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3-Benzyl-5-(4-hydroxyphenyl)-2-[4-(1-azi-2,2,2-trifluoroethyl)phenylacetamido]pyrazine, a Photoreactive Analogue of Coelenteramide. Synthesis and Photolysis

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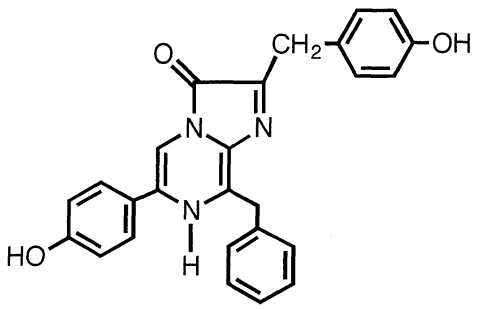
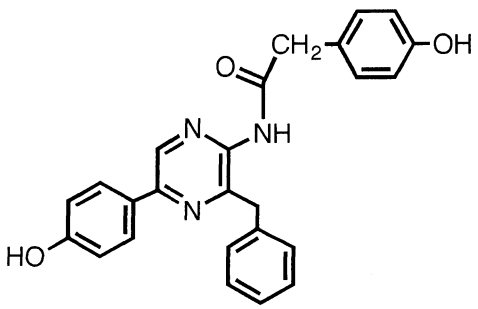
A photoreactive analogue of coelenteramide (light emitting species in aequorin bioluminescence) has been synthesized for photoaffinity labeling studies of aequorin. Photolysis of this compound in methanol gave a formal OH insertion product in 62% yield without damage of the main skeleton of coelenteramide, indicating a potential use for mapping the coelenterazine binding site in aequorin.

Aequorin (a photoprotein) extracted from jellyfish *Aequoria victoria* emitts light in the presence of Ca^{2+} and produces a blue fluorescent protein (BFP)[1] which is composed of coelenteramide (a light-emitting species) and apoaequorin. Aequorin is a protein-coelenterazine-O_2 complex[2] in which coelenterazine is tightly bound to the protein through a peroxide bridge.[3] Aequorin can be regenerated from apoaequorin by incubation with coelenterazine in the presence of molecular oxygen and an appropriate thiol.[4,5] Despite numerous studies have been carried out on aequorin bioluminescence, a true understanding of how the aequorin molecule functions, including the binding mode between coelenterazine and apoaequorin, and the nature of the active site of the chromophore and the binding site of calcium ion are still unknown.



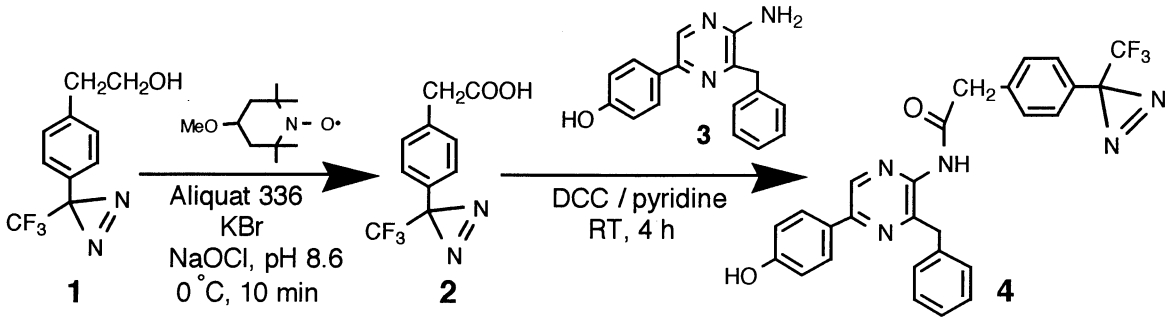
Coelenterazine



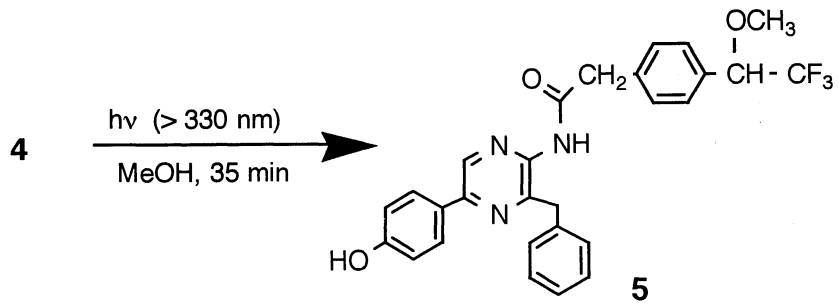
Coelenteramide

One may expect that x-ray crystallography is a powerful tool to clarify the binding site and active center. Unfortunately, however, a problem associated with the preparation of good crystals is the inherent instability of aequorin.[6,7] Shimomura et al[1] reported that BFP can also be regenerated from apoaequorin and coelenteramide in the presence of Ca^{2+} and coelenteramide is considered to be tightly bound in a noncovalent manner to apoaequorin. These results prompted us to synthesize a coelenteramide analogue with a photoreactive group for photoaffinity labeling studies to elucidate the active center of aequorin by regenarating BFP containing this photoreactive coelenteramide analogue followed by sequencing and analyzing the amino acid residues of the protein after irradiation. The photoreactive coelenteramide derivative **4** was designed by modification of native coelenteramide; the hydroxy group at the 4-hydroxyphenylacetamido moiety, which has been demonstrated to be unessential in aequorin bioluminescence previously by us[8] and other group,[9] was replaced by a trifluoromethyldiazirine group. In this paper, we reported the synthesis of the photoreactive coelenteramide **4** which was able to generate a reactive carbene intermediate photochemically.

The synthetic procedure of 3-Benzyl-5-(4-hydroxyphenyl)-2-[4-(1-azi-2,2,2-trifluoroethyl) -phenylacetamido]pyrazine **4** is summarized in Scheme 1. Coelenteramine **3** was synthesized according to the procedure of Kishi et al.[10] 3-Trifluoromethyl-3-[4-(2-hydroxyethyl)phenyl]diazirine **1** was prepared by adoption of the method published by Brunner.[11] 4-(1-Azi-2,2,2-trifluoro-ethyl)phenylacetic acid **2**[12,13] was prepared as follows: 0.35 M NaOCl aqueous solution at pH 8.6 (5.7 ml) was added to a mixture of 0.8 M CH_2Cl_2 solution of **1** (1 ml), 0.016 M CH_2Cl_2 solution of 4-methoxy-2,2,6,6-tetramethylpiperidinyl-1-oxy (0.5 ml),[14] 0.08 M CH_2Cl_2 solution of Aliquat 336® (tricaprylmethylammonium chloride) (0.5 ml) and 0.5 M KBr aqueous solution (0.16 ml) at 0 °C under argon. After allowed to stir for 10 min, the reaction mixture was adjusted to pH 2 by adding 2 M HCl aqueous solution, subsequently extracted with CH_2Cl_2, and dried over $MgSO_4$. After removal of the solvent the residue was purified by column chromatography (silica gel, 200:1 CH_3CO_2Et-CH_3CO_2H) and by recrystallization from n-hexane gave **2** as colorless needles (80 mg, 40%). The photoreactive analogue of coelenteramide **4** was synthesized by following procedure: A mixture of coelenteramine **3** (573 mg, 2.07 mmol), **2** (504 mg, 2.07 mmol), and DCC (1.37 g, 6.65



Scheme 1.



Scheme 2.

mmol) in pyridine (14 ml) was stirred at room temperature for 4 hr under argon. Precipitate formed in the reaction mixture was filtered off. The filtrate was evaporated to dryness. Purification by colmun chromatography (silica gel, 3:2 n-hexane-$(CH_3)_2CO$) and by recrystallization from ethanol affored the pure **4** as colorless crystals (0.64 g, 71%).[15]

An effective photoaffinity labeling reagent lies in having a pertinent photoreactive group. It is stable in the dark, but should have photoreactivity to produce a highly labile intermediate such as carbene, and form effectively corresponding OH or CH insertion products in an appropriate solvent. To verify the suitability of the synthetic photoreactive coelenteramide **4** for photoaffinity labeling experiment before practical applications in the bioluminescence system, a study on photolysis of the photoreactive coelenteramide **4** was performed (scheme 2). The solution of **4** (16 mg, 0.032 mmol) in 15 ml of methanol under argon was irradiated with a 500 W xenon lamp (cutoff filter for $\lambda <$ 330 nm) for 35 min monitoring by reverse phase HPLC. Products were separated by TLC (silica gel, 3:1 C_6H_6-CH_3CN). The main product (10 mg, 62%) was confirmed by spectral data to be the formal OH insertion product **5**, 3-benzyl-5-(4-hydroxyphenyl)-2-[4-(1-methoxy-2,2,2-trifluoroethyl)phenyl - acetamido]pyrazine[16] indicating that the main skeleton of the coelenteramide was not damaged during photolysis of **4**. This result indicates a potential use of the photoreactive coelenteramide **4** for mapping the coelenterazine binding site in aequorin. Studies on the practical application of the photoreactive compound to the aequorin bioluminescence system is in progress in our labotratory.

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13) Compound **2**: Mp 68-70 °C; λ_{max}(EtOH) / nm 224 (log ε 4.31), 270 (2.95), 335 (2.78); δ_H ($CDCl_3$) 3.67 (2H, s), 7.17 and 7.32 (4H, AB type, aromatic H); δ_C ($CDCl_3$) 28.3 (q, J=40.3 Hz), 40.5, 122.1 (q, J= 274.9 Hz), 126.8, 128.2, 129.9, 134.9, 176.9; m/z (positive-SIMS) 245 ($[M+H]^+$, 7%), 227(3), 216 (7); HRMS (in-beam EI) found $[M-N_2]^+$: 216.0414, calcd for $C_{10}H_7F_3O_2$, $[M-N_2]^+$: 216.0398; IR (KBr) ν / cm^{-1} 2200-3400, 1700, 1610, 1520, 1410, 1140, 1050, 935.
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15) Compound **4**: Mp 183-186 °C; λ_{max}(EtOH) / nm 275 (log ε 4.07), 293 (4.10), 331 (4.08); λ_{max}(DMSO) / nm 278 (log 4.04), 297 (4.08), 336 (4.07); δ_H (CD_3COCD_3) 3.83 (2H, s), 3.86 (OH, s), 4.14 (2H, s), 6.96 (2H, d, J= 8.8 Hz), 7.05-7.25 (5H, m), 7.27 (2H, d, J= 8.1 Hz), 7.54 (2H, d, J= 8.4 Hz), 7.97 (2H, d, J= 8.8 Hz), 8.73 (1H, s), 9.06 (NH, s); m/z (positive-SIMS) 504 ($[M+H]^+$, 9%), 476 (7), 304 (7), 276 (15); HRMS (positive-SIMS) found $[M+H]^+$: 504.1676, calcd for $C_{27}H_{21}F_3N_5O_2$, $[M+H]^+$: 504.1646; IR (KBr) ν / cm^{-1} 2200-3600, 1672, 1615, 1500, 1442, 1350, 1237, 1158.
16) Compound **5**: Mp 166-168 °C; λ_{max}(EtOH) / nm 277 (log ε 3.95), 296 (3.99), 334 (3.97); δ_H (CD_3COCD_3) 3.37 (3H, s), 3.81 (2H, s), 3.90 (1H, s), 4.15 (2H, s), 4.85 (1H, q, J= 7.0 Hz), 6.94 (2H, d, J= 8.8 Hz), 7.05-7.30 (5H, m), 7.40-7.60 (4H, m), 7.97 (2H, d, J= 8.8 Hz), 8.73 (1H, s), 9.11 (1H, s); m/z (in-beam EI) 507 (M^+, 62%), 304 (23), 277 (100); HRMS (in-beam EI) found M^+: 507.1754, calcd for $C_{28}H_{24}F_3N_3O_3$, M^+: 507.1767; IR (KBr) ν / cm^{-1} 2200-3600, 1662, 1610, 1485, 1430, 1172, 1132.

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