Synthesis, Chemi- and Bioluminescence Properties, and Photolysis of a Coelenterazine Analogue Having a Photoreactive Azido Group

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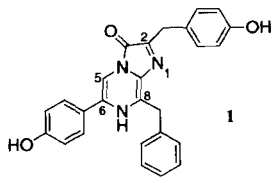
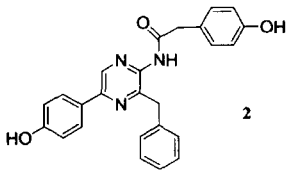
(Received September 6, 1999)

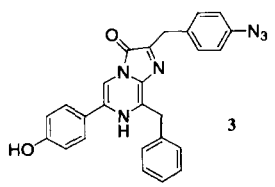
A photoreactive analogue of coelenterazine having an azido group was synthesized. The analogue showed similar chemi- and bioluminescence properties to those of the natural coelenterazine. Photolysis of the analogue in the presence of diethylamine gave a derivative azepine as the major product.

Aequorin (AQ) is a Ca^{2+}-binding photoprotein found in the margin of the umbrella of the jellyfish, *Aequorea victoria*. AQ is a molecular complex composed of coelenterazine (**1**) (organic substrate), molecular oxygen, and an apoprotein, apoaequorin (apoAQ), which is made up of 189 amino-acid residues in a single polypeptide chain.[1—3] Upon binding Ca^{2+}, an intramolecular reaction takes place in which AQ is converted into a luciferase, which then catalyzes the oxidation of coelenterazine (**1**) into coelenteramide (**2**) by the bound oxygen (Chart 1), yielding light (λ_{max} = 470 nm), CO_2, and a blue fluorescent protein (BFP). BFP consists of coelenteramide (**2**) bound to apoAQ. The excited-state of coelenteramide (**2**) is the light emitter in the AQ bioluminescence reaction.[4—6] AQ and BFP can be regenerated by incubating apoAQ with coelenterazine (**1**) and coelenteramide (**2**) respectively, under appropriate conditions (Scheme 1).[6,7]

Recent progress on the over expression[8] of apoAQ cDNA in *Escherichia coli* has made recombinant apoAQ possible to obtain in quantities. This extends the applications of AQ in biological systems,[9] and also makes it much easier to study the bioluminescence mechanism of AQ. To date, numerous studies have been carried out to clarify the bioluminescence mechanism by means of site-specific mutagenesis and semi-synthetic AQ techniques.[10—12] However, details for the active site and the binding mode of **1** in AQ remain unclear.[4,13,14] A promising method for clarifying the active site is photoaffinity labeling[15,16] by using a coelenterazine analogue with a photolabile group. Recently, we reported on the synthesis, photolysis, and chemi- and bioluminescence studies of a photoreactive coelenterazine analogue with a carbene precursor trifluoromethyldiazirine group.[17] A photolabile coelenterazine analogue **3** with an azido group can be another candidate for clarifying the active site of AQ. Photolysis of the aryl azide moiety in **3** can yield the corresponding aryl azepine functionality through a well-known process (Eq. 1).[16] Thus, cross-linking is expected to occur at a different nucleophilic site from the one labeled with the trifluoromethyl carbene. Herein we report on the synthesis, photochemical reaction, and chemi- and bioluminescence properties of **3**.







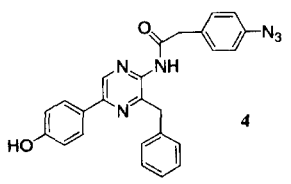
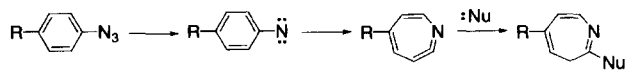


Chart 1.



(1)

Results and Discussion

Synthesis of Azido Coelenterazine Analogue. The synthesis of **3** is shown in Scheme 2. *p*-Azidophenylacetic acid (**6**)[18] was converted into the acid chloride **7** with thionyl chloride. The treatment of **7** with diazomethane gave the diazoketone **8** (88% from **6**). The reaction of **8** with HBr provided the bromo ketone **9** (90%). The treatment of **9** with silver nitrate afforded the nitrate **10** (86%). The treatment of **10** with sodium acetate trihydrate yielded the oxo aldehyde

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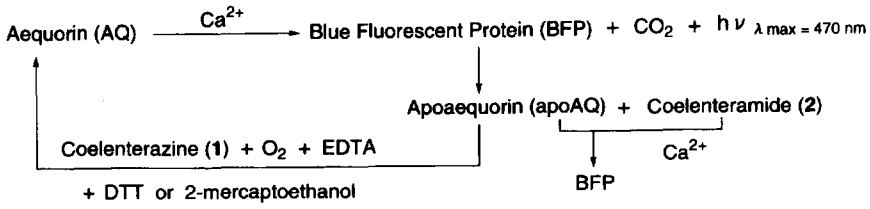
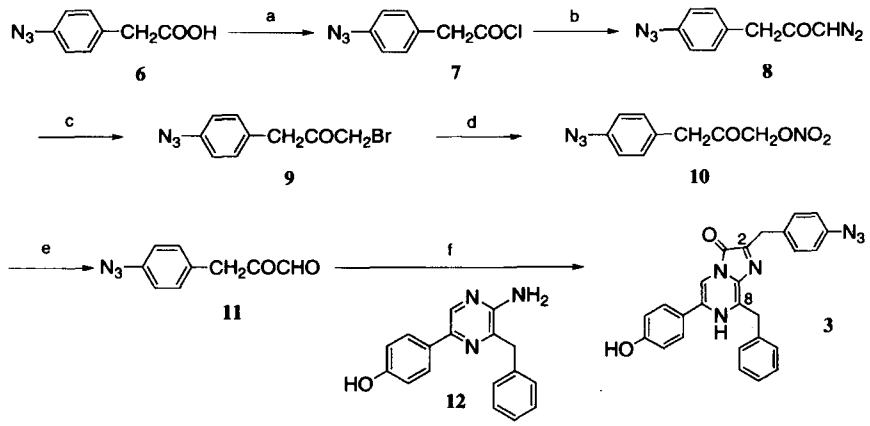


Scheme 1.



Scheme 2. Synthesis of the azido coelenterazine analogue **3**. Reagents and conditions: (a) $SOCl_2$, benzene, room temp; (b) CH_2N_2, ether, 0 °C; (c) HBr, ether, 0 °C; (d) $AgNO_3$, MeCN, room temp; (e) $NaOAc{\cdot}3H_2O$, DMSO, room temp; (f) aqueous HCl–EtOH, 55 °C.

11 quantitatively. Coupling **11** with coelenteramine (**12**)[19] in ethanol containing aq HCl at 55 °C furnished **3** (55%).

The azido coelenteramide analogue **4** was prepared by the acylation of coelenteramine (**12**) with the acid chloride **7**, followed by selective hydrolysis of the resulting phenolic ester group.

Chemiluminescence. The chemiluminescence of **3** was studied in order to examine the effect of the azido group on the luminescence behavior. The chemiluminescence of **3** was triggered upon the addition of DMSO under air. The chemiluminescence maximum (CL_{max}) of **3** and the fluorescence maximum (FL_{max}) of the spent solution after the chemiluminescence reaction of **3** are shown in Table 1. The fluorescence maximum (FL_{max}) of the spent solution coincided with that of the synthesized **4** (Table 1). This behavior of **3** is similar to that of native coelenterazine (**1**),[20] indicating that the chemiluminescence of **3** in DMSO with CL_{max} around 480 nm is due to the excited state of the amide anion of **4**, and that the fluorescence of the spent solution with FL_{max} around 415 nm is originated from the neutral form of **4**. After the chemiluminescence reaction, the azido coelenteramide **4** was obtained from the resulting mixture in 72% yield without any damage to the azido group, which was identified by a comparison of spectral data with those of the synthesized sample. The chemiluminescence efficiency of **3** was 49% of that of native coelenterazine (**1**) (Table 1).

Bioluminescence. The azido coelenterazine analogue **3** was incubated with recombinant apoAQ[8] in Tris-HCl buffer (pH 7.6) containing EDTA and dithiothreitol[10] (DTT) for a certain time. To the regenerated semi-synthetic AQ solution was added a solution of $CaCl_2$ in Tris-HCl buffer (pH 7.6); the luminescence intensity was recorded with the TS-1000

Table 1. Luminescence Properties of Coelenterazine (**1**) and Photolabile Coelenterazine Analogue **3**

	CL_{max} in DMSO/nm[a)]	FL_{max} in DMSO/nm[b)]	Relative CL efficiency[c)]	Relative BL activity[d)]
1	474		100	100
3	480		49	30
2		410		
Spent solution after chemiluminescence of **1**		410		
4		415		
Spent solution after chemiluminescence of **3**		415		

a) Chemiluminescence (CL) wavelength maximum. b) Fluorescence (FL) wavelength maximum. c) Relative chemiluminescence efficiency obtained by integrating the total emitted light after injection of DMSO. d) Relative bioluminescence (BL) activity.

lumiphotometer. The maximum light intensity was proportional to the amount of the semi-synthetic AQ present. The semi-synthetic AQ showed a flash pattern similar to that of the native AQ.[11d] The time course of regeneration of the semi-synthetic AQ from **3** and that of native AQ from **1** under the same conditions is shown in Fig. 1. The maximum bioluminescence activity was observed after incubation for 3 h, and was 30% of that of native AQ (Table 1). The bioluminescence efficiency of **3** was 40-times that of the corresponding diazirine analogue.[17] This may be attributable to the binding of **3** to the active site to be more proper than the corresponding diazirine analogue with the relatively bulky trifluoromethyldiazirine appendage. This result suggests that **3** may bind to the same active site as coelenterazine (**1**) does in AQ.

Photolysis of 3 and 4. Photochemical reactions of **3** and **4** were investigated for their potential abilities in photoaffinity labeling. The irradiation of **4** (Scheme 3) in methanol containing diethylamine [0.4 M (1 M = 1 mol dm^{-3})] with a 100 W high-pressure Hg lamp (pyrex filter) under Ar gave the azepine **5** in 89% yield. The structure of the azepine **5** was established by comparing the ^{1}H NMR spectral data with those of similar azepine derivatives reported in the literature.[21] Photolysis of **3** under the same conditions as those of the photolysis of **4** also yielded the azepine **5** (47%) as the major product along with coelenteramine (**12**) (21%), indicating that the imidazopyrazinone skeleton in **3** is labile under photochemical conditions. In both cases, no corresponding aniline derivative of **4** was found. A similar photolability of the imidazopyrazinone skeleton was observed in the photolysis of **1** and diazirine coelenterazine analogue.[17] The formation of **5** by the photolysis of **3** and **4** in the presence of diethylamine indicated **3** and **4** to be potential photoaffinity probes.

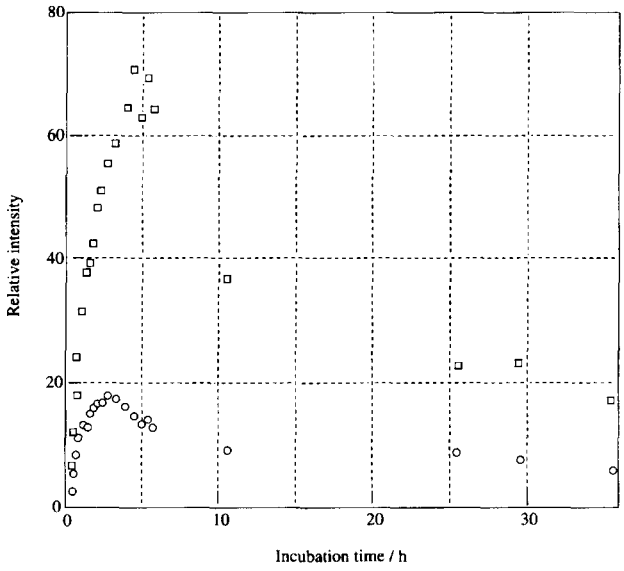


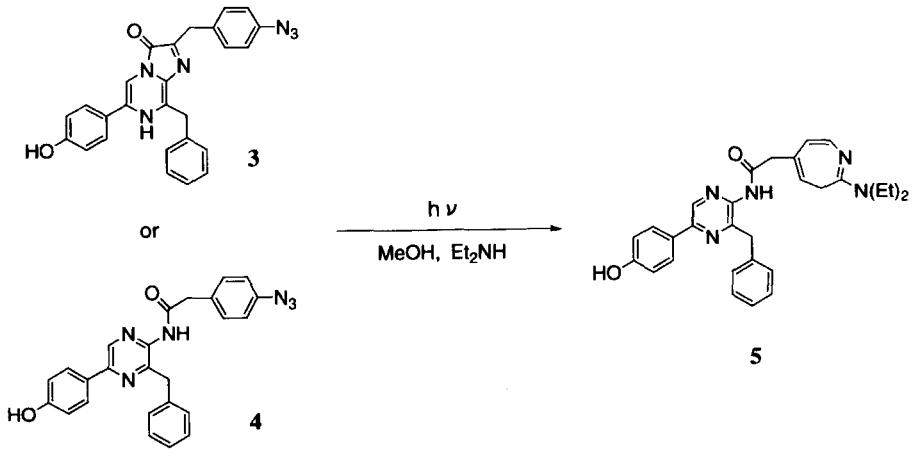
Fig. 1. Time course of regeneration of native AQ (□) and semi-synthetic AQ (○) by incubation of **1** and **3** with recombinant apoAQ at 0 °C, respectively.

In conclusion, the azido coelenterazine analogue **3** shows a similar chemiluminescence behavior to that of native **1**, can bind to the active site of apoAQ, and can be easily photoactivated. The azido coelenterazine analogue **3** may be a promising photoaffinity reagent for probing the binding site of coelenterazine in AQ.

Experimental

General Information. ^{1}H NMR and ^{13}C NMR spectra were measured on a JEOL JNM-GX270 (270 MHz) spectrometer. IR spectra were recorded on a JASCO IR-810 spectrometer, UV-vis spectra on a Hitachi Model 320 spectrophotometer, and FAB mass spectra on a Finnigan MAT TSQ-700 mass spectrometer. High-resolution mass spectra (FAB and EI) were obtained using a JEOL The MStation JMS-700AM. Fluorescence and chemiluminescence spectra were measured with a Hitachi Model F-4010 fluorescence spectrophotometer (for chemiluminescence spectra measurements the Xe lamp was turned off). The relative chemiluminescence efficiency was measured using a Labo Science Model TD-4000 lumiphotometer. The relative bioluminescence activity was obtained using a Labo Science Model TS-1000 lumiphotometer.

Spectral-grade DMSO was used for measuring the chemiluminescence and fluorescence spectra. The reagents used were generally of commercial grade. Anhydrous solvents were prepared by distillation with appropriate drying methods. The glassware used for anhydrous conditions was flame-dried and immediately cooled prior to use. The reactions were checked by thin-layer chromatog-



Scheme 3.

raphy.

Measurement of Chemiluminescence and Fluorescence Spectra. DMSO (1.5 mL) was added to a methanol solution (50 μL) of **1** or **3** [1.0×10^{-3} M (1 M = 1 mol dm^{-3})] in a quartz cuvette, initiating a chemiluminescence reaction. The chemiluminescence spectra of **1** and **3** in DMSO were measured using a F-4010 fluorescence spectrophotometer (emission bandpass: 40 nm; scan speed: 60 nm min^{-1}) with the Xe lamp turned off. The fluorescence spectra of the spent solution of **1** and **3** were measured with the F-4010 fluorescence spectrophotometer (λ_{ex}: 330 nm; emission bandpass: 5 nm; excitation bandpass: 5 nm; scan speed: 60 nm min^{-1}).

Measurement of Relative Chemiluminescence Efficiency. DMSO (300 μL) was injected into a methanol solution (6 μL, 2.0×10^{-5} M) of **1** or **3**. The total light emitted was measured by integration using a TD-4000 lumiphotometer. The relative chemiluminescence efficiency of **3** was determined by the comparison of the total light emitted from **1** and **3**, respectively.

Measurement of Bioluminescence. Recombinant apoAQ (5 μg) and a 0.1 M solution of DTT in 30 mM Tris-HCl (pH 7.6)/10 mM EDTA buffer (1 μL) were added to 30 mM Tris-HCl (pH 7.6)/10 mM EDTA buffer (1 mL); the mixture was pre-incubated for 30 min at 0 °C. To the mixture was added a 2.4×10^{-3} M solution of the azido coelenterazine analogue **3** in absolute methanol (200 μL). After thorough mixing, the resulting mixture was allowed to stand in an ice bath for the required time. The bioluminescence was measured by injecting a 30 mM solution of $CaCl_2$ in 30 mM Tris-HCl (pH 7.6) buffer (0.4 mL) into a regenerated semi-synthetic AQ solution (10 μL) in a quartz tube on the TS-1000 lumiphotometer. The bioluminescence activity of **3** is the ratio of its maximum light intensity relative to that of **1** under similar conditions.

Isolation of 4 from the Spent Solution of 3 after the Chemiluminescence Reaction in DMSO. A solution of **3** (16.5 mg, 0.037 mmol) in DMSO (20 mL) was stirred at room temperature for 3 h under O_2. The chemiluminescence reaction mixture was poured into ice-water. The mixture was then saturated with NaCl, and the resulting mixture was extracted with AcOEt. The extracts were combined, washed with water and then with brine, dried over Na_2SO_4, and concentrated. The residue was purified by preparative TLC (silica gel, hexane/acetone (3 : 2)) to obtain the azido coelenteramide **4** (11.6 mg, 72%), which was identified by comparing its spectra data with those of the synthesized **4**.

Photolysis of 4 in the Presence of Diethylamine. A solution of **4** (9.8 mg, 0.022 mmol, 0.4×10^{-3} M) in CH_3OH (55 mL) containing diethylamine (140 mg, 1.91 mmol, 0.4 M) was irradiated at 0 °C with a 100 W high-pressure Hg lamp (pyrex filter) under Ar. The reaction was monitored by reverse-phase HPLC (column: Inertsil-ODS 4.6×250 mm; eluent: MeCN/H_2O (4 : 1); flow rate: 1.0 mL min^{-1} detected at λ = 300 nm). The reaction was completed after irradiation for 65 min. The solvent was evaporated under reduced pressure. The residue was purified by preparative TLC (silica gel, CH_2Cl_2/CH_3OH (100 : 15)) to give

***N*-[3-benzyl-5-(*p*-hydroxyphenyl)pyrazin-2-yl]-2-(2-diethylamino-3*H*-azepin-5-yl)acetamide (5)** (9.6 mg, 89%) as a colorless powder. Mp 112—113 °C (decomp); IR (KBr) ν 3410, 3270, 1670, 1610, 1570, 1500 cm^{-1}; ^{1}H NMR (270 MHz, CD_3OD) δ = 1.13 (6 H, br t, J = 6.9 Hz), 2.78 (2 H, br s), 3.26 (2 H, s), 3.38 (4 H, q, J = 6.9 Hz), 4.13 (2 H, s), 5.26 (1 H, t, J = 6.9 Hz), 5.86 (1 H, d, J = 8.2 Hz), 6.86 (2 H, d, J = 8.8 Hz), 6.89 (1 H, d, J = 8.2 Hz), 7.14—7.28 (5 H, m), 7.88 (2 H, d, J = 8.8 Hz), 8.69 (1 H, s); ^{13}C NMR (270 MHz, CD_3OD) δ = 13.50 (q), 31.66 (t), 41.14 (t), 43.37 (t), 45.07 (t), 114.45 (d), 115.03 (d), 116.79 (d), 126.29 (s), 127.48 (d), 128.43 (s), 129.41 (d), 129.45 (d), 130.20 (d), 136.47 (s), 138.05 (d), 138.08 (d), 139.54 (s), 143.88 (s), 151.75 (s), 152.58 (s), 160.63 (s), 173.04 (s); MS (FAB) m/z 482 (M+H)$^+$; HRMS (FAB) Calcd for $C_{29}H_{31}N_5O_2$: (M+H)$^+$, 482.2556. Found: m/z 482.2539.

Photolysis of 3 in the Presence of Diethylamine. The photolysis of **3** (2.6 mg, 0.006 mmol, 0.6×10^{-3} M) in CH_3OH (10 mL) containing diethylamine (30 mg, 0.41 mmol, 0.4 M) was carried out according to the same procedure as described for the photolysis of **4**. The reaction was completed after irradiation for 25 min. From the reaction mixture, **5** (1.3 mg, 47%) and **12** (0.6 mg, 21%) were isolated.

***p*-Azidophenylacetyl Chloride (7).** To a stirred solution of **6** (5.0 g, 28.24 mmol) in dry benzene (50 mL) was added thionyl chloride (8 mL) at 0 °C. The mixture was gradually warmed to room temperature and stirred for 18 h. The mixture was concentrated under reduced pressure. The residue was further dried in vacuo to give the crude acid chloride **7**. ^{1}H NMR (270 MHz, $CDCl_3$) δ = 4.12 (2 H, s), 7.03 (2 H, d, J = 8.4 Hz), 7.26 (2 H, d, J = 8.4 Hz).

1-(*p*-Azidophenyl)-3-diazo-2-propanone (8). The acid chloride **7** in dry ether (50 mL) was added to a solution of an excess of diazomethane in dry ether (280 mL) at 0 °C. After being maintained at 0 °C for 1 h, the reaction mixture was concentrated, and the residue was chromatographed (silica gel, CH_2Cl_2) to afford the diazo ketone **8** (5.0 g, 88% based on **6**) as a yellowish oil. IR (neat) ν 2100, 1640, 1510 cm^{-1}; ^{1}H NMR (270 MHz, $CDCl_3$) δ = 3.59 (2 H, s), 5.14 (1 H, s), 7.00 (2 H, d, J = 8.4 Hz), 7.23 (2 H, d, J = 8.4 Hz); MS (FAB) m/z 202 (M+H)$^+$, 174 (M+H−N_2)$^+$; HRMS (EI) Calcd for $C_9H_7N_5O$: (M)$^+$, 201.0651. Found: m/z 201.0623.

1-(*p*-Azidophenyl)-3-bromo-2-propanone (9). To an ice-cold solution of the diazo ketone **8** (2.1 g, 10.45 mmol) in dry ether (35 mL) was passed over a steady stream of dried HBr for 20 min with stirring. The resulting mixture was washed successively with water, aqueous $NaHCO_3$, and water. The organic phase was separated, dried over Na_2SO_4, filtered, and concentrated. The residue was chromatographed (silica gel, CH_2Cl_2/hexane (2 : 1)) to provide the bromo ketone **9** as a yellowish solid (2.4 g, 90%). IR (KBr) ν 3100—3700, 2100, 1718, 1500 cm^{-1}; ^{1}H NMR (270 MHz, $CDCl_3$) δ = 3.91 (2 H, s), 3.95 (2 H, s), 7.01 (2 H, d, J = 8.6 Hz), 7.22 (2 H, d, J = 8.6 Hz); MS (FAB) m/z 255 (M)$^+$, 253 (M)$^+$, 227 (M−N_2)$^+$, 225 (M−N_2)$^+$; HRMS (EI) Calcd for $C_9H_8BrN_3O$: (M)$^+$, 252.9851. Found: m/z 252.9849.

3-(*p*-Azidophenyl)-2-oxopropyl Nitrate (10). To a stirred solution of the bromo ketone **9** (2.0 g, 7.87 mmol) in MeCN (11 mL) was added dropwise a solution of $AgNO_3$ (3.8 g, 22.4 mmol) in MeCN (15 mL). After being stirred at room temperature for 28 h, the reaction mixture was filtered through a pad of Celite; the filtrate and washings were then combined and concentrated. To the residue were added water and ether, and the insoluble materials were filtered off. The organic phase was separated, washed with water and then with saturated brine, dried over Na_2SO_4, filtered, and concentrated. Recrystallization from hexane yielded **10** (1.6 g, 86%) as colorless needles. Mp 63—64 °C; IR (KBr) ν 2100, 1720, 1630, 1500 cm^{-1}; ^{1}H NMR (270 MHz, $CDCl_3$) δ = 3.77 (2 H, s), 4.97 (2 H, s), 7.03 (2 H, d, J = 8.6 Hz), 7.22 (2 H, d, J = 8.6 Hz); MS (FAB) m/z 236 (M)$^+$, 208 (M−N_2)$^+$; HRMS (EI) Calcd for $C_9H_8N_4O_4$: (M)$^+$, 236.0546. Found: m/z 236.0528.

3-(*p*-Azidophenyl)-2-oxopropanal (11). To a solution of the nitrate **10** (476.8 mg, 2.02 mmol) in DMSO (20 mL) was portionwise added powdered sodium acetate trihydrate (274 mg, 2.0 mmol). After the mixture had been stirred at room temperature for 1 h, ice-cold water was added to the reaction mixture, and the aqueous layer was saturated with NaCl. The mixture was extracted with

ether. The extracts were combined, washed successively with water, aqueous $NaHCO_3$, and water, dried over Na_2SO_4, filtered, and concentrated to afford the oxo aldehyde **11** as a yellowish solid (372 mg, quantitatively). Mp 113—116 °C (decomp); IR (KBr) ν 3000—3700, 2110, 1670 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ = 6.14 (1 H, s), 6.60 (1 H, s), 7.07 (2 H, d, J = 8.6 Hz), 7.85 (2 H, d, J = 8.6 Hz), 9.24 (1 H, s); MS (FAB) m/z 190 $(M+H)^+$, 162 $(M+H-N_2)^+$; HRMS (EI) Calcd for $C_9H_7N_3O_2$: $(M)^+$, 189.0538. Found: m/z 189.0554.

2-(*p*-Azidobenzyl)-8-benzyl-6-(*p*-hydroxyphenyl)imidazo-[1, 2-*a*]pyrazin-3(7*H*)-one (3). To a mixture of the oxo aldehyde **11** (118.2 mg, 0.625 mmol), coelenteramine (**12**) (116.7 mg, 0.421 mmol), and water (407 μL) in ethanol (8 mL) was added concd HCl (8.6 μL) under Ar. The reaction mixture was gradually warmed to 55 °C and stirred for 8 h. After being cooled to room temperature, the mixture was concentrated, and the residue was chromatographed (silica gel, CH_2Cl_2/CH_3OH (33 : 2) and then AcOEt/CH_3OH (20 : 1)). Precipitation from AcOEt/hexane gave **3** (104.7 mg, 55%) as a yellow powder. Mp 126—128 °C (decomp); IR (KBr) ν 2300—3700, 2110, 1665, 1610, 1565, 1510 cm^{-1}; ^1H NMR (270 MHz, CD_3OD) δ = 4.15 (2 H, s), 4.40 (2 H, s), 6.88 (2 H, d, J = 8.5 Hz), 6.98 (2 H, d, J = 8.5 Hz), 7.18—7.40 (7 H, m), 7.47 (2 H, d, J = 8.2 Hz), 7.61 (1 H, br s); UV-vis λ_{max} (CH_3OH) 260 (log ε = 4.33), 350 (3.62), 435 (3.80) nm; MS (FAB) m/z 447 $(M-1)^-$ and 419 $(M-1-N_2)^-$; HRMS (FAB) Calcd for $C_{26}H_{20}N_6O_2$: $(M+H)^+$, 449.1726. Found: m/z 449.1717.

***N*-[3-Benzyl-5-(*p*-hydroxyphenyl)pyrazin-2-yl]-2-(*p*-azidophenyl)acetamide (4).** To a solution of coelenteramine (**12**) (125.3 mg, 0.45 mmol), pyridine (0.5 mL) in dry CH_2Cl_2 (2.0 mL) at 0 °C was slowly added the acid chloride **7** in dry CH_2Cl_2 (15 mL). After stirring the mixture at room temperature for 4 h, the reaction was quenched by the addition of saturated aqueous $NaHCO_3$. The resulting mixture was extracted with CH_2Cl_2. The extracts were combined and washed with water and then with brine. The organic phase was separated, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by preparative TLC (silica gel, CH_2Cl_2/CH_3OH (15 : 1)) to give an O, N-diacyl product as a colorless powder. To a solution of the diacyl product in CH_3OH/1, 4-dioxane (35 mL/50 mL) at room temperature was added aqueous 1 M NaOH (12 mL). After stirring for 1 h, the reaction mixture was adjusted to pH 4 by adding aqueous HCl, and then extracted with AcOEt. The extracts were combined, dried over Na_2SO_4, and concentrated. The residue was purified by preparative TLC (silica gel, CH_2Cl_2/CH_3OH (10 : 1)). Precipitation from CH_2Cl_2/hexane yielded **4** (84.5 mg, 43%, from **12**) as a colorless powder. Mp 196—197 °C (decomp); IR (KBr) ν 3250, 2130, 1670, 1610, 1540, 1500 cm^{-1}; ^1H NMR (270 MHz, DMSO-d_6) δ = 3.67 (2 H, s), 4.03 (2 H, s), 6.87 (2 H, d, J = 8.8 Hz), 7.03—7.22 (7 H, m), 7.39 (2 H, d, J = 8.2 Hz), 7.93 (2 H, d, J = 8.5 Hz), 8.82 (1 H, s), 9.87 (1 H, s), 10.47 (1 H, s); UV-vis λ_{max} (DMSO) 277 (log ε = 4.14), 293 (4.13), 337 (4.08) nm; MS (FAB) m/z 437 $(M+H)^+$; HRMS (FAB) Calcd for $C_{25}H_{20}N_6O_2$: $(M+H)^+$, 437.1726. Found: m/z 437.1704.

Financial support from the Ministry of Education, Science, Sports and Culture (Grants-in-Aid of Scientific Research Nos. 09041100 and 10680502), the Naito Foundation, and Shorai Foundation is gratefully acknowledged. The authors are grateful to Dr. Hiraki Toshikazu, Central Customs Laboratory, Ministry of Finance, for the measurement of HRMS.

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