Chemi- and Bio-luminescence of Coelenterazine Analogues with Phenyl Homologues at the C-2 Position

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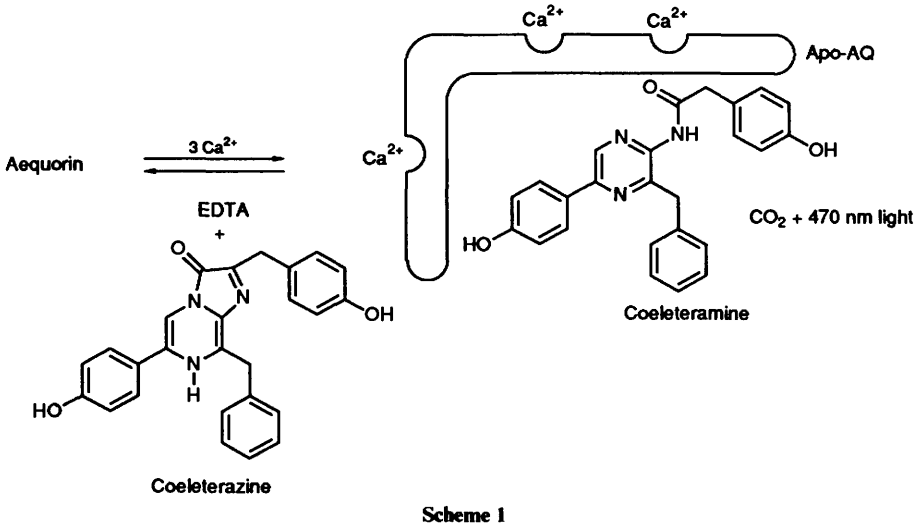
A series of phenyl homologues of coelenterazine substituted at the C-2 position were synthesized and their bio- and chemi-luminescence properties were investigated including the measurement of chemiluminescence spectra in various media. The light emitting species of each analogue was found to be a neutral form of a coelenteramide derivative in diethylene glycol dimethyl ether (DGM) containing a trace amount of acetate buffer (pH 5.60), while a monoanion was found only in dimethyl sulfoxide (DMSO) and a dianion was observed in DMSO containing a trace amount of aqueous sodium hydroxide. Based on pseudo first-order reaction kinetics, chemiluminescence rate constants were obtained in DGM containing a trace amount of acetate buffer. Each of the synthetic coelenterazine analogues was incorporated into recombinant apoaequorin to obtain a series of semi-synthetic aequorins. Measurements of bioluminescence activities of the aequorins revealed that a benzyl group in the C-2 position was essential for efficient luminescence activity. A two-step incubation procedure was used to determine why some analogues gave less luminescence activity than the benzyl analogue and natural coelenterazine.

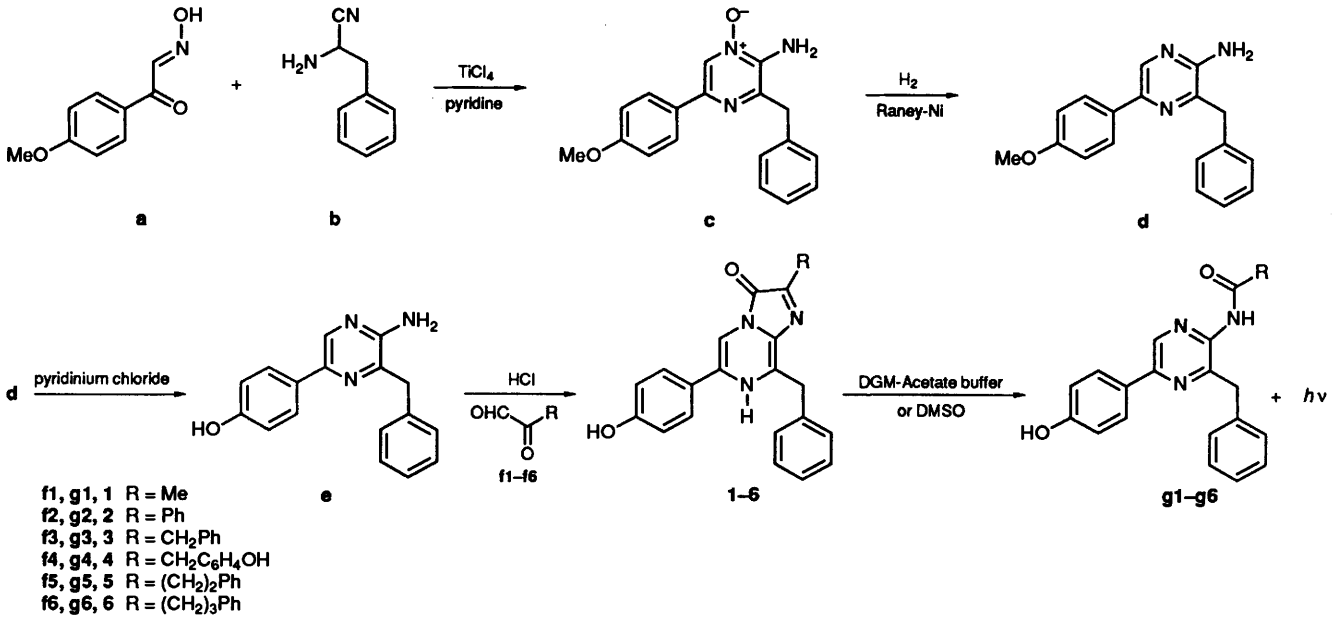
In 1962 Shimomura *et al.* discovered the luminescent protein aequorin from the jellyfish *Aequorea victoria* and showed that aequorin consists of a complex of apoaequorin (apoprotein), coelenterazine (chromophore) and molecular oxygen.[1] Since then the mechanism of the bioluminescence reaction and applications of the protein have been extensively studied. Such studies have revealed that the binding of calcium ion to the protein triggers the reaction of the already dioxygenated protein to yield carbon dioxide, coelenteramide and light (Scheme 1).[2] The excited state of coelenteramide anion bound to apoaequorin is considered to be the emitter in the reaction.[3] Shimomura and Johnson also established that aequorin can be regenerated from apoaequorin by incubation in TRIS [tris-(hydroxymethyl)aminomethane]–HCl buffer (pH 7.6) with coelenterazine, dissolved oxygen, ethylenediamine-tetraacetic acid (EDTA) and 2-mercaptoethanol (Scheme 1).[4] Since aequorin has high specificity and sensitivity to calcium ion, it has been used as an indicator for the presence of the calcium ion in biological systems,[5] Shimomura *et al.* have also reported on the use of coelenterazine analogues to improve the sensitivity of semi-synthetic aequorins to calcium ion.[6–8] The primary structure of apoaequorin has been deduced from its nucleotide sequence and consists of 189 amino acid residues in a single polypeptide chain.[9] However, a true understanding of the mode of binding between coelenterazine and apoaequorin, the nature of the active site, and the nature of the excited state during bioluminescence are still unknown. This paper deals with the influence of substituents at the C-2 position of coelenterazine on the chemiluminescence and bioluminescence of semi-synthetic aequorins containing coelenterazine analogues with a series of phenyl homologues at the C-2 position.

Results and Discussion

Syntheses of Coelenterazine Analogues.—Coelenterazine analogues with a series of phenyl homologues as substituents at the



Scheme 1



Scheme 2

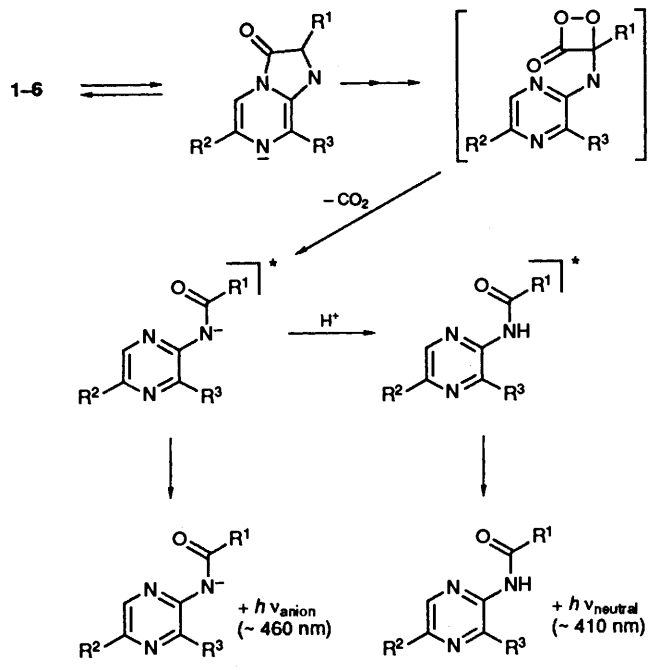
C-2 position were synthesized according to Kishi *et al.*[10] The synthetic procedure and structures of the analogues are presented in Scheme 2. The absorption spectra of analogues **3**, **5** and **6** are quite similar to that reported for coelenterazine **4**.[11] The methyl substituent at the C-2 position causes a slight blue shift, while the phenyl substituent causes a red shift due to the resonance of the additional phenyl group with the parent imidazopyrazine moiety. The tendency of shifts in the fluorescence maximum is in accord with that observed in absorption.

Relative Chemiluminescence Yields and Rate Constants.—Chemiluminescence yields were obtained by integrating the total light emitted after injection of diethylene glycol dimethyl ether (DGM) containing 0.66% of acetate buffer (pH 5.60) into a solution of each of the analogues in methanol. The results are shown in Table 1 with the relative light yields expressed as a percentage of that of coelenterazine. The results demonstrate that the light yields for almost all of the synthetic analogues are larger than that of coelenterazine, except for analogue **2** which shows a negligible light emission. Chemiluminescence rate constants under similar conditions were obtained according to a published procedure[12] in which the rate constants are calculated based on pseudo first-order reaction kinetics. The results are given in Table 1. The results do not show a great deal of difference between the rate constants of the synthetic analogues and coelenterazine, except for analogue **2** which showed a much larger rate constant than the others, although the total light yield of **2** was far less than that of the others.

Chemiluminescence Spectra and Light Emitting Species.—The emission maxima of the chemiluminescence under a variety of conditions are listed in Table 1. In DMSO (dimethyl sulfoxide) containing 0.5% of 0.1 mol dm^{-3} aqueous sodium hydroxide the chemiluminescence wavelength maximum of 523–545 nm matched well the fluorescence wavelength maximum of the corresponding spent reaction mixture except in the case of analogue **2**. The slight difference in wavelength between fluorescence and chemiluminescence is due to the difference in experimental conditions. In order to identify the light emitting species, 3-benzyl-5-(*p*-hydroxyphenyl)-2-(phenylacetamido)-pyrazine **g3** was synthesized. Under experimental conditions similar to that for chemiluminescence, the fluorescence and excitation spectra of **g3** were quite similar to those of the spent reaction mixture of analogue **3**. A similar result has been reported by Cormier and co-workers[13] for the chemiluminescence of analogue **1** in *N*,*N*-dimethylformamide (DMF) containing potassium *tert*-butoxide, where both the chemiluminescence and fluorescence of the spent reaction mixture exhibit maxima at 530 nm. Cormier *et al.* attributed the light emitting species to the dianion of 2-(acetamido)-3-benzyl-5-(*p*-hydroxyphenyl)pyrazine **g1**. Considering the close similarity of the present results with those of Cormier *et al.*, we conclude that in the chemiluminescence of analogues **3**–**6** the light emitting species in DMSO containing 0.5% of 1 mol dm^{-3} sodium hydroxide is the dianion of the corresponding coelenteramide analogue.

The wavelength maxima for chemiluminescence and fluorescence are apparently dependent on the base concentration, *i.e.* the proton concentration in the media. The chemiluminescence spectrum of each coelenterazine analogue **1**–**6** (except **2**) shows an emission maximum at 411–422 nm in DGM containing 0.66% of 0.1 mol dm^{-3} acetate buffer (pH 5.60), which is identical to the fluorescence spectrum of the spent reaction mixture of the corresponding analogue. In contrast, the chemiluminescence maxima of the analogues in DMSO are at 470–474 nm, being different from the fluorescence maxima (408–410 nm) of the spent solutions of the analogues, which correspond to those of the neutral coelenteramide analogues.

These results may be interpreted on the basis of a chemiluminescence mechanism proposed by Goto,[14] *i.e.* the p*K*a value of 8.3 of coelenterazine suggests that the NH of the pyrazine group dissociates first to give an anion which is oxidized by molecular oxygen and decomposed *via* an aminodioxetanone intermediate into coelenteramide anion in a singlet excited state. In neutral DMSO the excited state of the anion emits light before it protonates and consequently the emitting species is the amide anion of coelenteramide as shown in Scheme 3,[14,15] whereas the fluorescence spectrum of the spent reaction mixture is the neutral form of coelenteramide. In DGM containing 0.66% of acetate buffer (pH 5.6), proton-transfer prior to light emission takes place in the excited state of the anion to give the excited state of neutral coelenteramide, while in DMSO containing 0.5% of 1 mol dm^{-3} aqueous sodium hydroxide not only the pyrazine NH but the phenolic hydroxy



Scheme 3

Table 1 Chemiluminescence of coelenterazine analogues

	Analogue					
	1	**2**	**3**	**4**	**5**	**6**
In DGM–Acetate						
Rate const./10^{-3} s^{-1}	1.9	4.9	2.2	2.7	1.4	1.8
Relative light yield	1.72	0.004	1.44	1.00	1.46	1.72
Φcl/10^{-5} [a]	4.1	0.009	3.4	2.3	3.4	4.0
CL_{max}(nm) [b]	411	(570) [d]	421	422	415	411
FL_{max}(nm) [c]	415	(570)	416	416	415	415
In DMSO						
CL_{max}(nm) [b]	470	(472, 575) [d]	472	474	471	472
FL_{max}(nm) [c]	408	(414, 556)	410	410	408	409
In DMSO–NaOH						
Φcl/10^{-4} [a]	10	0.004	7.2	7.1	8.8	8.9
CL_{max}(nm) [b]	523	(597) [d]	545	530	530	543
FL_{max}(nm) [c]	540	(547, 590)	543	547	538	538

[a] Quantum yields of chemiluminescence at 25 °C (reference luminol). [b] Chemiluminescence wavelength maxima (emission bandpath 40 nm). [c] Fluorescence wavelength maxima of the spent reaction mixtures (emission bandpath 5 nm). [d] Very weak and barely detectable emission maxima in parentheses.

group also dissociates at the first stage and the excited state of the dianion species (or trianion in the case of **4**) is formed in the process of oxidation. Under the alkaline conditions the dianion (or trianion) form is preferable both in the ground and in the excited states, since the fluorescence spectrum of the spent solution corresponds to that of the dianion species. These reactions are summarized in Schemes 3 and 4. This spectral behaviour is in accord with our previous interpretation in which the intensity of fluorescence of a neutral form relative to that of an anion form correlated linearly with the proton concentration in the media and the lifetime of the corresponding coelenteramide anion.[16]

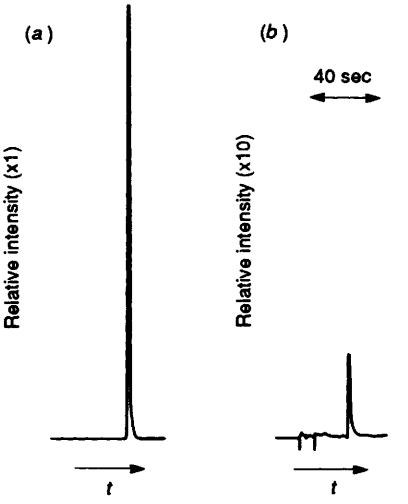
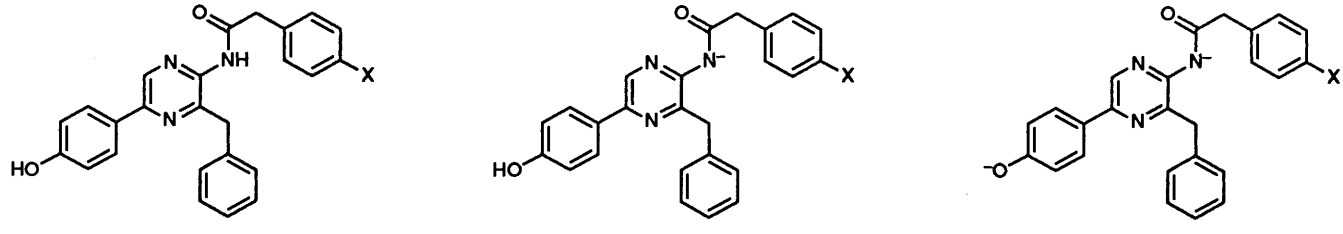


Fig. 1 Recording of calcium-triggered flash luminescence of a recombinant (wild type) aequorin (*a*) and a semi-synthetic aequorin containing a coelenterazine analogue **5** (*b*)

Bioluminescence Activities.—Apoaequorin was obtained by overexpressing the cDNA for apoaequorin in *Escherichia coli* and purifying (>95%) the expressed protein.[17] The coelenterazine analogue was incubated with the recombinant apoaequorin in TRIS–HCl buffer pH 7.60, containing 2-mercaptoethanol, dissolved oxygen, and EDTA.[18] Recombinant semi-synthetic aequorins were formed which exhibited bioluminescence in TRIS–HCl buffer pH 7.60 when calcium chloride was added. The flash patterns of the semi-synthetic aequorins triggered by calcium ion (Fig. 1) were virtually identical to that of recombinant (wild type) aequorin. This result suggests that the luminescence and regeneration mechanisms must be closely similar, if not the same, for all of the semi-synthetic aequorins studied. The relative luminescence activities of the recombinant aequorin and semi-synthetic aequorins are summarized in Table 2. From Table 2, it is evident that the hydroxybenzyl group at the C-2 position of coelenterazine(analogue **4**) gives the highest bioluminescence activity and its close structural analogue **3** also shows relatively high bioluminescence activity. In order to determine the reason for the low bioluminescence activities of the semi-synthetic aequorins containing coelenterazine analogues **1**, **2**, **5** and **6** relative to aequorin containing either coelenterazine or its close structural analogue **3**, the chemiluminescence activities of the analogues were compared with those of bioluminescence. The



Scheme 4

Table 2 Bioluminescence of coelenterazine analogues

	Analogue					
	1	2	3	4	5	6
Relative activity	0.01	0.00	0.37	1.00	0.01	0.02
Two-step incubation (%)	—	105	—	100	25	37

total light emitted by each analogue in DGM containing acetate buffer is summarized in Table 1. Since the chemiluminescence activities of analogues **1**, **3**, **5** and **6** are higher than that of coelenterazine, the low bioluminescence activities must be due to their low abilities to generate semi-synthetic aequorin and not to the luminescent activities of the chemically generated species in the singlet state. Analogue **1** has also been reported to chemiluminesce strongly in DMF and to luminesce with Renilla luciferase whose natural substrate is coelenterazine.[13,19]

Two-Step Incubation Procedure.—There are two possible explanations for the lack of light emission with analogue **2** in semi-synthetic aequorins, one is the failure of analogue **2** to generate aequorin and the other is a low chemiluminescence activity of **2**, even if the semi-synthetic aequorin with **2** can be regenerated. In order to clarify this point we carried out a two step experiment in which recombinant aequorin was regenerated by incubation with **2** in the presence of EDTA and 2-mercaptoethanol for 2 h, followed by incubation with coelenterazine for an additional 2 h, after which calcium chloride was added and luminescence activity measured.[20] If compound **2** can form semi-synthetic aequorin, *i.e.* occupy the active centre of apoaequorin and coelenterazine added could not form recombinant aequorin, the solution should show no or reduced luminescence activity, whereas if **2** is unable to interact with apoaequorin, the newly added coelenterazine would regenerate aequorin and the solution should emit light with the same intensity as recombinant aequorin. Experiments with analogues **5** and **6** were also carried out in a similar manner and the results are shown in Table 2.

In the case of semi-synthetic aequorins containing analogues **5** and **6** the emissions are dramatically reduced indicating that these compounds are not simply excluded but they occupy the active centre of the apoaequorin and cannot be easily displaced by the subsequent addition of coelenterazine. This conclusion is further supported by the findings shown in Table 2 that analogues **5** and **6** can form semi-synthetic aequorins, but their bioluminescence activities are extremely low. On the other hand, analogue **2** gave almost the same bioluminescence activity as coelenterazine, indicating that **2** either cannot be incorporated into semi-synthetic aequorin or, if incorporated, it is easily displaced by the coelenterazine added. Thus, we concluded in the series of phenyl homologues at the C-2 position, the benzyl group is of critical importance in eliciting optimal bioluminescence by coelenterazine.

Experimental

M.p.s were measured on a Yamato melting point apparatus MP-21 and are uncorrected. UV spectra were recorded with a Hitachi Model 320 spectrophotometer. ^{1}H NMR spectra were obtained with a JEOL JNM-GX 270 spectrometer or a Hitachi Model R-24 spectrometer, *J* values are given in Hz. In-beam EI mass spectra were determined with a Hitachi Model M-80B with a M-0101 mass data system. High resolution mass spectra were measured on a JEOL Model JMS-D300 with a JMS-2000 mass data system.

Bioluminescence intensity was measured with a Labo Science Model TD-8000 Lumiphotometer with a Pantos Nippon Denshi Kagaku Unicorder U-212 recorder. Chemiluminescence intensity was measured with a Labo Science Model TD-4000 Lumiphotometer.

Syntheses of Coelenterazine Analogues.—Coelenterazine and its analogues with various substituents at the C-2 position were synthesized by the synthetic routes of Goto and Kishi.[10,11] Pyruvic aldehyde **f1** and phenyl glyoxal **f2** were obtained from Tokyo Kasei Co. and used without further purification. 3-Benzyl-5-(*p*-hydroxyphenyl)pyrazin-2-amine **e** and 3-(*p*-hydroxyphenyl)-2-oxopropanal **f4** was prepared according to Shimomura *et al.*[7] 2-Oxo-3-phenylpropanal **f3** was synthesized according to Dakin and Dudley.[21] 2-Oxo-4-phenylbutanal **f5** and 2-oxo-5-phenylpentanal **f6** were prepared according to the method of Dakin and Dudley.[22]

General Method.—α-Oxophenylalkanal. Magnesium (0.9 g, 37.5 mmol) activated by heating under nitrogen was placed in a three-necked flask fitted with a dropping funnel, a refluxing condenser and a rubber stopper. Dried diethyl ether (16 cm^3) and phenylalkyl bromide (33 mmol, distilled under reduced pressure) were introduced into the flask. After 30 min the reaction ceased. The concentration of the RMgBr thus formed was determined to be 2.31 mmol cm^{-3} by titration. To a solution of diethoxyacetopiperidine[23] (5.4 g, 25.1 mmol) in dried diethyl ether, phenylalkylmagnesium bromide (10.5 cm^3) in diethyl ether (2.39 mmol cm^{-3}) was added dropwise at room temp. under nitrogen. After 30 min, saturated ammonium chloride solution (50 cm^3) was added and the mixture was extracted with diethyl ether. After removal of the solvent, the acetal of α-oxophenylalkanal was obtained as a colourless oil.

To a flask, fitted with a fractionating column connected to a condenser, aqueous α-oxophenylalkanal acetal (10.5 cm^3; 2.39 mmol cm^{-3}) and 2% H_2SO_4 (50 cm^3) were added. The mixture was heated under reflux for several hours while the ethanol formed in the reversible reaction was removed by fractional distillation, and the mixture extracted with diethyl ether. The organic layer was dried ($MgSO_4$) and evaporated to dryness, and the residue was distilled under reduced pressure.

2-Oxo-5-phenylpentanal **f6**; b.p. 84 °C/0.5 mmHg, yield 17%, $\delta_H(CDCl_3)$ 2.71 (2 H, td, *J* 7.0 and 1.1), 2.83 (2 H, t, *J* 7.3), 5.47 (1 H, t, *J* 7.2, enolic CH), 5.91 (1 H, br s, enolic OH), 7.10–7.36 (5 H, m) and 9.08 (1 H, s); *m/z* 176 (M^+, 6%), 174 (88) and 91 (100).

2-Oxo-4-phenylbutanal **f5**; b.p. 72 °C/0.5 mmHg, yield 21%, $\delta_H(CDCl_3)$ 3.72 (2 H, d, *J* 7.7), 5.65 (1 H, td, *J* 7.7 and 1.5, enolic CH), 6.03 (1 H, dd, *J* 1.1 and 1.1, enolic OH), 7.10–7.45 (5 H, m) and 9.14 (1 H, d, *J* 1.1); *m/z* 162 (M^+, 8%), 133 (41) and 91 (100).

4-(*p*-Acetoxyphenyl)-2-oxopropanal **f4**-acetate was prepared by using the synthetic route described in the literature.[24,25]

Coelenterazine Analogues.—A mixture of 3-benzyl-5-(*p*-hydroxyphenyl)pyrazin-2-amine **e** (0.173 mmol), 2-oxoaldehyde (0.260 mmol), ethanol (4 cm^3), and 36% aqueous hydrogen chloride (50 mm^3) was heated for 4 h with stirring at 80 °C. After cooling to room temp., the solvent was removed under reduced pressure. The residue was dissolved in methanol, separated by preparative TLC, eluted with methylene chloride–methanol (46/4 v/v) and crystallized from methanol.

8-*Benzyl*-6-*hydroxyphenyl*-2-*phenylimidazo*[1,2-a]*pyrazin*-3-*one* **2**. Yield 78%, m.p. 188–191 °C (decomp.) (Found: M^+ 393.1494. Calc. for $C_{25}H_{19}N_3O_2$; *M*, 393.1478); λ_{max}(MeOH)/nm 268 (log ε 4.39), 319 (4.15) and 470 (4.14); $\delta_H(CD_3COCD_3)$ 4.49 (2 H, s), 6.92 (2 H, d, *J* 8.8), 7.22–7.56 (11 H, m) and 8.61 (2 H, d, *J* 8.8); *m/z* 393 (M^+, 35%), 391 (3), 364 (57), 261 (100), 144 (9) and 115 (20).

8-*Benzyl*-6-*hydroxyphenyl*-2-(2-*phenylethyl*)*imidazo*[1,2-a]-

pyrazin-8-one **5**. Yield 52%, m.p. 131–134 °C (decomp.) (Found: M^+, 421.1805. Calc. for $C_{27}H_{23}N_3O_2$; *M*, 421.1790); λ_{max}(MeOH)/nm 260 (log ε 4.20), 350 (3.56) and 433 (3.77); $\delta_H(CD_3OD)$ 3.04–3.25 (4 H, m), 4.56 (2 H, s), 6.91 (2 H, d, *J* 8.8), 7.14–7.42 (11 H, m) and 7.73 (2 H, d, *J* 8.8); *m/z* 421 (M^+, 60%), 419 (77), 390 (20), 330 (92), 261 (100), 144 (22) and 115 (39).

8-*Benzyl-6-hydroxyphenyl-2-(3-phenylpropyl)imidazo*[1,2-a]-*pyrazin-3-one* **6**. Yield 40%, m.p. 100–103 °C (decomp.) (Found: M^+, 435.1950. Calc. for $C_{28}H_{25}N_3O_2$; *M*, 435.1947); λ_{max}(MeOH)/nm 260 (log ε 4.31), 351 (3.65) and 433 (3.94); $\delta_H(CD_3OD)$ 2.05–2.16 (2 H, m), 2.71 (2 H, t, *J* 7.7), 2.87 (2 H, t, *J* 7.7), 4.39 (2 H, s), 6.88 (2 H, d, *J* 8.8) and 7.09–7.51 (13 H, m); *m/z* 435 (M^+, 40%), 433 (48), 402 (18), 331 (49), 261 (100), 144 (20) and 115 (40).

Measurement of Bioluminescence.—A mixture of apoaequorin (100 ng), the coelenterazine analogue (1 μg mm^{-3} MeOH), 2-mercaptoethanol (1 mm^3) and EDTA (2 μmol dm^{-3}) in TRIS–HCl Buffer (pH 7.6, 200 mm^3) was allowed to incubate in an ice bath for 2 h. The regeneration mixture (50 mm^3) was injected with 4.5 μmol dm^{-3} $CaCl_2$ (1.5 cm^3) in TRIS–HCl buffer, pH 7.6, using a syringe and the maximum light intensity was recorded with the Lumiphotometer.

Measurement of Bioluminescence in a Two-step Procedure.—After the incubation of apoaequorin with the coelenterazine analogue for 2 h as mentioned above, coelenterazine (1 μg mm^{-3} MeOH) was added and allowed to incubate further for 2 h. Then 4.5 μmol dm^{-3} $CaCl_2$ in TRIS–HCl buffer (1.5 cm^3), pH 7.6, was injected into the mixture and the maximum light intensity was read as above.

Measurement of Chemiluminescence.—To a MeOH solution (0.6 μmol dm^{-3}) of the analogue (0.001 mol dm^{-3}), DGM (300 mm^3) containing 0.1 mol dm^{-3} acetate buffer (pH 5.6) (0.66%) or DMSO containing 0.5% of 1 mol dm^3 NaOH aqueous solution was added and the total light emission over a 1 h period was integrated using the Lumiphotometer.

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