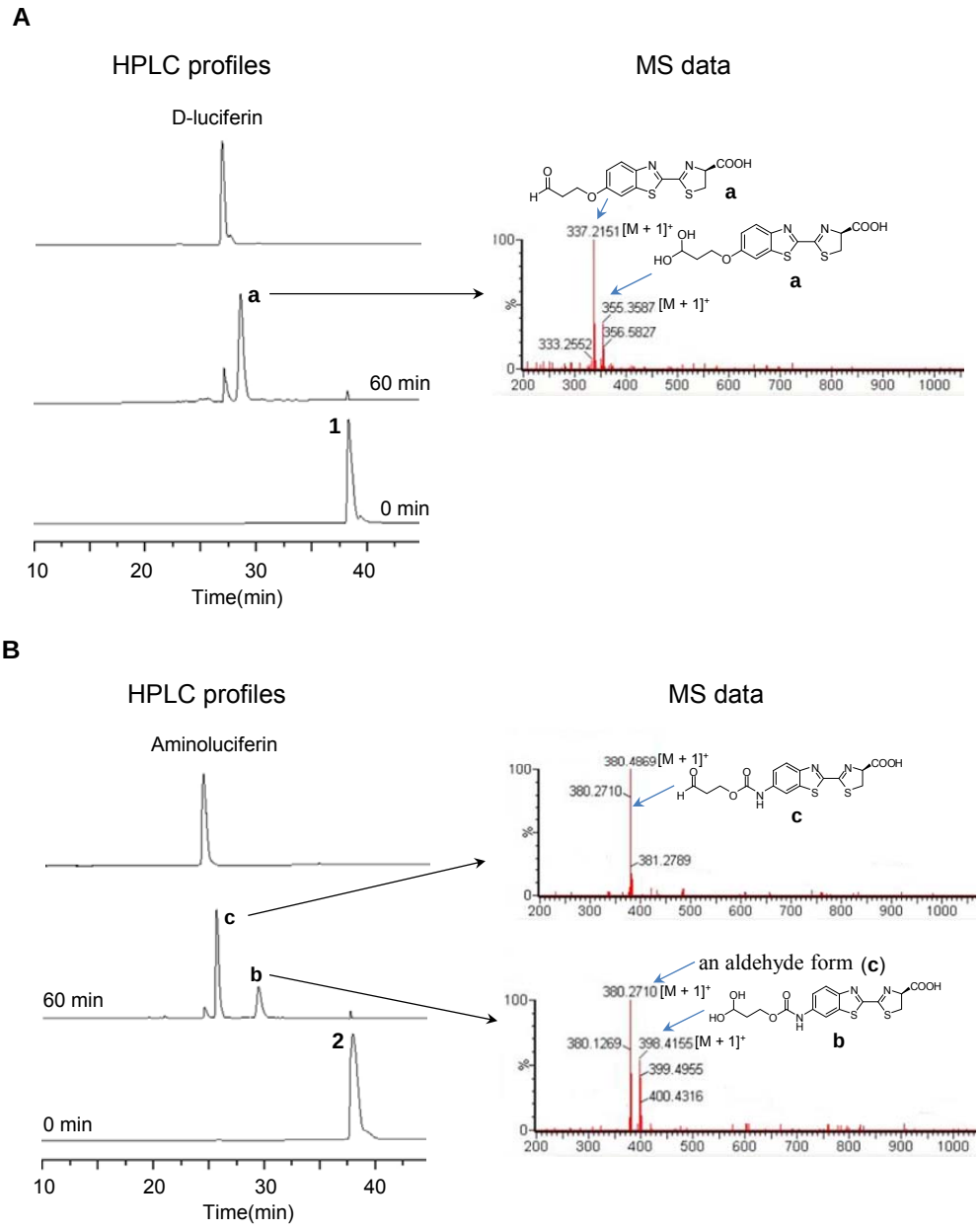


Fig. S2. HPLC profiles and MS data of products of reaction of probe (A) **1** and (B) **2** with ozone. MS data of **b** in (B) indicate that a hydrate form **b** is partially converted to its aldehyde form **a** during acquisition of mass data.

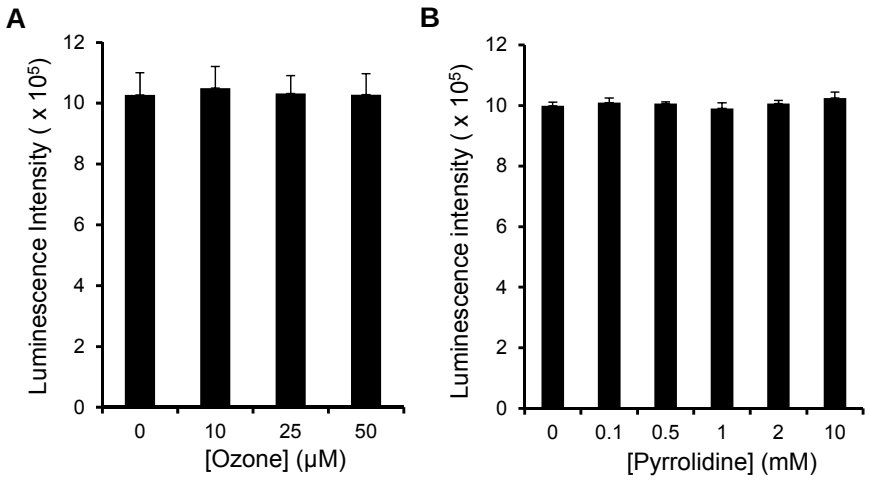


Fig. S3. Effect of ozone and pyrrolidine on the luciferase activity. Luciferase (100 μg/mL) in assay buffer was added to a solution of D-luciferin (2 μM) in potassium phosphate buffer (5 mM, pH 7.0) containing (A) 0–50 μM ozone and (B) 0–10 mM pyrrolidine. Bioluminescence intensity was measured by using a bioluminescent microplate reader (error bar: mean ± s.d., n = 3).

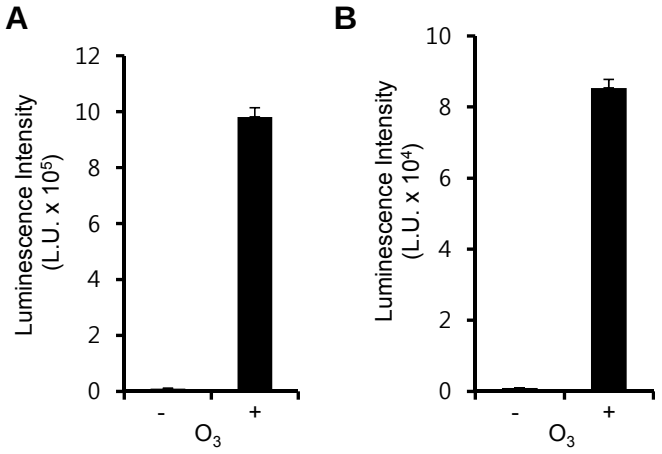


Fig. S4. Changes in bioluminescence intensity after treatment of probe (A) **1** and (B) **2** with ozone. A solution of each probe (2 μM) in potassium phosphate buffer (5 mM, pH 7.0) containing 0.5% DMSO was treated with 10 μM ozone. The mixture was treated with 2 mM pyrrolidine for 5 min and then luciferase (100 μg/mL) in assay buffer was added to the mixture. Bioluminescence intensity was measured by using a bioluminescence microplate reader (error bar: mean ± s.d., n = 3).

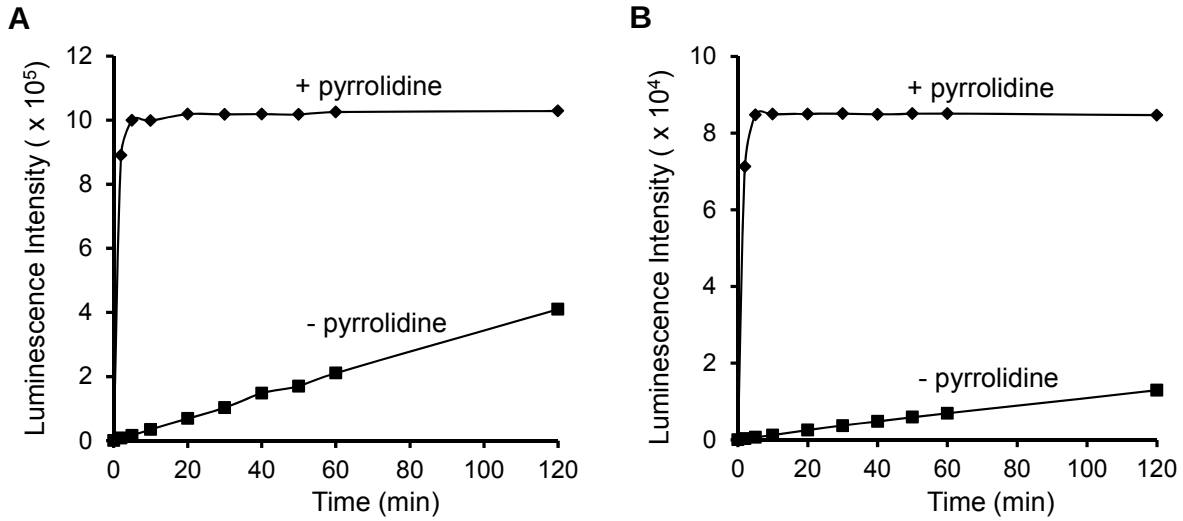


Fig. S5. Time-dependent bioluminescent detection of ozone using probe (A) **1** and (B) **2**. A solution of each probe (2 μM) in potassium phosphate buffer (5 mM, pH 7.0) containing 0.5% DMSO was treated with ozone (10 μM) in the absence or presence of 2 mM pyrrolidine at 37°C. After incubation for the indicated time, luciferase (100 μg/mL) in assay buffer was added to the mixture and then time-dependent bioluminescence intensity was measured by using a bioluminescence microplate reader.

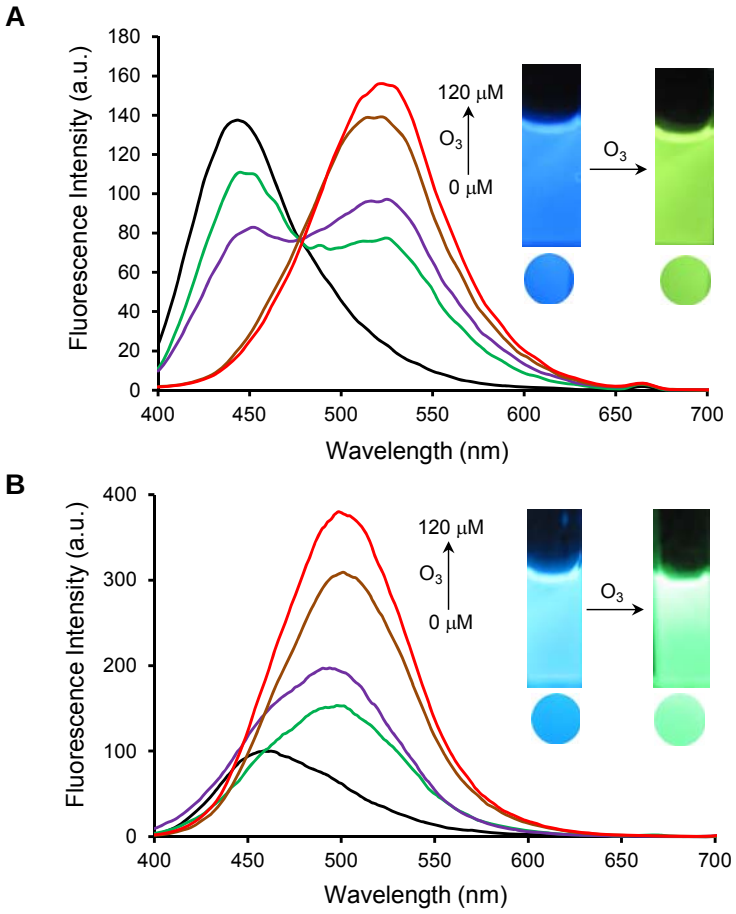


Fig. S6. Fluorescence spectra after treatment of probe (A) **1** and (B) **2** with ozone. A solution of each probe (40 μM) in potassium phosphate buffer (5 mM, pH 7.0) containing 0.5% DMSO was treated with various concentrations of ozone and then the mixture was treated with 2 mM pyrrolidine for 5 min (**1**; λ_{ex} = 330 nm, **2**; λ_{ex} = 350 nm).

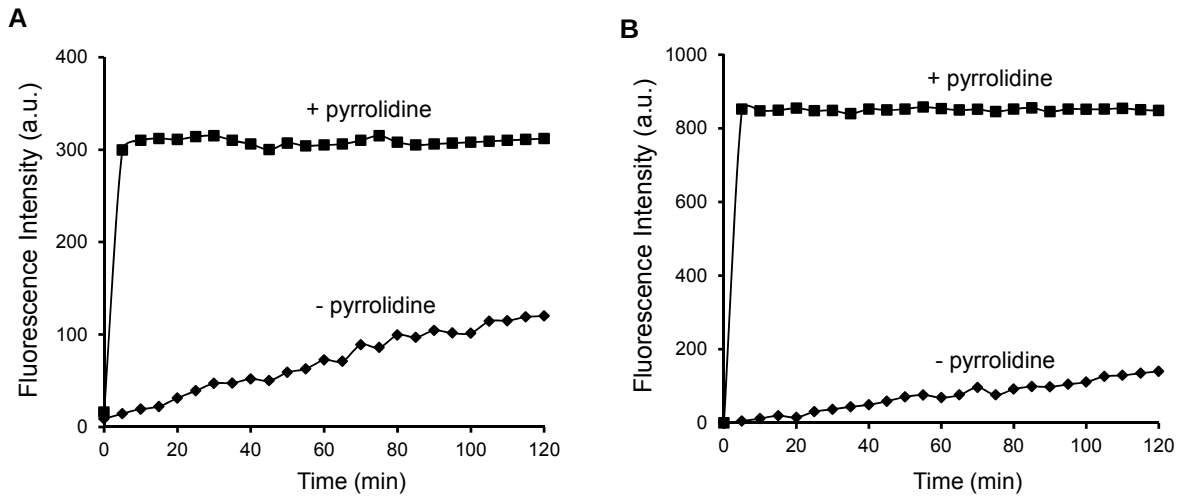


Fig. S7. Time-dependent changes in fluorescence intensity after treatment of probe (A) **1** and (B) **2** with ozone. A solution of each probe (40 μM) in potassium phosphate buffer (5 mM, pH 7.0) containing 0.5% DMSO was treated with 20 μM ozone in the absence or presence of 2 mM pyrrolidine. Time-dependent fluorescent intensity was measure by using a fluorescence microplate reader at 37˚C (**1**; λ_{ex} = 330 nm, λ_{em} = 530 nm, **2**; λ_{ex} = 350 nm, λ_{em} = 500 nm).

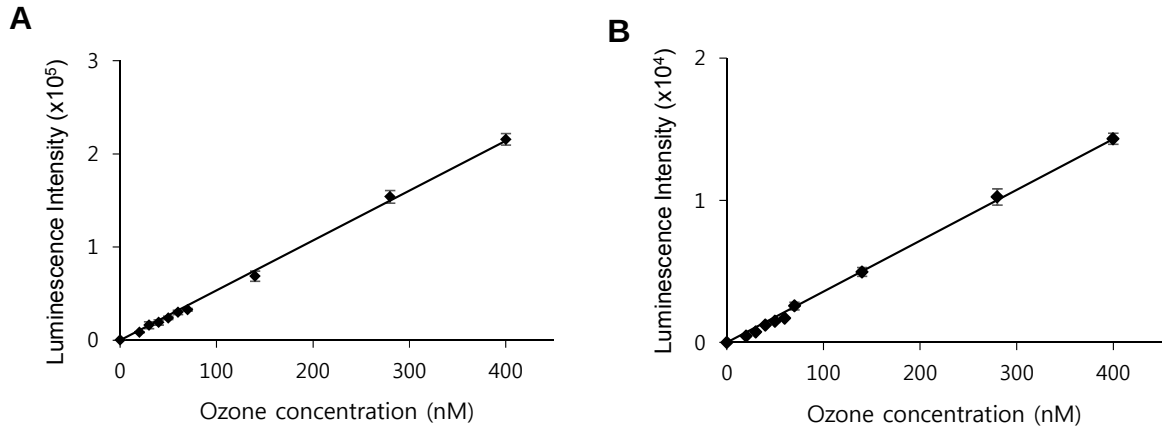


Fig. S8. Ozone concentration dependent bioluminescence intensity of (A) **1** and (B) **2**. A solution of each probe (2 μM) in potassium phosphate buffer (5 mM, pH 7.0) containing 0.5% DMSO was treated with ozone (0-400 nM) in potassium phosphate buffer at 25°C. The mixture was treated with 2 mM pyrrolidine for 5 min and then luciferase (100 μg/mL) in assay buffer was added to the mixture. Bioluminescence intensity was measured by using a bioluminescence microplate reader (error bar: mean ± s.d., n = 3).

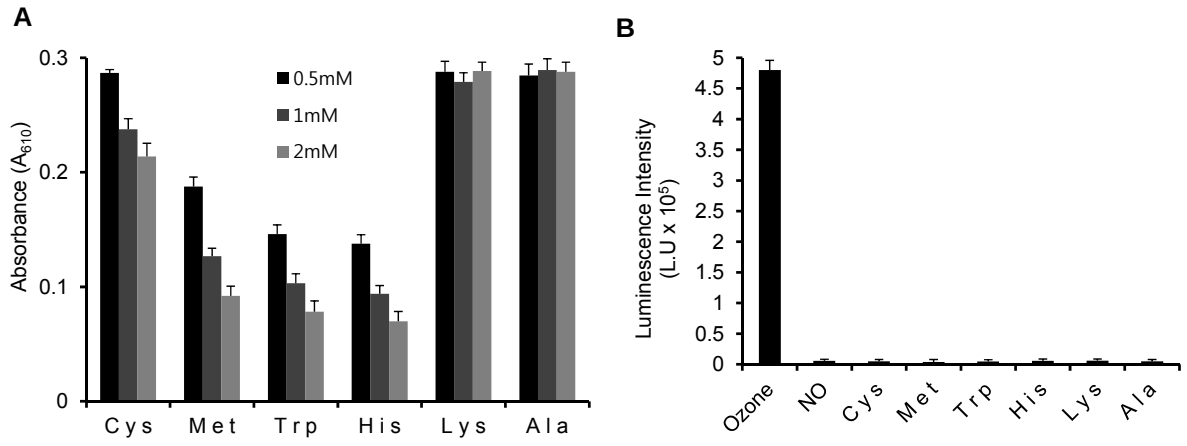


Fig. S9. (A) A solution of each amino acid, 6-FP (100 μM) and indigo carmine (60 μM) was irradiated for 5 min by using a UV lamp. Loss of indigo carmine was monitored by measuring its absorbance at 610 nm (error bar: mean ± s.d., n = 3). (B) Changes in bioluminescence intensity. A solution of probe **1** (20 μM), 6-FP (100 μM) and each amino acid was irradiated for 5 min by using a UV lamp. Ozone (1 μM) was used as a positive control and no treatment (NO) is a negative control. The mixture was treated with 2 mM pyrrolidine for 5 min and then luciferase (100 μg/mL) in assay buffer was added to the mixture. Bioluminescence intensity was measured by using a bioluminescence microplate reader (error bar: mean ± s.d., n = 3).

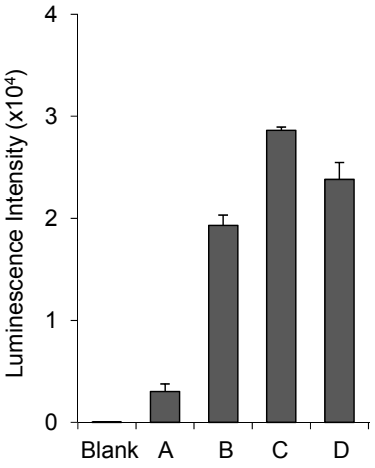
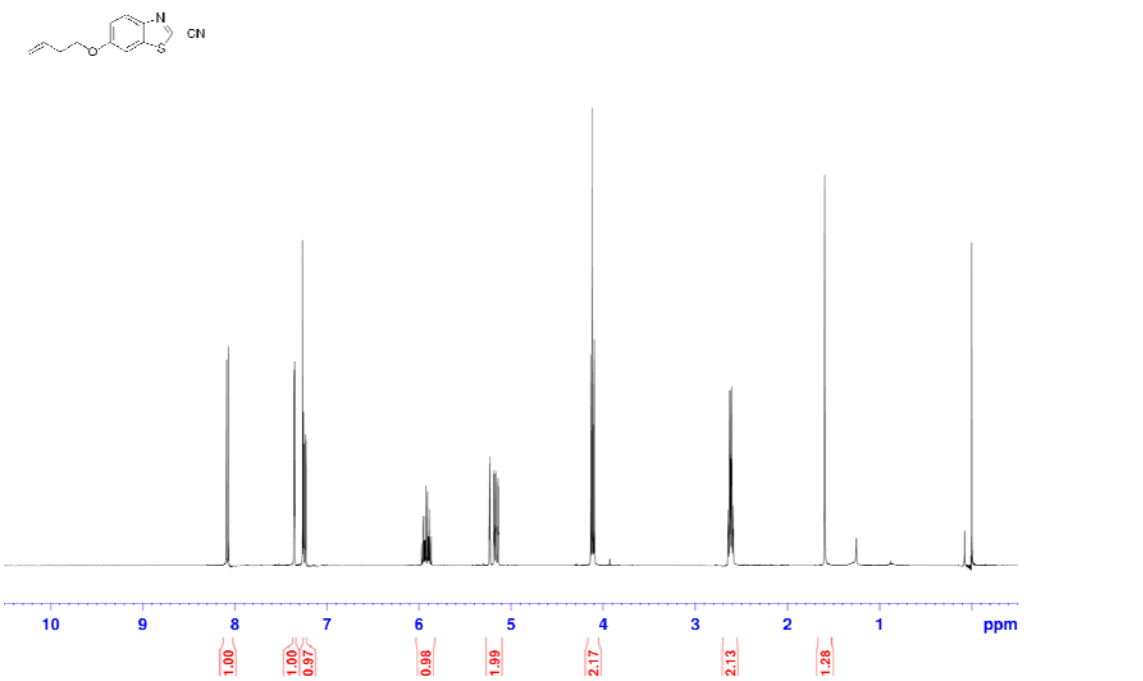


Fig. S10. Ozone detection in environmental samples using probe **1**. Open, wide mouth glass bottles containing 5 mM potassium phosphate buffer (pH 7.0) were placed at four different locations for 8 h: A, indoor Science building of Yonsei University; B, the road nearby three-way intersection of the Yeonhui-ro; C, the main gate of Yonsei University; D, the back gate of the Ewha Women's University. Blank contains 5 mM potassium phosphate buffer (pH 7.0) without ozone (error bar: mean ± s.d., n = 3). The ozone concentration of each sample was calculated by using a calibration curve shown in Figure S8A.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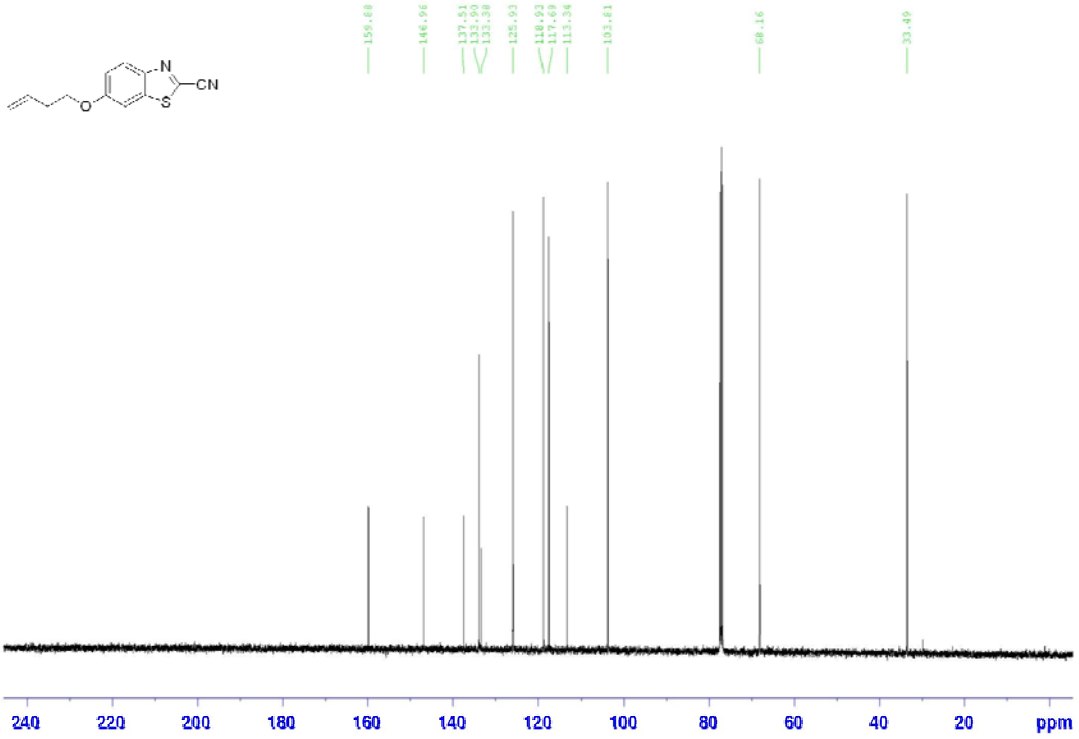
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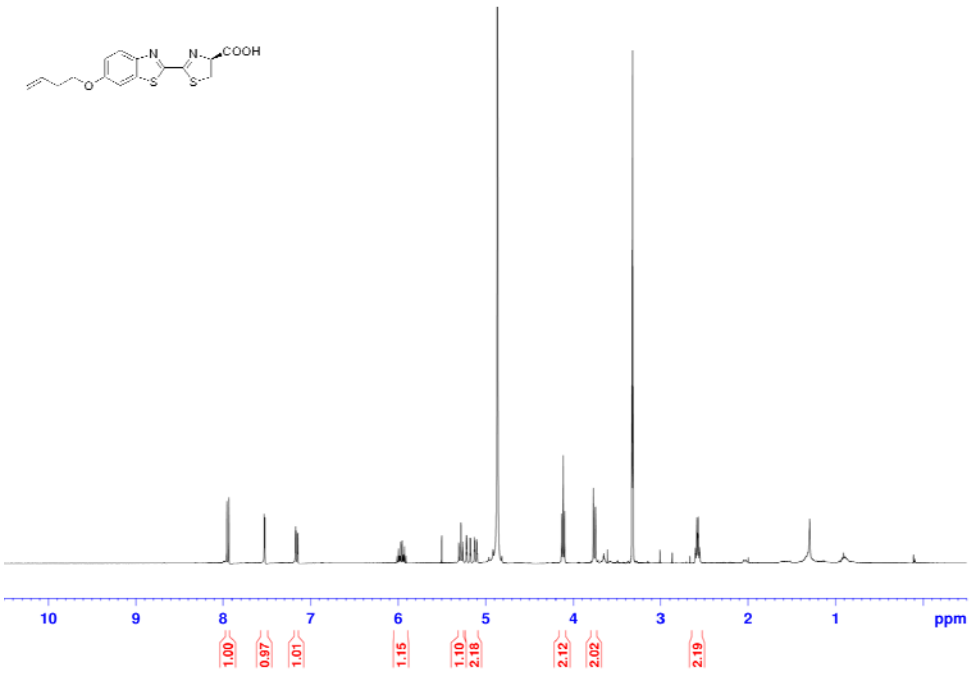
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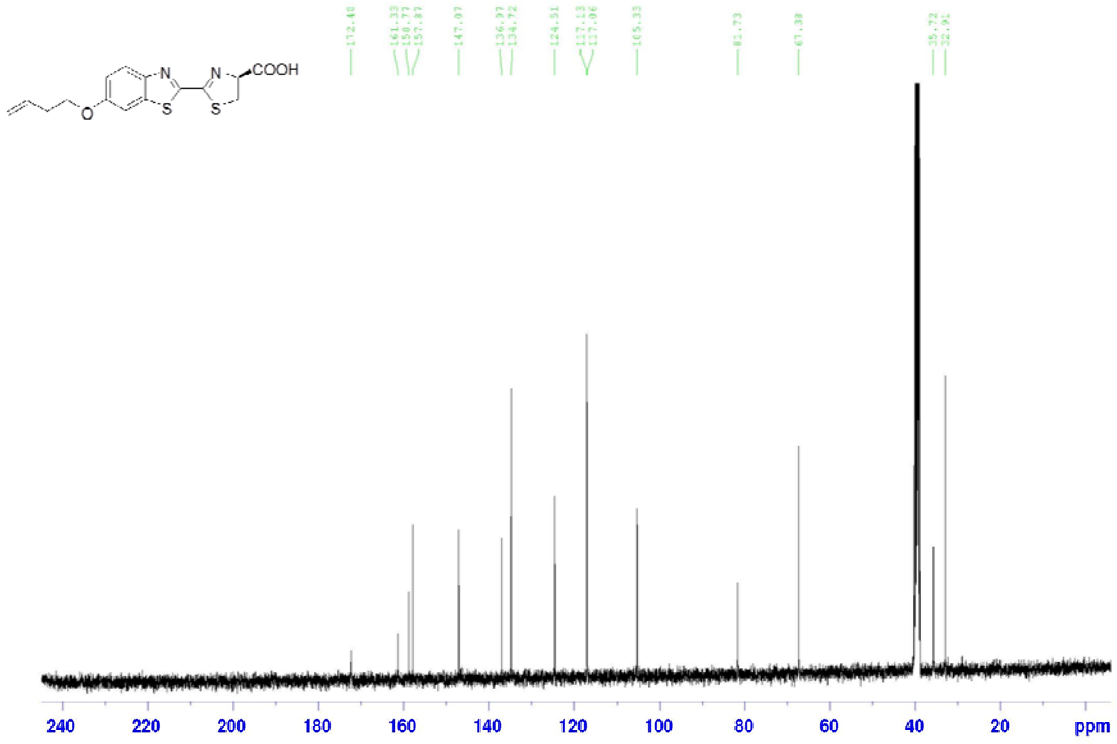
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20
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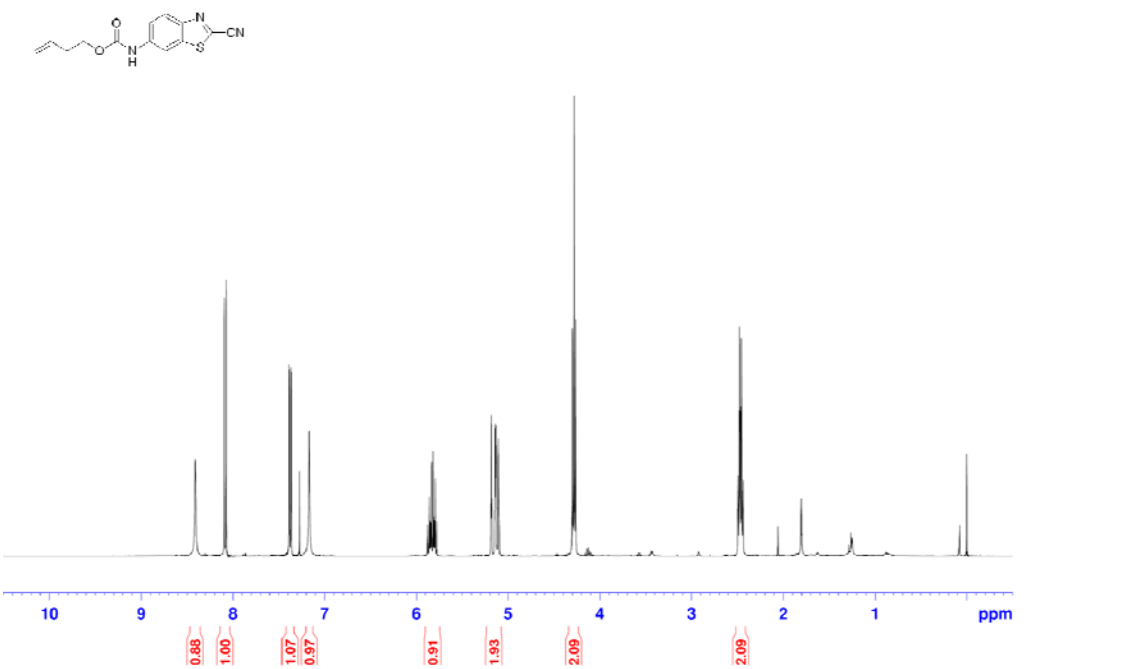
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H
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9
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7
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1.07
0.97
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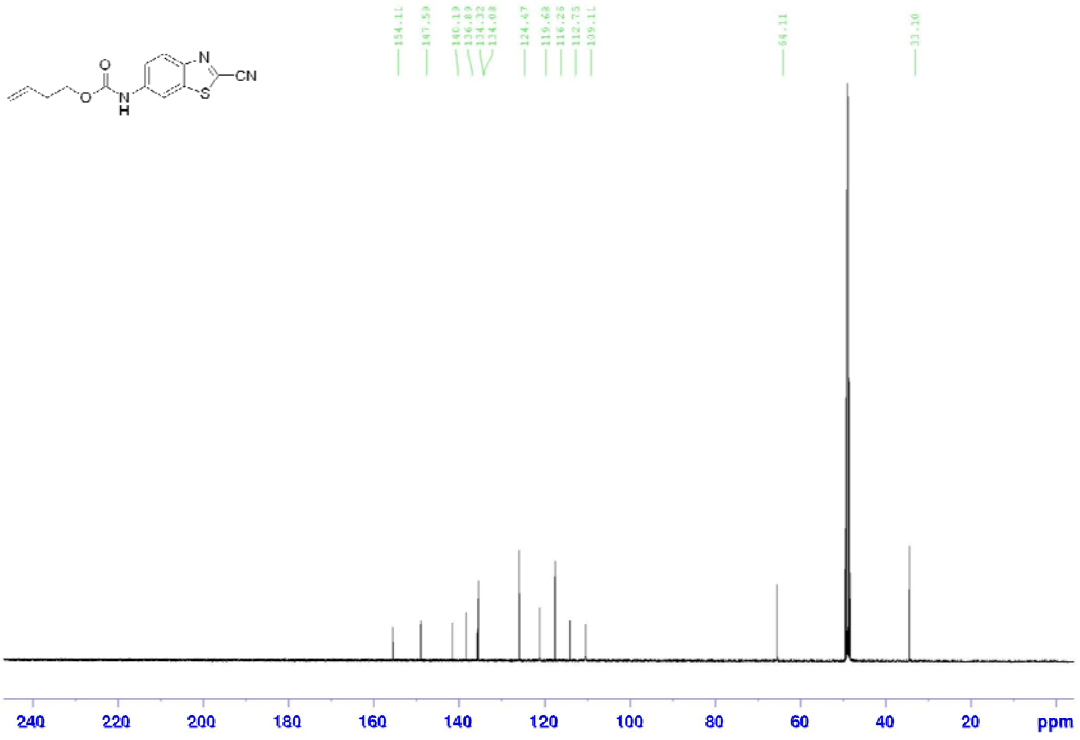
2.09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140.19
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134.08
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109.11
64.11
33.30
N
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O
S
O
N
H
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200
180
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140
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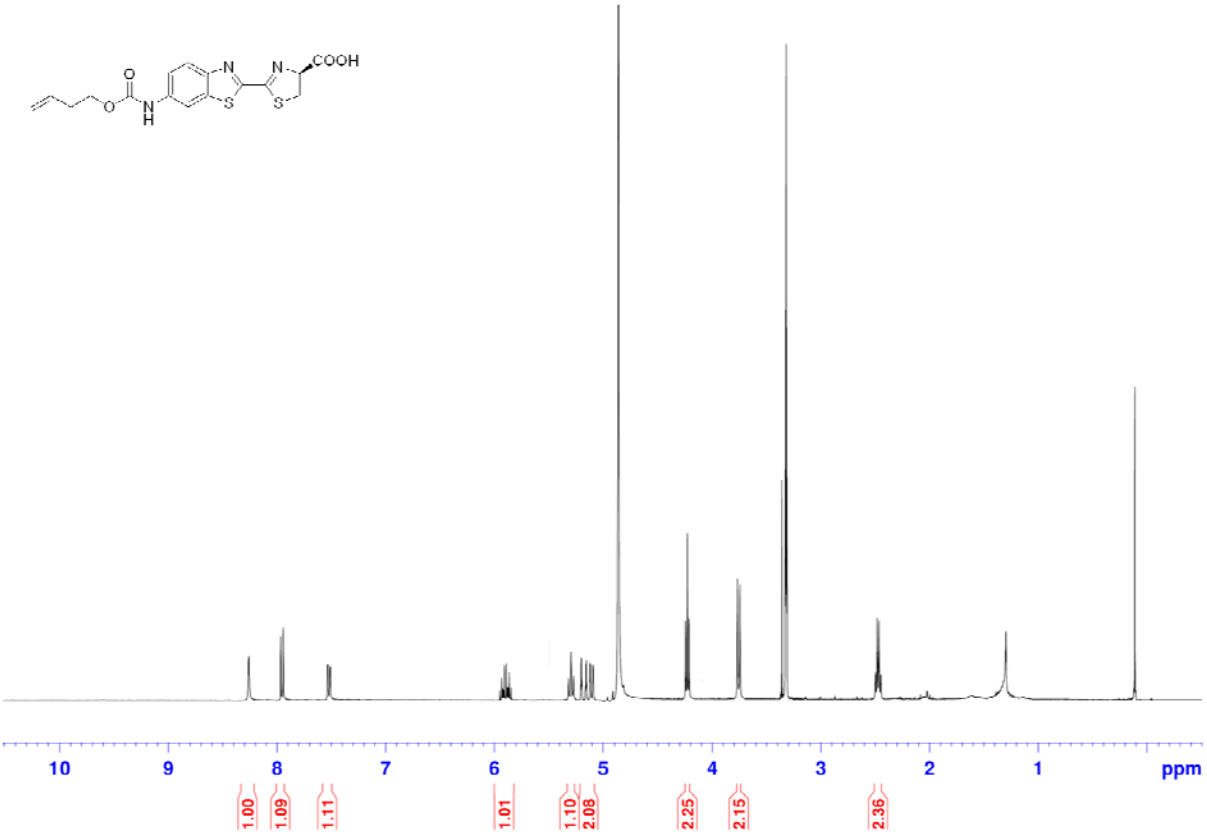
20
ppm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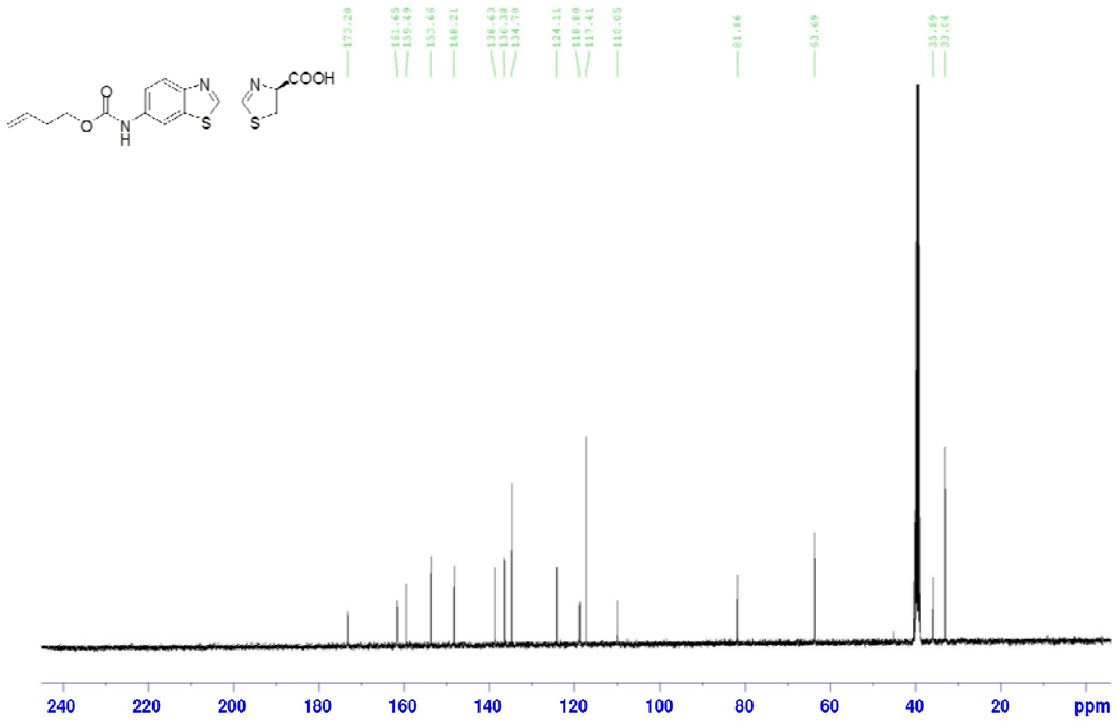
COOH
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200
180
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20
ppm