Synthesis and Preliminary Chemi- and Bio-luminescence Studies of a Novel Photolabile Coelenterazine Analogue with a Trifluoromethyl Diazirine Group

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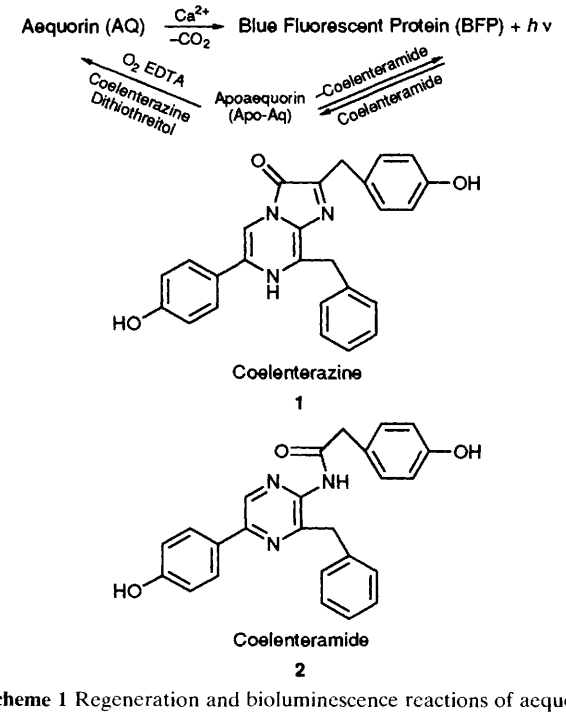
A novel photolabile analogue of coelenterazine with a trifluoromethyl diazirine group is successfully synthesized for photoaffinity labelling of the active site of aequorin; preliminary studies on its chemi- and bio-luminescence demonstrate that the photolabile analogue shows the same luminescence properties and kinetics as those of natural coelenterazine and thus, it is deduced that both compounds occupy the same active site of aequorin.

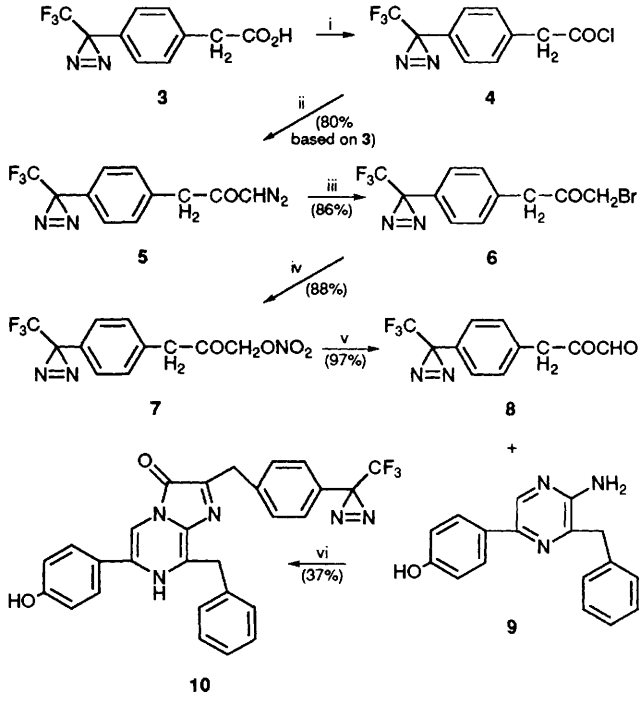
The photoprotein aequorin (AQ)[1] is known as a complex which is composed of apoaequorin (Apo-Aq), O_2 and a chromophore (coelenterazine **1**). In the presence of Ca^{2+}, the protein is converted into an oxygenase which catalyses the oxidation of **1** by the bound oxygen to produce light, CO_2 and a blue fluorescent protein (BFP). Under appropriate conditions, BFP can be separated into Apo-Aq and coelenteramide **2** (a light-emitting species), and restored by mixing the two components in the presence of Ca^{2+}. AQ can also be regenerated from Apo-Aq and coelenterazine in the presence of dissolved oxygen, EDTA, and an appropriate thiol (Scheme 1).[2–7] To date numerous studies have been carried out to examine the bioluminescence mechanism and the structure–function relationship of AQ such as modifications of the functional part of **1** chemically or the enzyme part Apo-Aq by means of site-specific mutagenesis techniques.[5–9] Despite many efforts, the nature of the active site and the binding mode between **1** and Apo-Aq are still obscure.[3,10,11] Recently photoaffinity labelling has become an attractive methodology to characterize and identify functional domains and active sites of biomacromolecules.[12,13] We believe that such a study will assist in clarifying the unclear points existing in AQ since the primary structure of Apo-Aq has been known.[14,15] In our previous paper,[16] we reported studies on the synthesis and photolysis of a photoreactive analogue of coelenteramide to regenerate BFP for photoaffinity labelling study. To continue our study on this objective, herein we report a synthesis and chemi- and bio-luminescence of a novel photolabile analogue of coelenterazine which is proved to be a potential photoaffinity reagent for labelling the active site of AQ.



Scheme 1 Regeneration and bioluminescence reactions of aequorin

The photolabile analogue **10** of coelenterazine was designed by placing a diazirine group in place of the benzyl hydroxy group at the C-2 position according to a systematic study of coelenterazine derivatives which shows that this group can be modified chemically with retention of bioluminescence activity.[6,7] The choice of the trifluoromethyl diazirine group as a photoreactive group results from its several advantages over others such as azido or diazo groups.[16–19] The diazirine bears chemically remarkable stability, longer wavelength absorption and 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 light with wavelengths shorter than 300 nm which is harmful to biological molecules. Furthermore, it has been known that both diazo and azido groups are easily reduced by thiols, which are necessary to regenerate the semi-synthetic AQ.

The synthetic procedure is outlined in Scheme 2. Phenyl acetic acid **3** with the diazirine group was prepared by our previously established method.[16] Diazoketone **5** was synthesized *via* two steps: (*i*) acid **3** was converted into its acid chloride **4** by reaction of **3** with thionyl chloride in benzene at room temp. for 14 h; (*ii*) **4** was dissolved in diethyl ether and



Scheme 2 Synthesis of photolabile analogue of coelenterazine with a diazirine group. *Reagents and conditions*: i, $SOCl_2$, benzene, room temp. 14 h; ii, CH_2N_2, ether, 0 °C, 1 h; iii, HBr, 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, 80 °C, 3 h.

then treated with diazomethane at 0 °C for 1 h to afford the diazoketone **5** (80% based on **3**). Diazoketone **5** in ether was passed over a steady stream of dried HBr at 0 °C for 1 h to give bromoacetone **6** in an isolated yield of 86%. Transformation of **6** into the 2-oxo-propyl nitrate derivative **7** was accomplished by reaction with silver nitrate in acetonitrile at room temp. for 38 h in a high yield (88%). 2-Oxo-aldehyde **8** was formed by slow treatment of **7** in DMSO with sodium acetate, with the mixture allowed to continue the reaction for 40 min (crude yield 97%). Finally, coupling of **8** with coelenteramine **9**[20] in ethanol containing aq. HCl at 80 °C for 3 h yielded the expected product **10** in 37% yield. All new compounds gave consistent spectral data and satisfactory elemental analyses by HRMS.

Chemiluminescence spectra and luminescence rates of **10** were investigated to examine the effect of the diazirine group on the luminescence behaviour and kinetics of the coelenterazine chromophore. We found that in DMSO under air, the chemiluminescence spectrum (λ_{max} 475 nm) of **10** is not consistent with either the fluorescence spectrum of its corresponding spent solution of the reaction or synthesized amide,[16] while the later two have the same maximum wavelength (λ_{max} 418 nm). These results coincide with those of natural coelenterazine in similar conditions:[7] the chemiluminescence spectrum around 475 nm is attributed to the emission from the anion of its corresponding amide which emits light before it protonates, whereas the fluorescence centred at 418 nm is assigned to the neutral form of the amide.[21] A pseudo first-order dependence of light production on the amount of **10** was observed in systems of both DMSO and DGM (diglyme) containing 0.66% of acetate buffer (0.1 mol dm^{-3}, pH 5.6) at 25 °C under air. The luminescence rates were obtained by the published procedure by Goto[22] to be $k_{DMSO} = 1.9 \times 10^{-2}$ s^{-1} and $k_{DGM} = 2.6 \times 10^{-3}$ s^{-1} respectively which are quite close to those of **1** ($k_{DMSO} = 2.7 \times 10^{-2}$ s^{-1}; $k_{DGM} = 2.7 \times 10^{-3}$ s^{-1}). From the investigations above, we can conclude that the chemiluminescence behaviour and kinetics of **10** and **1** are essentially the same.

Whether the synthetic photolabile coelenterazine analogue **10** can be used as a photoaffinity reagent for labelling the active site of AQ is determined by its ability to regenerate a bioluminescent semi-synthetic AQ and occupy the same binding site as with natural coelenterazine. In order to investigate this point, regeneration reaction and bioluminescence studies on **10** were carried out. **10** was incubated with recombinant Apo-Aq[23] in the presence of EDTA, dissolved O_2 and dithiothreitol (DTT) in an ice bath for a certain time, and then bioluminescence activity was measured upon addition of Ca^{2+} to the regenerated semi-synthetic AQ solution. As expected, it emits light with a flash pattern identical to that of native one and the time course of regeneration shows that it takes about 4 h to reach maximal light intensity. These facts indicate that the regeneration and luminescence mechanism of the semi-synthetic AQ from **10** is virtually identical to that of the natural one, and that **10** occupies the same active site as coelenterazine does in AQ.

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