DOI 10.5012/bkcs.2011.32.8.3109

Coumarin-Benzothiazoline Conjugate as a Fluorescence Turn-On Probe for Reactive Oxygen Species and its Cellular Expression†

Dong-Nam Lee,‡ Do Yeon Kim,§ Sung Ho Ghil,§ and Hae-Jo Kim#,*

‡*Department of Chemistry, Kyonggi University, Suwon 443-760, Korea*
§*Department of Life Science, Kyonggi University, Suwon 443-760, Korea*
#*Department of Chemistry, Hankuk University of Foreign Studies, Yongin 449-791, Korea.* **E-mail: haejkim@hufs.ac.kr*
Received January 19, 2011, Accepted February 11, 2011

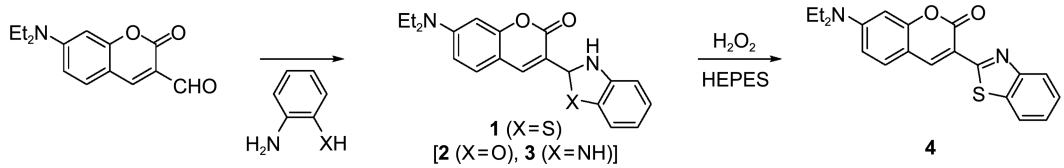
Key Words : Coumarin, Fluorescence, ROS

Many diseases associated with human aging such as cancer[1] and neurodegenerative diseases[2] have a strong oxidative stress component. Oxidative stress is a result of unregulated production of reactive oxygen species (ROS), and cellular mismanagement of ROS via oxidation-reduction chemistry can trigger subsequent oxidative damage to tissues and organs.[3] Therefore, expression of a mitochondrial peroxide detoxifying enzyme such as catalase can increase life span in mouse models.[4]

Although several excellent fluorescent probes based on boronate-[5] or benzyl ether-fluorophore[5a, 6] conjugates have been described for H_2O_2 detection in living cells for visualization, new imaging methods that allow visualization of localized production of mitochondrial ROS including hydrogen peroxide within living samples are potentially useful for disentangling the complex contributions of the ROS to both healthy and diseased states. Recently, Matthews and coworkers tried to monitor a cellular uptake of calixarene-NBD conjugate without any external stimuli, but just observed its nonspecific uptake into the cell cytoplasm.[7] Herein, we report a fluorescent probe for natural ROS without any external addition of ROS and its plausible localization in the specific cellular organelle in living cells.

In an effort to elucidate the role of ROS such as hydrogen peroxide in living cells, we have developed a new thiazoline compound (**1**) based on a fluorescent coumarin, which has shown both good water compatibility and cell permeability, and then fluorescence turn-on property in living cells.

Previously we prepared the 7-diethylamino-3-formyl coumarin and observed the fluorescence turned off upon the addition of homocysteine or cysteine due to the possible photo-induced electron transfer (PeT) from the sp^3 hybridized nitrogen electrons to the coumarin fluorophore.[8] To reverse the luminescent property, we have prepared a weakly fluorescent thiazoline **1** and expected its fluorescence to be turned on by the chemical transformation from sp^3 nitrogen to sp^2 hybridized nitrogen. Treatment of 2-aminobenzenethiol to the solution of 7-diethylamino-3-formylcoumarin in ethanol gave rise to **1** in 85% yield. Syntheses of analogous compounds were also tried



Scheme 1. Syntheses of the latent fluorophores.

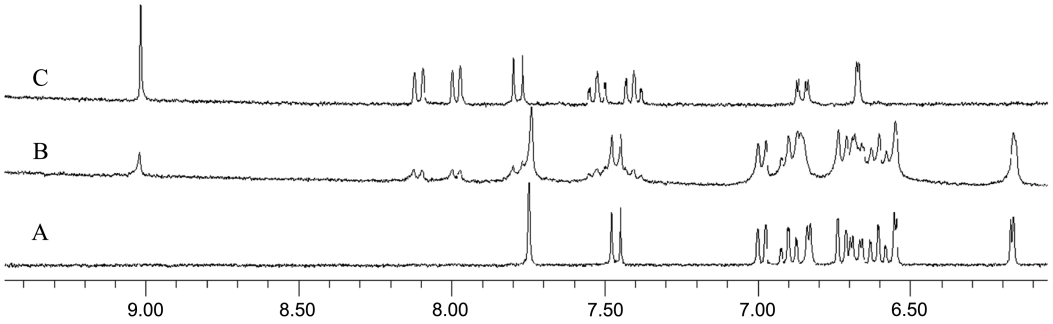


Figure 1. Partial ^{1}H NMR spectra of **1** (10 mM) upon the addition of H_2O_2 (20 equiv) in DMSO-d_6. (A) **1** only, (B) **1**, 1 h, (C) **4**.

†This paper is dedicated to Professor Eun Lee on the occasion of his honourable retirement.

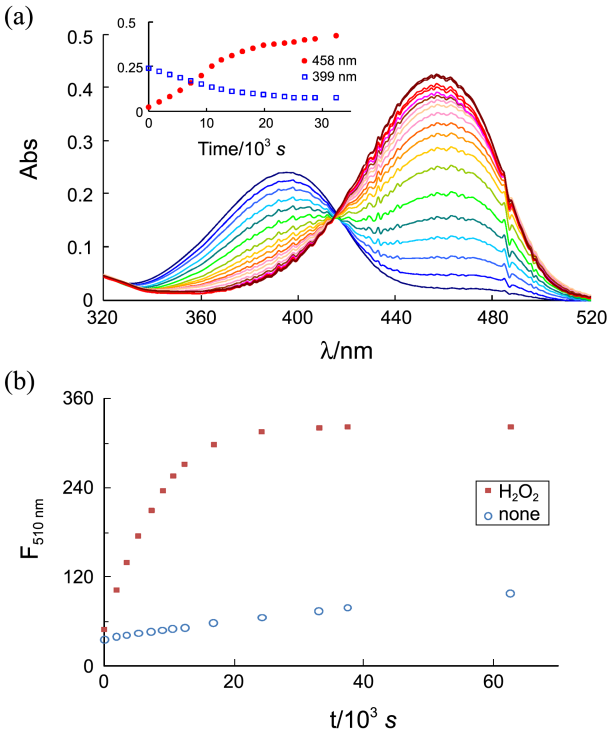


Figure 2. (A) UV-vis spectral changes upon addition of 500 equiv H_2O_2 to **1** (10 μM) in aqueous isopropanol (^{i}PrOH/water, 2/3, *v*/*v*). (B) Its fluorescence kinetics (λ_{ex} = 458 nm and λ_{em} = 510 nm).

with the corresponding amino phenol (**2**) or diaminobenzene (**3**) in a similar manner. However, compared to **1**, the oxazolidine (**2**) and the imidazolidine (**3**) derivatives were easily hydrolized in aqueous solution in the following order of stability of the heterocyclic structure for X, O < NH < S.[9] So we chose **1** as an appropriate probe to detect peroxides in living cells.

The chemical transformation was monitored after the addition of H_2O_2 to **1** in DMSO-d_6 by ^{1}H NMR spectroscopy. The methine (6.17 ppm) and aromatic (7.76 ppm) proton peaks of **1** disappeared while a new set of aromatic proton peaks were appearing with significant downfield shift up to 9.04 ppm (Fig. 1). The downfield proton signals indicate a large change occurred in the electronic structure of **1** with an electron withdrawing group.

Mass spectral data showed corroborative evidence to the NMR observation. The molecular ion peak of $[\mathbf{1}+H]^+$ (*m*/*z* 353.3) was changed to a new ion peak at *m*/*z* 351.3 ($[\mathbf{4}+H]^+$), two mass unit less than **1** upon the addition of H_2O_2. This indicates a hydrogen molecule from **1** was removed by the addition of hydrogen peroxide.

Probe **1** has a UV-vis maximum centered at 399 nm (ε = 3.2 × 10^4 $M^{-1}cm^{-1}$) and exhibits a weak fluorescence in aqueous isopropanol (IPA/H_2O, 2/3, *v*/*v*). The addition of hydrogen peroxide triggers a prominent bathochromic shift of the absorption maximum to 458 nm (ε = 5.7 × 10^4 $M^{-1}cm^{-1}$) with a pseudo-isosbestic point at 417 nm. The rate constant was measured under the pseudo first-order reaction conditions (500 equiv H_2O_2) by fluorescence spectroscopy, giving a half-life of τ 1.3 h, whereas the auto oxidation by aerial oxygen was

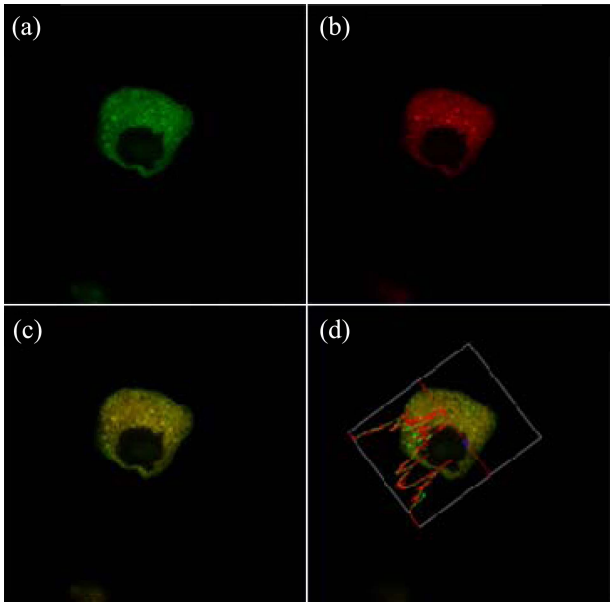


Figure 3. Confocal laser scanning micrographic images of **1** and MitoTracker Red in CHO cell. (A) green filter, (B) red filter, (C) merged fluorescence image, (D) its correlated signals (green line = **1**, red line = Mito Tracker Red).

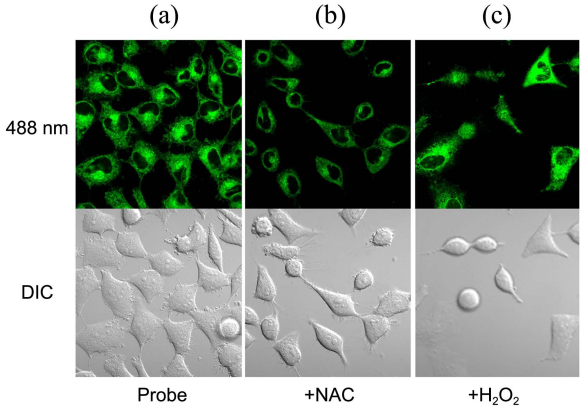


Figure 4. Confocal laser scanning microscopic images of HeLa cells by **1** (λ_{ex} 488 nm). (A) **1** (2.5 μM, 0.5 h), (B) NAC (5 mM, 24 h) + **1**, (C) H_2O_2 (0.1 mM, 0.5 h) + **1**.

30 times slower (τ 42 h) than H_2O_2 (Fig. 2). It is noticeable that **1** as a solid form did not show any detectable auto oxidation after 16 months at rt, permitting the probe to be available in a thiazoline form for a long time.

As expected, the benzothiazole (**4**) has a strong green fluorescence emission around 510 nm, whereas the thiazoline (**1**) shows a weak fluorescence due to the plausible PeT from the nitrogen lone pair electrons of the thiazoline ring. The fluorescence of **1** was turned on by the addition of H_2O_2 and reached the fluorescence intensity of commercially available coumarin 6 (**4**) within 1 h.

According to the pH profile of **1**, the probe was stable and showed a significant fluorescence enhancement around the biological pH.[10] To elucidate the practical role of probe **1** in living cells, we performed the cellular uptake experiments with Chinese hamster ovary (CHO) cell, breast cancer cell (MCF-

7), or human embryonic kidney cell (293T) lines. 293T cells showed no fluorescence without **1**, whereas a strong green fluorescence was observed upon the administration of **1** as low as 10 μM. Bright field measurements indicate that the cells are viable throughout the imaging experiments. Probe **1** was then tested for its ability to target the mitochondria, where cell respiration takes place and therefore ROS are rich.[11] CHO cells were incubated with 300 nM of MitoTracker (MitoTracker® Red FM) for 10 min, subsequently with 30 μM of **1** for 30 min at 37 °C. Confocal laser scaning microscopic images showed significant green fluorescence of **1** in discrete subcellular organelles, cytoplasma, whose signals were plausibly correlated with Mito Tracker Red (Fig. 3). Surprisingly, probe **1** in cytoplasma is highly sensitive enough to detect the natural ROS in the cytoplasm, without any addition of external hydrogen peroxide. Comparatively N-Acetylcysteine (NAC), a scavenger of ROS,[12] shows a significant decrease in the green fluorescence whereas external H_2O_2 exhibits a slight increase (Fig. 4).

In conclusion, we prepared a water-soluble and fluorescence turn-on probe to detect the natural cellular ROS. From cellular experiments, we found probe **1** plays a role of ROS probe in living cells with a good cell permeability and a significant fluorescence turn-on property, even without external stimuli. We expect that this modular probe would prove useful for targeting disease-related cells and further research is on progress.

Experimental Section

Materials and Methods. All reactants and solvents, unless otherwise stated, were purchased from commercial sources and used as received. Flash chromatography was carried out on silica gel (230-400 mesh). ^{1}H NMR and ^{13}C NMR spectra were recorded using 200/300 MHz. Chemical shifts were expressed in ppm and coupling constants (J) in Hz. Mass spectra were obtained using JEOL JMS-700 Mstation spectrometers. The absorption and PL spectra in solutions were measured by using an Agilent-8453 UV-vis spectrometer and Jasco FP-6500 PC fluorescence spectrophotometer, respectively.

General Procedure for the Synthesis of Compounds 1-3. 7-Diethylamino-3-formylcoumarin (63.6 mg, 0.25 mmol) and 2-aminobenzenthiol for **1**, 2-aminophenol for **2**, 1,2-phenylenediamine for **3** (0.3 mmol each) were dissolved in 1.0 mL of ethanol respectively. To a stirred solution of 7-diethylamino-3-formylcoumarin in EtOH, ortho-substituted aniline solution was slowly added. The reaction mixture was stirred for 3 h at rt. Resulting precipitates were filtered and washed with EtOH.

1 as a yellow solid in 85% yield.

^{1}H NMR (DMSO-d_6, 300 MHz): δ 7.76 (s, 1H), 7.48 (d, J = 9.0 Hz, 1H), 7.00 (d, J = 7.2 Hz, 1H), 6.92 (t, J = 7.8 Hz, 1H), 6.86 (d, J = 2.7 Hz, 1H), 6.74 (d, J = 7.8 Hz, 1H), 6.70 (dd, J = 9.0 Hz, J = 2.1 Hz, 1H), 6.63 (t, J = 7.2 Hz, 1H), 6.56 (d, J = 2.1 Hz, 1H), 6.17 (d, J = 2.7 Hz, 1H), 3.46 (q, J = 6.9 Hz, 4H), 1.14 (t, J = 6.9 Hz, 6H)

^{13}C NMR (DMSO-d_6, 75 MHz): δ 161.09, 155.73, 150.88, 147.66, 137.90, 130.04, 125.76, 125.60, 122.19, 121.84, 119.53, 110.04, 109.64, 107.74, 96.81, 63.89, 44.50, 12.75 (18 carbon peaks)

MS (ESI$^+$, MeOH) *m/z* calculated for $C_{20}H_{20}N_2O_2S$: 352.1, observed $[M+H]^+$: 353.4

HRMS (FAB$^+$, m-NBA) *m/z* calculated for $C_{20}H_{21}N_2O_2S$: 353.1324, observed: 353.1319

2 as a scarlet solid in 74% yield.

^{1}H NMR (DMSO-d_6, 300 MHz): δ 8.96 (s, 1H), 8.82 (s, 1H), 8.66 (s, 1H), 7.52 (d, J = 9.0 Hz, 1H), 7.19 (d, J = 7.8 Hz, 1H), 7.07 (t, J = 7.5 Hz, 1H), 6.90 (d, J = 7.8 Hz, 1H), 6.82 (m, 2H), 6.52 (s, 1H), 3.53 (q, J = 6.9 Hz, 4H), 1.18 (t, J = 6.9 Hz, 6H).

3 as a red solid in 78% yield.

^{1}H NMR (DMSO-d_6, 300 MHz): δ 8.75 (s, 1H), 8.56 (s, 1H), 7.62 (d, J = 9.0 Hz, 1H), 7.06 (d, J = 7.8 Hz, 1H), 6.96 (t, J = 7.5 Hz, 1H), 6.82 (d, J = 9.0 Hz, 1H), 6.72 (d, J = 7.8 Hz, 1H), 6.62 (s, 1H), 6.56 (t, J = 7.5 Hz, 1H), 5.29 (s, 2H), 3.50 (q, J = 6.9 Hz, 4H), 1.18 (t, J = 6.9 Hz, 6H).

Synthesis of 4. The weighed compound **1** (78.3 mg, 0.222 mmol) was dissolved in 5.0 mL of dichloromethane. Excess hydrogen peroxide (30% aq solution, 20 equiv) was added to the solution of **1**. The solution was refluxed at 40 °C for 5 h. Resulting solution was cooled to rt, and evaporated *in vacuum*. Purification of the residue by column chromatography on silica gel with dichloromethane as an eluent afforded product **4** as a yellow solid in 70% yield (R_f = 0.57).

^{1}H NMR (DMSO-d_6, 300 MHz): δ 9.04 (s, 1H), 8.13 (d, J = 7.8 Hz, 1H), 8.00 (d, J = 8.1 Hz, 1H), 7.81 (d, J = 9.0 Hz, 1H), 7.56 (t, J = 8.1 Hz, 1H), 7.44 (t, J = 7.8 Hz, 1H), 6.88 (dd, J = 9.0 Hz, J = 2.1 Hz, 1H), 6.69 (d, J = 2.1 Hz, 1H), 3.56 (q, J = 6.9 Hz, 4H), 1.19 (t, J = 6.9 Hz, 6H)

^{13}C NMR (DMSO-d_6, 75 MHz): δ 163.44, 160.29, 157.24, 152.74, 152.61, 142.99, 135.82, 132.14, 126.80, 125.00, 122.47, 122.24, 111.02, 110.81, 108.54, 96.66, 44.89, 12.87 (18 carbon peaks)

MS (ESI$^+$, MeOH) *m/z* calculated for $C_{20}H_{18}N_2O_2S$: 350.1, observed $[M+H]^+$: 351.3.

ROS Detection by Fluorescence and UV-vis Spectroscopy. All of solvents were utilized after degassing by sonification and subsequent N_2 blowing for 30 min. 10 mM stock solution of **1** was prepared in DMSO. The stock solution of **1** was diluted up to 10 μM in aqueous isopropanol (^{i}PrOH/water = 2/3, v/v, pH 7.4 HEPES buffer). 500 mM H_2O_2 solution was prepared from the commercially available 30% solution of H_2O_2. Time-dependency of absorbance and fluorescence changes for samples (auto-oxidation vs. oxidation by H_2O_2) were comparatively monitored, where λ_{ex} = 458 nm. Rate constants for various ROS were measured as follow: To each solution of **1** (10 μM, 2.0 mL), 4.0 μL of 100 mM stock solutions (H_2O_2, TBHP, NaOCl) were added. Superoxide (O_2^-) was added as solid KO_2 (14 mM). Hydroxyl radical (·OH) and *tert*-butoxy radical (·O^{t}Bu) were generated by reaction of 1.0 mM Fe^{2+} with 200 μM H_2O_2 or TBHP, respectively. Then, 10 mM stock solution of **1** (4.0 μL) was administered to the radical solutions. Time-dependent fluorescence intensities of each solutions were measured.

Biological Cell Imaging. Human breast cancer cell line, MCF-7 cells and human embryonic kidney cell line, 293T cells, were maintained in Dulbecco's modified Eagle's medium

(DMEM, Hyclone, South Logan, UT) supplemented with 10% (v/v) fetal bovine serum (Hyclone), 100 units/mL penicillin and 100 μg/mL streptomycin (Hyclone) at 37 °C in a humidified atmosphere of 5% CO_2 incubator (Sanyo, Tokyo, Japan). Live cells were placed on coverslips (coated with collagen and poly-D-Lysine) at a density of 5.0×10^3 per coverslip, and cultured for 1 day in growth media. Washed with ice-cold PBS buffer three times, cells were treated with **1** and MitoTracker probe (MitoTracker®Red FM, Invitrogen, Eugene, OR) for different time points. Images were acquired using a confocal laser scanning microscope (LSM 710, Carl Zeiss MicroImaging GmbH, Jena, Germany).

Acknowledgments. This research was supported by the National Research Foundation of Korea (2010-0008165) for financial support. We thank Professor Hong for the generous permission of access to the NMR spectroscopy at Seoul National University.

References

1. Oshima, H.; Tatemichi, M.; Sawa, T. *Arch. Biochem. Biophys*. **2003**, *417*, 3.
2. (a) Barnham, K. J.; Masters, C. L.; Bush, A. I. *Nat. Rev. Drug Discovery* **2004**, *3*, 205. (b) Connor, J. R. *Metals and Oxidative Damage in Neurological Disorders*; Plenum Press: New York, 1997.
3. Beckman, K. B.; Ames, B. N. *Physiol. Rev.* **1998**, *78*, 547.
4. Schriner, S. E.; Jinford, N. J.; Martin, G. M.; Treuting, P.; Ogburn, C. E.; Emond, M.; Coskun, P. E.; Ladiges, W.; Wolf, N.; van Remmen, H.; Wallace, D. C.; Rabinovitch, P. S. *Science* **2005**, *308*, 1909.
5. (a) Srikun, D.; Miller, E. W.; Domaille, D. W.; Chang, C. J. *J. Am. Chem. Soc.* **2008**, *130*, 4596. (b) Dickinson, B. C.; Chang, C. J. *J. Am. Chem. Soc.* **2008**, *130*, 9638. (c) Albers, A. E.; Okreglak, V. S.; Chang, C. J. *J. Am. Chem. Soc.* **2006**, *128*, 9640. (d) Miller, E. W.; Albers, A. E.; Pralle, A.; Isacoff, E. Y.; Chang, C. J. *J. Am. Chem. Soc.* **2005**, *127*, 16652. (e) Chang, M. C. Y.; Pralle, A.; Isacoff, E. Y.; Chang, C. J. *J. Am. Chem. Soc.* **2004**, *126*, 15392.
6. Koide, Y.; Urano, Y.; Kenmoku, S.; Kojima, H.; Nagano, T. *J. Am. Chem. Soc.* **2007**, *129*, 10324.
7. Lalor, R.; Baillie-Johnson, H.; Redshaw, C.; Matthews, S. E.; Mueller, A. *J. Am. Chem. Soc.* **2008**, *130*, 2892.
8. Kim, T.-K.; Lee, D.-N.; Kim, H.-J. *Tetrahedron Lett.* **2008,** *49*, 4879.
9. (a) Lambert, J. B.; Majchrzak, M. W. *J. Am. Chem. Soc.* **1980**, *102*, 3588. (b) Lambert, J. B.; Wang, G.; Huseland, D. E.; Takiff, L. C. *J. Org. Chem.* **1987**, *52*, 68. (c) Fülöp, F.; Mattiene, J.; Pihlaja, K. *Tetrahedron* **1990**, *46*, 6545.
10. In order to know the correlaton between pH and fluorescence intensity of the probe, we measured the fluorescence intensity at different pH and obtained a pH profile of **1**. In the neutral pH range of pH 7 ~ 9, the fluorescence intensity was significantly increased upon the addition of H_2O_2 to probe **1**.
11. (a) Dröge, W. *Physiol. Rev.* **2002**, *82*, 47. (b) Hamasaki, T.; Kashiwagi, T.; Imada, T.; Nakamichi, N.; Aramaki, S.; Toh, K.; Morisawa, S.; Shimakoshi, H.; Hisaeda, Y.; Shirahata, S. *Langmuir* **2008**, *24*, 7354. (c) Meany, D. L.; Thompson, L.; Arriaga, E. A. *Anal. Chem.* **2007**, *79*, 4588.
12. Kim, K. S.; Choi, H. W.; Yoon, H. E.; Kim, I. Y. *J. Biol. Chem.* **2010**, *285*, 40294.