Chemi- and Bio-luminescence of Coelenterazine Analogues. Effect of Substituents at the C-2 Position

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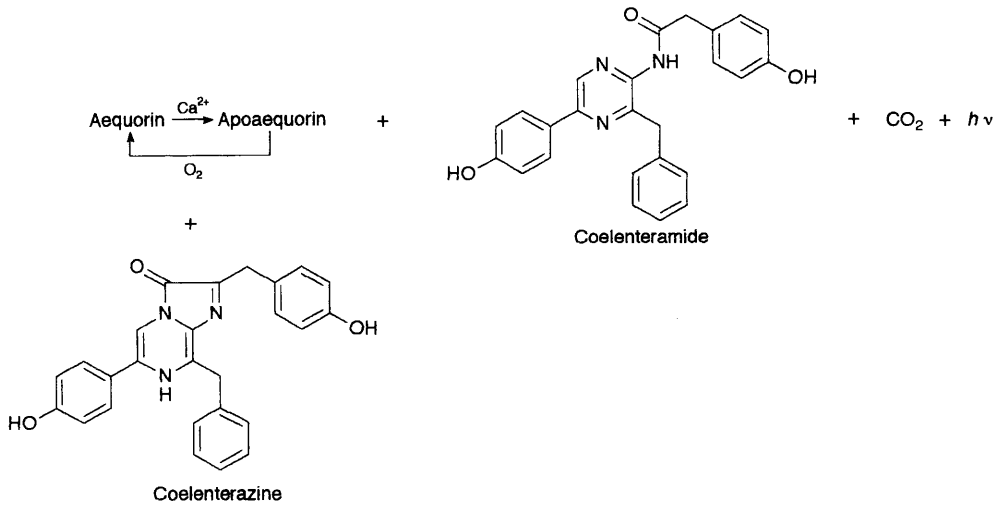
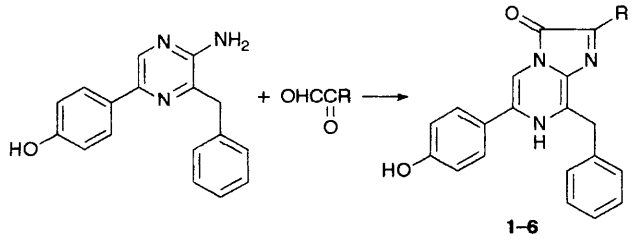
Light intensity measurements of recombinant aequorin and semisynthetic aequorins containing coelenterazine analogues with different substituents at the C-2 position showed that a benzyl group in the C-2 position is essential for efficient luminescence activity as revealed by a two-step incubation procedure used to determine why some analogues gave lower luminescence activities than others.

Since the discovery by Shimomura and Johnson of the luminescent protein aequorin[1] from the jellyfish *Aequorea victoria*, the mechanism of the bioluminescence reaction and applications of the protein have been extensively studied. Aequorin consists of a complex of apoprotein (apoaequorin), coelenterazine (chromophore) and molecular oxygen. In the presence of calcium ion, the protein is converted to a luciferase (oxygenase), which then catalyses the oxidation of colenterazine by the molecular oxygen to yield carbon dioxide and coelenteramide (Scheme 1). The excited state of coelenteramide bound to apoaequorin is the emitter in the reaction. Aequorin can be regenerated from apoaequorin by incubation in Tris–HCl buffer (pH 7.6) with coelenterazine, dissolved oxygen, ethylenediaminetetraacetic acid (EDTA) and 2-mercaptoethanol (Scheme 1). Aequorin shows high specificity and sensitivity to calcium ion and is used as an indicator for the presence of the ion in biological systems. However, a true understanding of how the aequorin molecule functions, including the mode of binding between coelenterazine and apoaequorin, and the nature of the active site, are still unknown. Recent reports by Shimomura and Kishi[2–4] on the use of coelenterazine analogues to improve the sensitivity of semisynthetic aequorins to calcium ion have prompted us to report our findings on this interesting protein. The paper deals with the effects of coelenterazine structure on aequorin activity.

The coelenterazine analogues with various substituents at the C-2 position were synthesized by the method of Kishi



Scheme 1



	R	Ref.
1	Me	8
2	Ph	*a*
3	CH_2Ph	3
4	$CH_2C_6H_4OH(p\text{-})$	*a*
5	$(CH_2)_2Ph$	*a*
6	$(CH_2)_3Ph$	*a*

[a] This work.

Scheme 2

Table 1 Relative activities of chemi- and bio-luminescence of coelenterazine and its analogues

	Compound					
	1	**2**	**3**	**4**	**5**	**6**
Bioluminescence[a]	0.01	0.00	0.37	1.00	0.01	0.02
Chemiluminescence[b]	1.72	0.00	1.44	1.00	1.46	1.72

[a] Measurement of bioluminescence: A mixture of apoaequorin (100 ng), the colenterazine analogue (1 μg/μl MeOH), 2-mercaptoethanol (1 μl) and EDTA (2 μmol dm^{-3}) in Tris buffer (pH 7.6) (200 μl) was allowed to incubate on ice for 2 h. To the regeneration mixture (50 μl) was injected 4.5 μmol dm^{-3} $CaCl_2$ in Tris buffer (1.5 ml) with a syringe and the maximum light intensity was recorded using a lumiphotometer (Labo Science TD-8000). [b] Measurement of chemiluminescence: To a MeOH solution (0.6 μl) of the analogue (10^{-3} mol dm^{-3}) was added a mixture of 300 μl of DGM containing 0.1 mol dm^{-3} acetate buffer (pH 5.6) (0.66%) and the total light emission over a 1 h period was integrated using a lumiphotometer (Labo Science TD-7000).

et al.[5] The synthetic procedure and structures of the analogues are presented in Scheme 2. Apoaequorin was obtained by overexpressing the cDNA for apoaequorin in *Escherichia coli* and purifying (>95%) the expressed protein.[6] The coelenterazine analogue was incubated with the recombinant apoaequorin in Tris– HCl buffer, pH 7.6, containing 2-mercaptoethanol, dissolved oxygen, and EDTA.[7] Recombinant semisynthetic aequorins were formed which elicited bioluminescence in Tris–HCl buffer, pH 7.6, when $CaCl_2$ was added. The flash patterns of the semisynthetic aequorins triggered by Ca^{2+} 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 aequorins studied. Table 1 summarized relative luminescence activities of the recombinant aequorin and semi-synthetic aequorins. From an inspection of Table 1, it is evident that the hydroxy benzyl group at the C-2 position of coelenterazine **4** gives the highest bioluminescence activity.

In order to determine the reason for the low bioluminescence activities of the semisynthetic aequorins containing coelenterazine analogues **1, 2, 5** and **6**, compared to aequorin containing either coelenterazine **4** or its close structure analogue **3**, relative chemiluminescence activities were measured. When a coelenterazine analogue is dissolved in diethylene glycol dimethyl ether (DGM, diglyme) containing acetate buffer, pH 5.6, the solution immediately begins to emit light.[8] The total light emitted by each analogue is summarized in Table 1, along with corresponding bioluminescence activities. It is seen that, since the chemiluminescence activities of analogues **1, 3, 5** and **6** are higher than that for coelenterazine **4**, the low bioluminescence activities must be due to their low ability to generate semisynthetic aequorin and not to the luminescent activities of the chemically generated species in the singlet excited state. Analogue **1** has also been reported to chemiluminescence strongly in dimethylformamide and to bioluminesce with *Renilla* luciferase whose natural substrate is coelenterazine.[9,10]

There are two possible explanations for the lack of light emission with analogue **2** in semisynthetic aequorin: one is a failure of **2** to generate aequorin and the other is a low chemiluminescence activity of **2**, even if the semisynthetic aequorin with **2** can be regenerated. In order to clarify this point we carried out a two-step experiment in which recombinant apoaequorin was regenerated 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 $CaCl_2$ was added and luminescence activity measured. If **2** had occupied the active centre of apoaequorin, the coelenterazine added could not form recombinant aequorin and thus 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 as follows; **2** 105%; **4** 100%; **5** 25%; **6** 37%.

Considering the case of the semisynthetic aequorins containing analogues **5** and **6**, the emissions are seen to be dramatically reduced indicating that these compounds are not simply excluded but they occupy the active centre of the apoaequorin molecule and cannot be easily displaced by the subsequent addition of coelenterazine. This conclusion is further supported by the findings in Table 1 which show that analogues **5** and **6** can form semisynthetic 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 semisynthetic aequorin or, if incorporated, it is readily displaced by the coelenterazine added. Furthermore, since **2** does not chemiluminescence in DGM, it may be presumed that compound **2** will not form active semisynthetic aequorin even if it is incorporated (Table 1). Thus, we conclude that in the series of phenyl homologues at the C-2 position, the benzyl group is of critical importance in eliciting optimal bioluminescence by coelenterazine, probably by contributing to the proper steric orientation of the molecule for subsequent catalysis.

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