Anal. Chem. **2001,** *73,* 1967−1973

Bioimaging of Nitric Oxide with Fluorescent Indicators Based on the Rhodamine Chromophore

Hirotatsu Kojima,[†] Miki Hirotani,[†] Naoki Nakatsubo,[†] Kazuya Kikuchi,[†] Yasuteru Urano,[†] Tsunehiko Higuchi,[†] Yasunobu Hirata,[‡] and Tetsuo Nagano*[,†]

Graduate School of Pharmaceutical Sciences, and Second Department of Internal Medicine, Faculty of Medicine, the University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Diaminofluoresceins are widely used for detection and imaging of nitric oxide (NO), but for biological applications, they have the disadvantages that the fluorescence of the fluorescein chromophore is pH-sensitive and overlaps the autofluorescence of cells. We have developed a membrane-permeable fluorescent indicator for NO based on the rhodamine chromophore, DAR-4M AM, which can be excited with 550-nm light. The fluorescence quantum yield of the product after reaction with NO is 840 times higher than that of DAR-4M. The detection limit of NO was 7 nM, and the fluorescence showed no pH dependency above pH 4. DAR-4M AM was successfully applied to practical bioimaging of NO produced in bovine aortic endothelial cells.

Although nitric oxide (NO) is well established to be a signal transmitter in vivo,[1−5] many of the details remain controversial. Several methods of detecting NO have been developed in order to investigate its diverse physiological and pathophysiological roles in vivo.[6−9] We have been developing fluorescent indicators for NO, such as the diaminofluoresceins (DAFs).[10] The vicinal aromatic diamine on fluorescein is transformed to the corresponding highly fluorescent triazole by reaction with NO. The actual reaction species are thought to be NO^+ equivalents, such as nitric anhydride (N_2O_3), which are generated by rapid autoxidation of NO. The diacetyl derivatives of these indicators, which can permeate into cells, where they are hydrolyzed by intracellular esterases, enabled bioimaging of NO production in living cells and tissues. The advantages of bioimaging with fluorescent indicators include highly sensitive detection with fine temporal and spatial resolution under microscopic observation. In bioimaging studies, longer wavelength excitation is desirable owing to the fluorescence of various cell components (autofluorescence) and the cytotoxicity caused by UV excitation. That was why we chose fluorescein as the fluorophore of our diamine indicators. Although the practical usefulness of DAFs for functional studies of NO was confirmed, DAFs lack some other desirable characteristics of NO indicators. To obtain higher photostability, longer excitation wavelength, and applicability over a wider pH range, we have examined the suitability of rhodamine, which is also widely used in biological studies, as the fluorophore. It is considered that the quenching mechanism of DAFs is based on photoinduced electron transfer from the diaminobenzene moiety to the xanthene moiety. We anticipated that this mechanism would also be applicable to the rhodamine fluorophore.

EXPERIMENTAL SECTION

Synthesis of DARs. ^{1}H NMR spectra were recorded on a JEOL JNM-LA300 instrument at 300 MHz; δ values are given in ppm relative to tetramethylsilane. Electron ionization mass spectrometry (EI-MS) spectra were measured with a JEOL JMS-DX 300 mass spectrometer. Fast atom bombardment mass spectrometry (FAB-MS) spectra were measured with a JEOL SX-102A mass spectrometer. The synthetic pathways employed to obtain the new dyes are outlined in Figure 1.

1 → 3. A mixture of **1** (2.95 g, 10 mmol)[10] and *N*,*N*-diethyl-3-aminophenol (3.33 g, 20 mmol) was stirred at 180 °C for 2 h. After cooling, the black amorphous solid was refluxed in 2 M HCl (200 mL) for 2 h. The mixture was neutralized with sodium hydroxide, and the precipitate was collected and dried. Extraction with dichloromethane could also be used for collection. The products were purified by silica gel chromatography with methanol/dichloromethane (5:95 v/v) to afford **3** (2.97 g, 59% yield).

3 → DAR-1. **3** (2.97 g, 5.9 mmol) was reduced with sodium sulfide and sodium hydrosulfide as previously described,[10] to give DAR-1 (0.51 g, 18%), with recovery of the starting material (2.22 g): mp 145 °C; ^{1}H NMR (dimethyl sulfoxide (DMSO)-d_6) δ 1.09 (t, 12H, CH_3, J = 6.8 Hz), 3.33 (m, 8H, CH_2), 4.98 (s, 2H, NH_2), 5.86 (s, 2H, NH_2), 6.06 (d, 1H, ArH, J = 7.7 Hz), 6.37−6.41 (m, 4H, ArH), 6.55 (d, 2H, ArH, J = 8.6 Hz), 6.78 (d, 1H, ArH, J = 7.7 Hz); EI-MS m/z 472 (M^+). Elemental Anal. Calcd for $C_{28}H_{32}N_4O_3 \cdot 0.5H_2O$: C, 69.83; H, 6.91; N, 11.64. Found: C, 69.85; H, 6.83; N, 11.44.

* Corresponding author: (e-mail) tlong@mol.f.u-tokyo.ac.jp; (fax) 81-3-5841-4855.

[†] Graduate School of Pharmaceutical Sciences.

[‡] Second Department of Internal Medicine, Faculty of Medicine.

(1) Murad, F. *Angew. Chem., Int. Ed. Engl.* **1999**, *38*, 1856−1868.
(2) Furchgott, R. F. *Angew. Chem., Int. Ed. Engl.* **1999**, *38*, 1870−1880.
(3) Ignarro, L. J. *Angew. Chem., Int. Ed. Engl.* **1999**, *38*, 1882−1892.
(4) Nathan, C.; Xie, Q. *Cell* **1994**, *78*, 915−918.
(5) Blottner, D. *J. Neurosci. Res.* **1999**, *58*, 139−151.
(6) Akaike, T.; Maeda, H. *Methods Enzymol.* **1996**, *268*, 211−221.
(7) Yoshimura, T.; Yokoyama, H.; Fujii, S.; Takayama, F.; Oikawa, K.; Kamada, H. *Nat. Biotechnol.* **1996**, *14*, 992−994.
(8) Barker, S. L. R.; Zhao, Y.; Marletta, M. A.; Kopelman, R. *Anal. Chem.* **1999**, *71*, 2071−2075.
(9) Meineke, P.; Rauen, U.; de Groot, H.; Korth, H.-G.; Sustmann, R. *Chem. Eur. J.* **1999**, *5*, 1738−1747.
(10) Kojima, H.; Nakatsubo, N.; Kikuchi, K.; Kawahara, S.; Kirino, Y.; Nagoshi, H.; Hirata, Y.; Nagano, T. *Anal. Chem.* **1998**, *70*, 2446−2453.

10.1021/ac001136i CCC: $20.00
Published on Web 03/24/2001

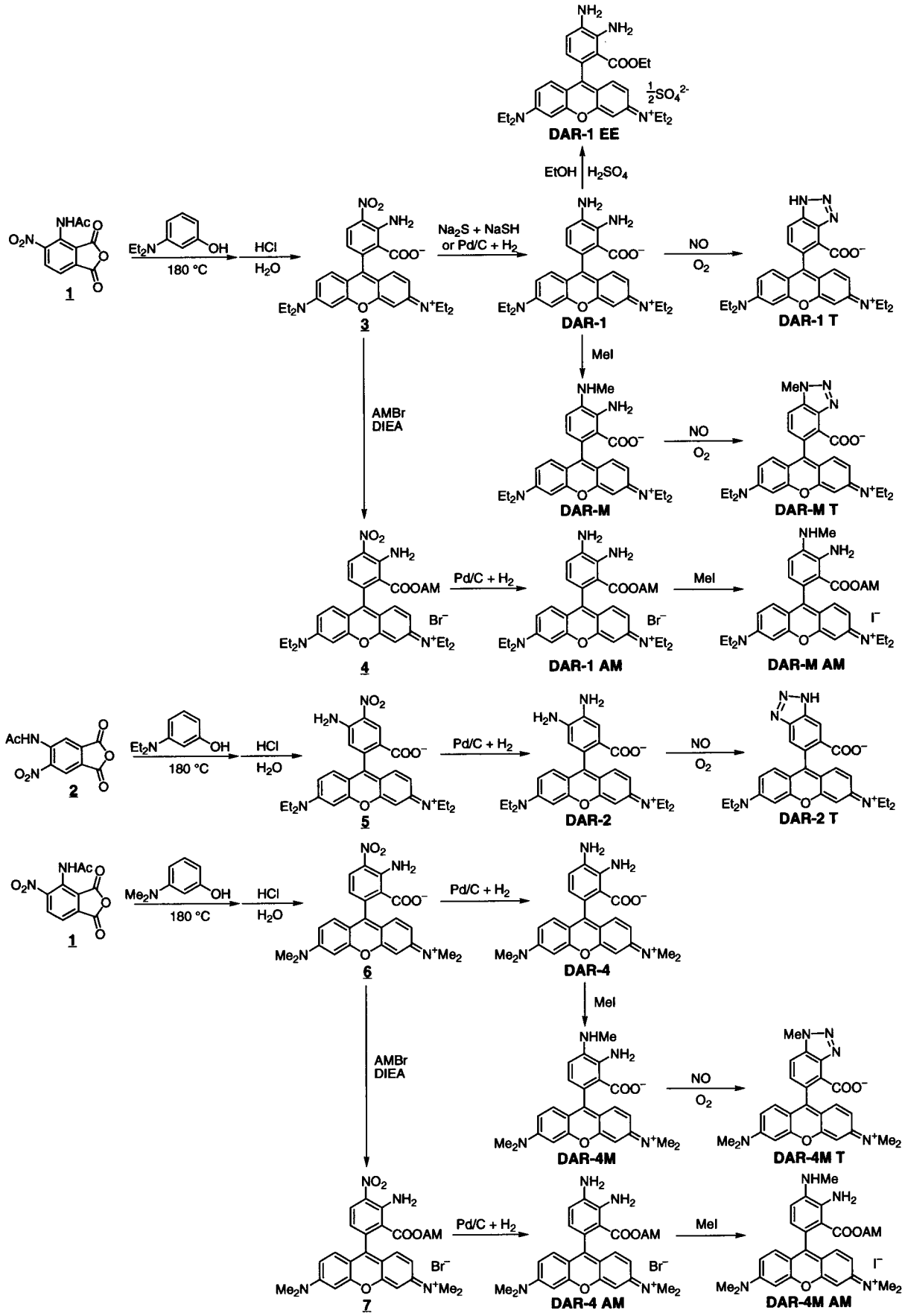

Figure 1. Synthetic scheme of DARs: Ac, acetyl; Et, ethyl; AM, acetoxymethyl; Me, methyl; DIEA, diisopropylethylamine.

DAR-1 → DAR-M. Methyl iodide (100 μL) was added in several portions to a solution of DAR-1 (231 mg, 0.489 mmol) in ethanol (10 mL) at 80 °C over 6.5 h. The product was purified by silica gel chromatography with methanol/dichloromethane (1:9 v/v) and by preparative TLC to afford DAR-M (25 mg, 11%), with recovery of the starting material: mp 150−154 °C; ^{1}H NMR ($CDCl_3$) δ 1.13 (t, 12H, CH_3, J = 7.0 Hz), 2.86 (s, 3H, NCH_3), 3.33 (q, 8H, CH_2, J = 7.0 Hz), 6.37−6.43 (m, 5H, ArH), 6.75 (d, 1H, ArH, J = 7.9 Hz), 6.81 (d, 2H, ArH, J = 9.0 Hz); FAB-MS m/z 487 ($(M + H)^+$).

DAR-1 → DAR-1 EE. Concentrated H_2SO_4 (0.1 mL) was added to an ethanol solution of DAR-1 (113 mg, 2.39 mmol), and the solution was refluxed for 2 h. After cooling, ethanol was removed in vacuo and the residue was neutralized with sodium hydroxide to collect the resulting precipitate. After drying, it was purified by silica gel chromatography with methanol/dichloromethane (8:92 v/v) to give DAR-1 EE (26 mg, 20%), with recovery of the starting material (62 mg): ^{1}H NMR ($CDCl_3$) δ 0.66 (t, 3H, CH_3, J = 7.1 Hz), 1.33 (t, 12H, CH_3, J = 7.1 Hz), 3.61 (q, 8H, NCH_2, J = 7.1 Hz), 4.19 (q, 2H, CH_2, J = 7.1 Hz), 4.55 (s, 2H, NH_2), 6.05 (s, 2H, NH_2), 6.33 (d, 1H, ArH, J = 7.9 Hz), 6.74 (d, 2H, ArH, J = 2.2 Hz), 6.83 (dd, 2H, ArH, J = 9.7, 2.2 Hz), 6.94 (d, 1H, ArH, J = 7.8 Hz), 7.45 (d, 1H, ArH, J = 9.7 Hz); EI-MS m/z 501 (M^+).

3 → 4. Acetoxymethyl bromide (AMBr; 100 μL) was added to a 1,2-dichloroethane solution (5 mL) of **3** (0.49 g, 0.98 mmol) and diisopropylethylamine (DIEA; 200 μL). The mixture was stirred in a stoppered vessel at room temperature. After 24 h, 100 μL of AMBr and 200 μL of DIEA were added and stirring was continued for 18 h. Dichloromethane was added and the solution was washed with water. After removal of the solvent, the product was purified by silica gel chromatography with methanol/dichloromethane (6:94 v/v) to give **4** (124 mg, 19%), with recovery of the starting material (359 mg).

4 → DAR-1 AM. Compound **4** (0.38 g, 0.58 mmol) was reduced with Pd/C (10% Pd, 98 mg) in EtOH (90 mL) under hydrogen (1 atm) at room temperature for 2 h. The product was purified by silica gel chromatography with methanol/dichloromethane (1:9 v/v) to give DAR-1 AM (0.29 mg, 80%): ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 12H, CH_3, J = 7.0 Hz), 1.67 (s, 3H, $COCH_3$), 3.59 (q, 8H, CH_2, J = 7.0 Hz), 5.34 (s, 2H, OCH_2O), 6.24 (d, 1H, ArH, J = 7.9 Hz), 6.71 (d, 2H, ArH, J = 2.6 Hz), 6.82 (dd, 2H, ArH, J = 9.5, 2.6 Hz), 6.93 (d, 1H, ArH, J = 7.9 Hz), 7.41 (d, 2H, ArH, J = 9.5 Hz); FAB-MS m/z 545 (M^+).

DAR-1 AM → DAR-M AM. A methyl group was introduced into DAR-1 AM (300 mg, 0.48 mmol) by a method similar to that used to obtain DAR-M, to afford DAR-M AM (68 mg, 21%) with recovery of DAR-1 AM (bromide 87 mg and iodide 67 mg): mp 147−150 °C; ^{1}H NMR ($CDCl_3$) δ 1.34 (t, 12H, CH_3, J = 7.0 Hz), 1.67 (s, 3H, $COCH_3$), 2.98 (s, 3H, NCH_3), 3.63 (q, 8H, CH_2, J = 7.0 Hz), 5.36 (s, 2H, OCH_2O), 6.42 (d, 1H, ArH, J = 8.0 Hz), 6.66 (d, 1H, ArH, J = 8.0 Hz), 6.75 (d, 2H, ArH, J = 2.4 Hz), 6.84 (dd, 2H, ArH, J = 9.3, 2.4 Hz), 7.43 (d, 2H, ArH, J = 9.3 Hz); FAB-MS m/z 559 (M^+).

2 → 5. Compound **5** was synthesized from **2** by a method similar to that used to obtain **3** (yield 7.7%).

5 → DAR-2. Compound **5** was reduced by a method similar to that used to obtain DAR-1 AM, to afford DAR-2 (quantitative yield): mp 158−163 °C; ^{1}H NMR (DMSO-d_6) δ 1.08 (t, 12H, CH_3, J = 6.8 Hz), 4.95 (br, 2H, NH_2), 5.52 (br, 2H, NH_2), 6.09 (s, 1H, ArH), 6.37 (d, 2H, ArH, J = 2.4 Hz), 6.41 (dd, 2H, ArH, J = 8.8, 2.4 Hz), 6.51 (d, 2H, ArH, J = 8.8 Hz), 6.89 (s, 1H, ArH); FAB-MS m/z 472 (M^+).

1 → 6. Compound **6** was synthesized from **1** and *N*,*N*-dimethyl-3-aminophenol by a method similar to that used to obtain **3** (yield 66.4%).

6 → DAR-4. Compound **6** was reduced by a similar method (solvent: dichloromethane/ethanol 7:13 v/v) to that used to obtain DAR-1 AM, to afford DAR-4 (65.4%): ^{1}H NMR ($CDCl_3$) δ 2.97 (s, 12H, CH_3), 5.03 (br, 2H, NH_2), 6.34 (d, 1H, ArH, J = 7.7 Hz), 6.40 (dd, 2H, ArH, J = 8.8, 2.6 Hz), 6.46 (d, 2H, ArH, J = 2.6 Hz), 6.75 (d, 2H, ArH, J = 8.8 Hz), 6.90 (d, 1H, ArH, J = 7.7 Hz); FAB-MS m/z 417 ($(M + H)^+$).

DAR-4 → DAR-4M. A methyl group was introduced into DAR-4 (0.55 g, 1.3 mmol) by a method similar to that used to obtain DAR-M, to afford DAR-4M (0.14 g, 24.5%) with recovery of DAR-4 (0.25 g): mp 219 °C; ^{1}H NMR ($CDCl_3$) δ 2.92 (s, 3H, CH_3), 2.97 (s, 12H, CH_3), 4.99 (br, 2H, NH_2), 6.40 (dd, 2H, ArH, J = 8.6, 2.6 Hz), 6.47 (d, 2H, ArH, J = 2.6 Hz), 6.47 (d, 1H, ArH, J = 8.0 Hz), 6.75 (d, 2H, ArH, J = 8.6 Hz), 6.85 (d, 1H, ArH, J = 8.0 Hz); FAB-MS, m/z 431 ($(M + H)^+$).

6 → 7. An acetoxymethyl group was introduced into **6** (2.44 g, 5.46 mmol) by a method similar to that used to obtain **4**, to afford **7** (0.445 g, 13.6%) with recovery of **6** (1.70 g).

7 → DAR-4 AM. Compound **7** was reduced by a similar method (solvent: dichloromethane/ethanol 1:1 v/v) to that used to obtain DAR-1 AM, to afford DAR-4 AM (50.9%):^{1}H NMR ($CDCl_3$) δ 1.69 (s, 3H, $COCH_3$), 3.33 (s, 12H, CH_3), 4.81 (br, 2H, NH_2), 5.32 (s, 2H, OCH_2O), 6.29 (d, 1H, ArH, J = 7.9 Hz), 6.47 (br, 2H, NH_2), 6.78 (d, 2H, ArH, J = 2.6 Hz), 6.91 (dd, 2H, ArH, J = 9.5, 2.6 Hz), 6.94 (d, 1H, ArH, J = 7.9 Hz), 7.45 (d, 2H, ArH, J = 9.5 Hz).

DAR-4 AM → DAR-4M AM. A methyl group was introduced into DAR-4 AM (133 mg, 0.234 mmol) by a method similar to that used to obtain DAR-M, to afford DAR-4M AM (40.5 mg, 27.5%) with recovery of DAR-4 AM (iodide 70 mg): mp 177−178 °C; ^{1}H NMR ($CDCl_3$) δ 1.69 (s, 3H, $COCH_3$), 2.99 (d, 3H, NCH_3, J = 4.6 Hz), 3.36 (s, 12H, CH_3), 5.06 (q, 1H, NH, J = 4.6 Hz), 5.33 (s, 2H, OCH_2O), 6.34 (s, 2H, NH_2), 6.46 (d, 1H, ArH, J = 7.9 Hz), 6.69 (d, 1H, ArH, J = 7.9 Hz), 6.79 (d, 2H, ArH, J = 2.6 Hz), 6.91 (dd, 2H, ArH, J = 9.5, 2.6 Hz), 7.48 (d, 2H, ArH, J = 9.5 Hz); FAB-MS m/z 503 (M^+).

Triazole Compounds of DARs. NO gas was introduced into a methanol solution of each DAR (a mixture of methanol and dichloromethane was used as the solvent only in the case of DAR-4M) to yield the corresponding triazole compound (DAR-1 T, DAR-2 T, DAR-M T, DAR-4M T). Each triazole compound was purified by silica gel chromatography with methanol/dichloromethane (1:9 v/v, a small amount of acetic acid (0.02% v/v) was added only in the case of purification of DAR-4M T): (DAR-1 T) mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 1.10 (t, 12H, CH_3, J = 6.4 Hz), 3.34 (m, 8H, CH_2), 6.50−6.71 (m, 7H, ArH), 8.01 (d, 1H, ArH, J = 8.1 Hz); FAB-MS m/z 484 ($(M+H)^+$). (DAR-2 T) mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 1.08 (t, 12H, CH_3, J = 7.0 Hz), 6.38 (dd, 2H, ArH, J = 8.2, 2.2 Hz), 6.44 (d, 2H, ArH, J = 2.2 Hz), 6.49 (d, 2H, ArH, J = 8.6 Hz), 7.63 (s, 1H, ArH), 8.57 (s, 1H, ArH);

EI-MS m/z 483 (M^+). (DAR-M T) mp 155−160 °C; ^{1}H NMR ($CDCl_3$) δ 1.12 (t, 12H, CH_3, J = 7.1 Hz), 3.32 (q, 8H, CH_2, J = 7.1 Hz), 4.37 (s, 3H, NCH_3), 6.31 (dd, 2H, ArH, J = 9.0, 2.5 Hz), 6.43 (d, 2H, ArH, J = 2.5 Hz), 6.58 (d, 2H, ArH, J = 9.0 Hz), 7.26 (d, 1H, ArH, J = 8.6 Hz), 7.83 (d, 1H, ArH, J = 8.6 Hz); FAB-MS m/z 498 ($(M + H)^+$). (DAR-4M T) mp 242−245 °C; ^{1}H NMR ($CDCl_3$) δ 2.98 (s, 12H, CH_3), 4.41 (s, 3H, CH_3), 6.36 (dd, 2H, ArH, J = 9.0, 2.6 Hz), 6.50 (d, 2H, ArH, J = 2.6 Hz), 6.62 (d, 2H, ArH, J = 9.0 Hz), 7.24 (d, 1H, ArH, J = 8.5 Hz), 7.77 (d, 1H, ArH, J = 8.5 Hz); FAB-MS m/z 442 ($(M + H)^+$).

Fluorometric Analysis. A fluorescence spectrophotometer (F4500, Hitachi, Tokyo, Japan) was used. The slit width was 2.5 nm for both excitation and emission. The photomultiplier voltage was 950 V. DARs were dissolved in DMSO (for fluorometric analysis; Dojin, Kumamoto, Japan) to obtain 10 mM stock solutions. Relative quantum efficiencies of fluorescence of DARs and their triazole forms were obtained by comparing the area under the corrected emission spectrum of the test sample at 535-nm excitation with that of a solution of rhodamine B in ethanol, which has a quantum efficiency of 0.97 according to the literature.[11] NO solution was prepared as described before.[10]

HPLC Analysis. The HPLC unit consisted of a pump (PU980, Jasco, Tokyo, Japan), a multiwavelength detector (MD910, Jasco), and a column (Inertsil ODS-2, 4.0 × 150 mm, GL Sciences Inc., Tokyo, Japan). Gradient elution was employed. (DAR-1 EE: sodium phosphate buffer (pH 7.4, 10 mM)/acetonitrile 25:75 v/v → 0:100 for 15 min, DAR-1 AM: 0.1% H_3PO_4/MeOH 60:40 v/v → 40:60 for 10 min). The flow rate was 1.0 mL/min. Absorbance was monitored in the range of 500−600 nm. Aliquots of 10 μL were injected for analysis.

Hydrolysis by Rat Brain Homogenate. Male Sprague−Dawley rats (8 weeks old) were decapitated with a guillotine, and the whole brains were removed. A 5-fold excess (by weight) of tris-HCl buffer (0.1 M, pH 7.6) containing 0.1% Triton X-100 was added, and homogenization was carried out. The supernatant after centrifugation of the homogenate at 40000*g* for 10 min was used for hydrolysis of the ester derivatives of dyes (10 μM). The incubation was carried out at 37 °C.

Imaging Procedures. Primary cultured endothelial cells from bovine aorta were passaged in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics. Cells between the 14th and 16th passages on glass-bottomed culture dishes (P35G-0-1-C; MatTek Corp., Ashland, MA) were used for these experiments. The cells were incubated for 30 min at 37 °C in DMEM containing 10 μM DAR-4M AM (serum-free, final 0.2% DMSO) for loading, and postincubation with DMEM was carried out for 1 h. Then, the medium was switched to phosphate-buffered saline (PBS)(+). The cells were mounted on an inverted fluorescence microscope (IX70: Olympus, Tokyo, Japan) equipped with an objective lens (×20), an excitation filter (520−550 nm), a dichroic mirror (565 nm), and a long-pass emission filter (580 nm). The air temperature was maintained at 37 °C with a warming box (IX-IBM; Olympus). Bradykinin and L-NAME were purchased from Sigma (St. Louis, MO). Bradykinin (1 mM, 1 μL) was carefully added to 1 mL of PBS(+) over the cells. Optical signals were recorded at 10-s intervals with an Argus 50 (Hamamatsu Photonics, Shizuoka, Japan), which is an imaging system including a cooled charge-coupled device (CCD) camera.

RESULTS AND DISCUSSION

We designed DAR-1 and DAR-2, based on the highly fluorescent Rhodamine B fluorophore, and synthesized them from phthalic anhydride derivatives (**1**, **2**) and *N*,*N*-diethyl-3-aminophenol (Figure 1). The same alkaline reduction system of a nitro group with sulfide and hydrosulfide ions that we had employed to synthesize DAFs was used.[10] However, the yield was low due to the poor solubility of **3** in water. We found that catalytic hydrogenation with Pd in an organic solvent afforded higher yields of the desired diamines.

It was confirmed that the reaction of DARs and NO affords the corresponding fluorescent triazole, as in the case of DAFs. To image NO in living cells, DARs should be membrane-permeable. We therefore introduced an ethyl group into the carboxyl group in DAR-1, anticipating that the ethyl ester, DAR-1 EE, would be hydrolyzed by cytosolic esterases after permeation through the cell membrane. Then, we applied this dye to imaging of NO produced by activated rat aortic smooth muscle cells, using a procedure similar to that reported in the previous paper.[10] There was almost no autofluorescence of the cells due to the longer wavelength excitation, as expected. Although the NO imaging with DAR-1 EE was successful, we encountered a difficulty in loading. It took a longer time to obtain a stable fluorescence intensity in the cells than in the case of DAF derivatives. This presumably occurred because the ethyl ester, which was attached to a bulky acid, was not readily hydrolyzed. DAR-1 EE remaining in the cytosol might be distributed to the fatty membrane in cells, and fluorescent rhodamines generally show higher fluorescence intensity in organic solvents, such as ethanol, than in water. We therefore examined the hydrolysis of DAR-1 EE with rat brain homogenate as an esterase preparation, by means of HPLC. During a 30-min incubation, DAR-1 EE was not hydrolyzed at all. Therefore, most of the loaded DAR-1 EE was probably localized on membranes in the smooth muscle cells, in unhydrolyzed form.

Next, we synthesized and examined the acetoxymethyl ester of DAR-1 (DAR-1 AM; Figure 1), because acetoxymethyl esters were reported to be easily hydrolyzed by intracellular esterases.[12] The esterification was carried out before reduction of the nitro group, because diamine is reactive to acetoxymethyl bromide. We found that 27% of DAR-1 AM was hydrolyzed in 30 min by rat brain homogenate. So, we decided to utilize this ester.

We expected that the fluorescence of DAR-1 T would be independent of physiological pH change, because the rhodamine fluorophore of DAR-1 T does not have phenolic hydroxyl groups. However, the triazole proton of DAR-1 T affected the fluorescence due to its pK_a value, 6.69.[13] Therefore, we introduced a methyl group into DAR-1.[13] The fluorescence intensity of the triazole form of DAR-M (DAR-M T; Figure 1) was stable above pH 4, while that of DAR-1 T or DAR-2 T was unstable around pH 7 (Figure 2).

For bioimaging applications, an acetoxymethyl ester was introduced into DAR-M. DAR-M AM (Figure 1) was applied to NO imaging in bovine aortic endothelial cells. However, the

(11) Nishikawa, Y.; Hiraki, K. *Analytical Methods of Fluorescence and Phosphorescence*; Kyoritsu Publishing Co.: Tokyo, 1984; pp 76−80.

(12) Tsien, R. Y. *Nature* **1981**, *290*, 527−528.

(13) Kojima, H.; Hirotani, M.; Urano, Y.; Kikuchi, K.; Higuchi, T.; Nagano, T. *Tetrahedron Lett.* **2000**, *41*, 69−72.

Table 1. Absorbance and Fluorescence Properties of Dyes[a]

dye[b]	extinction coeff and absorption max ($\times 10^4$ M^{-1} cm^{-1}, nm) diamine	extinction coeff and absorption max ($\times 10^4$ M^{-1} cm^{-1}, nm) triazole	fluoresc max of triazole form (nm)	rel quantum efficiencies diamine	rel quantum efficiencies triazole
DAN	0.5, 336	0.6, 357	433	0.51	0.83
DAN-1	0.6, 340	0.6, 360	447	0.002	0.63
DAF-2	7.9, 486	7.3, 491	513	0.005	0.92
DAF-5	9.8, 499	7.9, 503	523	0.007	0.70
DAF−FM	8.4, 487	7.3, 495	515	0.005	0.81
DAR-1	12, 550	8.7, 556	575	0.004	0.25
DAR-2	10, 549	7.1, 552	571	0.006	0.34
DAR-M	9.8, 550	7.5, 558	574	0.007	0.29
DAR-4M	7.8, 543	7.6, 554	572	0.0005	0.42

[a] All data were obtained at 20 °C in 0.1 M sodium phosphate buffer, pH 7.4. [b] DAN, 2,3-diaminonaphthalene; DAN-1, 4-[[(3-amino-2-naphthalenyl)amino]methyl]-benzoic acid.[15]

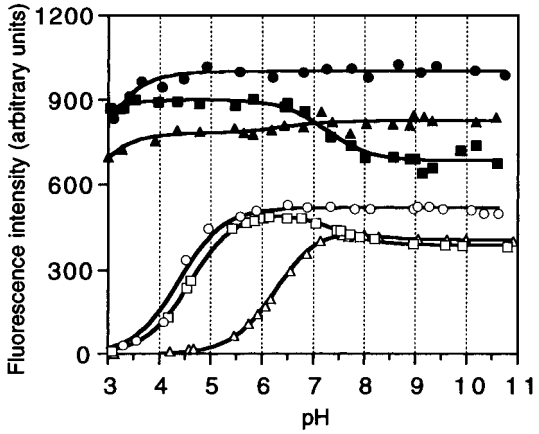

Figure 2. pH profiles of the indicators after reaction with NO. Triazole forms of DAR-1, DAR-2, DAR-M, DAF-2, DAF-5, and DAF-FM (final 1 μM) were added to sodium phosphate solution adjusted to various pH values. The pH was measured after mixing. The fluorescence intensities of DAR-1 T (closed triangle), DAR-2 T (closed square), DAR-M T (closed circle), DAF-2 T (open triangle), DAF-5 T (open square), and DAF-FM T (open circle) were determined at 580, 575, 575, 515, 520, and 515 nm with excitation at 565, 565, 560, 495, 505, and 495 nm, respectively. The photomultiplier voltage was 950 (DARs) or 400 V (DAFs). The curves were fitted to the following equations: (DAR-1 T) intensity = $783/(1 + 10^{2.13-pH}) + 42.7/(1 + 10^{6.69-pH})$, $R = 0.956$; (DAR-2 T) intensity = $899/(1 + 10^{1.62-pH}) - 218/(1 + 10^{7.27-pH})$, $R = 0.987$; (DAR-M T) intensity = $1000/(1 + 10^{2.39-pH})$, $R = 0.951$; (DAF-2 T) intensity = $460/(1 + 10^{6.27-pH}) - 55.6/(1 + 10^{7.94-pH})$, $R = 1.00$; (DAF-5 T) intensity = $505/(1 + 10^{4.59-pH}) - 119/(1 + 10^{7.41-pH})$, $R = 1.00$; (DAF−FM T) intensity = $518/(1 + 10^{4.38-pH})$, $R = 0.994$.

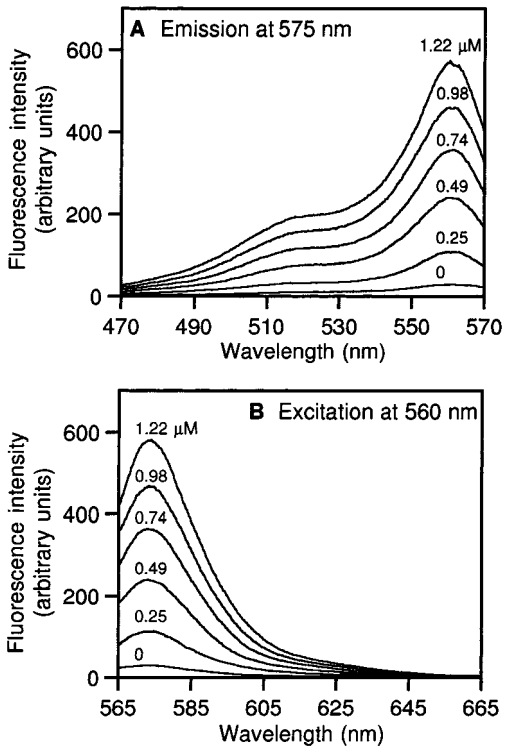

Figure 3. Excitation (A) and emission (B) spectra for DAR-4M at 37 °C in sodium phosphate buffer (0.1 M, pH 7.4) with NO values ranging from 0 to 1.22 μM. NO solution was added to 10 μM DAR-4M solution under aerobic conditions. The spectra were obtained from an average of five accumulations after addition of NO solution.

difficulty in loading was hardly improved, compared to DAR-1 EE. We thought that this might be because DAR-M AM, which has four ethyl groups, is too hydrophobic to be stably distributed to the cytosol. Therefore, we changed the fluorophore to tetramethylrhodamine and examined the suitability of DAR-4M AM for bioimaging (Figure 1). The fluorescence spectra of DAR-4M are shown in Figure 3 and the properties of the dyes that have been developed are summarized in Table 1. It was found that the quantum efficiency of DAR-4M T is the highest of all DARs examined. Judging from NO standard curves calibrated with the indicators, the sensitivity of DAR-4M to NO is twice that of DAR-1. The detection limit of NO by DAR-4M was 7 nM. DAR-4M should offer a good signal-to-noise ratio in the examination of biological samples because of the low background fluorescence with the longer wavelength excitation, even though the quantum yield of DAR-4M T is lower than that of fluorescein dyes. Moreover, as we mentioned above, DAR-4M permitted NO detection above pH 4, while DAF-FM, a fluorescein dye, can only be applied above pH 5.8.[14]

We applied DAR-4M AM to the imaging of NO in bovine aortic endothelial cells, for which a high sensitivity to NO is required. The loading was easier than with any other DAR examined. The cells were incubated in DMEM containing 10 μM DAR-4M AM

(14) Kojima, H.; Urano, Y.; Kikuchi, K.; Higuchi, T.; Hirata, Y.; Nagano, T. *Angew. Chem., Int. Ed. Engl.* **1999**, *38*, 3209−3212.
(15) Kojima, H.; Sakurai, K.; Kikuchi, K.; Kawahara, S.; Kirino, Y.; Nagoshi, H.; Hirata, Y.; Akaike, T.; Maeda, H.; Nagano, T. *Biol. Pharm. Bull.* **1997**, *20*, 1229−1232.

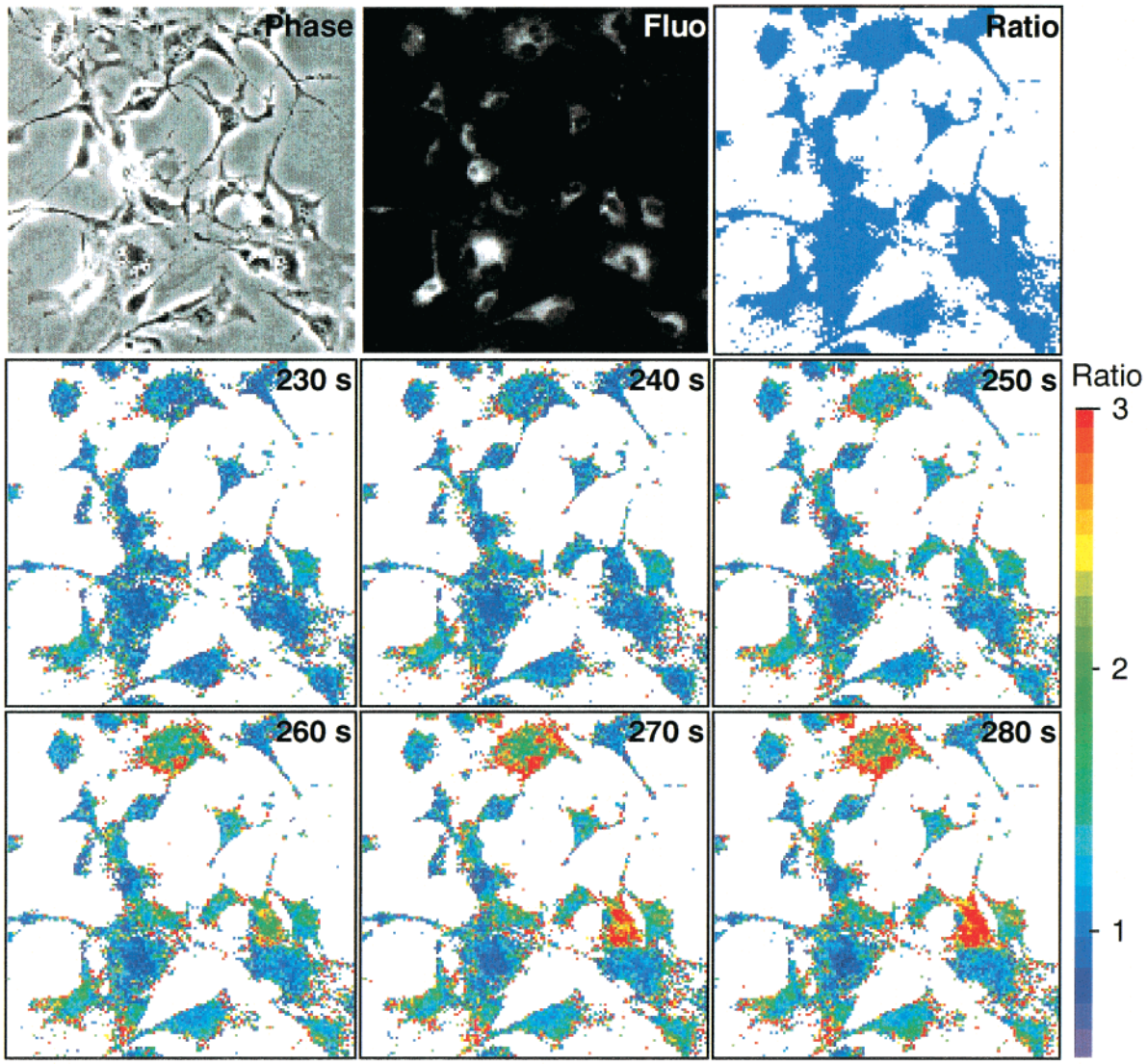

Figure 4. Bright-field and fluorescence images of cultured bovine aortic endothelial cells loaded with DAR-4M AM. The upper images are the bright-field, the fluorescence, and the fluorescence ratio images at the start of measurement, respectively. The middle and the lower images are fluorescence ratio images at the indicated times after the start of measurement. The fluorescence ratio images indicate the ratio of the intensity to the initial intensity at the start. However, areas where the fluorescence intensity was low were excluded and are shown in white. These images correspond to the fluorescence intensity data in Figure 5A.

at 37 °C for 30 min. After this incubation, they were postincubated with DMEM without the dye for 1 h to allow stabilization of the fluorescence intensity due to hydrolysis and distribution of the dye.

Our diamine indicators, before trapping NO, have weak fluorescence. When using DAFs, it is sometimes difficult to tell whether the cells are loaded with the dye, because autofluorescence of the cells overlaps the fluorescence of DAFs. On the other hand, we could easily distinguish the cells loaded with DAR-4M, because the unloaded cells had almost no fluorescence. Moreover, DARs are more photostable than DAFs, as we mentioned previously.[13]

The fluorescence image shows that the dye was successfully loaded into cells and was distributed to the cytosol, although it was hardly distributed to the nucleus, as shown in "Fluo" in Figure 4. The fluorescence changes are shown in pseudocolored pictures (Figure 4) and graphs (Figure 5). The fluorescence intensity of the cells is shown as the ratio of the intensity to the initial intensity

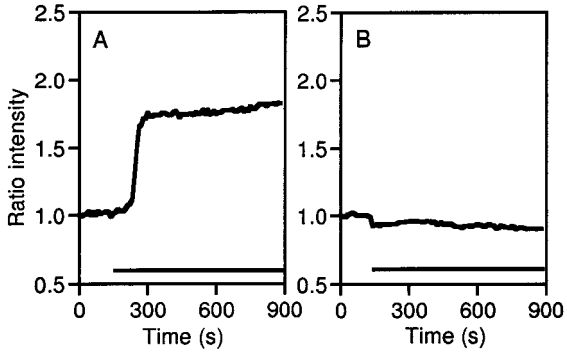

Figure 5. Fluorescence response of DAR-4M AM-loaded cultured bovine aortic endothelial cells, reflecting NO production in response to the addition of 1 μM bradykinin at 150 s. (A) The data corresponding to the images in Figure 4. (B) Control data in the presence of a NOS inhibitor, L-NAME (1 mM). The medium was switched to medium containing L-NAME just before measurement. The line shows mean values of the average fluorescence intensity in seven areas containing a cell.

Table 2. Comparison of DAF- and DAR-Type Indicators

	DAF	DAR
suitable excitation range	blue (~490 nm)	green (~550 nm)
applicable range of intracellular pH	above pH 6	above pH 4
detection limit of NO	3 nM (DAF−FM)	7 nM (DAR-4M)
photostability of the corresponding triazole [a]	severely photobleached (more than 72%)	not photobleached
distribution to nucleus and cytosol	equal	cytosol > nucleus

[a] Determined by measuring the fluorescence intensity after exposure to sunlight for 3 h.[13,14]

before stimulation for ease of comparison. A fluorescence increase in cells was observed after stimulation with 1 μM bradykinin, which raises the intracellular Ca^{2+} level and thereby activates NOS, and this increase was suppressed by addition of a NOS inhibitor (1 mM L-NAME; Figure 5). Therefore, this fluorescence increase was caused by NO production in the cells. The fluorescence intensity of all cells was increased, but it was observed that the NO productivity varied from cell to cell (Figure 4). This fine spatial resolution is one of the major advantages of our method. Thus, we succeeded in highly sensitive imaging of NO with a Rhodamine-type indicator. The characteristics of DAF and DAR are compared in Table 2. DAR-4M AM should be useful for bioimaging in samples that have strong autofluorescence in the case of 490-nm excitation or whose intracellular pH may fall below 6.

ACKNOWLEDGMENT

This work was supported by Grants-in-Aid for Scientific Research on Priority Areas, 09304061, 10169215, and 10557243, from the Ministry of Education, Science, Sport, and Culture of Japan.

Received for review September 22, 2000. Accepted February 18, 2001.

AC001136I