Cellular Imaging

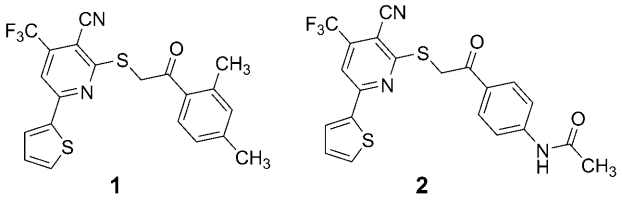
DOI: 10.1002/anie.201100935

A Mitochondrial Surface-Specific Fluorescent Probe Activated by Bioconversion**

Yoshinori Kawazoe, Hiroki Shimogawa, Ayato Sato, and Motonari Uesugi**

A number of fluorescent small molecule probes have been developed that stain selectively organelles or indicate the status of cells or specific cellular components, including second messengers,[1] pH,[2] reactive oxygen species,[3] metal ions,[4] nitric oxide,[5] sugar chains,[6] nucleic acids,[7] and cell differentiation.[8] Such probes have made significant contributions to biomedical research. However, many cellular components remain to be explored by small molecule probes, and the demand for reagents that specifically stain different components of living cells is increasing.[9] To address this need, we screened 12000 chemicals, relatively enriched in aromatic structures, to identify fluorescent chemicals that stain specific components of cellular architecture.

HeLa cells were seeded onto 96-well plates and incubated with each chemical at a concentration of 20 $\mu g\,mL^{-1}$ for 2 h. The cells were then observed under a fluorescence microscope. Our screening identified thirty-one candidate probes. Herein, one of the candidates is described (molecule **1**; Scheme 1), which exhibited a distinct staining pattern. We chemically synthesized nine analogues of molecule **1** (Supporting Information, Figure S1) and examined their cellular staining. Several analogues exhibited similar staining to that of molecule **1**, although strengths and selectivities of staining varied. Many of them also produced small fluorescent dots in the images, possibly owing to low water solubility. We subsequently focused on molecule **2** (Scheme 1), which did not produce dots and exhibited a simpler staining pattern in confocal microscopic images.



Scheme 1. Chemical structures of molecules **1** and **2**.

To determine which cell component was stained by molecule **2**, we conducted double staining experiments using molecule **2** and commercially available, organelle-specific fluorescent probes, namely LysoTracker Red, MitoTracker Red, and ER-Tracker Blue-White DPX (Invitrogen). The staining patterns of molecule **2** observed by a confocal microscope were similar to those of MitoTracker Red (Figure 1). Further magnification revealed circular staining by molecule **2** around areas stained by MitoTracker Red (Figure 1 d,e). In merged images, mitochondria stained by MitoTracker Red were completely surrounded by staining from molecule **2** (Figure 1 f), suggesting that molecule **2** stains mitochondrial surfaces. Although many fluorescent probes that stain mitochondria are known, no fluorescent probes have been reported for visualizing mitochondrial surfaces.

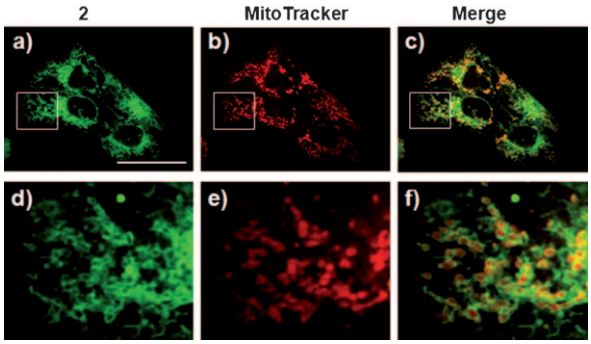


Figure 1. Confocal images of HeLa cells treated with molecule **2** (a,d) or MitoTracker Red (b,e). c) Merged picture of (a) and (b). d)–f) Magnifications of areas indicated by white boxes in a)–c). Scale bar = 25 μm.

To confirm that molecule **2** stains mitochondrial surfaces, we performed organelle fractionation of stained cells. The cells were treated with molecule **2** (5 $\mu g\,mL^{-1}$) and then lysed with a Dounce homogenizer. After removal of the nuclei by low-speed centrifugation, the nucleus-free extracts were loaded on a 5–50% sucrose gradient and ultracentrifuged. The resulting layers were fractionated, and the fluorescent fraction was analyzed using SDS-PAGE (Supporting Information, Figure S2). Major protein bands in the fluorescent fraction were excised and sequenced. Peptide sequence analysis showed that these were mitochondrial membrane proteins, including porin isoform 1, pyrroline-5-carboxylate reductase 1, solute carrier family 25, member 5 (SLC25A5), prohibitin, and electron transfer flavoprotein. The co-purifi-

[*] Dr. Y. Kawazoe, Dr. H. Shimogawa, Dr. A. Sato, Prof. M. Uesugi
Institute for Chemical Research, Kyoto University
Uji, Kyoto 611-0011 (Japan)
Fax: (+81)774-38-3226
E-mail: kawazoe@scl.kyoto-u.ac.jp
Prof. M. Uesugi
Institute for Integrated Cell-Material Sciences
Kyoto University, Uji, Kyoto 611-0011 (Japan)
E-mail: uesugi@scl.kyoto-u.ac.jp

[**] This work was supported by the JST, the Uehara Memorial Foundation, and the Global COE program.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201100935.

cation of molecule **2** with mitochondria supports our notion that molecule **2** stains mitochondrial surfaces. However, there is also a possibility that molecule **2** interacts with a cellular component tightly bound to mitochondrial surface.

In the course of organelle fractionation, we noticed that molecule **2** underwent structural conversion within the cells. HPLC analysis of molecule **2** produced a single peak at a retention time of 24 min (Figure 2). In contrast, organic extracts derived from cells treated with molecule **2** produced

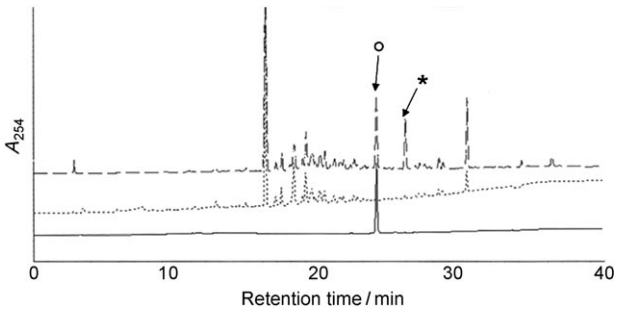


Figure 2. HPLC analyses of molecule **2** alone (——), organic extract from untreated HeLa cells (•••••), and organic extract from HeLa cells treated with molecule **2** (-----). The peak for molecule **2** is indicated by a circle (24 min) and for its metabolite by an asterisk (26 min).

two peaks: a peak at 24 min indicating molecule **2**, and a peak at 26 min (Figure 2, indicated by an asterisk). Organic extracts of untreated cells produced no significant peak. The substance that produced the peak at 26 min was considered to be a metabolite of molecule **2**.

To determine whether molecule **2** or its metabolite was the source of fluorescence signals in cells, we isolated the metabolite from the cells treated with molecule **2** and examined its fluorescence properties. The results showed that fluorescence spectra of the metabolite were red-shifted and much stronger than those of molecule **2**. Molecule **2** displayed maximum fluorescence excitation at 296 nm and maximum fluorescence emission at 419 nm, while the maximum excitation and emission wavelengths of the metabolite were both red-shifted, to 469 and 551 nm, respectively (Figure 3). Our confocal microscope has a 488 nm Ar laser for excitation and a 500–540 nm band path filter for green color fluorescence, and it is not capable of detecting the weak 419 nm fluorescence of molecule **2**. Therefore, the observed mitochondrial surface signals appeared to be produced by the metabolite of molecule **2**.

To determine the chemical structure of the metabolite, we treated 80 L of cultured HeLa S3 cells with 5 μg mL^{-1} of molecule **2** for 2 h. The cells were then harvested and extracted with organic solvents. The fluorescent metabolite was purified by HPLC, approximately 1 mg of the metabolite was successfully isolated. The purified metabolite was subjected to high-resolution mass spectrometry. The parent ion of the metabolite was observed at *m/z* 462.0540, thus indicating that the elemental composition of the metabolite was $C_{21}H_{15}F_3N_3O_2S_2$, which exactly matches that of molecule **2** (calculated mass = 462.0558, observed mass = 462.0587, com-

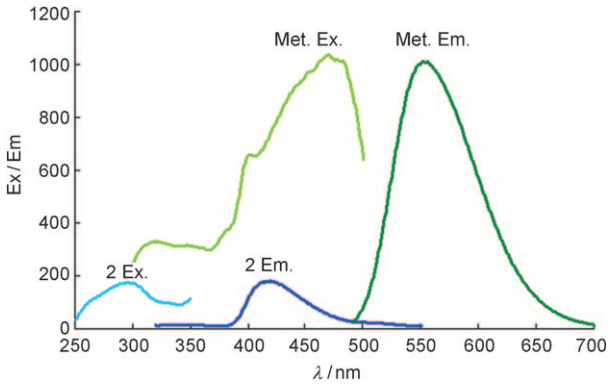


Figure 3. Excitation (Ex) and fluorescence emission spectra (Em) of molecule **2** and its metabolite (Met). Fluorescence spectra for each molecule were obtained at a concentration of 20 μg mL^{-1}.

position: $C_{21}H_{15}F_3N_3O_2S_2$). We conducted ^{1}H NMR spectroscopic experiments to compare the chemical structures of molecule **2** and the metabolite. Thiophene and *N*-acetylaniline groups were intact in both compounds, but the methylene group in the linker region of molecule **2** appeared to be lost in the metabolite (Figure 4). When quenched by D_2O, the peak at 10.3030 ppm diminished, and the peak at 7.6764 ppm disappeared completely (Supporting Information, Figure S3), suggesting that the metabolite has hydrogen atoms bound to heteroatoms.

Based on the ^{1}H NMR results, we hypothesized that molecule **2** was cyclized to molecule **3** (Figure 5a). We

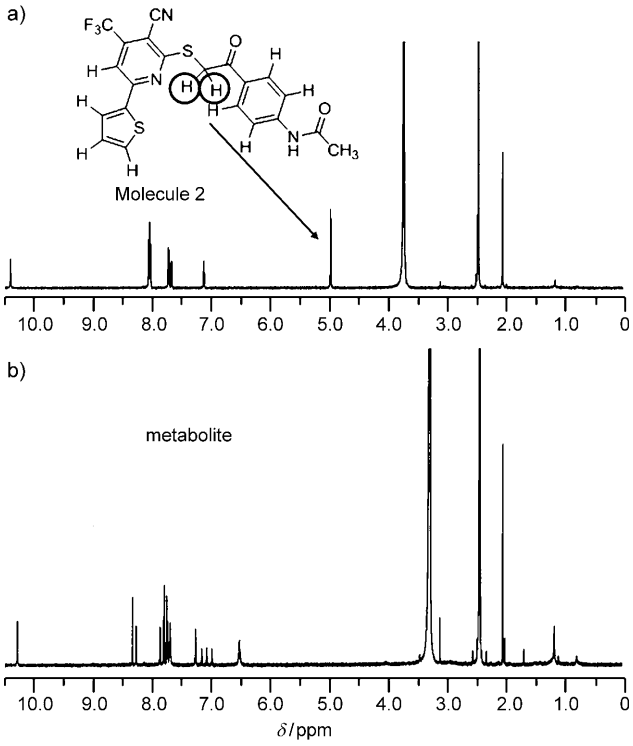


Figure 4. NMR spectra of a) molecule **2** and b) its metabolite. An arrow indicates a signal from the α protons in molecule **2**, which is lost in the metabolite.

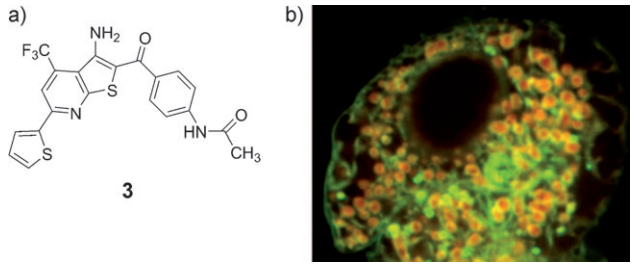


Figure 5. a) The chemical structure of molecule **3**, the metabolite of molecule **2**. b) HeLa cells that were double-stained with molecule **3** (20 μg mL^{-1}) and MitoTracker Red and then observed by a confocal microscope.

chemically synthesized molecule **3** and compared its physical and chemical properties to those of the metabolite. The synthesized compound showed exactly the same HPLC profile and fluorescence spectrum as the metabolite, and the signatures in its absorbance and ^{1}H NMR spectra matched those of the metabolite (Supporting Information, Figure S4 and NMR chart). Both molecule **3** and the metabolite could be monoacetylated to yield a substance with a molecular mass of 525.80, which is consistent with the presence of a free amino group.

Incubation of HeLa cells with fluorescent molecule **3** resulted in similar staining of mitochondrial surfaces as did incubation with molecule **2** (Figure 5b), suggesting that molecule **3**, not molecule **2**, is the molecule that generates fluorescent images. Nevertheless, the staining pattern of molecule **3** was more complex and had higher background than that of molecule **2** at the same concentration. Molecule **2** is likely to penetrate cell membrane and gradually become fluorescent through bioconversion within the cells, lowering background levels. Another possible explanation would be in situ bioconversion of molecule **2** in the mitochondrial matrix. The pH of the mitochondrial matrix in cells has been estimated to be 8.05 ± 0.11.[10] Model reactions of molecule **2** at the pH range of 6.0–8.0 in 150 mM sodium phosphate buffers showed faster conversion of molecule **2** in alkaline conditions (see the Supporting Information for details). At pH 8.0, 65.8 % of molecule **2** was converted into molecule **3** in 4 h, while a conversion of only 8.4 % and 1.5 % of molecule **2** into molecule **3** at pH 7.0 and pH 6.0, respectively, was observed. Perhaps an acidic α proton of molecule **2** is gradually released at pH 8.0 to give an enolate, which undergoes cyclization. Hydrolysis of an ester within cells has been used for controlling the properties of fluorescent small molecule probes.[11] This example suggests that a similar control of a fluorescent probe may be possible through cyclization of non-fluorescent molecules within cells.

Direct molecular targets of molecule **3** remain unknown. Selectivity of molecule **3** may be achieved by direct physical association with some of the many proteins in the mitochondrial surface. Alternatively, molecule **3** may be recognized by non-proteins or influenced by the environmental status of the mitochondrial membrane. We examined the staining of molecule **2** in the presence of CCCP, an uncoupling reagent that disrupts the mitochondrial membrane potential (Supporting Information, Figure S5). The staining pattern of molecule **2** remained the same in the presence of CCCP, indicating that the staining properties of molecule **2** are independent of the membrane potential. We also performed staining experiments with additional cell types: human embryonic kidney HEK 293, prostate carcinoma DU 145, hepatocyto carcinoma HepG2, breast adenocarcinoma SK-BR-3, and mouse myoblast C2C12. The results showed that staining patterns of molecule **2** in these cell lines are similar to each other (Supporting Information, Figure S6). These results suggest that molecule **2** is recognized by mitochondrion-associated cellular components common in a range of cell types.

In conclusion, we have developed a novel fluorescent probe specific for mitochondrial surfaces. To our knowledge, this molecule is the first such probe. Mitochondria play an important role not only in energy metabolism but also in apoptosis.[12] Furthermore, recent studies revealed that mitochondria interacts with endoplasmic reticulum and supports direct transfer of lipids and Ca^{2+} ions.[13] Molecule **2** or its analogues may allow the visualization of cross-talk between mitochondria and other organelles and for mitochondrial surface dynamics in response to various stimuli. Cell-based image screening of larger chemical libraries enriched in aromatic or fluorescent structures will almost certainly discover additional cell-permeable probes with various properties,[14] and some may undergo bioconversion within cells to achieve their selectivities.

Experimental Section

The chemical library of 12000 molecules consists of relatively large molecules enriched in aromatic groups that were purchased from several chemical library companies. HeLa cells were cultured in DMEM supplemented with 10 % fetal bovine serum. Each compound was screened at 20 μg mL^{-1} by incubation of cells for 2 h, and the signals were checked in situ by fluorescence microscopy. The details of the cell-based screening are provided in the Supporting Information.

The synthesis of molecules **2** and **3** and other analogues is described in the Supporting Information. To purify the metabolite, 80 L of cultured HeLa S3 cells were treated with 5 μg mL^{-1} of molecule **2** for 2 h. The cells were extracted twice with acetonitrile and then lyophilized. The resulting powder was extracted with chloroform and lyophilized again. The extracts were fractionated by sequential column chromatography with Inertsil ODS-3 (GL Sciences, Japan) and COSMOSIL Chlester (Nacalai Tesque, Japan), using a Shimadzu LC2010C HPLC system.

Fluorescence spectra for each compound were obtained with a Hitachi F-7000 fluorescence spectrometer at 20 μg mL^{-1}. ^{1}H NMR spectra were recorded in deuterated solvents at 600 MHz (JOEL JNM-ECA 600). High-resolution mass spectra were obtained on a JEOL JMS-700 spectrometer.

Received: February 7, 2011
Published online: May 3, 2011

Keywords: bioconversion · cyclization · fluorescent probes · live-cell imaging · mitochondria

[1] S. Adams, A. Harootunian, Y. Buechler, S. Taylor, R. Tsien, *Nature* **1991**, *349*, 694 – 697.

[2] a) D. Perez-Sala, D. Collado-Escobar, F. Mollinedo, *J. Biol. Chem.* **1995**, *270*, 6235 – 6242; b) E. Adie, S. Kalinka, L. Smith, M. Francis, A. Marenghi, M. Cooper, M. Briggs, N. Michael, G. Milligan, S. Game, *Biotechniques* **2002**, *33*, 1152 – 1154.

[3] a) H. Zhang, J. Joseph, C. Felix, B. Kalyanaraman, *J. Biol. Chem.* **2000**, *275*, 14038 – 14045; b) T. Iuchi, M. Akaike, T. Mitsui, Y. Ohshima, Y. Shintani, H. Azuma, T. Matsumoto, *Circ. Res.* **2003**, *92*, 81 – 87.

[4] a) Y. Tsien, T. Rink, M. Poenie, *Cell Calcium* **1985**, *6*, 145 – 157; b) P. Negulescu, A. Harootunian, R. Tsien, T. Machen, *Cell Regul.* **1990**, *1*, 259 – 268; c) S. Burdette, G. Walkup, B. Spingler, R. Tsien, S. Lippard, *J. Am. Chem. Soc.* **2001**, *123*, 7831 – 7841; d) K. Kikuchi, K. Komatsu, T. Nagano, *Curr. Opin. Chem. Biol.* **2004**, *8*, 182 – 191.

[5] H. Kojima, K. Sakurai, K. Kikuchi, S. Kawahara, Y. Kirino, H. Nagoshi, Y. Hirata, T. Akaike, H. Maeda, T. Nagano, *Biol. Pharm. Bull.* **1997**, *20*, 1229 – 1232.

[6] S. Wang, Y. T. Chang, *Chem. Commun.* **2008**, 1173 – 1175.

[7] a) S. Feng, Y. K. Kim, Y. T. Chang, *Chem. Commun.* **2010**, *46*, 436 – 438; b) Q. Li, Y. Kim, J. Namm, A. Kulkami, G. R. Rosania, Y. H. Ahn, Y. T. Chang, *Chem. Biol.* **2006**, *13*, 615 – 623.

[8] C. N. Im, N. Y. Kang, H. H. Ha, X. Bi, J. J. Lee, S. J. Park, S. Y. Lee, M. Vendrell, Y. K. Kim, J. S. Lee, J. Li, Y. H. Ahn, B. Feng, H. H. Ng, S. W. Yun, Y. T. Chang, *Angew. Chem.* **2010**, *122*, 7659 – 7662; *Angew. Chem. Int. Ed.* **2010**, *49*, 7497 – 7500.

[9] a) R. Tsien, *Nat. Rev. Mol. Cell. Biol.* **2003**, Suppl, SS16 – 21; b) M. Vendrell, J. S. Lee, Y. T. Chang, *Curr. Opin. Chem. Biol.* **2010**, *14*, 383 – 389.

[10] M. F. C. Abad, G. D. Benedetto, P. J. Magalhaes, L. Filippin, T. Pozzan, *J. Biol. Chem.* **2004**, *279*, 11521 – 11529.

[11] a) G. Grynkiewicz, M. Poenie, R. Y. Tsien, *J. Biol. Chem.* **1985**, *260*, 3440 – 3450; b) S. Izumi, Y. Urano, K. Hanaoka, T. Terai, T. Nagano, *J. Am. Chem. Soc.* **2009**, *131*, 10189 – 10200.

[12] a) D. R. Green, J. C. Reed, *Science* **1998**, *281*, 1309 – 1312; b) N. A. Thornberry, Y. Lazebnik, *Science* **1998**, *281*, 1312 – 1316; c) A. Gross, J. M. McDonnell, S. J. Korsmeyer, *Genes Dev.* **1999**, *13*, 1899 – 1991.

[13] a) R. Rizzuto, P. Pinton, W. Carrington, F. S. Fay, K. E. Forgarty, L. M. Lifshitz, R. A. Tuft, T. Pozzan, *Science* **1998**, *280*, 1763 – 1766; b) S. J. Stone, J. E. Vance, *J. Biol. Chem.* **2000**, *275*, 34534 – 34540; c) C. Giorgi, D. D. Stefani, A. Bononi, R. Rizzuto, P. Pinton, *Int. J. Biochem. Cell Biol.* **2009**, *41*, 1817 – 1827.

[14] E. Kim, M. Koh, J. Ryu, S. B. Park, *J. Am. Chem. Soc.* **2008**, *130*, 12206 – 12207.

