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Emission under Hypoxia: One-Electron Reduction and Fluorescence Characteristics of an Indolequinone–Coumarin Conjugate

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A characteristic feature of the reactivity of indolequinone derivatives, substituents of which can be removed by one-electron reduction under hypoxic conditions, was applied to the development of a new class of fluorescent probes for disease-relevant hypoxia. A reducing indolequinone parent molecule conjugated with fluorescent coumarin chromophores could suppress efficiently the fluorescence emission of the coumarin moieties by an intramolecular electron-transfer quenching mechanism and a conventional internal-filter effect. Under hypoxic conditions, however, the conjugate, denoted IQ-Cou, underwent a one-electron reduction triggered by X irradiation or the action of a reduction enzyme to release a fluorescent coumarin chromophore, whereupon an intense fluorescence emission with a maximum intensity at 420 nm was observed. The one-electron reduction of IQ-Cou was suppressed by molecular oxygen under aerobic conditions. IQ-Cou also showed intense fluorescence in a hypoxia-selective manner upon incubation with a cell lysate of the human fibrosarcoma cell line HT-1080. The IQ-Cou conjugate has several unique properties that are favorable for a fluorescent probe of hypoxia-specific imaging.

Introduction

Most cellular functions rely on the continuous and adequate supply of oxygen molecules from blood vessels. A stable oxygen supply is preserved in normal tissues by so-called oxygen homeostasis. An inadequate oxygen supply to cells induces hypoxia, which is one of the well-known pathophysiological characteristics of cardiac ischemia,[1] inflammatory diseases,[2] and solid tumors.[3] Tumor hypoxia is of particular importance, as it has been associated closely with the malignant phenotype of cancer cells, resistance to cancer therapies, and the low mortality rate of cancer patients.[3] Therefore, there has been increasing demand for hypoxia-specific molecular probes as useful indicators for the pathophysiological analysis of diseases.

We have developed prodrugs of the well-documented antitumor agents 5-fluorouracil (5-FU) and 5-fluorodeoxyuridine (5-FdUrd), which release 5-FU and 5-FdUrd, respectively, upon hypoxic X irradiation.[4] One 5-FdUrd prodrug with an indolequinone structure showed strong cytotoxicity against hypoxic tumor cells.[4a] Indolequinone derivatives have been identified by other research groups as a new class of hypoxia-specific prodrugs that can be activated to eliminate cytotoxic substituent components (active drugs) selectively by bioreduction or radiolytic reduction under hypoxic conditions.[5] These findings prompted us to investigate the development of hypoxia-imaging molecular probes containing a reducing indolequinone skeleton. Two coumarin chromophores were thus conjugated with an indolequinone unit through a 2,6-bis(hydroxymethyl)-*p*-cresol linker[6] to produce IQ-Cou, the indolequinone unit of which undergoes one-electron reduction to liberate three functional components through the spontaneous cyclization of a free-amine intermediate and the rearrangement of the resulting phenol derivative to the corresponding 1,4-quinone methide (Scheme 1). IQ-Cou itself showed weak fluorescence, because the fluorescent excited singlet state of the coumarin unit is quenched efficiently by the indolequinone unit located intramolecularly in close proximity. Upon the one-electron reduction of the indolequinone unit, the coumarin chromophore was eliminated and no longer affected by the fluorescence-quenching action of the indolequinone unit; thus, an intense fluorescence emission was observed. With these reaction characteristics, IQ-Cou might be applicable as a fluorescent probe for the molecular imaging of disease-relevant hypoxia.

Results and Discussion

The synthetic route to IQ-Cou is outlined in Scheme 2. The diol **3** was prepared by a previously described method[6] and cou-

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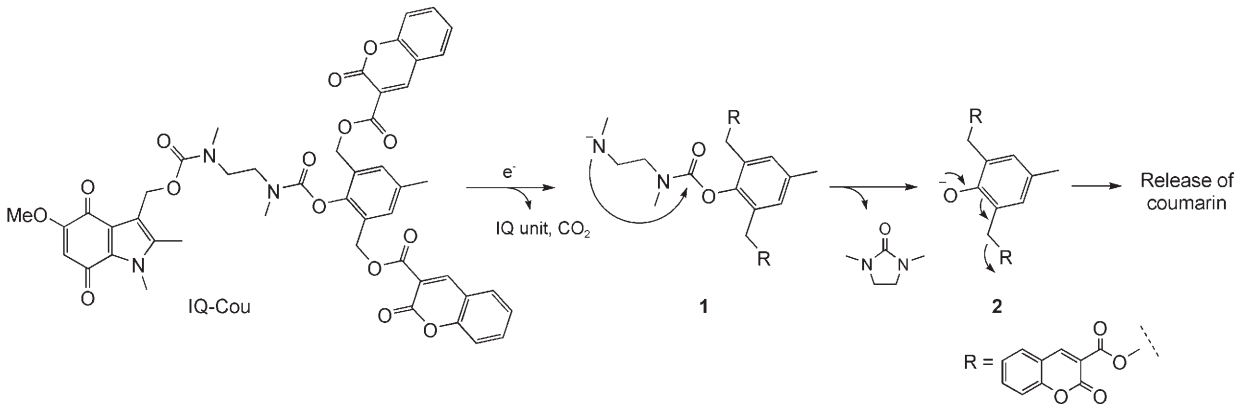
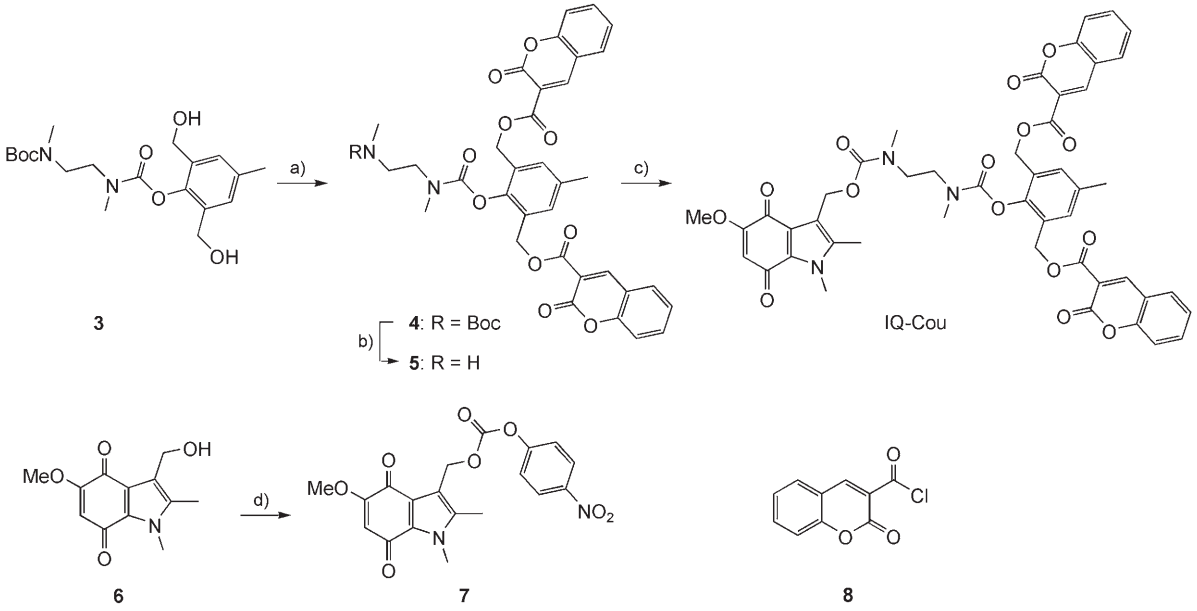


Scheme 1. Mechanism for the activation of IQ-Cou to release a fluorescent coumarin chromophore under reductive conditions.

pled with the acid chloride **8** to form **4**. The diester **4** was converted into the free amine under acidic conditions and then coupled with the indolequinone derivative **7** (prepared from the alcohol **6**[7]) to give IQ-Cou.

We first compared the fluorescence spectrum of IQ-Cou with that of the reference compound coumarin-3-carboxylic acid (Figure 1 A). Whereas coumarin-3-carboxylic acid showed an intense fluorescence emission at about 420 nm upon excitation at a wavelength of 300 nm, the apparent fluorescence intensity of IQ-Cou was extremely weak: The formal fluorescence quantum yields (Φ_F) of coumarin-3-carboxylic acid and IQ-Cou were 0.042 and 0.002, respectively. These results indicate that the fluorescence of the coumarin chromophore in IQ-Cou is quenched intramolecularly by the neighboring indolequinone unit and predict that fluorescence emission will be restored upon the one-electron reduction of IQ-Cou to release a coumarin-3-carboxylic acid fragment.

The suppressed fluorescence emission of IQ-Cou is attributable to two modes of action of the indolequinone unit. First, a conventional internal filter effect due to the presence of the indolequinone unit could lead to a decrease in the apparent fluorescence quantum yield of the counterpart coumarin chromophore by about a third in view of the similarity of the molar extinction coefficients at 300 nm of coumarin-3-carboxylic acid and the indolequinone **6** ($\varepsilon = 6206$ and $5863\ M^{-1}cm^{-1}$, respectively).[8] Second, a photoinduced electron-transfer (PET) process[9] should be operative as a key mechanism for modulating the fluorescence properties of IQ-Cou. The PET that leads to fluorescence quenching can occur between the excited states of an electron-withdrawing (electron-donating) fluorophore



Scheme 2. Reagents and conditions: a) **8**, Et_3N, CH_2Cl_2, room temperature, 62%; b) HCl, MeOH, room temperature; c) **7**, Et_3N, CH_2Cl_2, room temperature, 39% (two steps); d) 4-nitrophenyl chloroformate, Et_3N, CH_2Cl_2, 0 °C, 37%. Boc = *tert*-butoxycarbonyl.

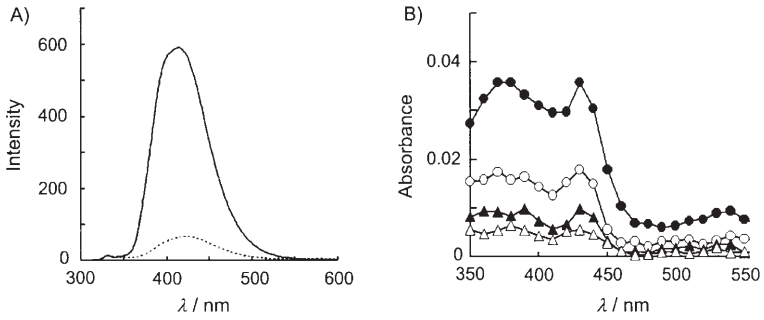


Figure 1. A) Fluorescence spectra of IQ-Cou (10 μM; ——) and coumarin-3-carboxylic acid (10 μM; ····) in acetonitrile. The fluorescence spectra were measured with excitation at 300 nm. B) Transient absorption spectra observed upon the excitation at 355 nm of IQ-Cou (30 μM) in acetonitrile; 1 (●), 10 (○), 20 (▲), and 30 μs (△) after laser flash photolysis.

coumarin-3-carboxylic acid increased with increasing irradiation dose upon hypoxic X irradiation of IQ-Cou. The fluorescence after 720 Gy of X irradiation was three times as intense as that of IQ-Cou in the absence of irradiation (Figure 2 A). These results indicate clearly that IQ-Cou is activated to release coumarin-3-carboxylic acid from the indolequinone quencher by radiolytic one-electron reduction by e_{aq}^- with the restoration of fluorescence.

A smaller enhancement in fluorescence emission occurred upon aerobic irradiation: The sample solution showed only a 1.5-fold increase in fluorescence intensity upon exposure to 720 Gy of X irradiation (Figure 2 B). This oxygen effect on the activation of

and an electron-donating (electron-withdrawing) quencher. The feasibility of the process depends on the relative ordering of the energy levels of the highest occupied molecular orbitals (HOMO) and the lowest unoccupied molecular orbitals (LUMO) of the fluorophore and the quencher.[10] The MO energy levels of 3-methoxycarbonylcoumarin and the indolequinone **6** were estimated by ab initio calculations at the B3LYP/6-31G(d) level, which revealed that the LUMO energy level of **6** (−2.94 eV) is lower than that of coumarin (−2.25 eV), whereas the HOMO energy level of **6** (−6.15 eV) is higher than that of coumarin (−6.77 eV). These calculation results suggest strongly that an intramolecular electron transfer from coumarin in the excited state to the indolequinone unit in the ground state is feasible thermodynamically in IQ-Cou.

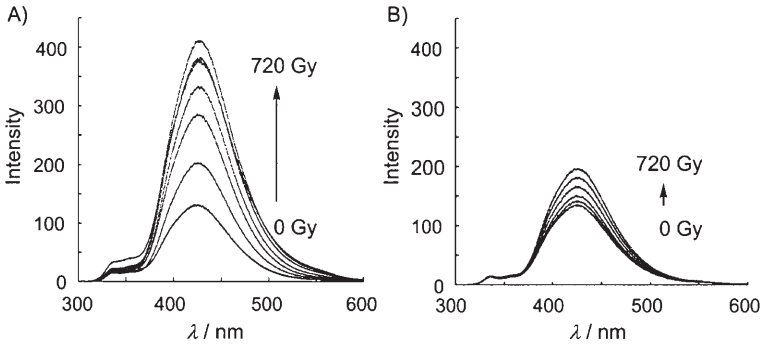


Figure 2. Changes in the fluorescence spectrum of IQ-Cou upon X irradiation. IQ-Cou (100 μM) was irradiated, and then the fluorescence spectra were measured with excitation at 300 nm. A) Fluorescence spectra observed after hypoxic irradiation of IQ-Cou (from bottom to top: 0, 72, 144, 288, 528, 720 Gy). B) Fluorescence spectra observed after aerobic irradiation of IQ-Cou (from bottom to top: 0, 72, 144, 288, 528, 720 Gy).

We also carried out laser flash photolysis studies to gain further insight into the suppressed fluorescence emission of IQ-Cou. Figure 1 B shows the transient absorption spectra observed in the laser flash photolysis at 355 nm of IQ-Cou in acetonitrile. The transient absorption appeared 1 μs after the laser flash. An absorption in the region 360–390 nm was assigned to the semiquinone radical anion.[4a,5d] We also observed a transient absorption at 410–440 nm that may be assigned to the coumarin radical cation.[11] This absorption decayed in a similar way to that of the semiquinone radical anion. These results indicate that the photolysis of IQ-Cou induces electron transfer from the excited coumarin unit to the indolequinone unit. Thus, we concluded that indolequinone could suppress efficiently the fluorescence emission of coumarin by an intramolecular electron-transfer mechanism.

We measured the changes in the fluorescence spectrum of IQ-Cou upon X irradiation. An argon-purged, aqueous solution of IQ-Cou in the presence of excess 2-methylpropan-2-ol was used for these experiments. Under these conditions, IQ-Cou undergoes one-electron reduction, whereby the indolequinone unit, with a lower LUMO energy level, may capture reducing hydrated electrons (e_{aq}^-) generated as a primary intermediate of water radiolysis.[12,13] As shown representatively in Figure 2, the intensity of the fluorescence at about 420 nm assigned to IQ-Cou can be accounted for by the reactivity of molecular oxygen, which captures efficiently reducing e_{aq}^- species to form a superoxide anion radical ($O_2^{\bullet-}$). Furthermore, recent studies on the reductive activation of indolequinone prodrugs suggest that a semiquinone anion radical intermediate generated by the one-electron reduction of the indolequinone unit could reduce molecular oxygen to form the original indolequinone and $O_2^{\bullet-}$, a process that would lead to a decrease in the net yield of the released drug.[4a] Because of the reaction characteristics of molecular oxygen, the described radiolytic activation of IQ-Cou to enhance the fluorescence emission is likely to occur substantially under hypoxic conditions.

We also monitored the course of the radiolytic reduction of IQ-Cou and release of coumarin-3-carboxylic acid with time by a reversed-phase HPLC (Figure 3). Upon the hypoxic X irradiation of an argon-purged, aqueous solution of IQ-Cou in the presence of excess 2-methylpropan-2-ol, the concentrations of decomposed IQ-Cou and released coumarin-3-carboxylic acid increased with increasing radiation dose. *G* values[13] of 218.9 and 15.2 nmol J^{-1} were found for the decomposition of IQ-Cou and the release of coumarin, respectively.[14] Consistent with the smaller change in fluorescence intensity in the aerobic irradiation of IQ-Cou, the decomposition of IQ-Cou was suppressed to a *G* value of 16.5 nmol J^{-1} and the release of cou-

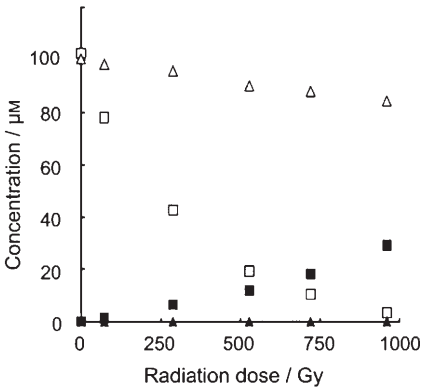


Figure 3. X-Radiolysis (4.0 Gy min^{-1}) of IQ-Cou (100 μM; open symbols) in aqueous solution containing 30% 2-methylpropan-2-ol at ambient temperature under hypoxic (square) and aerobic (triangle) conditions, with the release of coumarin-3-carboxylic acid (filled symbols).

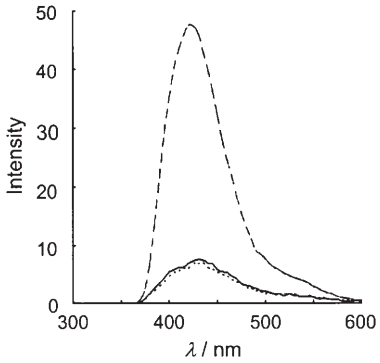


Figure 4. Fluorescence spectra of IQ-Cou after treatment with reductase. IQ-Cou (500 μM) was incubated with NADPH:cytochrome P450 reductase (10.6 μg mL^{-1}) and β-NADPH (2 mM) at 37 °C for 45 min at three different oxygen concentrations (< 0.5 mg L^{-1}: -----; 8.2 mg mL^{-1}: ——; > 20 mg mL^{-1}: ····).[16] The fluorescence spectra were measured with excitation at 300 nm.

marin-3-carboxylic acid was not detected under aerobic conditions. Thus, the concentration change of coumarin-3-carboxylic acid upon irradiation correlates well with the change in fluorescence intensity.

To characterize the biological one-electron reduction of IQ-Cou, we subjected IQ-Cou to enzymatic reduction. NADPH:cytochrome P450 reductase is an electron-donating protein that catalyzes the one-electron reduction of quinone derivatives to semiquinone anion radicals. Evidence that NADPH:cytochrome P450 reductase is expressed in many pathological tissues[15] stimulated us to carry out the bioreduction of IQ-Cou. We incubated IQ-Cou with NADPH:cytochrome P450 reductase and the cofactor β-NADPH at three different oxygen concentrations (<0.5, 8.2, and > 20 mg L^{-1}). An intense fluorescence emission was observed for the solution of IQ-Cou incubated with < 0.5 mg L^{-1} oxygen as a model of hypoxia (Figure 4). At an oxygen concentration of 8.2 mg L^{-1}, as under aerobic conditions, the extent of enhanced fluorescence intensity diminished significantly as a result of the competitive scavenging by molecular oxygen of hydrated electrons, e_{aq}^-, and the semiquinone radical to inhibit partially the reductive fragmentation of IQ-Cou. Thus, IQ-Cou was activated by NADPH:cytochrome P450 reductase in a hypoxia-selective manner, in accord with the results of radiolytic reduction. We also confirmed the hypoxia-selective release of coumarin-3-carboxylic acid from IQ-Cou upon treatment with NADPH:cytochrome P450 reductase, as monitored by HPLC (see the Supporting Information).

To further assess the function of IQ-Cou, we studied the reaction of IQ-Cou upon treatment with a cell lysate. IQ-Cou was incubated at 37 °C for 4 h under hypoxic and aerobic conditions with a lysate of the human fibrosarcoma cell line HT-1080. After incubation, the samples were filtered and analyzed by fluorescence spectrophotometry and HPLC. An intense fluorescence emission at about 420 nm was observed for the sample incubated under hypoxic conditions (Figure 5A). The intensity of the emission was four times as strong as that of the sample incubated under aerobic conditions. The difference in the fluorescence intensity of the two samples correlates well with the corresponding yields of coumarin-3-carboxylic acid from IQ-Cou, as quantified independently by HPLC (Figure 5B). We also confirmed that the incubation of IQ-Cou in a buffer solution resulted in a similar fluorescence intensity to that observed for the sample incubated aerobically. These results indicate strongly that IQ-Cou undergoes one-electron reduction by intracellular reductase to release fluorescent coumarin-3-carboxylic acid under hypoxic conditions.

In this study, we developed a new type of hypoxia-specific fluorescence imaging probe. IQ-Cou is activated efficiently by intracellular reductase under hypoxic conditions. Although low expression of NADPH:cytochrome P450 reductase was reported for the HT-1080 cell line,[17] an intense fluorescence emission was observed for IQ-Cou even upon treatment with HT-1080 cell lysate under hypoxic conditions. IQ-Cou may therefore be expected to show more intense fluorescence emission when incorporated in certain hypoxic tumor cells with higher reductase expression. In view of the hypoxia-specific fluorescence emission of IQ-Cou, the one-electron reduction of indolequinone derivatives could be a promising strategy for the detection of disease-relevant hypoxia. However, IQ-Cou has some drawbacks: First, an inner-filter effect due to the overlapping of the absorption of indolequinone with that of coumarin leads to the apparent suppression of fluorescence emission. Therefore, fluorescent molecules that absorb in a wavelength region that does not coincide with the absorption of indolequinone should be employed to establish a highly sensitive hypoxia-specific fluorescence imaging probe. Second, the fluorescence spectrum of coumarin-3-carboxylic acid occurs at around 420 nm, that is, at wavelengths that are too short to be applied to the imaging of deep-seated malignant tissues. One of the key strategies for in vivo optical imaging is the employment of near-infrared (NIR) light, because hemoglobin, as a principle absorber of visible light, and water and lipids, as principle absorbers of infrared light, have their lowest absorption in the NIR region, at around 650–900 nm.[18] Moreover, tissue autofluorescence observed in the NIR region has a minimum intensity. From this point of view, it is essential to apply dyes with fluorescence at NIR wavelengths to the present strategy with indolequinone derivatives.

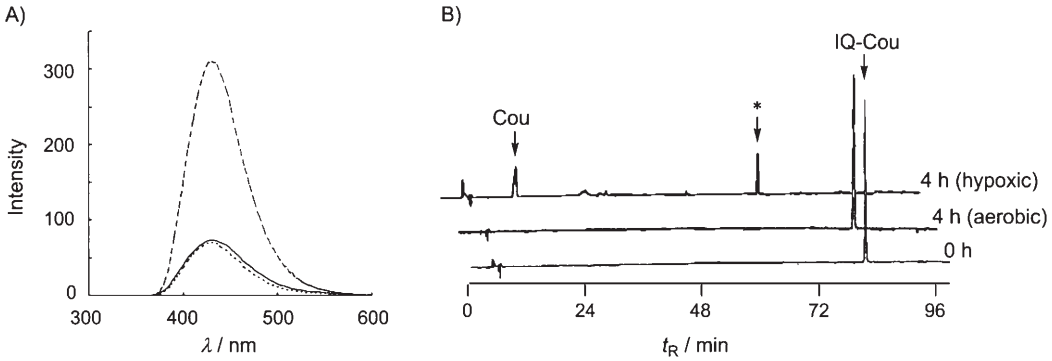


Figure 5. Treatment of IQ-Cou with a cell lysate obtained from the human fibrosarcoma cell line HT-1080. IQ-Cou (500 μM) was incubated with the cell lysate for 4 h at 37 °C under hypoxic or aerobic conditions. A) The fluorescence spectra[16] of IQ-Cou upon treatment with the cell lysate under hypoxic (-----) or aerobic conditions (——). IQ-Cou was also incubated alone in a buffer solution as a control (····). The fluorescence spectra were measured with excitation at 300 nm. B) HPLC profiles of IQ-Cou (500 μM) upon treatment with the cell lysate for 0 and 4 h under hypoxic or aerobic conditions. The HPLC signal indicated "Cou" was identified as coumarin-3-carboxylic acid. The HPLC signal indicated with the symbol "*" possibly corresponds to a reaction intermediate; however, the compound could not be identified because of its low stability and prompt degradation.

Conclusions

In summary, we have characterized the reactivity by one-electron reduction of IQ-Cou, which was synthesized as a hypoxia-specific fluorescence probe. IQ-Cou consists of a hypoxia-sensitive oxidizing indolequinone parent unit, two fluorescent coumarin chromophores, and a 2,6-bis(hydroxymethyl)-*p*-cresol linker. Both radiolytic and enzymatic one-electron reduction under hypoxic conditions lead to the efficient decomposition of IQ-Cou, with the release of coumarin-3-carboxylic acid accompanied by intense fluorescence. A similar enhancement in fluorescence emission was also observed in a hypoxia-selective manner upon the incubation of IQ-Cou with an HT-1080 cell lysate. Thus, it appears that IQ-Cou can be activated by intracellular reductase. Although IQ-Cou is a promising candidate as a hypoxia-specific fluorescence imaging tool, the absorption and emission of the coumarin fluorophore at relatively short wavelengths is a disadvantage. Furthermore, it is necessary to characterize the reactivity of the probe with potentially reactive species in living cells in detail to establish a molecular system for hypoxia imaging. Our current studies focus on the construction of a hypoxia-specific imaging probe that is sensitive to NIR light and thereby applicable to in vivo optical imaging.

Experimental Section

General methods: NMR spectra were recorded on a 270-MHz JMN-AL-270 (JEOL), 300-MHz JMN-AL-300 (JOEL), or 400-MHz JMN-AL-400 (JOEL) spectrophotometer at ambient temperature. Chemical shifts are reported in ppm relative to the residual solvent peak. Mass spectra were recorded on a JMS-SX102 A (JOEL) mass spectrometer, with a glycerol or *m*-nitrobenzylalcohol (NBA) matrix as an internal standard. All reactions were carried out under a dry nitrogen atmosphere with freshly distilled solvents, unless otherwise noted. Reagents were purchased from Aldrich, Wako Pure Chemical Industries, or Nacalai Tesque and used without purification. NADPH:cytochrome P450 reductase and β-NADPH coenzyme were obtained from Oxford Biomedical Research and Oriental Yeast Co., respectively. Tetrahydrofuran was distilled under a nitrogen atmosphere from sodium/benzophenone ketyl immediately prior to use. Ultrapure water was obtained from a Yamato WR-600 A water purifier. Precoated TLC (Merck silica gel 60 F_{254}) plates were used for monitoring the reactions. Wako gel (C-300, Wako Pure Chemical Industries) was used for column chromatography. A Rigaku Radioflex-350 X-ray generator was employed for X radiolysis at ambient temperature. High-performance liquid chromatography (HPLC) was carried out with a Shimadzu HPLC system (SPD-10A UV/Vis detector, CT0-10A column oven, two LC-10AS pumps, C-R6A chromatopac). Sample solutions were injected onto a reversed-phase column (Inertsil ODS-3, GL Science Inc., ø 4.6×150 nm). The following solvent program was used: of 88.9% B (20 min) followed by 88.9–16.7% B (a linear gradient over 70 min; solution A: 95% acetonitrile; solution B: 5% acetonitrile containing 0.1 M triethylammonium acetate (TEAA) buffer, pH 5). Fluorescence spectra were recorded with excitation at 300 nm on a Shimadzu RF-5300PC spectrofluorophotometer at ambient temperature, whereby a UV-33 glass filter (Toshiba Glass Co.) was set on the detector side to cut off the excitation peak at 300 nm. The amount of dissolved oxygen (DO) was measured with an OM-51 DO meter (Horiba) at 37 °C. Compound **7** was prepared from compound **6** as reported elsewhere.[7] The human fibrosarcoma cell line, HT-1080, was purchased from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco Modified Eagle Medium (Invitrogen) containing fetal bovine serum (10%) in a humidified incubator with 5% CO_2, 95% air at 37 °C.

Preparation of compound 4: Coumarin-3-carboxylic acid (510 mg, 2.68 mmol) was dissolved in thionyl chloride (5 mL), and the resulting mixture was stirred at 100 °C for 2.5 h. The mixture was then concentrated by evaporation to give the acid chloride **8**, which was used immediately, without purification, in the next step.

The crude acid chloride **8** was added as a solution in dichloromethane (2 mL) and Et_3N (1 mL) to a solution of **3** (254 mg, 0.66 mmol) in dichloromethane (3 mL), and the resulting mixture was stirred at 0 °C for 15 min, and then at ambient temperature for 3 h. The reaction mixture was then diluted with saturated aqueous sodium hydrogen carbonate and extracted with chloroform. The organic layer was washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (SiO_2, 33% hexane/ethyl acetate) to give **4** (300 mg, 62%) as a brown oil. ^{1}H NMR (300 MHz, $CDCl_3$; as a result of restricted rotation about the amide bond adja-

cent to the linker, two signals were observed for some atoms): $\delta =$ 8.49 and 8.46 (s, 2H), 7.57–7.49 (m, 4H), 7.29–7.17 (m, 6H), 5.22 (s, 4H), 3.54–3.23 (m, 4H), 3.08, 2.83, and 2.67 (s, 6H), 2.28 (s, 3H), 1.32 and 1.29 ppm (s, 9H); ^{13}C NMR (68 MHz, $CDCl_3$; as a result of restricted rotation about the amide bond adjacent to the linker, two signals were observed for some atoms): $\delta =$ 162.0, 156.3, 154.9, 154.0, 149.0, 148.9, 145.9, 136.0, 135.9, 134.3, 131.7, 131.4, 129.5, 128.4, 124.7, 117.6, 117.4, 117.3, 116.5, 79.6, 62.6, 62.5, 47.5, 47.1, 46.3, 35.5, 35.3, 34.9, 29.6, 28.4, 20.9, 14.2 ppm; FABMS (NBA/$CHCl_3$): *m/z* 727 $[M+H]^+$; HRMS: *m/z* calcd for $C_{39}H_{39}N_2O_{12}$: 727.2498 $[M+H]^+$; found: 727.2505.

Preparation of IQ-Cou: Compound **4** (155 mg, 0.21 mmol) was added to 0.5 M HCl/MeOH (2 mL), and the mixture was stirred for 14 h at ambient temperature. The solvent was then removed under reduced pressure to give **5** (181 mg) as a colorless oil. The crude product **5** was dissolved in *N*,*N*-dimethylformamide (DMF; 1 mL).

The indolequinone derivative **7** (177 mg, 0.44 mmol) in DMF (1 mL) was added to the solution of **5** in DMF, and the resulting mixture was stirred at ambient temperature for 8 h. The mixture was then diluted with saturated aqueous sodium hydrogen carbonate and extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (SiO_2, from 16.7% ethyl acetate/hexane to 100% ethyl acetate) to give IQ-Cou (72.1 mg, 39%) as an orange oil. ^{1}H NMR (300 MHz, $CDCl_3$, room temperature; as a result of restricted rotation about the amide bonds, two signals were observed for some atoms): $\delta =$ 8.55 and 8.51 (s, 2H), 7.58–7.53 (m, 4H), 7.31–7.20 (m, 6H), 5.52–5.39 (m, 1H), 5.24–5.04 (m, 6H), 3.83–3.66 (m, 6H), 3.58–2.74 (m, 10H), 2.31 (s, 3H), 2.24 and 2.19 ppm (s, 3H); ^{1}H NMR (400 MHz, [D_6]DMSO, 120 °C): $\delta =$ 8.62 (s, 2H), 7.83 (d, $J =$ 0.02 Hz, 2H), 7.72 (t, $J =$ 0.02 Hz, 2H), 7.38 (t, $J =$ 0.02 Hz, 6H), 5.61 (s, 1H), 5.27–5.13 (br, 4H), 5.11 (s, 2H), 3.74 (s, 3H), 3.71 (s, 3H), 3.48–3.33 (m, 5H), 2.92–2.69 (m, 5H), 2.36 (s, 3H), 2.19 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$, room temperature; as a result of restricted rotation about the amide bonds, two signals were observed for some atoms): $\delta =$ 178.4, 177.0, 162.1, 159.3, 156.4, 155.0, 154.1, 149.3, 149.0, 145.7, 138.1, 136.0, 134.3, 131.4, 129.6, 129.0, 128.5, 124.7, 121.6, 117.77, 117.75, 117.3, 116.6, 106.5, 62.5, 57.5, 56.3, 47.2, 46.7, 35.7, 35.3, 35.0, 32.3, 21.0, 9.6, 9.4 ppm; ^{13}C NMR (100 MHz, [D_6]DMSO, 120 °C): $\delta =$ 177.3, 176.3, 163.0, 161.5, 158.9, 155.1, 154.2, 152.8, 147.8, 145.1, 137,5, 134.6, 133.8, 129.8, 129.5, 128.3, 127.8, 124.2, 120.6, 117.2, 117.1, 115.6, 115.1, 106.2, 61.4, 56.7, 55.8, 46.3, 45.8, 34.2, 33.9, 31.3, 19.8, 8.3 ppm; FABMS (NBA/$CHCl_3$): *m/z* 888 $[M+H]^+$; HRMS: *m/z* calcd for $C_{47}H_{42}N_3O_{15}$: 888.2610 $[M+H]^+$; found: 888.2604.

Measurement of extinction coefficients: The extinction coefficients (ε) of compound **6** and coumarin-3-carboxylic acid were calculated according to the Beer–Lambert law. UV spectra of the samples in acetonitrile (final concentration: 120 μM) were recorded with a Jasco V-530 UV/Vis spectrometer.

Fluorescence spectrophotometry: Fluorescence spectra of IQ-Cou and coumarin-3-carboxylic acid in acetonitrile (final concentration: 10 μM) were recorded with a Shimadzu RF-5300PC fluorescence spectrophotometer with excitation at 300 nm.

Measurement of fluorescence quantum yield: The fluorescence quantum yield (Φ_F) was determined by using Coumarin 153, with a known Φ_F value of 0.42 in methanol, as a reference. The area of the emission spectrum was integrated by using instrumentation software, and the quantum yield was calculated according to Equation (1), in which $\Phi_{F(S)}$ and $\Phi_{F(R)}$ are the fluorescence quantum yields of the sample and the reference, respectively, the terms $A_{(S)}$ and $A_{(R)}$ refer to the area under the fluorescence spectra, $(Abs)_{(R)}$ and $(Abs)_{(S)}$ are the optical densities of the sample and reference solutions at the excitation wavelength, and $n_{(S)}$ and $n_{(R)}$ are the refractive indices of the solvents used for the sample and the reference.

$$\Phi_{F(S)}/\Phi_{F(R)} = \frac{A_{(S)}}{A_{(R)}}\frac{(Abs)_{(R)}}{(Abs)_{(S)}}\frac{{n_{(S)}}^2}{{n_{(R)}}^2} \quad (1)$$

Laser flash photolysis: Laser flash photolysis was carried out with a Unisoku TSP-601 flash spectrometer. A continuum Surelite-I Nd-YAG (Q-switched) laser with the fourth harmonic at 355 nm was employed for flash photoirradiation. The probe beam from a Hamamatsu 150-W xenon short-arc lamp was guided with an optical fiber scope to a perpendicular orientation with respect to the excitation laser beam. The probe beam was monitored with a Unisoku MD200 photomultiplier tube through a Hamamatsu DG535 image-intensifier controller (1024 photodiodes). The timing of the excitation pulse laser, the probe beam, and the detection system was achieved through a Tektronix model TDS 3012 digital phosphor oscilloscope interfaced to an IBM Windows XP computer. A solution of IQ-Cou (30 μM) in acetonitrile was deaerated by bubbling argon through it prior to laser flash photolysis.

Radiolytic reduction: To establish hypoxia, an aqueous solution of IQ-Cou (100 μM) containing 2-methyl-2-propanol (30%) was purged with argon for 30 min and then irradiated in a sealed glass ampoule at ambient temperature with an X-ray source (4.0 Gy min^{-1}). After irradiation, aliquots were taken at appropriate time intervals for fluorescence spectrophotometry, and then diluted by 33% with Milli-Q water for analytical HPLC. A control air-saturated sample solution was irradiated and analyzed in a similar manner.

Bioreduction by NADPH–P450 reductase: To establish hypoxia, a solution of NADPH:cytochrome P450 reductase (final concentration: 10.6 μg mL^{-1}) and β-NADPH (final concentration: 2 mM) in phosphate buffer (25 mM, pH 7.4) was purged with argon for 10 min at 37 °C. IQ-Cou (final concentration: 500 μM) was added to the resulting solution, which was then incubated at 37 °C. For analytical HPLC, aliquots were taken at appropriate time intervals and diluted by 10% with Milli-Q water/acetonitrile (1:1). The reaction mixture was extracted with ethyl acetate to remove β-NADPH coenzyme, and florescence spectra of the organic layer were measured with excitation at 300 nm. A control aerobic sample solution was irradiated and analyzed in a similar manner.

Bioreductive activation of IQ-Cou by HT-1080 cell lysate: HT-1080 cells were cultured in six dishes (90% confluent in dishes of 100 mm in diameter) and washed twice with ice-cold phosphate-buffered saline. The cell lysate was then harvested with ice-cold CelLytic M cell lysis reagent (2 mL; Sigma–Aldrich), maintained at ambient temperature for 15 min, and centrifuged at 14000 rpm for 5 min to remove the cell debris. The resulting supernatant was kept in a Bactron II anaerobic environmental chamber (Sheldon Manufacturing, Cornelius, OR; 94% N_2, 5% CO_2, 1% H_2) at 37 °C for 22 h for hypoxic treatment. IQ-Cou (final concentration: 500 μM) was added to the hypoxic lysate, which was then incubated at 37 °C for 4 h. Aliquots were taken at appropriate time intervals, diluted by 10% with a 1:1 mixture of Milli-Q water and acetonitrile, and then diluted by 50% with acetonitrile. All solutions were filtered with a Cosmonice Filter S (Nacalai Tesque, Kyoto, Japan) for analytical HPLC and fluorescence spectrophotometry. Normoxic

lysate, which was kept in a well-oxygenated incubator (95% air, 5% CO_2, 37 °C), and lysis buffer alone were mixed in a similar way with IQ-Cou and analyzed as negative controls.

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Keywords: enzymes · fluorescent probes · hypoxia · indolequinones · one-electron reduction

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