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Chemi- and Bioluminescence of Coelenterazine Analogues Possessing an Adamantylmethyl Group

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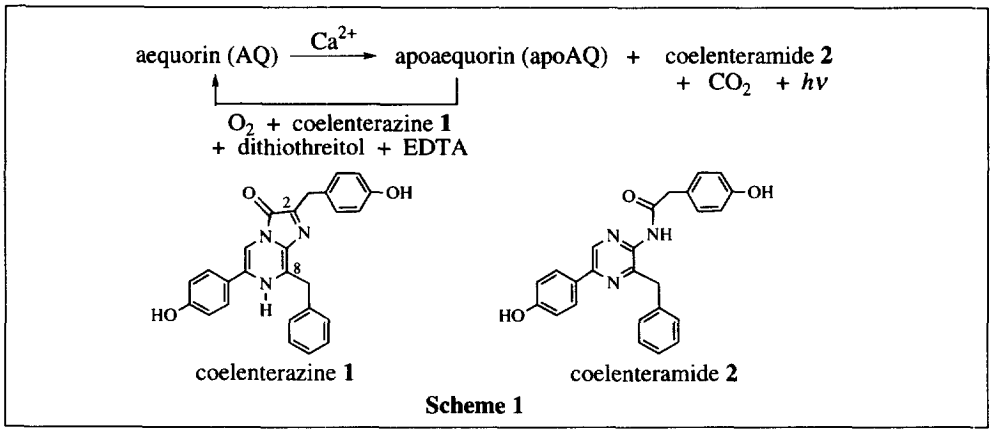
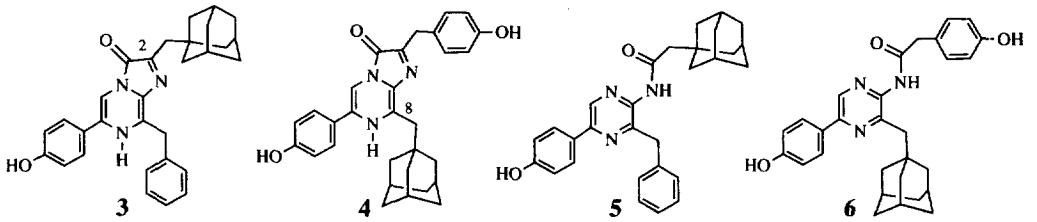
Abstract: Coelenterazine analogues possessing the adamantylmethyl group at the C2 or C8 position were prepared to study their effects on chemi- and bioluminescence. Stability of the excited state coelenteramide analogues was significantly affected by the substitutions, resulting in a neutral amide emission of chemiluminescence in diglyme-acetate buffer and in a blue-shifted emission of bioluminescence in Tris-HCl buffer. Substitution of the adamantylmethyl group in the C8 position caused bioluminescence intensity to double. The 8-adamantylmethyl group may serve to orient the coelenterazine skeleton in a suitable position in the active site for efficient bioluminescence activity. Results with semi-synthetic AQs containing 8-adamantylmethyl analogues, and those of semi-synthetic AQs containing 2-benzyl and 2-methyl analogues, indicate that apoAQ and apoAQ C145,152,180S have the ability to recognize the C2 and C8 side-chains in coelenterazine during AQ regeneration.

Introduction

The photoprotein aequorin (AQ), isolated from the luminescent jellyfish *Aequorea victoria*, emits light by an intramolecular reaction on binding calcium ions.[1, 2] The photoprotein is made up of an apoprotein (apoaequorin, apoAQ), coelenterazine **1**, and molecular oxygen, bound together in a complex. During light emission **1**, serving as a substrate, is oxidized to coelenteramide **2** by the bound oxygen, yielding as products light, a blue fluorescence protein (BFP) and carbon dioxide. BFP consists of **2** bound to apoAQ and **2** is the fluorescent chromophore in BFP.[3] AQ may be prepared by incubating **1** with apoAQ in the presence of EDTA, a thiol reducing agent and dissolved oxygen (Scheme 1).[4] In order to modify the luminescence reactivity of AQ, Shimomura et al [5] prepared semi-synthetic AQ by incubating apoAQ with various analogues of coelenterazine **1**. The resulting semi-synthetic AQs showed novel properties, such as two emission maxima (400 nm and 460 nm) in the case of semi-synthetic *e*- AQ.[5a-c] Thus, a study of semi-synthetic AQs may help us understand how molecular (substrate) recognition takes place in AQ and how apoAQ functions.[6] A high efficiency (ca. 23%) is one of the characteristics of the AQ bioluminescence reaction.[7] The chemiluminescence of coelenterazine **1** in organic solvents containing O_2 leads to coelenteramide **2** and CO_2 as oxidized products. However, the quantum yield of chemiluminescence of **1** is lower than that of bioluminescence of AQ.[8] The hydrophobic character of the active site of apoAQ presumably provides a favorable environment for obtaining efficient luminescence with **1**.[9] Like **1**, the analogues of coelenterazine possessing a hydrophobic group may be



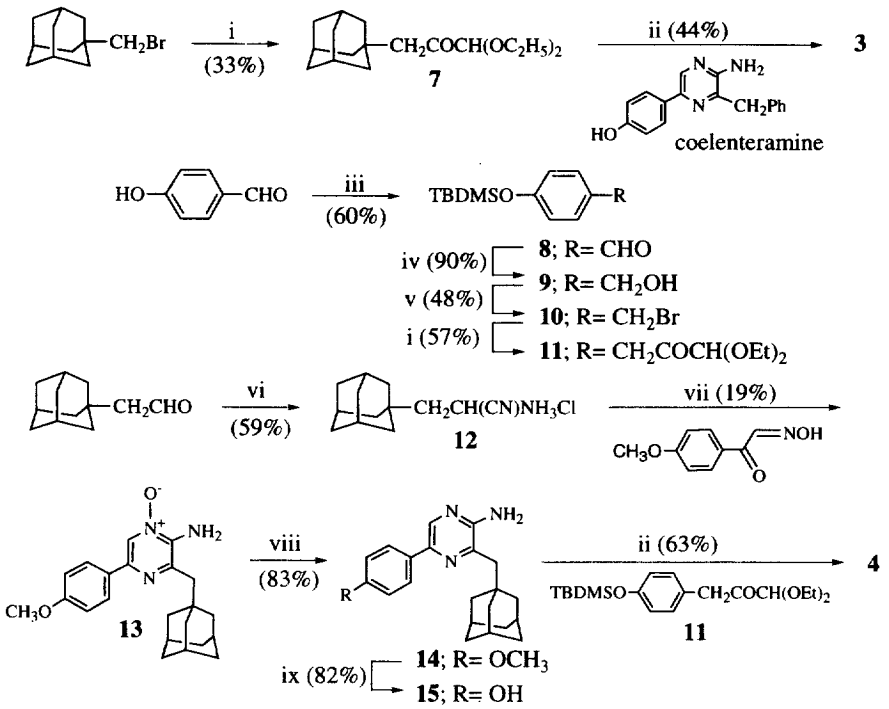
Scheme 1



expected to produce efficient bioluminescence when incorporated into the hydrophobic site of apoAQ. Replacing one of the substituents of **1** with a hydrophobic group should also affect the chemiluminescence properties of **1** in organic solvents. This paper reports (a) the preparation of coelenterazine analogues **3** and **4** with 2- and 8-adamantylmethyl groups, respectively, as hydrophobic substituents, (b) preparation of their corresponding products **5** and **6**, and (c) the investigation of the chemi- and bioluminescence properties of **3**, **4**, **5**, and **6** compared to **1**.[10]

Results and Discussion

Preparation of Adamantylmethyl Coelenterazine Analogues. The 2- and 8-adamantylmethyl analogues **3** and **4**, respectively, were prepared by the procedure as shown in Scheme 2. Coupling of 1-(diethoxyacethyl)piperizine with adamantylmethyl magnesium bromide[11] gave keto acetal **7**. Condensation of **7** with coelenteramine[12] in aqueous HCl / dioxane at 100 °C afforded analogue **3**. For preparing analogue **4**, keto acetal **11** was synthesized from 4-hydroxybenzaldehyde in four steps. The 3-adamantylmethyl coelenteramine analogue **15** was prepared by condensation of aminonitrile **12** with 2-(4-methoxyphenyl)-2-oxoactaldehyde oxime, followed by reduction with Raney Ni and demethylation, as described by Kishi et al.[12] Coupling **11** with **15** in HCl / dioxane gave **4** in 63% yield. Keto acetal **11** was found to be a useful reagent in place of 4-acetoxybenzylglyoxal in preparing coelenterazine **1**.[13] 2-Benzyl- and 2-methyl-8-adamantylmethy coelenterazine analogues **4Bn** and **4Me** were prepared by coupling of 3-adamantylmethyl coelenteramine analogue **15** with 3-phenyl-2-oxopropanal diethyl acetal and pyruvic aldehyde under acidic conditions, respectively. Coelenteramide analogues **5** was synthesized by coupling coelenteramine with adamantylacetyl chloride and **6** was prepared from **15** and 4-acetoxyphenylacetic acid using $BrP[N(CH_3)]_3PF_6$.



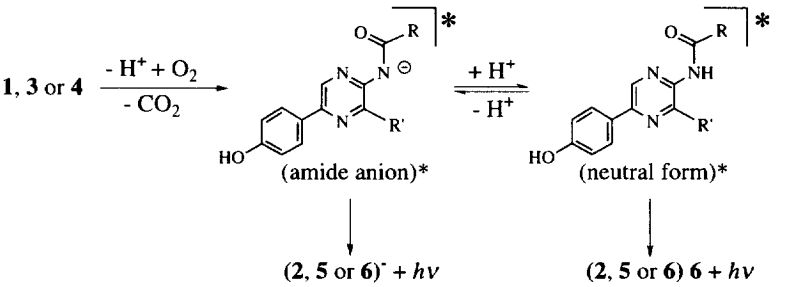
Scheme 2 *Reagents and conditions:* i, (1) Mg, Et_2O, (2) $C_5H_{10}NCOCH(OEt)_2$, (3) H_3O^+; ii, HCl-dioxane, 100-110 °C; iii, TBDMSCl, imidazole, DMF; iv, $NaBH_4$, MeOH; v, CBr_4, PPh_3; vi, (1) $NaHSO_3$, (2) NH_3, (3) NaCN, (4) HCl; vii, $TiCl_4$, pyridine, 80 °C; viii, Raney Ni (W2), H_2, EtOH, reflux; ix, pyridinium chloride, 200 °C

Chemiluminescence. When a solution of **3** or **4** in methanol (100 μl) was mixed with 2 ml of DMSO or diglyme containing 0.20 mol dm^{-3} acetate buffer (pH 5.6, 0.66% v/v) under air, the solution began to emit light. The chemiluminescence maxima (CL_{max}) and the relative efficiencies (relative Φ_{cl}) of **3** and **4** in these solvents and the fluorescence maxima (FL_{max}) of the spent solutions following the chemiluminescence reaction are shown in Table 1, together with those of **1**. The fluorescence maxima (FL_{max}) of the chemiluminescence reaction products of **3** and **4** present in the spent reaction mixture coincided with those of synthesized **5** and **6**, respectively. As reported previously,[14-17] the chemiluminescence of **3** and **4** in DMSO with CL_{max} around 470 nm came from excited states of the amide anions of **5** and **6** (Scheme 3). The CL_{max} values of **3** and **4** in DMSO are similar to that of **1**. Further, in diglyme containing acetate buffer, the chemiluminescence spectra of **3** and **4** were different from that of **1** as shown in Fig. 1. The emission of **3** with CL_{max} at 405 and 445 nm came from excited states of both the neutral and amide anion forms of **5**, respectively, and that of **4** came from only the excited neutral form of **6**, although **1** chemiluminesced from the excited amide anion of **2** (Scheme 3). When the pH value of the acetate buffer added to diglyme was decreased, the relative intensity of the 400 nm band increased while the intensity of the 460 nm band decreased, as shown in Fig. 1. These results indicate that the substitution of the adamantylmethyl group and increased proton concentration accelerate the formation of the excited neutral form shown in Scheme 3.[18] As reported previously,[17] the ratio of chemiluminescence intensity

Table 1. Chemiluminescence of coelenterazine **1** and its analogues **3** and **4** in DMSO and in diglyme containing acetate buffer.

	compounds		
	1	**3**	**4**
in DMSO			
CL_{max} / nm[a]	475	480	467
relative Φcl[b]	1.0	0.3	0.6
FL_{max} / nm[c]	410	410	405
in diglyme-acetate buffer[d]			
CL_{max} / nm[a]	455	405 and 445	400
relative Φcl[b]	1.0	0.8	0.7
FL_{max} / nm[c]	403	409	405

[a] CL_{max}; chemiluminescence maxima, [b] relative Φcl; relative quantum yields of chemiluminescence, [c] FL_{max}; fluorescence maxima of the spent reaction mixtures, [d] diglyme containing 0.20 mol dm^{-3} acetate buffer (pH 7.6, 0.66% v/v).



Scheme 3

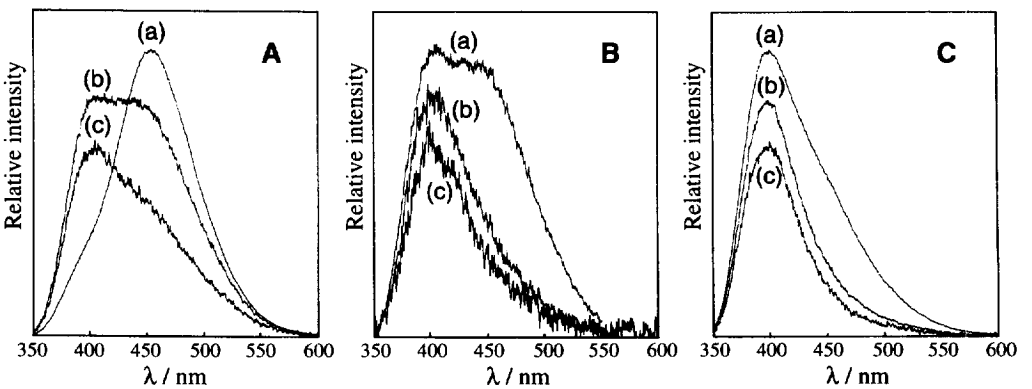


Figure 1 Chemiluminescence spectra of **1** (A), **3** (B) and **4** (C) in diglyme containing acetate buffer under air. The pH values of acetate buffer added to diglyme were 5.6 (a), 4.5 (b), and 4.0 (c).

from a neutral form to that from an amide anion is expressed as a function of the fluorescence quantum yield ratio of the species, fluorescence life time of the amide anion, proton concentration and rate of protonation to an amide anion. The excited state of an amide anion may be destabilized intramolecularly by the hydrophobic adamantylmethyl group, accelerating the rate of protonation to the amide anion to give the excited neutral form (Scheme 3). The slight decrease in the relative chemiluminescent efficiencies (relative Φ_{cl}) of **3** and **4** in DMSO and in diglyme containing acetate buffer may also be caused by destabilization of the excited states of the amide anions.

Bioluminescence. The 2- and 8-adamantylmethyl coelenterazine analogues **3** and **4** were incubated with recombinant wild type apoAQ in Tris-HCl buffer (pH 7.6) containing EDTA and dithiothreitol, as described previously.[19] Of the two analogues, only the 8-adamantylmethyl coelenterazine analogue **4** yielded an active semi-synthetic aequorin. Incubation of this analogue with apoAQ resulted in a rapid increase in bioluminescence activity, which could be measured by mixing an aliquot of the incubation mixture with aqueous $CaCl_2$ and reading the resulting light intensity with a photometer. The maximal light intensity was proportional to the amount of semi-synthetic aequorin present. Semi-synthetic aequorin containing **4** showed a bioluminescence flash pattern similar to that of the wild type AQ.[6a] The time course of regeneration of semi-

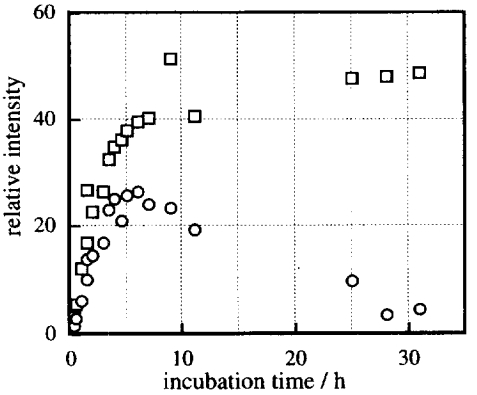
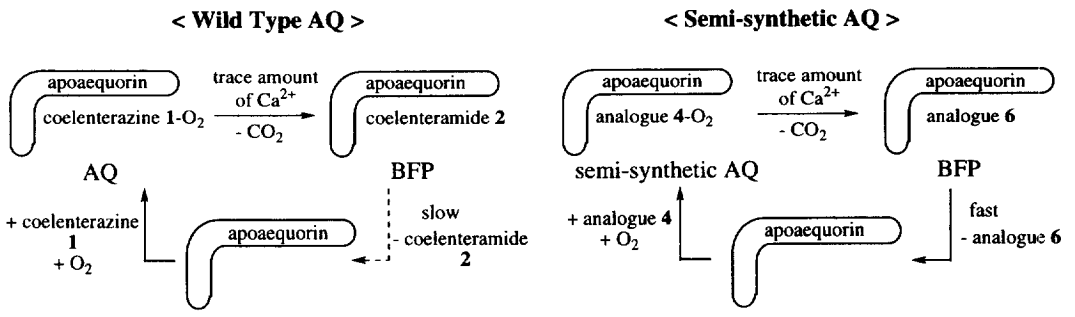


Figure 2 Regeneration of wild type AQ (O) and semi-synthetic AQ (□) by incubation of **1** and **4** with recombinant apoAQ at 0 °C, respectively.



Scheme 4

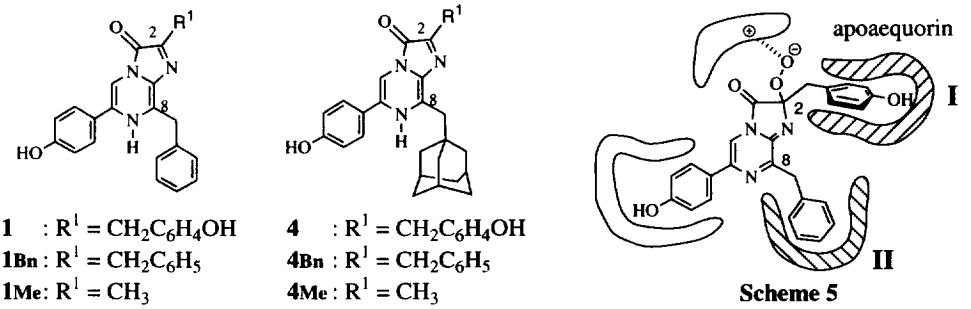
synthetic aequorin containing **4** and that of wild type AQ containing **1** under the same conditions is shown in Fig. 2. The regeneration of wild type AQ occurred rapidly during the initial 5 h, reaching a peak at around 6 h and then gradually decreased over the next 24 h. In contrast, the regeneration of semi-synthetic AQ containing **4** was similar to that of wild type AQ during the initial 5 h, but the curve assumed a plateau thereafter. The decrease in the activity curve of wild type AQ after 5 h may be attributed to bioluminescence since AQ is known to emit light with trace amounts of calcium ions.[20] During the bioluminescence reaction, a blue fluorescence protein (BFP) is formed which consists of coelenteramide **2** and apoAQ (Scheme 4). In the case of wild type BFP, the replacement of **2** by **1** at the active site of apoAQ of BFP may take place at a rate slower than the bioluminescence initiated by trace amounts of calcium ions, resulting in a net decrease in the amount of AQ. In contrast, the regeneration of semi-synthetic AQ containing **4** reached a steady state, suggesting that BFP containing coelenteramide analogue **6** is unstable and **6** is easily diffusible from the active site of apoAQ of BFP, making apoAQ available for regeneration with **4** (Scheme 4). With **6** easily diffusible from the active site of apoAQ in BFP and the regeneration of semi-synthetic AQ taking place at a faster rate than the oxidation of semi-synthetic AQ, a steady state could be reached for semi-synthetic AQ containing **4**. This explanation is supported by earlier studies in which fluorescence measurements were taken on BFP following completion of the bioluminescence reaction.[7,21] After bioluminescence of wild type AQ, the spent reaction mixture containing BFP showed an intense blue fluorescence, whereas the spent reaction mixture after bioluminescence of semi-synthetic AQ containing **4** showed no fluorescence. This result also suggested that in BFP the adamantylmethyl group of coelenteramide analogue **6** made the binding of **6** by apoAQ unfavorable, causing **6** to diffuse from the active site of apoAQ.

In order to obtain information on the lack of activity of 2-adamantylmethyl analogue **3**, a two-step incubation experiment was carried out with **3**.[6a,22] Analogue **3** was incubated with apoAQ for 3 h, after which coelenterazine **1** was added to the incubation mixture. After 3 more hours of incubation, a solution of $CaCl_2$ was added to the regeneration mixture and no bioluminescence activity was observed. This suggested that **3** possessing the 2-adamantylmethyl group had become bound irreversibly to the active site of apoAQ, although **3** bound to the active site of apoAQ had an unfavorable orientation for initiating the bioluminescence reaction. The binding of **3** to the active site may prevent the regeneration of AQ from apoAQ by incorporation of the added coelenterazine **1**.

To investigate how molecular recognition and incorporation of coelenterazine and its analogues take place in apoAQ, bioluminescence activities of semi-synthetic AQs containing 8-adamantylmethyl analogue **4**, **4Bn** and **4Me** were compared with those of the wild type AQ and semi-synthetic AQs containing **1Bn** and **1Me**.[6a] Coelenterazine **1** and its analogues **1Bn**,**1Me**, **4**, **4Bn** and **4Me** were incubated with recombinant wild type apoAQ in Tris-HCl buffer (pH 7.6) containing EDTA, dithiothreitol and dissolved oxygen for 3 h in an ice bath. To compare the activity of wild type apoAQ with that of apoAQ with its cysteine residues 145, 152, and 180 substituted with serine (apoAQC145,152,180S), semi-synthetic AQC145,152,180S was also prepared by the same procedure.[23] The relative maximum light intensities, determined by adding $CaCl_2$ in Tris-HCl buffer to the regeneration mixtures, are summarized in Table 2. The best bioluminescence intensities were obtained with semi-synthetic AQ and semi-synthetic AQC145,152,180S containing analogue **4**. Replacement of the 2-(4-hydroxyphenyl)methyl group of **4** with either benzyl or methyl group decreased bioluminescence intensity. The ratio of the bioluminescence intensities **4**:**4Bn**:**4Me** of semi-synthetic AQ matches that of **1**:**1Bn**:**1Me** of AQ, indicating that the side-chains at C2 and C8 of coelenterazine independently determine the bioluminescence

Table 2 Relative bioluminescence intensities of coelenterazine **1** and its analogues **1Bn**, **1Me**, **4**, **4Bn**, and **4Me**.

compounds	Relative bioluminescence activity	
	AQ	AQC145,152,180S
1	1.0	1.0
1Bn	0.35	0.14
1Me	0.01	0.01
4	2.0	2.4
4Bn	0.73	0.67
4Me	0.02	0.01>



1 : $R^1 = CH_2C_6H_4OH$ **4** : $R^1 = CH_2C_6H_4OH$
1Bn : $R^1 = CH_2C_6H_5$ **4Bn** : $R^1 = CH_2C_6H_5$
1Me: $R^1 = CH_3$ **4Me** : $R^1 = CH_3$

Scheme 5

activity. These ratios are also similar for the bioluminescence intensities of semi-synthetic AQC145,152,180S, showing that replacing cysteine in apoAQ with serine does not change the properties of the active site in recognizing the structure of the coelenterazine molecule. Thus, apoAQ and apoAQC145,152,180S independently recognize the side-chains at C2 and C8 of coelenterazine at sites I and II for regeneration of AQ, as shown in Scheme 5.

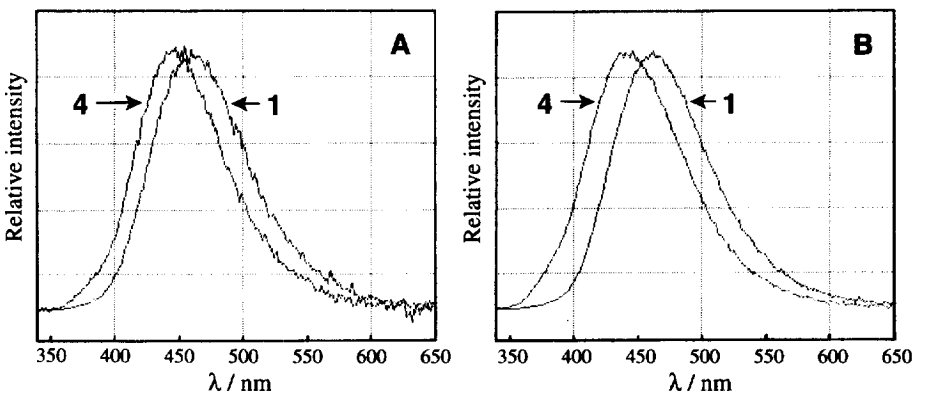


Figure 3 Bioluminescence emission spectra of AQ, semi-synthetic AQ and mutant AQC145,152,180S. A: Bioluminescence spectra of AQ and semi-synthetic AQ which were regenerated by incubating **1** and **4** with recombinant apoAQ in an ice bath for 3 h, respectively. B: Bioluminescence spectra of AQC145,152,180S mutants which were obtained by incubating **1** and **4** with apoAQC145,152,180S under the same conditions as apoAQ in A.

Bioluminescence emission spectra of semi-synthetic AQ containing **4** and semi-synthetic AQC145,152,180S containing **4**, showed maxima at 445 nm (Fig. 3A) and 442 nm (Fig. 3B), respectively, which were at shorter wavelengths than those containing **1** (λ_{max} ca. 460 nm) (Fig. 3). Shimomura et al have previously reported that the bioluminescence emission maximum of semi-synthetic AQs containing 8-cyclohexylmethyl and 8-cyclopentylmethyl coelenterazine analogues is at around 445 nm.[5b-e,g] The structure of the excited coelenteramide **2** in aequorin bioluminescence, which emits ca. 460 nm light, has been assigned as an amide anion[5g] or a phenolate anion.[20] Therefore, it is suggested that the hydrophobic character of 8-adamantylmethyl and 8-cycloalkylmethyl groups of the corresponding coelenteramide analogues intramolecularlly decreases the stability of the excited states of these anion species in BFP, and induces a blue-shifted emission.

In conclusion, the above results show that the adamantylmethyl group at the C2 and C8 positions of the coelenterzine molecule influences the chemi- and bioluminescence characteristics of the excited state coelenteramide molecule. The hydrophobic character of the adamantylmethyl group may destabilize the excited states of the anion species of coelenteramide analogues **5** and **6**, resulting in a neutral amide emission of chemiluminescence in diglyme-acetate buffer and a blue-shifted emission of bioluminescence in Tris-HCl buffer. Bioluminescence intensity of semi-synthetic AQ containing 8-adamantylmethyl analogue **4** was higher than that of wild type AQ. This result and the inhibition of regeneration of wild type apoAQ by the 2-adamantylmethyl analogue **3** provide strong support for the belief that, by undergoing hydrophobic interaction with hydrophobic regions of apoAQ, the adamantylmethyl group of **3** and **4** occupy the active center of apoAQ. Further, to achieve high bioluminescence activity (Table 2), the 8-adamantylmethyl group of **4** may orient the coelenterazine skeleton in the active site of apoAQ in such a way as to increase bioluminescence efficiency. In contrast, coelenteramide analogue **6** in BFP, produced by the bioluminescence reaction, is more diffusible due to weakening of hydrophobic interactions at the active site. The results with semi-synthetic AQs containing 8-adamantylmethyl analogue **4**, **4Bn** and **4Me**, including those with wild type AQ and semi-synthetic AQs containing **1Bn** and **1Me**, indicate that apoAQ and apoAQ C145,152,180S are able to recognize the adamatylmethyl side-chains at C2 and C8 of coelenterazine during AQ regeneration.

Experimental

General. ^{1}H NMR spectra were recorded using a JEOL JNM-GX270 (270 MHz) spectrometer. IR spectra were obtained using a JASCO IR-810 spectrometer. High- and low-resolution electron impact (EI) mass spectra were obtained using a Hitachi Model M-80B mass spectrometer with a Hitachi M-0101 data system. UV-visible absorption spectra were measured with a Hitachi Model 320 spectrophotometer. Fluorescence and chemiluminscence emission spectra were read using a Hitachi Model F-4010 fluorescence spectrophotometer (for chemiluminescence spectra measurements the Xe lamp was turned off). For measuring bio- and chemiluminescence emission spectra, a Hamamatsu PMA-10 image intensification-multiphoton counting system was used. Relative bioluminescence activities were obtained using a Labo Science Model TS-1000 lumiphotometer. Spectral grade DMSO was used for measurement of chemiluminescence and fluorescence spectra. Diglyme was dried over CaH_2, and distilled under reduced pressure.

Measurement of chemiluminescence and fluorescence spectra. Two milliliters of DMSO or diglyme containing acetate buffer (pH 4.0-5.6, 0.66% v/v) was mixed with a methanol solution (100 μl) of **1** or

its analogues (1.8×10^{-3} M) in a quartz cuvette, initiating the chemiluminescence reaction (1 M = 1 mol dm^{-3}). Chemiluminescence spectra of **1** and its analogues in DMSO were measured with the PMA-10 image intensification-multiphoton counting system and those in diglyme containing acetate buffer was recorded using the F-4010 fluorescence spectrophotometer (emission bandpass, 20 nm; scan speed, 60 nm/min) with the Xe lamp turned off. Total light emitted was obtained by integration using the respective instruments. The fluorescence spectra of the spent reaction mixture of **1** and its analogues were measured with the F-4010 fluorescence spectrophotometer (excitation bandpass, 5 nm; emission bandpass, 3 nm; scan speed, 60 nm/min).

Measurement of bioluminescence. Recombinant wild type apoAQ and mutant apoAQC145,152,180S were prepared as previously described.[23,24] ApoAQ and apoAQC145,152,180S were purified chromatographically and gave a single band on SDS-PAGE (12.5%), indicating a purity of > 95% .

A solution (20 μl) of coelenterazine **1** or its analogue (2.4×10^{-3} M) in methanol was added to a solution (1.0 ml) of recombinant apoAQ or mutant apoAQC145,152,180S (0.5 μg) in Tris-HCl buffer (0.03 M, pH 7.6) containing dithiothreitol (0.002 M) and EDTA (0.010 M), and the AQ or semi-synthetic AQ regenerated by incubation in an ice bath. Assay for bioluminescence activity was carried out in a quartz tube by mixing 20 μl of the incubation mixture with 0.4 ml of $CaCl_2$ (0.03 M) in Tris-HCl buffer (pH 7.6) and reading the light intensity using the TS-1000 lumiphotometer. The flash patterns observed by adding $CaCl_2$ to regenerated AQ, semi-synthetic AQ and mutant apoAQC145,152,180S were similar to those reported previously[6a] and the regeneration curves for wild type AQ and semi-synthetic AQ containing **4** are shown in Fig. 2 . The bioluminescence activities of the coelenterazine analogues listed in Table 2 and are the ratios of their maximum light intensities relative to **1** after their AQs were regenerated for 3h. The bioluminescence emission spectra were measured after 3h of incubation using the PMA-10 image intensification-multiphoton counting system, while the fluorescence spectra of the spent solution were measured using the F-4010 fluorescence spectrophotometer (excitation bandpass, 5 nm; emission bandpass, 10 nm; scan speed, 60 nm/min). The two-step incubation experiment s were performed according to a previously described procedure.[6a,21]

3-Adamantyl-2-oxopropanal diethyl acetal 7. To Mg turnings (2.01 g, 83 mmol) in 60 ml of anhydrous ether, a solution (80 ml) of adamantylmethyl bromide (1.98 g, 8.6 mmol) in ether was added dropwise over 6 h at room temperature under Ar. The mixture was stirred for an additional 2 h and a solution (60 ml) of 1-(diethoxyacetyl)piperidine (2.00 g, 12 mmol) in ether was added dropwise to the solution of adamantylmethyl magnesium bromide.[11] The reaction mixture was refluxed for 3 h and then poured into saturated aqueous NH_4Cl. After extraction with ether (800 ml), the ether layer was washed with brine, dried over Na_2SO_4, concentrated and purified by silica gel column chromatography (elution with *n*-hexane, *n*-hexane / ether (98:2) and *n*-hexane / ether (95:5)) to give 0.80 g (33%) of **7.** A colorless oil; IR (neat) 2976, 2901, 2846, 1728, and 1455 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 1.24 (t, J = 7.0 Hz, 6H), 1.65 (m, 12 H), 1.94 (br s, 3 H), 2.33 (s, 2 H), 3.50-3.61 (m, 2 H), 3.61-3.73 (m, 2 H), and 4.46 (s, 1 H); MS (EI) *m/z* 280 (M^+, 4), 279 (10), 135 (47) and 103 (100). HRMS (In beam-EI) Found: *m/z* 280.2058. Calcd for $C_{17}H_{28}O_3$: M, 280.2038.

Preparation of 2-adamantylmethyl coelenterazine analogue 3. A solution of coelenteramine (55 mg, 0.20 mmol) and **7** (114 mg, 0.41 mmol) in a mixture of 1,4-dioxane (4 ml) and H_2O (2 ml) containing 10% aqueous HCl (1 ml) was heated for 3 h at 100 ℃ under N_2. The reaction mixture was diluted with H_2O

(50 ml) and extracted with AcOEt (100 ml). The organic layer was washed with brine, dried over $MgSO_4$, concentrated and purified by silica gel column chromatography (elution with AcOEt / CH_3CN / CH_3OH (40:6:2)) to give **3** (41 mg, 44%) as a yellow powder: mp 115-156 ℃ (dec.); IR (KBr) 2890, 2840, 1733, 1538, and 1513 cm^{-1}; ^1H NMR (CD_3OD) δ = 1.66 (m, 12 H), 1.95 (br s, 3 H), 2.61 (s, 2 H), 4.42 (s, 2 H), 6.89 (m, 2 H), 7.28 (m, 5 H), 7.43 (m, 2 H), and 7.43 (br s, 1 H); UV-vis λ_{max} (CH_3OH) 263 (log ε = 4.42), 350 (3.76), and 432 (4.00) nm. HRMS (In beam-EI) Found: *m/z* 465.2425. Calcd for $C_{30}H_{31}N_3O_2$: M, 465.2416.

4-(*tert*-Butyldimethylsiloxy)benzaldehyde 8. A solution of 4-hydroxybenzaldehyde (25.6 g, 0.21 mol), *tert*-butyldimethylchlorosilane (36.9 g, 0.25 mol) and imidazole (34.8 g, 0.51 mol) in 50 ml of DMF was stirred at room temperature for 2 days. The reaction mixture was diluted with H_2O (300 ml) and extracted with *n*-hexane (700 ml). The organic layer was washed with 10% aqueous HCl and H_2O, dried over $MgSO_4$, concentrated and purified by distillation to give **8** (29.8 g, 60%) as a colorless oil: bp 102-110 ℃ (0.5 mmHg); IR (neat) 2950, 2920, 2850, 1700, 1600, 1500, and 1280 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 0.25 (s, 6 H), 0.99 (s, 9 H), 6.95 (m, 2 H), 7.79 (m, 2 H), and 9.89 (s, 1 H); MS (EI) *m/z* 236 (M^+, 19) and 179 (100). HRMS (EI) Found: *m/z* 236.1235. Calcd for $C_{13}H_{20}O_2Si$: M, 236.1233.

4-(*tert*-butyldimethylsiloxy)phenylmethanol 9. To a solution of **8** (29.8 g, 0.13 mol) in CH_3OH (330 ml), $NaBH_4$ (6.0 g, 0.16 mol) was added at room temperature. After stirring overnight at room temperature, the reaction was quenched by adding saturated aqueous NaCl (250 ml). The reaction mixture was concentrated by evaporation and the aqueous residue was extracted with ether (700 ml). The ether layer was washed with 5% aqueous HCl and H_2O, dried over $MgSO_4$ and concentrated *in vacuo* to give **9** (27.0 g, 90%) as a colorless oil; IR (neat) 3325, 2950, 2930, 2852, 1610, 1512, 1473, and 1260 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 0.19 (s, 6 H), 0.98 (s, 9 H), 1.62 (br s, 1 H), 4.61 (s, 2 H), 6.83 (m, 2 H), and 7.23 (m, 2 H); MS (EI) *m/z* 238 (M^+, 32), 181 (100), 151 (58) and 75 (24). HRMS (EI) Found: *m/z* 238.1400. Calcd for $C_{13}H_{22}O_2$ Si: M, 238.1389.

4-(bromomethyl)-1-(*tert*-Butyldimethylsiloxy)benzene 10. A mixture of **9** (37.3 g, 156 mmol), CBr_4 (54.6 g, 165 mmol), and PPh_3 (43.2 g, 165 mmol) was stirred at room temperature over night. After concentrating the reaction mixture *in vacuo*, triphenylphosphine oxide was filtered and washed with *n*-hexane and benzene. The filtrate was concentrated *in vacuo* and purified by silica gel column chromatography (elution with *n*-hexane / ether (2:3)) and distillation, to give **10** (22.2 g, 48%). A colorless oil: bp 105-110 ℃ (0.25 mmHg); ^1H NMR ($CDCl_3$) δ = 0.20 (s, 6 H), 0.98 (s, 9 H), 4.49 (s, 2 H), 6.79 (m, 2 H), and 7.26 (m, 2 H). Bromide **10** was unstable and should be used for the next reaction as soon as possible.

4-(*tert*-Butyldimethylsiloxy)phenyl-2-oxopropanal diethyl acetal 11. To Mg turnings (258 mg, 11 mmol) in 3 ml of anhydrous ether, **10** (1.47 g, 4.9 mmol) was added dropwise over 1 h at 0 ℃ under N_2. A solution (10 ml) of 1-(diethoxyacetyl)piperidine (0.90 g, 4.2 mmol) in ether was added dropwise to the solution of Grignard reagent over a period of 30 min. After being stirred for 3 h at room temperature, the reaction mixture was poured into saturated NH_4Cl aqueous solution and extracted with ether. The ether layer was washed with saturated aqueous $NaHCO_3$ and brine, and dried over Na_2SO_4. After removing the solvent *in*

vacuo, the residue was purified by silica gel column chromatography (elution with *n*-hexane / ether (4:1)) to give **11** (0.84 g, 57%). A colorless oil; IR (neat) 2950, 2920, 2880, 2850, 1740, 1610, 1505, 1260, and 1060 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 0.18 (s, 6 H), 0.97 (s, 9 H), 1.23 (t, J = 7.0 Hz, 6 H), 3.50-3.56 (m, 2 H), 3.62-3.71 (m, 2 H), 3.80 (s, 2 H), 4.62 (s, 1 H), 6.77 (m, 2 H), and 7.07 (m, 2 H); MS (EI) *m/z* 352 (M^+, 3), 324 (4), 261 (6), 221 (12), 164 (10), 149 (9), 103 (100) and 75 (29). HRMS (EI) Found: *m/z* 352.2068. Calcd for $C_{19}H_{32}O_4Si$: M, 352.2070.

1-Adamantyl-2-cyanoethylammonium chloride 12. A solution (10 ml) of adamantylacetaldehyde (5.42 g, 31 mmol) in THF was added dropwise to an aqueous solution (10 ml) of $NaHSO_3$ (3.18 g, 31 mmol) with vigorous stirring for 10 min at room temperature, to give a white precipitate. A 28% aqueous NH_3 (32 mmol) was added to the precipitate and the mixture was heated at 60 °C for 1 h. After cooling in an ice bath, the precipitate was dissolved in H_2O (10 ml), giving a pH of 10. An aqueous solution (7 ml) of NaCN (1.35 g, 27.5 mmol) was added dropwise to the solution over 30 min while being kept at 10 °C. The reaction mixture was stirred at 30 °C for an additional 2 h and extracted with ether (500 ml). The ether layer was dried over $MgSO_4$ and filtered. To the filtrate kept in an ice bath, 10 ml of anhydrous ethanol containing HCl (3.3 M) was added giving a precipitate. Filtration gave **12** (4.30 g, 59%) as colorless needles: mp 146-148 °C (dec.); IR (KBr) 2906, 2846, 1708, and 1448 cm^{-1}; ^1H NMR (CD_3OD) δ = 1.55 (dd, J = 2.6 and 13.9 Hz, 1 H), 1.67 (d, J = 2.2 Hz, 6 H), 1.76 (m, 6 H), 1.91 (dd, J = 11.4 and 13.9 Hz, 1 H), 2.02 (br s, 3 H), and 4.48 (dd, J = 2.6 and 11.5 Hz, 1 H).

3-Adamantylmethy-2-amino-5-(4-methoxyphenyl)pyrazine 1-oxide 13. To a solution of **12** (838 mg, 3.5 mmol) and 2-(4-methoxyphenyl)-2-oxoactaldehyde oxime (808 mg, 4.5 mmol) in anhydrous pyridine (12 ml) kept in an ice bath, $TiCl_4$ (120 µl, 1.1 mmol) was added and the reaction mixture heated at 80 °C for 5 h. The reaction mixture was filtered through Celite and the filtrate was concentrated *in vacuo* to remove pyridine. The residue was extracted with CH_2Cl_2 and the CH_2Cl_2 layer was dried over $MgSO_4$, concentrated *in vacuo* and purified by silica gel column chromatography (elution with benzene / AcOEt (1:1)) to give **13** (0.24 g, 19%) as a colorless powder: mp 196-198 °C; IR (KBr) 3346, 2896, 2841, 1613, 1518, and 1483 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 1.59-1.73 (m, 12 H), 1.99 (br s, 3 H), 2.59 (s, 2 H), 3.86 (s, 3 H), 5.52 (br s, 2 H), 6.99 (m, 2 H), 7.80 (m, 2 H), and 8.33 (s, 1 H); MS (EI) *m/z* 366 (M^++1, 17), 365 (M^+, 87), 349 (50), 348 (100), 214 (21) and 135 (44). HRMS (EI) Found: *m/z* 365.2072. Calcd for $C_{22}H_{27}N_3O_2$: M, 365.2103.

3-Adamantylmethy-5-(4-methoxyphenyl)pyrazinamine 14. A mixture of **13** (526 mg, 1.4 mmol) and Raney Ni (W2) (1.5 g) in ethanol (50 ml) was refluxed under H_2 for 9 h. After being cooled, the reaction mixture was filtered through Celite and the filtrate was concentrated *in vacuo* and purified by silica gel column chromatography (elution with CH_2Cl_2 / AcOEt (5:1)) to give **14** (415 mg, 83%) as a pale yellow powder: mp 136-138 °C; IR (KBr) 2901, 2846, 1613, 1513, and 1458 cm^{-1}; ^1H NMR ($CDCl_3$) δ = 1.68 (m, 12 H), 1.98 (br s, 3 H), 2.55 (s, 2 H), 3.86 (s, 3 H), 4.96 (2 H, br s), 6.99 (2 H, m), 7.84 (2 H, m) and 8.19 (1 H, s); MS (EI) *m/z* 349 (M^+, 100) and 135 (53). HRMS (EI) Found: *m/z* 349.2139. Calcd for $C_{22}H_{27}N_3O$: M, 349.2154.

3-Adamantylmethy-5-(4-hydroxyphenyl)pyrazinamine 15. A mixture of **14** (100 mg, 0.29 mmol) and pyridinium chloride (2.12 g, 18 mmol) was heated at 200 ℃ for 30 min under Ar. After being cooled to room temperature, the reaction mixture was diluted with saturated aqueous $NaHCO_3$ and extracted with AcOEt (500 ml). The organic layer was washed with brine, dried over $MgSO_4$, concentrated *in vacuo* and purified by silica gel column chromatography (elution with CH_2Cl_2 and CH_2Cl_2 / AcOEt (2:1)) to give **15** (79 mg, 82%) as a pale yellow powder: mp 264-265 °C (dec.); IR (KBr) 3386, 2896, 2846, 1617, 1608, 1512, and 1435 cm^{-1}; ^1H NMR (CD_3OD) δ =1.72 (m, 12 H), 1.96 (br s, 3 H), 2.55 (s, 2 H), 6.85 (m, 2 H), 7.70 (m, 2 H), and 8.13 (s, 1 H); MS (EI) *m/z* 335 (M^+, 100). HRMS (EI) Found: *m/z* 335.2016. Calcd for $C_{21}H_{25}N_3O$: M, 335.1998.

Preparation of 8-adamantylmethyl coelenterazine analogue 4. A solution of **15** (36 mg, 0.11 mmol) and **11** (50 mg, 0.14 mmol) in a mixture of 1,4-dioxane (2 ml) and H_2O (1 ml) containing 10% aqueous HCl (0.5 ml) was heated at 110 ℃ for 4.5 h under Ar. After being cooled to room temperature, the reaction mixture was diluted with H_2O (100 ml) and extracted with AcOEt (200 ml). The organic layer was washed with brine, dried over $MgSO_4$, concentrated and purified by silica gel column chromatography (elution with CH_3CN / CH_2Cl_2 / CH_3OH (5:5:1)) to give **4** (33 mg, 63%) as a yellow powder: mp 168-169 ℃ (dec.); IR (KBr) 2900, 2840, 1733, 1538 and 1503 cm^{-1}; ^1H NMR (CD_3OD) δ = 1.69 (m, 12 H), 1.98 (br s, 3 H), 2.88 (s, 2 H), 4.05 (s, 2 H), 6.67 (m, 2 H), 6.93 (m, 2 H), 7.13 (m, 2 H), and 7.49 (m, 3 H); UV-vis λ_{max} (CH_3OH) 262 (log ε = 4.44), 343 (3.80), and 430 (4.02) nm. HRMS (In beam-EI) Found: *m/z* 481.2367. Calcd for $C_{30}H_{31}N_3O_3$: M, 481.2365.

8-Adamantylmethyl-2-benzyl-3,7-dihydro-6-(4-hydroxyphenyl)imidazo[1,2-a]pyrazin-3-one 4Bn. A solution of **15** (22 mg, 0.06 mmol) and 3-phenyl-2-oxopropanal diethyl acetal (14 mg, 0.06 mmol) in a mixture of 1,4-dioxane (2 ml) and H_2O (1 ml) containing 10% aqueous HCl (0.5 ml) was heated at 110 ℃ for 1 h under Ar. The reaction mixture was diluted with H_2O (60 ml) and extracted with AcOEt (300 ml). The organic layer was dried over $MgSO_4$, concentrated *in vacuo* and purified by silica gel column chromatography (elution with CH_3CN / CH_2Cl_2 / CH_3OH (15:30:1)) to give **4Bn** (6.5 mg, 22%) as a yellow powder: mp 174-175 °C (dec.); IR (KBr) 2906, 2846, 1561, and 1513 cm^{-1}; ^1H NMR (CD_3OD) δ =1.69 (m, 12 H), 1.98 (br s, 3 H), 2.88 (s, 2 H), 4.16 (s, 2 H), 6.93 (m, 2 H), 7.16-7.33 (m, 5 H), and 7.47 (m, 3 H). HRMS (In beam-EI) Found: *m/z* 465.2447. Calcd for $C_{30}H_{31}N_3O_2$: M, 465.2416.

8-Adamantylmethyl-3,7-dihydro-6-(4-hydroxyphenyl)-2-methylimidazo[1,2-a]pyrazin-3-one 4Me. A solution of **15** (21 mg, 0.06 mmol) and 40% aqueous pyruvic aldehyde (25 μl, 0.4 mmol) in a mixture of 1,4-dioxane (2 ml) and H_2O (1 ml) containing 10% aqueous HCl (0.5 ml) was heated at 115 ℃ for 30 min under Ar. The reaction mixture was diluted with H_2O (50 ml) and extracted with AcOEt (200 ml). The organic layer was dried over $MgSO_4$, concentrated *in vacuo* and purified by silica gel column chromatography (elution with CH_3CN / CH_2Cl_2 / CH_3OH (10:10:1)) to give **4Me** (8.8 mg, 38%) as a yellow powder: mp 210-212 °C (dec.); IR (KBr) 2901, 2841, 1598, and 1518 cm^{-1}; ^1H NMR (CD_3OD) δ = 1.71 (m, 12 H), 1.99 (br s, 3 H), 2.44 (s, 3 H), 2.86 (s, 2 H), 6.93 (m, 2 H), and 7.48 (m, 3 H). HRMS (In beam-EI) Found: *m/z* 389.2110. Calcd for $C_{24}H_{27}N_3O_2$: M, 389.2103.

2-(Adamantylacetamido)-3-benzyl-5-(4-methoxyphenyl)pyrazine 5. To a solution of coelenteramine (51 mg, 0.18 mmol) in a mixture of $CHCl_3$ (2 ml) and pyridine (1 ml) kept in an ice bath, adamantylacetyl chloride (317 mg, 1.5 mmol) was added. After being stirred at room temperature for 1.5 h, the reaction was quenched by addition of saturated aqueous $NaHCO_3$ and the mixture was extracted with AcOEt (200 ml x 5). The organic layer was washed with brine and dried over $MgSO_4$. After removing the solvent, the residue containing the diacyl product was washed with methanol and suspended in a mixture of 1,4-dioxane (2 ml) and methanol (5 ml). To the suspension was added aqueous NaOH (1.0 M, 0.5 ml) and the reaction mixture was stirred at room temperature for 20 min. The pH of the solution was adjusted to 5 by adding aqueous HCl and the reaction mixture was extracted with AcOEt (100 ml x 3). After removing the solvent, the residue was purified by silica gel column chromatography (elution with $CHCl_3$ and $CHCl_3$ / AcOEt (5:1)), followed by recrystallization from methanol to give **5** (9.1 mg, 11%) as colorless needles: mp 224-225 ℃ (dec.); IR (KBr) 3260, 2910, 2840, 1688, 1603, and 1578 cm^{-1}; ^1H NMR (CD_3OD) δ = 1.72 (m, 12 H), 1.95 (br s, 3 H), 2.13 (s, 2 H), 4.27 (s, 2 H), 6.89 (m, 2 H), 7.22 (m, 5 H), 7.92 (m, 2 H), and 8.73 (s, 1 H); MS (EI) *m/z* 453 (M^+, 60) and 277 (100); UV-vis λ_{max} (DMSO) 283 (log ε = 4.00), 295 (3.99), and 342 (3.90) nm. HRMS (EI) Found: *m/z* 453.2451. Calcd for $C_{29}H_{31}N_3O_2$: M, 453.2416.

5-Adamantylmethyl-3-benzyl-2-(4-hydroxyphenylacetamido)pyrazine 6. To a suspension of **15** (21 mg, 0.06 mmol), 4-acetoxyphenylacetic acid (79 mg, 0.41 mmol) and $BrP[N(CH_3)]_3PF_6$ (148 mg, 0.38 mmol) in anhydrous CH_2Cl_2 (0.5 ml), *N, N*-diisopropylethylamine (140 µl, 0.8 mmol) was added at room temperature under Ar and the mixture was stirred at room temperature for 11 h. The reaction mixture was purified by silica gel column chromatography (elution with benzene / AcOEt (2:1)) to give a diacyl product as a colorless powder. To a solution of the diacyl product in a mixture of ethanol (1.5 ml) and 1,4-dioxane (1.5 ml), aqueous NaOH (1.0 M, 0.5 ml) was added at room temperature. After stirring for 30 min, the solution was concentrated *in vacuo*. The residue was suspended in H_2O (20 ml) and extracted with AcOEt (30 ml). The organic layer was dried over $MgSO_4$, concentrated and purified by preparative TLC (silica gel, CH_2Cl_2 / AcOEt (1:1)). Recrystallization from AcOEt / CH_2Cl_2 yielded **6** (14 mg, 46%) as a colorless powder: mp 180-181 °C (dec.); IR (KBr) 3250, 2895, 2845, 1652, 1609, and 1592 cm^{-1}; ^1H NMR (DMSO-d_6) δ = 1.28 (br s, 6 H), 1.43 (d, *J* = 11.6 Hz, 3 H), 1.55 (d, *J* = 11.6 Hz, 3 H), 1.78 (br s, 3 H), 2.39 (s, 2 H), 3.50 (s, 2 H), 6.73 (m, 2 H), 6.88 (m, 2 H), 7.19 (m, 2 H), 7.94 (m, 2 H), 8.78 (s, 1 H), 9.30 (s, 1 H), 9.86 (br s, 1 H), and 10.25 (s, 1 H); MS (EI) *m/z* 469 (M^+, 31) and 335 (100); UV-vis λ_{max} (DMSO) 277 (log ε = 4.18), 293 (4.18), and 335 (4.15) nm. HRMS (EI) Found: *m/z* 469.2371. Calcd for $C_{29}H_{31}N_3O_3$: M, 469.2365.

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