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Bioluminescence Activity of Coelenterazine Analogues after Incorporation into Recombinant Apoaequorin

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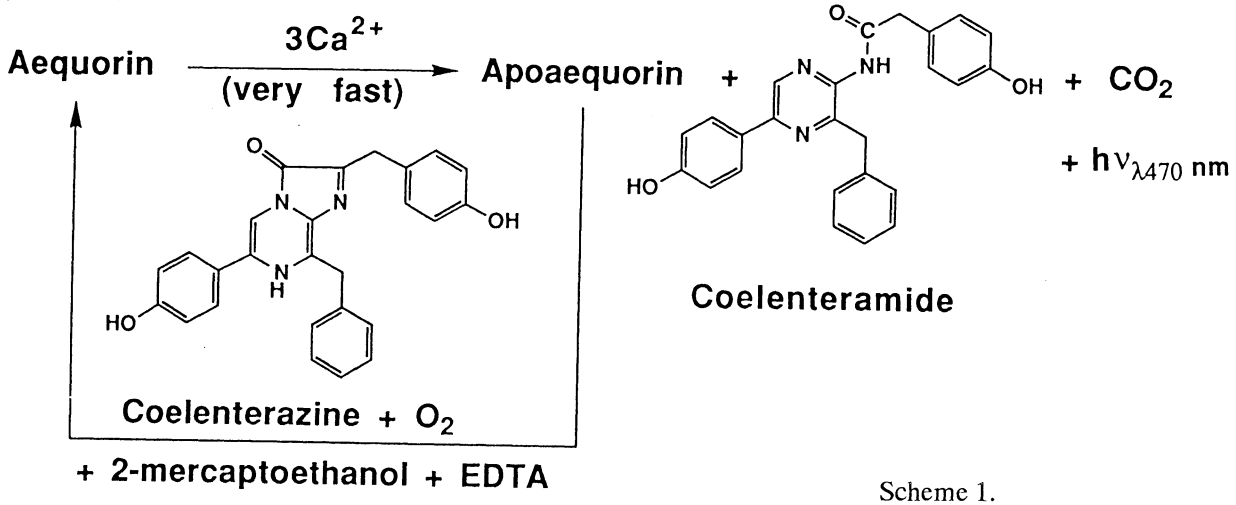
Of twenty-seven synthetic analogues of coelenterazine tested for bioluminescence activity with recombinant apoaequorin, eleven showed activity, but the best activity was obtained with celenterazine incorporated into wild type apoaequorin and into apoaequorin with all three cystein residues replaced by serine.

The jellyfish *Aequorea victoria* possesses in the margin of its umbrella a small (Mr = 21400) Ca^{2+}-binding protein called aequorin that emits light in the presence of Ca^{2+} or Sr^{2+}.[1,2] Aequorin (AQ) consists of a complex of 2-(p-hydroxybenzyl)-6-(p-hydroxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazine-3-one (coelenterazine), molecular oxygen, and apoaequorin (apoAQ) (apoprotein). ApoAQ is made up of 189 amino acid residues in a single polypeptide chain with 3 Ca^{2+}-binding sites.[3,4] The binding of Ca^{2+} activates an intramolecular reaction in which the protein is conformationally changed to an enzyme, which then catalyses the oxidation of coelenterazine (substrate) by the bound oxygen to yield light, coelenteramide and CO_2 (Scheme 1).

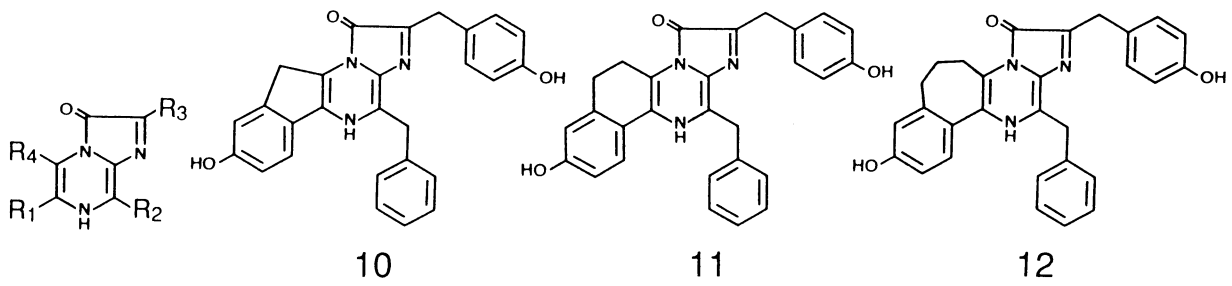


Scheme 1.

The electronically excited state of coelenteramide bound to apoAQ is the emitter in the reactio Coelenterazine may be incorporated into apoAQ by incubation with dissolved oxygen, 2-mercaptoethanol and ethylenediamine-tetraacetic acid (EDTA).[6,7]

Structure-function studies of AQ may be carried out by individually modifying either coelenterazine or apoAQ and measuring light emission with a photometer. Thus, glycine, cysteine, histidine, proline and tryptophan residues in apoAQ have been substituted to determine their role in light generation.[8-11] Coelenterazine has also been modified to obtain improved forms of AQ for use in Ca^{2+} assay using a natural AQ mixture,[12-14] to evaluate side-chain influence on coelenterazine activity,[15,16] and to increase chemiluminescence light yield.[17] This paper presents further data on the structural requirements of coelenterazine for bioluminescence by the use of recombinant apoAQ.

Coelenterazine analogues with various substituents in the R_1, R_2, R_3 and R_4 positions (Scheme 2) were synthesized by the method of Kishi et al.[18] Recombinant wild type apoAQ and apoAQ with cysteine residues 145, 152 and 180 replaced by serine (apoAQC145,152,180S) were prepared by overexpressing the cDNA for each protein in *Escherichia coli* and chromatographically purifying the protein (>95%).[19,20]



Scheme 2.

a: R_1 = C_6H_4OH(p-), R_2 = CH_2Ph, R_4. **b**: R_1 = C_6H_4OH(p-), R_2 = CH_2Ph, R_3 = $CH_2C_6H_4OH$(p-)
c: R_3 = $CH_2C_6H_4OH$(p-), R_4 = H. **d**: R_1 = $C_6H_4OCH_3$(p-), R_2 = CH_2Ph, R_4 = CH_3.
e: R_2 = CH_2Ph, R_4 = CH_3 **f**: R_2 = R_4 = H

Compound

1a: R_3 = $CH_2C_6H_4OH$(p-) **2a**: R_3 = CH_2Ph **3a**: R_3 = $CH(CH_3)CH_2CH_3$ **4a**: R_3 = CH_3 **5a**: R_3 = Ph
6b: R_4 = CH_3 **7b**: R_4 = CH_2CH_2OH **8c**: R_1 =$C_6H_4OCH_3$(p-) R_2 = CH_2Ph **9c**: R_1 = C_6H_4OH(p-),
R_2 = CH(OH)Ph **13d**: R_3 = $CH(CH_3)CH_2CH_3$ **14d**: R_3 = $CH_2CH(CH_3)_2$ **15d**: R_3 = $C(CH_3)_3$
16d: R_3 = CH_3 **17d**: R_3 = CH_2SH **18d**: R_3 = CH_2CH_2COOH **19d**: R_3 = $CH_2CH_2COOCH_3$
20e: R_1 = C_6H_4OH(p-),R_3 = $CH_2C_6H_4OH$(p-) **21e**: R_1 = $C_6H_4OCH_3$(p-), R_3 = $CH_2CH(CH_3)$
22f: R_1 = $C_6H_4N(CH_3)_2$,R_3 = $CH_2C_6H_4OH$(p-) **23f**: R_1 = C_6H_4OH(p-) R_3 = CH_2CH_2Ph
24f: R_1 = Ph,R_3 = CH_2CH_2Ph **25f**: R_1 = Ph, R_3 = CH_2Ph **26f**: R_1 = R_3 = Ph
27f: R_1 = H, R_3 = $CH(CH_3)_2$

Coelenterazine and coelenterazine analogues were incorported into recombinant apoAQ and apo-AQ-C145,152,180S by incubating 2 mg of the compound with 100 ng of apoprotein dissolved in 200 ml of 30 mM Tris-HCl, pH 7.6/10 mM EDTA containing 2 ml of 2-mercaptoethanol for 3 or 6 h in an ice bath.[6,7] The luminescence activity was determined by injecting 1.5 ml of 30 mM $CaCl_2$/30 mM Tris-HCl, pH 7.6, into 50 ml of the incubation mixture and measuring the initial maximal light intensity with a Labo Science (Tokyo) TD-8000 photometer. The spectral energy distributions of the light emitted by coelenterazine and coelenterazine analogues were virtually the same, as previously reported.[15,16]

Figure 1, upper panel, shows the luminescence activities of coelenterazine (**1a**) and eleven analogues incorporated into apoAQ. The best activities were obtained with **2a**, **6b**, **9c** and **11**, whereas **3a**, **4a**, **5a**, **7b**, **8c**, **10** and **12** gave low or no activity. The other 15 analogues were inactive (data not shown). The results with **2a**, **6b** and **11** confirm results previously published.[11-14] The lack of activity with **10** agrees with the finding of an earlier report.[12] Analogues **2a** and **9c** gave slightly higher activity than **6b**. The relatively good activity obtained with **9c** and the fact that good activities have been reported with R_2 modified,[13] suggest that latitude exists in altering this group. Analogue **11**, with an extra ethylene bridge, gave the second highest activity. The compound has been reported to produce higher light yield and to induce the formation of two excited states in aequorin bioluminescence.[11-14] In a parallel experiment, the same analogues were incubated with apoAQ for 6 h, then **1a** was added, and the incubation continued for an additional 6 h before being assayed for activity. All of the mixtures had reasonably good activity, except for mixture containing **10** which had minimal activity (data not shown). Thus, **10** may function as a specific inhibitor of aequorin regeneration.

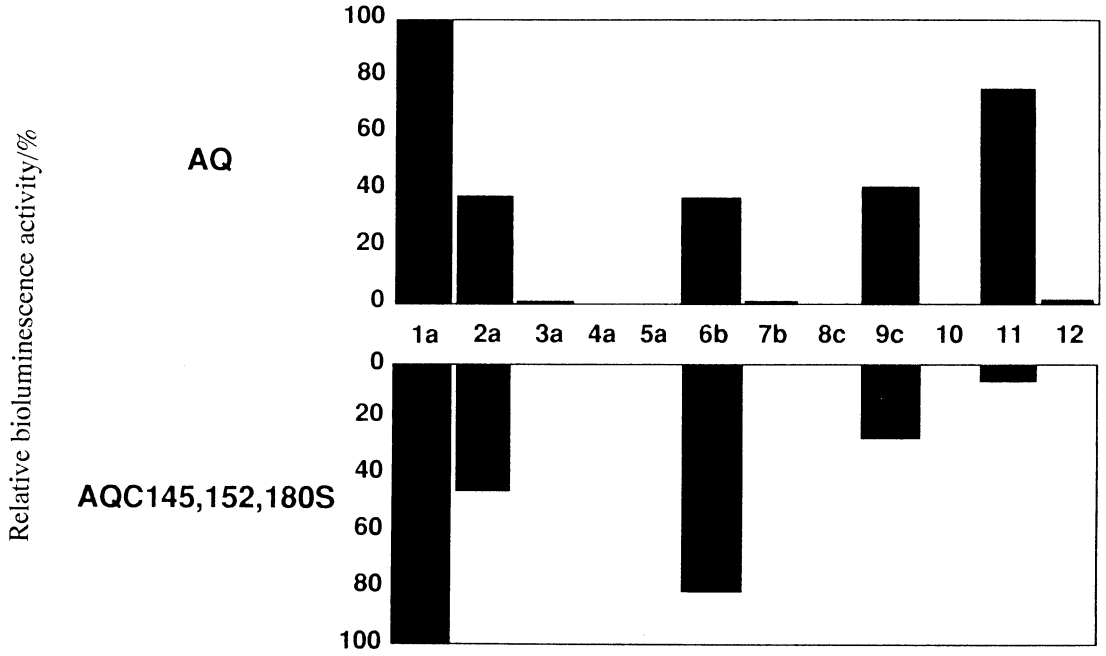


Fig. 1. Bioluminescence activities of coelenterazine and coelenterazine analogues incorporated into apoAQ and apoAQC145, 152,180S. Upper panel: Compounds **1a** (coelenterazine), **2a**, **3a**, **4a**, **5a**, **6b**, **7b**, **8c**, **9c**, **10**, **11** and **12** were incubated with apoAQ for 3 h and assayed for activity. Lower panel: the same compounds were incubated with apoAQC145,152,180s for 3 h and assayed for activity.

Figure 1, lower panel, shows the relative activities of the same analogues incorporated into apoAQ-C145,152,180S. Analogue **2a** had nearly the same activity as **2a** with apoAQ (upper panel), while **6b** had significantly higher activity than **6b** with apoAQ. In contrast, **9c** had less, and **11** had markedly lower, activity than the same compounds incorporated into apoAQ. Analogue **11** incorporated into apoAQW79F has been previously reported to yield more light than the wild type AQ control.[11]

The low activities observed for **8c** (Fig. 1) and for an analogue having $C_6H_4NH_2$(p-) at R_1[12] indicate that a C_6H_4OH(p-) at R_1 is essential for good activity. The results shown by **9c** with the two apoaequorins (Fig. 1) suggest that a CH_2Ph at R_2 is required for maximum activity. Shimomura et al. have shown that some aliphatic or alicyclic analogues at R2 have considerable activitiy in the case of the natural apoAQ mixture.[13] The low activities shown by **3a**, **4a** and **5a**, compared to relatively high activity by **2a** indicate that a $CH_2C_6H_4OH$(p-) or CH_2Ph is needed at R_3.[15,16] Finally, R_4 does not seem to be essential for activity.

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