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SYMPLECTOTEUTHIS BIOLUMINESCENCE (1)

STRUCTURE AND BINDING FORM OF CHROMOPHORE IN PHOTOPROTEIN OF A LUMINOUS SQUID

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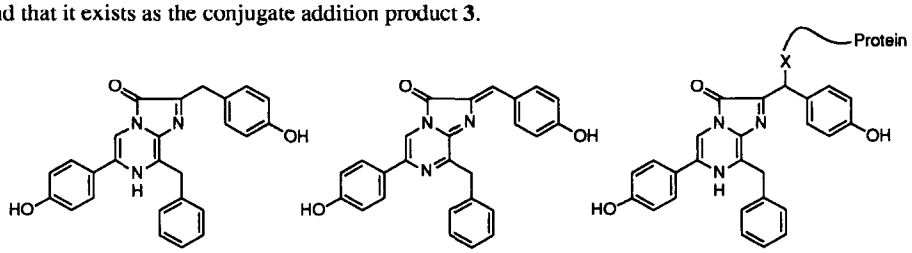
Abstract: The chromophore in the luminous squid, *Symplectoteuthis oualaniensis*, was identified to be dehydrocoelenterazine. This chromophore exists as conjugate adduct in the photoprotein.

Bioluminescence of marine organisms is widely recognized phenomenon because of the emission of visible light. Limited knowledge has been accumulated about luminous squids, and chemistry of the chromophore (luciferin) has been studied only on *Watasenia scintillans*, B[1] (Japanese name, Hotaru-Ika). Many of these luminescent systems involve a common chromophore, coelenterazine (**1**), which is found in other marine luminescent systems, e.g. *Oplophorus* luciferin (coelenterazine,[2] *Watasenia* preluciferin[3]), *Aequorea aequorea* and *A. victoria*.[4] This article describes the structure of chromophore involved in a different luminous squid, *Symplectoteuthis oualaniensis* L (Japanese name, Tobi-Ika),[5] with special reference to the binding form in this photoprotein. The occurrence of *S. oualaniensis* is very wide in tropical Pacific Ocean including South-West islands of Japan, where we collected the squids with approximate size of 20 cm length and 6 cm width. The yellow luminous organs are located on the back-forward of mantle in ovarian range (5 cm x 3 cm). In 1981, Tsuji *et al.* reported some biochemical aspects of luminescence of *S. oualaniensis*; thus, (i) sodium or potassium ions are necessary for the luminescence and (ii) essentially luminous molecules cannot be extracted from membrane structure.[6] No more report has appeared about this squid since then. We have just disclosed that the chromophore of *S. oualaniensis* is dehydrocoelenterazine (**2**)[7] and that it exists as the conjugate addition product **3**.



coelenterazine (1) *yellow (luminescent)* | **dehydrocoelenterazine (2)** *reddish purple (non luminescent)* | **bound form (3) in *S. oualaniensis***

Luciferins are usually unstable compounds to purify, so that Goto *et al.* recorded several examples about extraction of more stable oxyluciferins (luminescent products) instead of difficult luciferins.[3,8] We decided to employ his method to study the bioluminescence system of this squid. The luminescence spectrum of *S. oualaniensis* shows the maximum at 470 nm, that suggests the corresponding oxyluciferin would show blue fluorescence. The luminous organs (17 g) were separated at ca. 0°C from 30 bodies of *S. oualaniensis*. The organs were freeze-dried (5.8 g) and then homogenized at -78°C in acetone solvent (100 mL) under nitrogen atmosphere. The mixture was filtered through Büchner funnel and the residue was washed with cold acetone (50 mL) to give pale yellow acetone-powder (5 g). No fluorescent compound was found in acetone extracts. This acetone-powder showed bioluminescence activity when mixed with KCl solution and also showed chemiluminescence activity when suspended in aprotic polar solvent such as DMSO, DMF[8] in the presence of molecular oxygen.

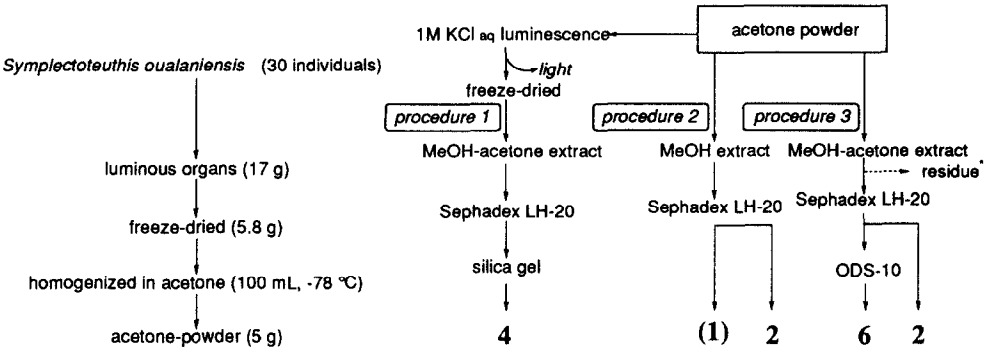


Fig. 1. Flow chart of extraction of chromophores.

The first extraction of the chromophore was followed as the *procedure 1* in Fig. 1. After mixing with 1M KCl to observe light, the acetone-powder was extracted with a mixture of MeOH and acetone (1:1) to give a fraction having blue fluorescence. This compound was separated by Sephadex LH-20 and then by SiO_2 chromatography to isolate a blue fluorescent compound which was identified to be coelenteramine **4** [^{1}H NMR (CD_3OD, 500 MHz) δ 4.15(2H, s), 6.86(2H, d, J = 10 Hz), 7.23(1H, m), 7.30(4H), 7.73(2H, d, J= 10), 8.22(1H, s) ppm; FAB-MS m/z 278 (M+1); Fluorescence Em_{max} 439 nm (Ex 356 nm)] by comparison of chromatographic and spectroscopic data with chemically synthesized authentic sample (**4**).[9] It implies that extraction of the acetone-powder gave **4** but not oxyluciferin equivalent to **5** even after addition of 1M KCl to the homogenate of the photogenic organs.[10] These negative experiments prompted us to extract anything retaining luminescent activity, that can be monitored with a chemiluminescence detector.[11]

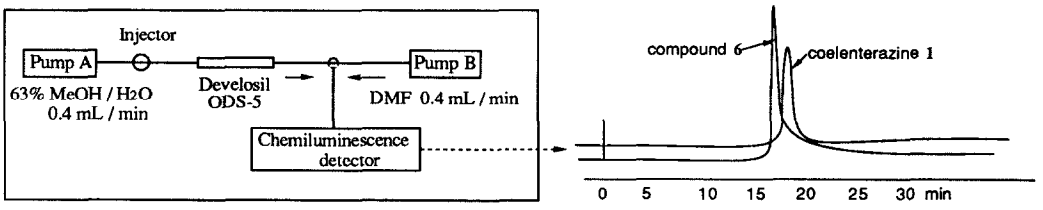
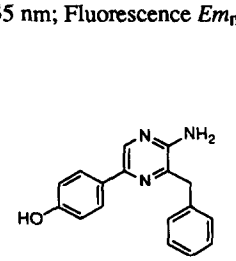


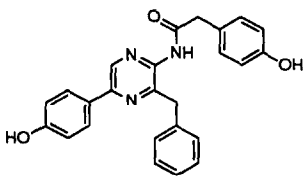
Fig. 2. Chemiluminescence detection and chromatogram of luminous compounds 1 and 6.

The *procedure 2* includes the direct extraction of the chromophore having luminescent ability. The acetone-powder of the photogenic organs of *S. oualaniensis* was extracted with MeOH, and the extract was separated by Sephadex LH-20 column chromatography. Two fractions showing fluorescence were separated. The first fraction showed green fluorescence and it was analyzed by TLC (silica gel, 10 % MeOH-CH_2Cl_2) and by HPLC on an ODS column equipped with chemiluminescence detector (retention time 18 min) as shown in Fig. 2. The results suggest the structures to be coelenterazine (**1**) in very trace amount (only detectable by chemiluminescence $<10^{-10}$ mol, but not detectable by UV monitor). The second fraction showed reddish purple color, that eluted slowly with interaction with Sephadex LH-20 gels, was identified to be dehydrocoelenterazine (**2**)[12] [FAB-MS m/z 422 (M+1); UV (MeOH) λ_{max} 280, 422, 530, 570, 610(sh) nm]. The dehydro body (**2**) had characteristic deep-red color and did not show luminescence activity. This compound (**2**) was also found in the following MeOH-acetone extracts (having luminescence activity) in smaller amount. But dehydrocoelenterazine (**2**) became non-detectable from the acetone-powder after treatment with 1M KCl and simultaneous observation of luminescence.

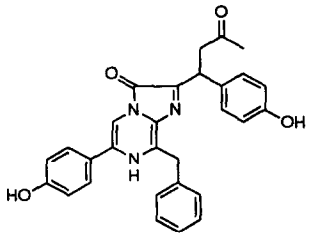
In the *procedure 3* (Fig. 1), the acetone-powder (5 g) was suspended in a mixture of MeOH and acetone (1:1, 100 mL) at room temperature for 10 min and then filtered through Büchner funnel. The residual powder was extracted once more with MeOH-acetone (1:1, 50 mL) and three times with MeOH (50 mL) to leave "residue*" (* this being used in the transformation of **2** into **6** in a later experiment). The extracts were combined and concentrated into 20 mL and the residue was allowed to stand for 1 hr at 0°C to form precipitates. After filtration, the filtrate was separated by a Sephadex LH-20 column (24 mm x 425 mm) using MeOH as eluant to collect yellow fraction showing chemiluminescence activity (with DMF, *vide supra*). The fraction was concentrated to dryness and suspended in aliquot of 10% H_2O-MeOH. Filtering the insoluble mass, the (luminescence-active) solution was applied onto an ODS column (Develosil ODS-10) with 90% MeOH as eluant to collect fractions having chemiluminescence activity, which were checked by the method in Fig. 2. The active fractions were combined and further separated with the same column with 65% MeOH (once) and then 60% MeOH (once) to yield a chemiluminescence fraction having different retention time (16 min) of the luminescence peak from coelenterazine (**1**)(18 min, see Fig 2). This luminous compound was assigned to have a structure **6**; ^1H NMR (CD_3OD, 500 MHz) δ 2.16(3H, s), 3.14(1H, dd, *J*= 17, 5.5 Hz), 3.74(1H, dd, *J*= 17, 10), 4.43(2H, AB), 4.75(1H, dd, *J*= 10, 5.5), 6.70(2H, d, *J*= 9.0), 6.88(2H, d, *J*= 9.0), 7.20(2H, d, *J*= 9.0), 7.25(1H), 7.32(2H), 7.4(3H, br), 7.43(2H, d, *J* = 9.0) ppm; ^{13}C NMR (CD_3OD, 150 MHz) δ 31.11, 34.36, 40.73, 49.35, 108.28, 117.10, 117.61, 128.92, 130.18, 130.50, 130.60, 130.90, 134.90, 139.21, 157.98, 160.92, 210.79 ppm. FAB-MS m/z 480 (M+1); UV (MeOH) λ_{max} 261, 435 nm; Fluorescence Em_{max} 537 nm (*Ex* 429 nm)].



coelenteramine (4)

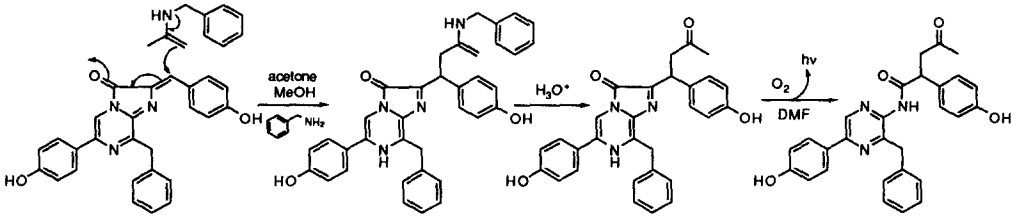


coelenteramide (5)



acetone adduct (6)

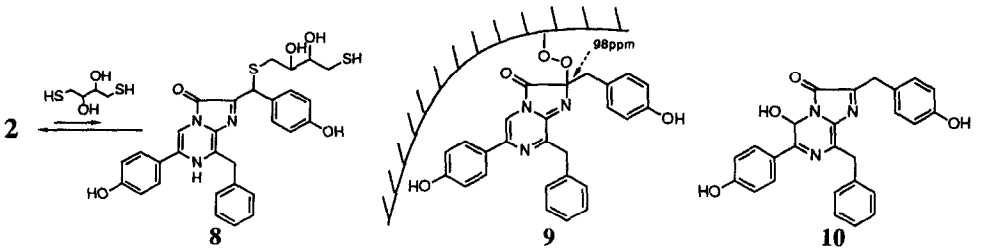
The structure **6** being adduct of solvent acetone to dehydrocoelenterazine (**2**) was confirmed by synthesis; thus, a solution of **2** (2.5 mg) dissolved in MeOH-acetone (1:1, 0.3 mL) containing benzylamine (4 mg) was stirred for 30 min at rt. TLC (SiO_2) analysis showed the presence of an unstable intermediate, which can be assigned to be the enamine (**6a**). To the reaction mixture was added 0.1*N* HCl to decompose the enamine, and then the mixture was separated by Sephadex LH-20 and then ODS-10 (*vide supra*) to afford **6** (0.3 mg, all spectra being identical with the sample as above) in ca. 15% yield. Alternatively, synthetic dehydrocoelenterazine (**2**, 8.0 mg) was suspended with the residue of MeOH-acetone extraction of the acetone-powder ("residue*" in flow chart in Fig. 1; 150 mg) in a mixture of MeOH-acetone (1:1, 25 mL) with stirring overnight at room temperature. The mixture was filtered and the filtrate was concentrated to give residue, which was purified with a Sephadex LH-20 column and then with an ODS-10 column to yield 3.1 mg of **6**. This adduct (**6**), when further treated with NaOH in DMF solvent with bubbling oxygen, yielded light and the oxyluciferin-type compound **7** (FAB-MS m/z= 468, M+1)(see Scheme 1).



2 **6a** **6** **7**

Scheme 1

Dehydrocoelenterazine-acetone adduct (**6**) having luminescence activity was extracted from the photogenic organs. Judging from the fact that similar extraction with a mixture of methyl ethyl ketone (instead of acetone in *procedure 3* Fig. 1) and MeOH afforded mixture of adducts of the ketone, compound **6** was concluded as an artifact which happened by addition of solvent acetone to dehydrocoelenterazine (**2**) at room temperature but not at -78°C. Dithiothreitol (DTT, 8.8 mg) was added to **2** (2.4 mg) in a mixture of MeOH-acetone (1:1, 0.3 mL), while the reddish color changed to yellow. The yellow product showed chemiluminescence ability when added into DMSO solvent. Attempted separation of this product by chromatography, however, did not give yellow compound but it decomposed in a column to give back dehydrocoelenterazine (**2**). The yellow sulfur-adduct existed only as constituents of equilibrium, since FAB-MS of this mixture (using DTT as a matrix for measurement) gave m/z 576 equivalent to M+1 of compound **8**.



8 **9** **10**

Scheme 2 jelly fish (*A. aequorea*) aequorin,[15] **5-coelenterazinol**

Aequorin, a well-known photoprotein found in jelly fish *Aequorea aequorea*, induces bioluminescence by addition of Ca^{++} and no other component is necessary for this luminescence system.[13] This system involves coelenterazine as built-in chromophore, that tightly binds with protein. Thus, apoprotein of aequorin binds with coelenterazine (**1**) luminescent chromophore prior to receive Ca^{++}. Shimomura *et al.* suggested the binding of coelenterazine to apoaequorin being through peroxide linkage as **9**.[14] In fact, no molecular oxygen is required for luminescence of this complex. Kishi *et al.* verified this linkage by means of NMR (the C-2 being at δ 98 ppm) to suggest its reduction product (yellow compound) to have 2-hydroxy group.[15] We have revised the yellow compound to have hydroxy group at the 5-position (**10**).[16] Cormier *et al.* suggested another mechanism without such peroxide linkage.[17] In the squid *S. oualaniensis*, on the other hand, required molecular oxygen and monovalent cations (e.g. Na^+, K^+) for bioluminescence,[6] suggesting involvement of a different photoprotein from aequorin.

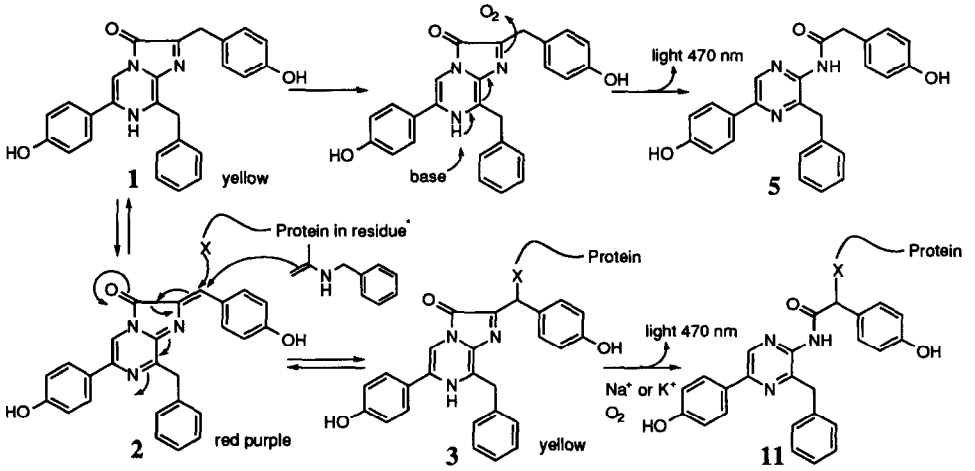


Fig. 3. Suggested form of chromophore in photoprotein of *S. oualaniensis* and possible mechanism for luminescence. Above example (1 ⟶ 5) is the usual mechanism for 1.

When the acetone-powder (Fig. 1) was extracted only with MeOH, dehydrocoelenterazine (**2**) was detected, its amount being estimated about 20% from the total luminescent light. None of **2** nor **6** was, however, detected in the extracts that once luminesced by addition of KCl, indicating that the bioluminescence of *S. oualaniensis* consumed dehydrocoelenterazine (**2**, see Fig. 3). Although dehydrocoelenterazine (**2**) itself did not exhibit any luminescence activity, some derivatives produced by addition (such as **6**, **8**) should retain the activity. Although dehydrocoelentera-

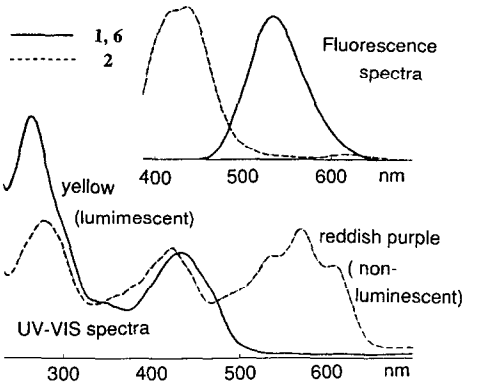


Fig. 4. UV-VIS and fluorescence spectra of compound 1, 2 and 6.

zine (**2**) is reddish purple, the photogenic organs are not red but yellow-brown (similar to the acetone adduct **6**) as shown in Fig. 4, indicating that the existing conjugate system in **2** is broken. This phenomenon can be interpreted by deconjugative addition of nucleophile in protein (e.g. a functional residue on lysine or cysteine). These facts led us to conclude that dehydrocoelenterazine (**2**) existed as adduct **3**. In Fig. 3, usual mechanism of coelenterazine (**1**) is described for comparison to the case of the adduct form of dehydrocoelenterazine (**2**), that would exist as **3** in protein. When **3** is extracted with solvent containing acetone, *protein-X* would exchange with acetone enamine through **2** existing as a small equilibrium. This adduct, after luminescence, still hold the chromophore in protein. This was proven by the finding that only a high molecular fraction (from Bio-Gel P-6 gel filtration chromatography using AcOH/AcONa buffer pH 5.0) of extracts with bioluminescence activity emitted light (470 nm) by addition of KCl and O_2. These facts suggest that the luminescence system of *S. oualaniensis* includes "photoprotein" and a new type of "binding form" of the chromophore as **3** (Fig. 3). Further studies on the details including protein are in progress.

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References and Notes

1. Inoue, S.; Kakoi, H.; Goto, T. *Tetrahedron Lett.* **1976**, *28*, 2971.
2. Hori, K.; Wampler, J. E.; Matthews, J. C.; Cormier, M. J. *Biochemistry* **1973**, *12*, 4463.
3. Inoue, S.; Sugiura, S.; Kakoi, H.; Hashizume, K.; Goto, T.; Iio, H. *Chem. Lett.* **1975**, 141.
4. Shimomura, O.; Johnson, F. H. *Nature* **1975**, *256*, 236.
5. Young, R. E. *Comp. Biochem. Physiol.* **1975**, *52B*, 141.
6. Tsuji, F. I.; Leisman, G. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 6719.
7. Goto, T.; Iio, H.; Inoue, S.; Kakoi, H. *Tetrahedron Lett.* **1974**, *26*, 2321.
8. a) Goto, T. *Pure Appl. Chem.* **1968**, *17*, 421. b) Goto, T.; Inoue, S.; Sugiura, S. *Tetrahedron Lett.* **1968**, 3873. c) Inoue, S.; Okada, K.; Tanino, H.; Kakoi, H. *Chem. Lett.* **1987**, 417.
9. Kishi, Y.; Tanino, H.; Goto, T. *Tetrahedron Lett.* **1972**, *27*, 2747.
10. To check any enzymatic digestion, an given amount of coelenteramide **5** (synthetic) was added to the homogenate and recovered unchanged **5** after the same extraction condition.
11. A chemiluminescence detector, Model 825-CL JASCO, Hachioji, Tokyo, Japan was used.
12. Inoue, S.; Taniguchi, H.; Murata, M.; Kakoi, H.; Goto, T. *Chem. Lett.* **1977**, 259.
13. Shimomura, O.; Johnson, F. H.; Saiga, Y. *J. Cell. Comp. Physiol.* **1962**, *59*, 223.
14. Johnson, F. H.; Shimomura, O. *Methods in Enzymol.* **1978**, *57*, 271.
15. Musicki, B.; Kishi, Y.; Shimomura, O. *J. Chem. Soc., Chem. Commun.* **1986**, 1566.
16. Teranishi, K.; Isobe, M.; Yamada, T.; Goto, T. *Tetrahedron Lett.* **1992**, *33*, 1303.
17. Ward, W. W.; Cormier, M. J. *Proc. Natl. Acad. Sci. USA* **1975**, *72*, 2530.