A potential photoaffinity probe for labelling the active site of aequorin: a photolabile coelenterazine analogue with a trifluoromethyldiazirine group

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In order to clarify the active site of aequorin, we have successfully synthesized a photolabile analogue of coelenterazine with a trifluoromethyldiazirine group as a photoaffinity probe. Our studies on the chemi- and bio-luminescence of this novel analogue indicate that its behaviour is almost identical with that of natural coelenterazine in terms of luminescence characteristics. Therefore, the analogue with the photolabile diazirine should be a useful photoaffinity label for probing the detailed structure of aequorin.

Aequorin (AQ), a photoprotein isolated from the photogenic cells in the umbrella of *Aequorea victoria* (jellyfish), emits a blue light with a wavelength maximum at 469 nm to produce carbon dioxide and a blue fluorescent protein (BFP) upon addition of calcium ions.[1] Since AQ is a highly sensitive, selective calcium-dependent photoprotein, it has been widely used as an indicator for the presence of calcium ions in various biological systems.[2] Studies on this photoprotein demonstrate that it is a complex composed of apoaequorin (apo-AQ), molecular oxygen, and coelenterazine **1** (a chromophore), in which coelenterazine is tightly bound to the protein part through a peroxide bridge.[3–6] AQ and BFP can be regenerated by incubating apo-AQ with coelenterazine and coelenteramide, respectively, under appropriate conditions (Scheme 1).[6,7]

Cloning and sequence analysis of the cDNA for apo-AQ[8] and direct sequencing[9] of the protein indicate that it is composed of 189 amino acid residues (M_r 21 400) with three EF-hand structures characteristic of a calcium-binding site. Over expression[10] of the apo-AQ cDNA in *Escherichia coli* has been successful and by this means a large amount of a single isotype of recombinant apo-AQ can be obtained. This extends the applications of AQ in biological systems and also makes it much easier to study the molecular structure and bioluminescence mechanism of AQ.

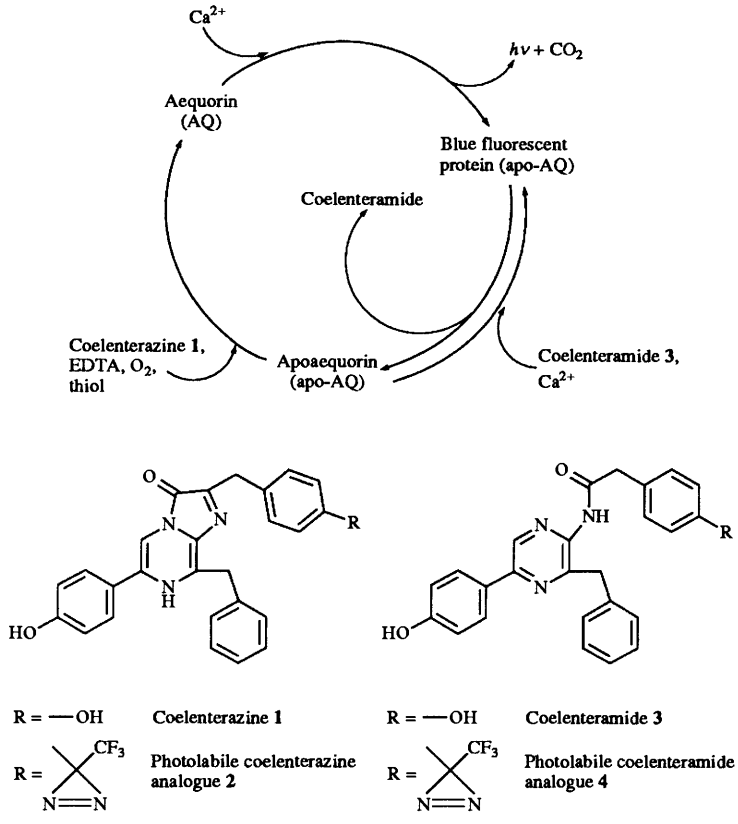
Recently the molecular structure, bioluminescence mechanism and structure–function relationships of AQ have been extensively studied by modification of the functional part[11] of coelenterazine chemically, or of apo-AQ[12] by means of the site specific mutagenesis technique. Despite many efforts, there are still numerous problems[5,10a,13] associated with AQ which remain to be clarified. Although a dioxetanone intermediate has been proposed to be involved in the bioluminescence reaction, the actual dioxetanone in the biological system has never been isolated experimentally. It has been suggested that the molecular oxygen in AQ exists in the form of a peroxide, however, the binding site of the oxygen atom in the protein is not known. It has been proposed that the coelenterazine is located in the hydrophobic region of AQ, but the detail is unknown and the binding mode and the binding site between coelenterazine and the protein are still unclear.

Our work focuses on the study of the active site in order to understand the detailed molecular structure of AQ. From investigations into the activity change of AQ modified by replacement of some of the amino acid residues at certain positions in the protein part, one can obtain some information about the active site. However, one problem associated with this approach is that it is difficult to decide whether a particular change is due to a specific interference with the binding ligand or to a change in the tertiary structure of the protein. Recently photoaffinity labelling[14] has become an attractive methodology for characterizing and identifying functional domains and active sites of biomacromolecules. If we can synthesize a photoaffinity coelenterazine analogue capable of regenerating a bioluminescent semi-synthetic AQ, then this can be used to help clarify the points raised above, since the primary structure of apo-AQ is known and large amounts of single recombinant apo-AQ can be obtained. Herein we present the results of studies on the synthesis and chemi- and bio-luminescence of a novel photolabile coelenterazine analogue with a trifluoromethyldiazirine group which has proved to be a useful photoaffinity probe for labelling the active site of AQ.

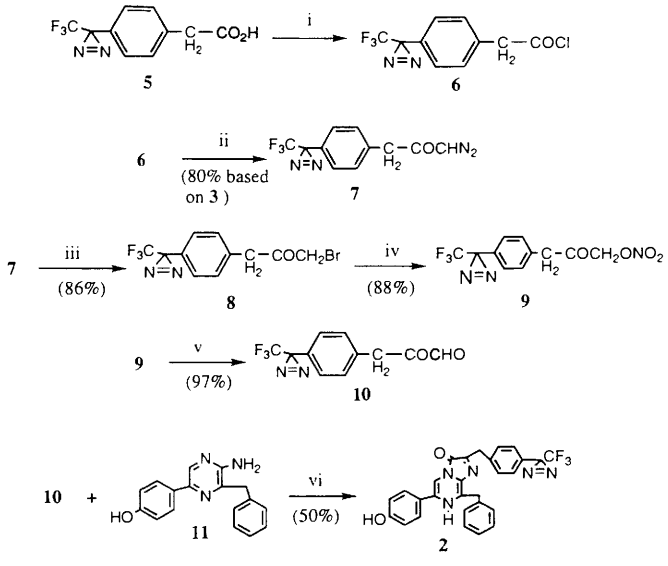
Results and discussion

Design and synthesis

The photoaffinity labelling technique requires the synthesis of a photolabile reagent that can occupy the same active site as the native substrate (*i.e.* it should have the same function as that of native substrate or inhibit the formation of a functional complex between the native substrate and a biomacromolecule when the photoaffinity reagent is added first). In addition it should produce a reactive intermediate, such as carbene and nitrene, which inserts rapidly into the amino acid residues of the biomacromolecule and tag the macromolecule to allow isolation and structural determination. Several photolabile candidates are widely utilized. Among these diazo ketones, diazo acetates, diazirines, and aromatic azides are typical examples. Of these moieties, diazirine has several advantages[15] over the others. Thus, diazirine has remarkable chemical stability, a long wavelength absorption and a relative ease of photolysis. The azido group usually produces a longer lifetime intermediate nitrene, which causes undesired side-reactions, whereas photolysis of the diazo group generally requires wavelengths shorter than 300 nm which are harmful to biological molecules. Furthermore, it is known that both diazo and azido groups are easily reduced by thiols, which are necessary to regenerate the semi-synthetic AQ. Studies on the effect of the substituents at the C-2 position of coelenterazine



Scheme 1 Regeneration and bioluminescence reactions of aequorin



Scheme 2 Synthesis of a photolabile analogue of coelenterazine with a diazirine group. *Reagents and conditions:* i, $SOCl_2$, benzene, room temp., 14 h; ii, CH_2N_2, diethyl ether, 0 °C, 1 h; iii, HBr, diethyl ether, 0 °C, 30 min; iv, $AgNO_3$, MeCN, room temp., 38 h; v, NaOAc, DMSO, room temp., 40 min; vi, EtOH containing aqueous HCl, 55 °C, 7.5 h.

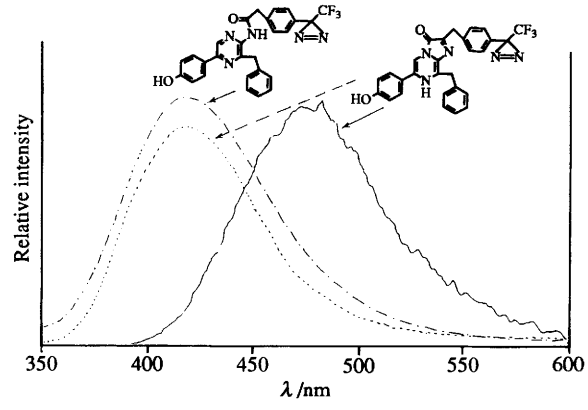


Fig. 1 Chemiluminescence spectra of the photolabile coelenterazine analogue **2** and fluorescence spectra of the spent solution of **2** and the synthetic corresponding amide **4** in DMSO. - · - · Fluorescence of the photolabile coeleteramide analogue **4**; – – – fluorescence of the spent solution of **2** in DMSO; — chemiluminescence of the photolabile coelenterazine analogue **2**.

on the bioluminescence activity of AQ indicate that the benzyl hydroxy group at the C-2 position can be modified chemically with retention of bioluminescence activity.[11b,d] Based on these results, a photolabile coelenterazine analogue was designed with a trifluoromethyldiazirine group in place of the benzyl hydroxy group at the C-2 position of coelenterazine.

The synthesis of the photolabile coelenterazine analogue **2** is shown in Scheme 2 and started from trifluoromethyldiazirine substituted phenylacetic acid **5** which was prepared by our previously established method.[16] Diazo ketone derivative **7** was prepared as follows: (i) the acid **5** was treated with thionyl chloride in benzene at room temperature for 14 h to afford its corresponding acid chloride **6**; (ii) **6** in diethyl ether was treated with diazomethane at 0 °C for 1 h to give the diazo ketone **7** in a high yield of 80% based on **5**. The diazo ketone **7** was dissolved in diethyl ether, and then a steady stream of dried hydrogen bromide was passed over it at 0 °C for 1 h. Separation of the reaction mixture yielded bromo ketone **8** in 86% yield. Transformation of **8** into the 2-oxopropyl nitrate derivative **9** was performed by treatment with silver nitrate in acetonitrile at room temperature for 38 h in a yield of 88%. 2-Oxo aldehyde **10** was obtained by treatment of **9** in dimethyl sulfoxide (DMSO) with sodium acetate, and the mixture was allowed to react for

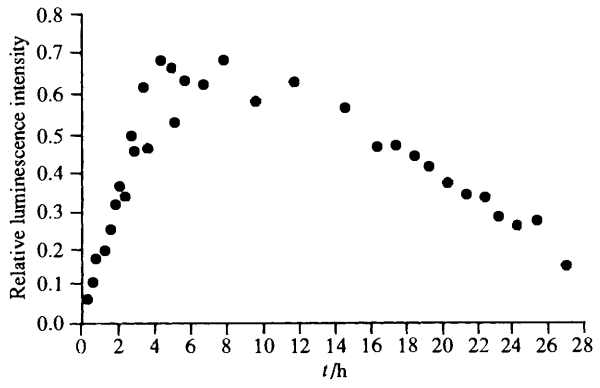


Fig. 2 Time course of regeneration of the semi-synthetic aequorin in the presence of dithiothreitol, photolabile coelenterazine analogue **2**, recombinant aequorin. *Regeneration:* a mixture of recombinant apoaequorin (5 μg), dithiothreitol (2 μmol), photolabile coelenterazine analogue (0.04 μmol) in Tris-HCl buffer (30 mmol dm^{-3}; 1.04 cm^3) at pH 7.6 containing EDTA (10 mmol dm^{-3}) was incubated in an ice bath for a certain time. *Assay:* the assay was carried out by injecting Tris-HCl buffer (30 mmol dm^{-3}; 400 mm^3) at pH 7.6 containing $CaCl_2$ (30 mmol dm^{-3}) into regenerated semi-synthetic aequorin solution (10 mm^3) and the maximum light intensity was recorded using a lumiphotometer (Labo Science TS-1000).

an additional 40 min. The desired product was obtained as a yellowish solid (crude yield 97%). Finally coupling of **10** with coelenteramine[17] **11** in ethanol containing aqueous HCl at 55 °C for 7.5 h yielded the expected product **2** in 50% yield. **2** was identified by the spectral data; the IR and NMR are consistent with the structure (see Experimental section). The UV–VIS spectrum of **2** shows three peaks at 260, 350 and 435 nm in methanol, quite similar to that of native coelenterazine. The absorption band around 435 nm is characteristic of the basic skeleton imidazopyrazinone of coelenterazine. The positive-ion FAB MS shows a quasi molecular peak at 516 $(M + 1)^+$ and a peak at 488 $(M + 1 - N_2)^+$ clearly indicating the presence of diazirine group.

Chemiluminescence

A chemiluminescence study on **2** was carried out to examine the effect of the diazirine group on the luminescence behaviour and kinetics. Chemiluminescence of **2** was triggered upon addition of DMSO. The chemiluminescence spectrum (λ_{max} 475 nm) of **2** is not consistent with either the fluorescence spectrum of its spent solution after the reaction or the synthesized photolabile amide[16] **4**; the last two have superimposed emission bands with the same maximum wavelength at λ_{max} 418 nm (Fig. 1). These results coincide with those of native coelenterazine in similar conditions:[11d] the chemiluminescence spectrum centred at 475 nm is attributed to the emission from the anion of the corresponding amide which is formed initially through the decomposition of an intermediate dioxetanone, and emits light before it protonates in the singlet excited state, whereas the fluorescence around 418 nm is assigned to the neutral form of the amide.[18] This conclusion was further supported by the observation of consistency between the absorption spectrum of **4** and the absorption spectrum and the excitation spectrum of the spent solution of **2** after completion of the luminescence reaction under the same conditions.

In order to obtain direct experimental evidence of the nature of the product of the luminescence reaction in DMSO and to investigate the stability of the photolabile diazirine group in the reaction, isolation of the products was performed by pouring the reaction mixture into ice–water, saturated with sodium chloride, and then extracting it with ethyl acetate. After purification, **4** was obtained in *ca.* 90% yield without damage to the diazirine group, which was identified by its identical spectral data with those of the sample synthesized by an independent method.

A pseudo first-order dependence of light production on the amount of **2** was observed in systems of both DMSO and diethylene glycol dimethyl ether (DGM) containing 0.66% of 0.1 mol dm^{-3} acetate buffer (pH 5.6) at 25 °C under air. The chemiluminescence rates were obtained by Goto's procedure[19] to be k_{DMSO} 1.9×10^{-2} s^{-1} and k_{DGM} 2.6×10^{-3} s^{-1}, respectively which are quite close to those of native coelenterazine (k_{DMSO} 2.7×10^{-2} s^{-1} and k_{DGM} 2.7×10^{-3} s^{-1}).

The chemiluminescence activity of **2** in DMSO was recorded to be 10% of that of native coelenterazine by integrating the total emitted light. Since conversion of **2** into **4** in the chemiluminescence reaction occurs in a high yield, the lower chemiluminescence activity of **2** possibly results from a lower fluorescence quantum yield of its corresponding amide **4**. To investigate this point, the quantum yield of the fluorescence of **4** in DMSO was measured by integrating the spectral emission range and was shown to be 12% of that of native coelenteramide **3** in the same solvent (Table 1). This result gives experimental evidence to support the hypothesis above.

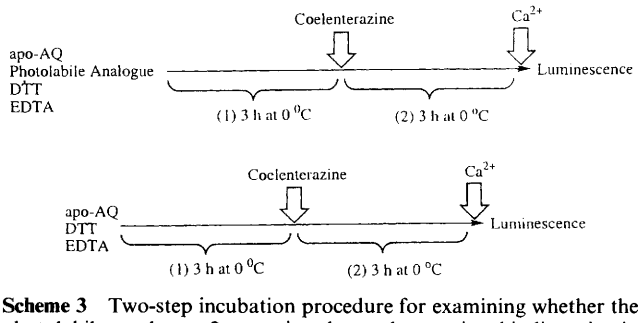
From the results above, we conclude that the chemiluminescence of **2** in DMSO follows the same mechanism as that of native coelenterazine. Although the substitution of the benzyl hydroxy group with a trifluoromethyldiazirine group at the C-2 position of coelenterazine leads to a reduction in luminescence activity, it does not change the chemiluminescence behaviour and kinetics.

Bioluminescence

Whether the synthetic photolabile coelenterazine analogue **2** can be used as a photoaffinity probe for labelling the active site of AQ is determined by its ability to regenerate a bioluminescent semi-synthetic AQ and to occupy the same binding site as native coelenterazine. In order to investigate this point, regeneration reactions and bioluminescence of **2** were studied. A mixture of recombinant AQ,[20] dithiothreitol (DTT), photolabile coelenterazine analogue **2** in tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) buffer at pH 7.6 containing ethylenediamine-*N*,*N*,*N*,*N*-tetraacetic acid (EDTA) was incubated in an ice bath for a certain time and then the bioluminescence activity was measured by adding a solution of $CaCl_2$ to the regenerated semi-synthetic AQ solution. As expected, it emits light with a flash pattern identical with that of the native one. The time course of regeneration (Fig. 2) shows that it takes about 4 h to reach a maximum light intensity, and that its bioluminescence activity is 0.7% of that of the native AQ under similar conditions.

Two-step incubation procedure

To clarify what factor causes the low bioluminescence activity of **2**, a two-step incubation experiment was carried out (Scheme 3) using the following procedure.



Scheme 3 Two-step incubation procedure for examining whether the photolabile analogue **2** occupies the coelenterazine binding site in aequorin

Table 1 Luminescence properties of the photolabile coelenterazine analogue **2** and its corresponding amide **4** (for comparison the properties of native products **1** and **3** are also listed)

	CL_{max} in DMSO (nm)[a]	FL_{max} in DMSO (nm)[b]	Relative CL yield[c]	Relative FL quantum yield[d]	Rate constant $k_{DMSO}/10^{-2}$ s^{-1}	Rate constant $k_{DGM}/10^{-3}$ s^{-1}	Relative BL activity[e]	Relative BL activity in a two-step incubation procedure
1	474		100		2.7	2.7	100	100[f]
2	475		10		1.9	2.6	0.7	15[g]
3		410		100				
Spent solution after luminescence of **1**		410						
4		418		12				
Spent solution after luminescence of **2**		418						

[a] Chemiluminescence (CL) wavelength maximum (emission bandpass 40 nm). [b] Fluorescence (FL) wavelength maximum (emission bandpass 5 nm). [c] Relative Chemiluminescence yield obtained by integrating the total emitted light after injection of DMSO. [d] Relative fluorescence quantum yield in DMSO by integrating the spectral emission range of the corresponding amide. [e] Relative bioluminescence (BL) activity. [f] Data was obtained without addition of **2** at the first step in the two-step incubation procedure. [g] Data was obtained with addition of **2** at the first step in the two-step incubation procedure.

After incubation of a mixture of recombinant AQ in Tris-HCl buffer at pH 7.6 containing EDTA, DTT and photolabile analogue **2** for 3 h in an ice bath, coelenterazine **1** was added to the regenerated semi-synthetic AQ solution, and it was incubated for an additional 3 h at this temperature, after which a solution of $CaCl_2$ in Tris-HCl buffer was added to it, and the luminescence was immediately measured. For comparison, a control experiment was performed in which **2** was not added at the first step in the two-step incubation procedure. If **2** had occupied the same active site of the photoprotein as with coelenterazine, then the coelenterazine subsequently added could not form recombinant AQ and thus the solution should show no or reduced luminescence activity, whereas if **2** is unable to interact with apo-AQ, the newly added coelenterazine would regenerate AQ with a luminescence activity equal to that of recombinant AQ in the control experiment. The experimental result indicates that the bioluminescence activity measured in the two-step procedure in the presence of **2** is only 15% of that measured in the absence of **2**. This fact clearly demonstrates that the photolabile coelenterazine analogue **2** is incorporated into the native chromophore binding site, and occupies a statistical average of 85% of the active site of the photoprotein. The lower bioluminescence activity of **2** is mainly due to its lower luminescence ability since **2** has a lower chemiluminescence activity in DMSO as mentioned above. The relatively low luminescence activity of **2** comes from the lower fluorescence quantum yield of its corresponding amide, rather than occupation of a different binding site in the protein.

The two-step incubation procedure was also applied to the photolabile coelenteramide analogue **4** to examine whether a semi-synthetic BFP containing **4** can be obtained or not. The experimental result demonstrates that **4** is able to regenerate the semi-synthetic BFP and occupy the same binding site as native coelenterazine, since the bioluminescence activity of AQ was completely lost when **4** was added at the first step in the two-step incubation procedure.

Photolysis of 2 in methanol

A solution of **2** in methanol was irradiated by means of a 500 W xenon lamp (cut-off filter for $\lambda < 330$ nm). After irradiation for 13 min **2** disappeared and several components were formed as observed by reversed-phase HPLC. The expected OH insertion product of **2** was not obtained, instead, the OH insertion product of the corresponding amide was identified as the main product along with a small amount of coelenteramine by comparing the data of mass spectrometry and the analysis of reversed-phase HPLC of the reaction mixture with those of the corresponding synthetic standard samples.

Although direct photolysis of **2** causes damage to the imidazolone skeleton of coelenterazine, an alternative method is available in a practical photoaffinity labelling experiment. This can be done by photolysis of the semi-synthetic AQ derived from **2** immediately after the completion of the bioluminescence reaction, since we have already demonstrated that photolysis[16] of the photolabile coelenteramide analogue **4** gave a high yield of OH insertion product (62%) and we also know that the conversion of **2** into **4** in the luminescence reaction was very high yielding (90%) and that a semi-synthetic BFP derived from **4** was indeed formed as mentioned above.

In summary, we have successfully synthesized a photolabile coelenterazine analogue **2**. The results from the studies on its chemi- and bio-luminescence demonstrate that **2** shows the luminescence behaviour and kinetics almost identical with those of native coelenterazine and is proved to be a potential photoaffinity probe for labelling the active site of AQ. Studies to clarify the active site of AQ using a photolabile analogue are in progress.

Experimental

Mps were determined with a Yamato Model MP-21 apparatus and are uncorrected. IR (infrared) spectra were recorded on a JASCO IR-810 spectrometer. Nuclear magnetic resonance spectra (^{1}H and ^{13}C NMR) were recorded on a JEOL JNM-GX 270 spectrometer; chemical shifts are reported relative to internal tetramethylsilane (TMS, δ 0.00) or the centre line of the deuteriated solvent used and coupling constants (J) are given in Hz. Negative-FAB mass spectra were recorded with a Finnigan MAT TSQ-700 mass spectrometer using *m*-nitrobenzyl alcohol (MNBA) as matrix, and high resolution mass spectra (EI mode) were obtained using a Hitachi Model M-80B mass spectrometer with a Hitachi M-0101 data system. UV–VIS spectra were recorded on a Hitachi Model 320 spectrophotometer. Fluorescence and chemiluminescence spectra were measured with a Hitachi Model F-4010 fluorescence spectrophotometer, and in the case of chemiluminescence measurement with the xenon lamp turned off. The measurement of relative chemiluminescence activities was performed on a Labo Science Model TS-4000 lumiphotometer. Relative bioluminescence activities were obtained using a Labo Science Model TS-1000 lumiphotometer.

Moisture and oxygen-sensitive reactions were run in pre-dried glassware under an inert atmosphere. Reagents used were generally commercial grade, and used as supplied unless otherwise stated. Solvents used were generally distilled or dried followed by distillation by standard procedures when necessary.

Coelenteramine **11** was synthesized by Kishi *et al.*'s method.[17] Coelenterazine **1** was prepared by a slight modification of a known procedure.[21] Preparation of coelenteramide **3** and its photolabile analogue **4** followed our previously established method.[16]

Measurements of chemiluminescence and fluorescence spectra
The chemiluminescence spectrum of the photolabile coelenterazine analogue **2** was measured immediately after the addition of DMSO (1.5 cm^3) to **2** (50 mm^3)† in methanol by means of a fluorescence spectrophotometer with the xenon lamp off (emission bandpass: 40 nm). The fluorescence spectrum of the spent solution of **2** after the luminescence reaction was measured with the same instrument (λ_{em} 330 nm, emission and excitation bandpass: 5 and 5 nm, respectively).

Relative chemiluminescence activities and rate constants
Relative chemiluminescence activities of **2** in DMSO and DGM containing 0.66% acetate buffer (0.1 mol dm^{-3}) at pH 5.6 were recorded by injecting the solvent (300 mm^3) into a solution of **2** in methanol (1.0×10^{-3} mol dm^{-3}; 0.6 mm^3) and the total emitted light was integrated using a lumiphotometer (coelenterazine was used as a standard). Rate constants of chemiluminescence in the two systems were obtained according to Goto's procedure.

Measurements of bioluminescence
A mixture of recombinant apo-AQ (5 μg), dithiothreitol (2 μmol), photolabile coelenterazine analogue **2** (4.0×10^{-2} μmol) in Tris-HCl (30 mmol dm^{-3}; 1.04 cm^3) buffer at pH 7.6 containing EDTA (10 mmol dm^{-3}) was incubated in an ice bath for 4 h. Bioluminescence was measured by injecting aq. $CaCl_2$ (30 mmol dm^{-3}; 400 mm^3) into the regenerated semi-synthetic AQ solution (10 mm^3) using a lumiphotometer. The relative bioluminescence activity of **2** was calculated by applying coelenterazine as a standard under the same conditions.

Two step incubation procedure
After incubation of a mixture of recombinant AQ in Tris-HCl buffer (30 mmol dm^{-3}) at pH 7.6 containing EDTA (10 mmol dm^{-3}; 1 cm^3, 5 μg cm^{-3}), DTT (20 mm^3) in the buffer above (0.1 mol dm^{-3}) and **2** in methanol (2 mmol dm^{-3}; 20 mm^3) for 3 h in an ice bath, native coelenterazine in methanol (2 mmol dm^{-3}; 20 mm^3) was added to the regenerated semi-synthetic AQ, and it was incubated for an additional 3 h at this temperature. After which, to the incubated solution (10 mm^3) was added Tris-HCl buffer (30 mmol dm^{-3}; 400 mm^3) containing $CaCl_2$ (30 mmol dm^{-3}), and the emitted light was immediately recorded. A control experiment, in which the first step in the two-step incubation procedure without the addition of **2** was carried out under the same conditions.

Isolation of products from the spent solution of 2 after the chemiluminescence reaction in DMSO
The photolabile coelenterazine analogue **2** (7.9 mg) was dissolved in DMSO (10 cm^3) and oxygen was bubbled through the resulting solution to accelerate the chemiluminescence reaction at room temperature. After 2.5 h the reaction mixture was poured into ice–water, which was then saturated with sodium chloride and the resulting mixture was extracted with ethyl acetate. The organic phase was washed with water, dried over anhydrous sodium sulfate and then the solvent was removed by evaporation. The residue was purified by PTLC (silica gel, hexane–acetone 3:2) to yield the photolabile coelenteramide analogue **4** (7 mg, 90%), which was identified by comparing its spectral data with those of the sample[16] prepared by an independent method.

† 1 $mm^3 \equiv 1$ μL.

Photolysis of 2 in methanol
Compound **2** (0.8 mg) was dissolved in methanol (1.5 cm^3) and the resulting solution was put in a quartz tube and then irradiated with a xenon lamp (Ushio Model UI-50IC, 500 W, cut-off filter for $\lambda < 330$ nm) at 0 °C. The reaction was monitored by reversed-phase HPLC (JASCO 880-PU Intelligent HPLC pump connected to a Shimadzu C-R6A chromatopac with a JASCO UVIDEC-100-IV spectrophotometer; column: Intertsil-ODS 4.6 × 250 mm; eluent: methanol–water, 80:20; flow rate: 0.9 cm^3 min^{-1} detected at $\lambda = 300$ nm). The starting material disappeared after irradiation for 13 min.

4-(3-Trifluoromethyldiazirin-3-yl)phenylacetyl chloride 6
A solution of 4-(3-trifluoromethyldiazirin-3-yl)phenylacetic acid[16] **5** (488 mg, 2 mmol) in benzene (2.5 cm^3) was cooled to −5 °C and then thionyl chloride (500 mm^3) was added dropwise to it. The reaction mixture was left to stand for 14 h, and then the solvent was removed by distillation under reduced pressure. The residue was further dried *in vacuo* to afford the crude acid chloride **6**, which was used for the next reaction without further purification, δ_H(270 MHz; $CDCl_3$) 4.17 (2 H, s) and 7.20 (2 H, d, *J* 8.1) and 7.32 (2 H, d, *J* 8.1, ArH).

1-Azo-3-[4-(3-trifluoromethyldiazirin-3-yl)phenyl]acetone 7
Acid chloride **6** in diethyl ether (8 cm^3) was added dropwise to a solution of an excess of diazomethane (20 cm^3) in diethyl ether at 0 °C. The reaction was maintained at this temperature for 50 min, and then the solvent was evaporated. The residue was purified by short column chromatography (silica gel, chloroform) to yield the desired product **7** as an oil (426 mg, 80% based on the starting material **5**), ν_{max}(neat)/cm^{-1} 2700–3200, 2100, 1640, 1515, 1518, 1420, 1360, 1345, 1230, 1180, 1150, 1075 and 1050; δ_H(270 MHz; $CDCl_3$) 3.62, (2 H, s), 5.15 (1 H, s) and 7.17 (2 H, d, *J* 8.4) and 7.28 (2 H, d, *J* 8.4, ArH); δ_C(67.9 MHz; $CDCl_3$) 28.3 (q, *J* 40.3), 47.3, 55.1, 122.1 (q, *J* 274.9), 126.9, 128.2, 129.9, 136.3 and 190.4; *m/z* (negative-FAB) 267 [$(M - 1)^-$] and 239 [$(M - 1 - N_2)^-$] [Found: $(M - N_2)^+$, 240.0500. $C_{11}H_7F_3N_2O$ requires $M - N_2$, 240.0509].

1-Bromo-3-[4-(3-trifluoromethyldiazirin-3-yl)phenyl]acetone 8
A steady stream of dried hydrogen bromide was passed over diazo ketone **7** (300 mg, 1.12 mmol) in diethyl ether (6 cm^3) at 0 °C for 30 min with stirring. The resulting mixture was washed with water, aqueous sodium hydrogen carbonate and water. After removal of the solvent, the residue was purified by column chromatography (silica gel, diethyl ether–hexane, 1:3) providing the expected product **8** as a yellowish solid (310 mg, 86%), mp 50–51 °C; ν_{max}(KBr)/cm^{-1} 3000–3200, 2940, 2880, 1738, 1605, 1520, 1400, 1385, 1345, 1323, 1230, 1180, 1140, 1050 and 1040; δ_H(270 MHz; $CDCl_3$) 3.91 (2 H, s), 3.99 (2 H, s) and 7.18 (2 H, d, *J* 8.4) and 7.27 (2 H, d, *J* 8.4, ArH); δ_C(67.9 MHz; $CDCl_3$) 28.3 (q, *J* 40.3), 33.4, 46.0, 122.1 (q, *J* 274.9), 126.9, 128.4, 130.0, 134.8 and 198.7; *m/z* (negative-FAB) 319 [$(M - 1)^-$], 321 [$(M + 2 - 1)^-$], 291 [$(M - 1 - N_2)^-$] and 293 [$(M + 2 - 1 - N_2)^-$] [Found: $(M - N_2)^+$, 291.9723. $C_{11}H_8BrF_3O$ requires $M - N_2$, 291.9711].

2-Oxo-3-[4-(3-trifluoromethyldiazirin-3-yl)phenyl]propyl nitrate 9
A solution of silver nitrate (117 mg, 0.69 mmol) in acetonitrile (0.6 cm^3) was added to a solution of bromo ketone **8** (100 mg, 0.3 mmol) in acetonitrile (0.35 cm^3). The mixture was stirred at room temperature for 18 h and then filtered. Water and diethyl ether were added to the solution. After filtration, the organic phase was dried over anhydrous magnesium sulfate and then the solvent was removed by distillation under reduced pressure. The ^1H NMR spectrum of the residue showed that the expected

product **9** was obtained almost pure (83 mg, 88%). The product was recrystallized from hexane to give colourless needles, mp 56–57 °C; ν_{max}(KBr)/cm^{-1} 3000–3100, 3000, 2920, 1735, 1645, 1635, 1520, 1410, 1385, 1343, 1330, 1285, 1230, 1185, 1140, 1080, 1045 and 1025; δ_H(270 MHz; $CDCl_3$) 3.81 (2 H, s), 4.97 (2 H, s) and 7.19 (2 H, d, *J* 8.4) and 7.27 (2 H, d, *J* 8.4, ArH); δ_C(67.9 MHz; $CDCl_3$) 28.2 (q, *J* 41.2), 45.6, 73.3, 122.0 (q, *J* 274.9), 127.1, 128.7, 129.9, 133.5 and 198.1; *m*/*z* (negative-FAB) 302 [(M − 1)$^-$] and 274 [(M − 1 − N_2)$^-$] [Found: (M − N_2)$^+$, 275.0436. $C_{11}H_8F_3NO_4$ requires *M* − N_2, 275.0405].

2-Oxo-3-[4-(3-trifluoromethyldiazirin-3-yl)phenyl]propanol 10

A solution of 2-oxopropyl nitrate **9** (61 mg, 0.2 mmol) in DMSO (2 cm^3) was treated slowly with sodium acetate trihydrate (28 mg). The resulting solution was stirred at room temperature for 40 min and then poured into ice–water. The resulting mixture was saturated with sodium chloride and then extracted with diethyl ether. The organic phase was washed with water, aqueous sodium hydrogen carbonate and then again with water. Removal of the solvent by distillation under reduced pressure followed by drying *in vacuo* gave the almost pure expected product **10** (yellowish solid, 50 mg, 97%), which was used for the next reaction without further purification, mp 88–90 °C; ν_{max}(KBr)/cm^{-1} 2700–3700, 1670, 1640, 1615, 1515, 1410, 1375, 1340, 1310, 1230, 1195, 1185 and 1163; δ_H(270 MHz; $CDCl_3$) 6.16 (1 H, s), 6.70 (1 H, s), 7.21 (2 H, d, *J* 8.4) and 7.86 (2 H, d, *J* 8.9, ArH) and 9.27 (1 H, s); δ_C(67.9 MHz; $CDCl_3$) 28.5 (q, *J* 41.2), 120.6, 122.0 (q, *J* 274.9), 126.6, 129.7, 130.5, 134.8, 149.3 and 188.1; *m*/*z* (negative-FAB) 255 [(M − 1)$^-$] and 227 [(M − 1 − N_2)$^-$] [Found: (M − N_2)$^+$, 228.0384. $C_{11}H_7F_3O_2$ requires *M* − N_2, 228.0397].

8-Benzyl-6-(4-hydroxyphenyl)-2-[4-(3-trifluoromethy(diazirin-3-yl)benzyl]-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one 2

A mixture of 2-oxo aldehyde **10** (54 mg, 0.21 mmol), coelenteramine **11** (41 mg, 0.15 mmol), ethanol (3.3 cm^3), 36% aqueous HCl (5 mm^3) and water (200 mm^3) was heated at 55 °C for 7.5 h under argon. After cooling the mixture to room temperature, the solvent was removed by evaporation and the residue further dried *in vacuo*. The residue was purified by column chromatography (silica gel, first with dichloromethane–methanol, 66:4 and then with ethyl acetate–methanol, 100:5) to yield the title compound **2** along with unchanged starting material **11** (20 mg). Further precipitation of the desired product from ethyl acetate–hexane gave a yellow powder (19.4 mg, 50%), mp 112–115 °C (decomp.); ν_{max}(KBr)/cm^{-1} 2200–3700, 1700, 1665, 1625, 1610, 1570, 1515, 1490, 1450, 1380, 1340, 1270, 1230, 1175, 1155, 1080, 1050 and 1030; λ_{max}(MeOH)/nm 260 (log ε 4.19), 350 (3.53) and 435 (3.71); δ_H(270 MHz; CD_3OD) 4.20 (2 H, s), 4.40 (2 H, s), 6.88 (2 H, d, *J* 8.4) and 7.05–7.80 (12 H, m); *m*/*z* (negative-FAB) 514 [(M − 1)$^-$] and 486 [(M − 1 − N_2)$^-$] [Found: (M − N_2)$^+$, 487.1430. requires $C_{28}H_{20}F_3N_3O_2$ *M* − N_2, 487.1506].

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References

1 O. Shimomura, F. H. Johnson and Y. Saiga, *J. Cell Comp. Physiol.*, 1962, **59**, 223.
2 (*a*) E. B. Ridgway and C. C. Ashley, *Biochem. Biophys. Res. Commun.*, 1967, **29**, 229; (*b*) J. R. Blinks, W. G. Eier, P. Hess and F. G. Prengergast, *Prog. Biophys. Mol. Biol.*, 1982, **40**, 1; (*c*) P. H. Cobbold and T. J. Rink, *Biochem. J.*, 1987, **248**, 229; (*d*) O. Shimomura, B. Musicki and Y. Kishi, *Biochem. J.*, 1989, **216**, 913; (*e*) M. R. Knight, A. K. Campbell, S. M. Smith and A. J. Trewavas, *Nature (London)*, 1991, **352**, 524; (*f*) J. Nakajima-Shimada, H. Iida, F. I. Tsuji and Y. Anyaku, *Proc. Natl. Acad. Sci. USA*, 1991, **88**, 6878.
3 O. Shimomura, F. H. Johnson and Y. Saiga, *Science*, 1963, **140**, 1339.
4 J. R. Blinks, P. H. Mattingly, B. R. Jewell, M. van Leeuwen, G. L. Harrer and D. G. Allen, *Meth. Enzymol.*, 1978, **57**, 292.
5 O. Shimomura and F. H. Johnson, *Proc. Natl. Acad. Sci. USA*, 1978, **75**, 2611.
6 O. Shimomura and F. H. Johnson, *Nature (London)*, 1975, **256**, 236.
7 O. Shimomura and F. H. Johnson, *Tetrahedron Lett.*, 1973, 2963.
8 S. Inouye, M. Noguchi, Y. Sakaki, Y. Takaki, T. Miyata, S. Iwanaka, T. Miyata and F. I. Tsuji, *Proc. Natl. Acad. Sci. USA*, 1985, **82**, 3154.
9 H. Charbonneau, K. A. Walsh, R. O. McCann, F. G. Prendergast, M. J. Cormier and T. C. Vanaman, *Biochemistry*, 1985, **24**, 6762.
10 (*a*) D. C. Prasher, R. O. McCann and M. J. Cormier, *Biochem. Biophys. Res. Commun.*, 1985, **12**, 1259; (*b*) S. Inuuye, Y. Sakaki, T. Goto and F. I. Tsuji, *Biochemistry*, 1986, **25** 8425; (*c*) S. Inouye, S. Aoyama, T. Miyata, F. I. Tsuji and Y. Sakaki, *J. Biochem.*, 1989, **10**, 473.
11 (*a*) O. Shimomura, B. Musicki and Y. Kishi, *Biochem. J.*, 1988, **251**, 405; (*b*) O. Shimomura, B. Musicki and Y. Kishi, *Biochem. J.*, 1989, **261**, 913; (*c*) F. Q. Chen, Y. Gomi, M. Ohashi, Y. Ohmiya and F. I. Tsuji, *J. Chem. Soc., Chem. Commun.*, 1991, 1307; (*d*) F. Q. Chen, Y. Gomi, T. Hirano, M. Ohashi, Y. Ohmiya and F. I. Tsuji, *J. Chem. Soc., Perkin Trans. 1*, 1992, 1607; (*e*) Y. Ohmiya, K. Teranishi, M. Akutagawa, M. Ohashi and F. I. Tsuji, *Chem. Lett.*, 1993, 2149.
12 (*a*) F. I. Tsuji, S. Inouye, T. Goto and Y. Sakaki, *Proc. Natl. Acad. Sci. USA*, 1986, **83**, 8107; (*b*) S. Inouye, Y. Sakaki and F. I. Tsuji, *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 80; (*c*) K. Kurose, S. Inouye, S. Sakaki and F. I. Tsuji, *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 80; (*d*) Y. Ohmiya, M. Ohashi and F. I. Tsuji, *FEBS Lett.*, 1992, **301**, 197.
13 (*a*) K. Hori, J. M. Anderson, W. W. Ward and M. J. Cormier, *Biochemistry*, 1975, **14**, 2371; (*b*) K. Teranishi, M. Isobe, T. Yamata and T. Goto, *Tetrahedron Lett.*, 1992, **33**, 1303; (*c*) K. Teranishi, M. Isobe and T. Yamata, *Tetrahedron Lett.*, 1994, **35**, 2565; (*d*) K. Teranishi, K. Ueda, H. Nakao, M. Hisamatsu and T. Yamata, *Tetrahedron Lett.*, 1994, **35**, 8181.
14 (*a*) H. Bayley and J. R. Knowles, *Meth. Enzymol.*, 1977, **46**, 69; (*b*) R. J. Guillory, *Pharmac. Ther.*, 1989, **41**, 1; (*c*) T. A. Nakayama and H. G. Khirana, *J. Biol. Chem.*, 1990, **265**, 15762; (*d*) A. J. Chavan, C. M. Ensor, P. Wu, B. E. Haley and H. H. Tai, *J. Biol. Chem.*, 1993, **268**, 16437; (*e*) H. Zhang, K. A. Lerro, T. Yamato, T. H. Lien, L. Sastry, M. A. Gawinowicz and K. Nakanishi, *J. Am. Chem. Soc.*, 1994, **116**, 10165.
15 (*a*) R. A. G. Smith and J. R. Knowles, *J. Chem. Soc., Perkin Trans. 2*, 1975, 686; (*b*) G. F. Bradley, B. L. Evans and I. D. R. Stevens, *J. Chem. Soc., Perkin Trans. 2*, 1977, 1214; (*c*) M. Nassal, *Liebigs Ann. Chem.*, 1983, 1510.
16 F. Q. Chen, T. Hirano, M. Ohashi, H. Nakayama, K. Oda and M. Machida, *Chem. Lett.*, 1993, 287.
17 Y. Kishi, H. Tanino and T. Goto, *Tetrahedron Lett.*, 1972, 2747.
18 T. Hirano, Y. Gomi, T. Takahashi, K. Kitahara, F. Q. Chen, I. Mizoguchi, S. Kyushin and M. Ohashi, *Tetrahedron Lett.*, 1992, **33**, 5771.
19 T. Goto, *Pure Appl. Chem.*, 1968, **17**, 421.
20 I. Inouye, S. Zenno, Y. Sakaki and F. I. Tsuji, *Protein Expression Purif.*, 1991, **2**, 122.
21 S. Inoue, S. Sugiura, H. Kakoi, K. Hasizume, T. Goto and H. Iio, *Chem. Lett.*, 1975, 141.

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