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Synthesis of ^{13}C-Dehydrocoelenterazine and Model Studies on *Symplectoteuthis* Squid Bioluminescence

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Abstract: *In the photoprotein of an Okinawan squid bioluminescence of Symplectoteuthis oualaniensis L a dehydrocoelenterazine has been assigned as a chromophoric precursor to its apoprotein. To prove this mechanism, we have established new synthetic route to ca. 100 %-^{13}C incorporated dehydrocoelenterazine and coelenterazine at the neighboring carbon of the 2-position of 2,3-dihydroimidazo-[1,2a]-pyrazinone skeleton. This ^{13}C enriched dehydrocoelenterazine readily converted in equilibrium between its adduct forms as a diastereomixture with glutathione (GSH) or dithiothreitol (DTT) compounds having sulfhydryl group. Structures of such adducts were fixed under acidic conditions and then discussed by NMR spectroscopy as well as absorbance and fluorescence spectra.*

Various new bioluminescent systems have recently been investigated.[1] In 1981, Tsuji and Leisman reported that a homogenate of luminous organ of the oceanic squid, *Symplectoteuthis oualaniensis* L., gives light in the presence of monocation such as Na^+, K^+ etc. and molecular oxygen at pH 7.8.[2] In 1993 we succeeded in the extraction of a photoprotein responsible for the luminescence into a 0.6M KCl solution from the same squid *S. oualaniensis* collected in Okinawa, Japan and we have reported that this photoprotein contains dehydrocoelenterazine (**1a**), a similar chromophore as coelenterate luciferin, and that the chromophore may chemically be bound with the photoprotein through covalent bond such as thioether.[3] The major evidence to this chromophore is that addition of dehydrocoelenterazine (**1a**) solution into apoprotein of this squid enhanced the luminescence in almost the same amount of natural light, but the addition of coelenterazine (**4**) solution did not give light. After this addition the reddish chromophore of **1a** instantaneously changes into yellowish color as the chromophore in **2** the same as coelenterazine (**4**).

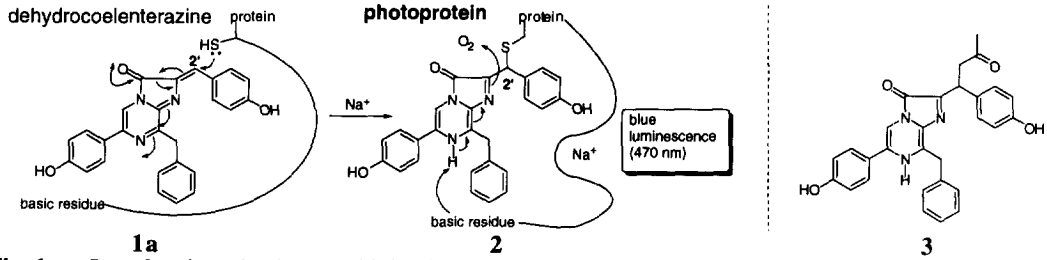

Fig. 1. Postulated mechanisms on bioluminescence of *Symplectoteuthis oualaniensis*.

Symplectoteuthis Bioluminescence Part 3; for Part 2, see Takahashi, H.; Isobe, M. *Chemistry Lett.*, **1994**, 843-846.[3,4]

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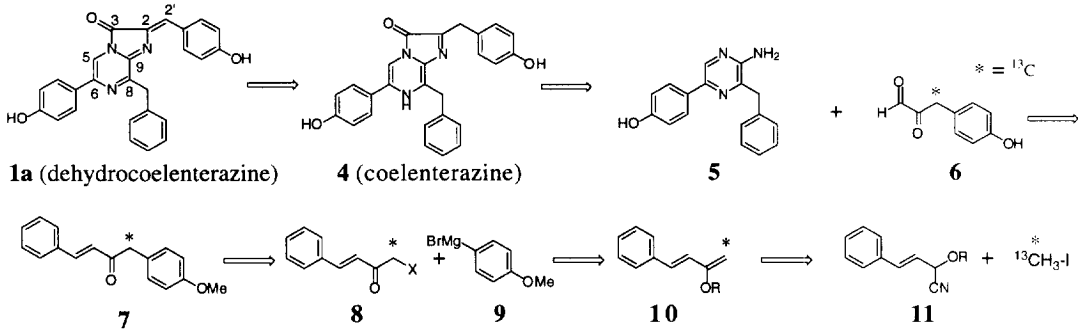
The connecting position of this chromophore has been suggested from an acetone adduct (**3**) to dehydrocoelenterazine (**1a**), that was isolated as an artifact from this photogenic organs.[3] Further experiments on time depending change of the absorbance and fluorescence spectra during luminescence of this photoprotein suggested that the chromophore would link with cysteine residue of the protein through thioether as illustrated in Fig. 1.[4] Molecular mechanism of further oxidative process has recently been demonstrated by synthesis of the ^{13}C-enriched *t*-butyl dioxetanone derivatives at low temperatures and by NMR studies.[5] This paper deals with the synthesis of ^{13}C-enriched dehydrocoelenterazine at the 2'-position and to prove this mechanism with a model sulfhydryl compounds such as glutathion (GSH) or dithiothreitol (DTT) through the analysis of ^{13}C-NMR and other spectroscopic methods as a mimic of protein interaction.

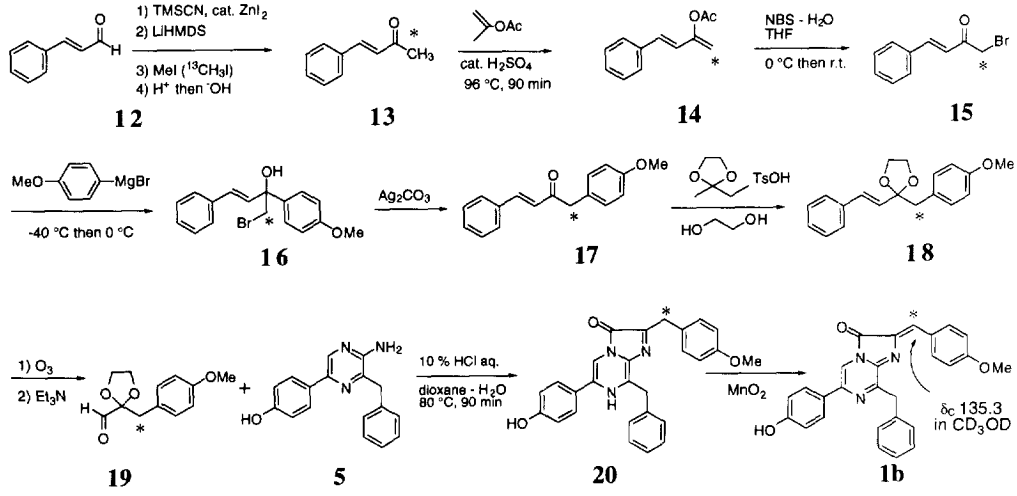
Retrosynthetic analysis for the ^{13}C enrichment of dehydrocoelenterazine (**1a**) is illustrated in Scheme 1; thus, it is obtained from coelenterazine (**4**) by MnO_2 oxidation.[6] The reported synthetic route to coelenterazine (**4**)[7] by coupling between aminopyrazine (**5**) and ketoaldehyde[8] suggested the synthesis of 3-(4'-hydroxyphenyl)-2-oxo-3-^{13}C-propanal (**6**). This ketoaldehyde is generated by ozonolysis of the α,β–unsaturated ketone (**7**), and the disconnection between the benzylic and phenyl carbon atoms give critical two precursors **8** and **9**. Due to the limitation of the commercially available ^{13}C sources, vinylether (or ester) **10** is necessary for the oxidation stage X in **8**. The cyanohydrin of cinnamaldehyde (**11**) was thus selected as the starting material for this purpose.



Scheme 1. Retrosynthetic analysis of ^{13}C-enriched coelenterazine.

Scheme 2 illustrates the synthetic route to 2'-^{13}C-dehydrocoelenterazine (**1a***). [In order to indicate and to distinguish ^{13}C enrichment from natural abundance isotope compounds the asterisk is attached to the formula number such as **N*** in text.] The cyanohydrin (**11** R= $SiMe_3$)[9] was in fact synthesized from cinnamaldehyde (**12**) and methylation with $^{13}CH_3I$ was followed by hydrolysis to afford the methylketone (**13***) in 63 % overall yield. For the oxidation of this methyl group of **13***, it was first converted into its vinylacetate (**14***, 80 %) with isopropenyl acetate in the presence of acid catalyst. Bromination with *N*-bromosuccinimide afforded the bromo-ketone (**15***, 75 %). Critical addition of the 4-methoxyphenylmagnesium bromide was carried out in diethyl ether solvent first at -40 °C and then at 0 °C to provide the bromohydrin (**16***) as only the product in 76 % yield.[10] The expected 1,2-rearrangement cleanly took place only with silver carbonate to give the α,β–unsaturated ketone (**17***, 77 %). A direct ozonolysis of **17*** gave only some over-oxidation products presumably due to its facile enolization. So the carbonyl group of **17*** was temporally protected as the ethyleneketal (**18***) and then subjected to ozonolysis. Incidentally, the

corresponding ozonide was best reduced by triethylamine[11] into the requisite ketal-aldehyde (**19***). These products were not isolated because of instability with silica gel column, so it was directly reacted with aminopyrazine (**5**) at 80 °C in a mixture of 10 % HCl and dioxane. The coelenterazine mono-methyl ether (**20***) was isolated in 34 % overall yield in 4 steps from the enone (**17***). Oxidation of **20*** with manganese dioxide in a mixture of ethanol and ether provided the dehydrocoelenterazine mono-methyl ether (**1b***, δ 2'-C 135.3 ppm)[12] in 97 % yield.



Scheme 2. Synthesis of ^{13}C-enriched and non-enriched dehydrocoelenterazine mono methyl ether.

Prior to the studies using these ^{13}C enriched compounds, the equilibrium of the addition of DTT as a sulfhydryl compound to dehydrocoelenterazine (**1a**) and its analogs (**1b-d**) with natural abundance isotopes (eq. 1) were studied with special reference to the equilibrium to **21a-d** under slightly alkaline media between pH 6.8 and 9.2 since the optimum pH is known to be 7.8 for the squid photoprotein.[2] The questions in eq. 1 are the effects of dissociation of the phenol groups in the equilibrium and also the relationship to the pKa being 8 for the dihydroimidazopyrazinone N-H proton in **21**. The latter is critical step for luminescence reaction with strong base such as potassium *t*-butoxide in DMSO solvent. When an aqueous solution of the compounds **21a** and **21b** were added to a solution of apoprotein of the squid photoprotein preparation (0.6 M KCl at pH 7.8 under air oxygen atmosphere)[4] both of them gave light (bioluminescence), and the latter afforded about 40 % of the light of the former. On the contrary, the light amount of the former (**21a**) that produced in DMSO with ^{t}BuOK (chemiluminescence) was less than 10 % of the latter (**21b**). This striking difference would be due to the dissociation of the phenol extended to the 2-position of the imidazopyrazinone ring; thus, strong alkaline condition for chemiluminescence directed the equilibrium for elimination of the SH (**Fig. 2**).

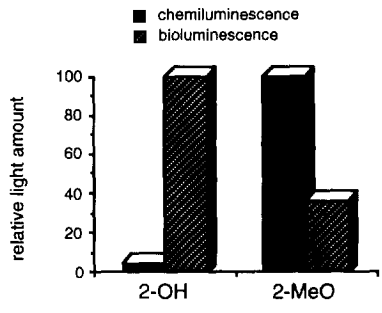
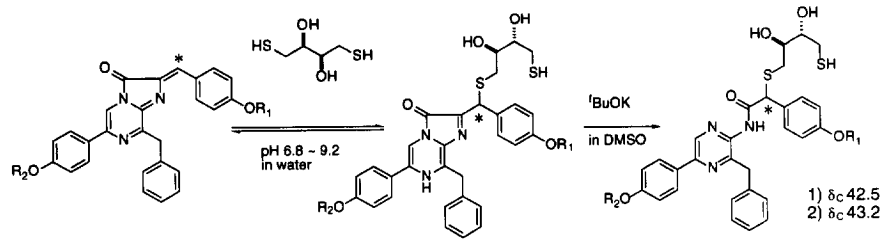


Fig. 2. Relative light amount by chemi- and bioluminescence of **21a** (2-OH) and **21b** (2-MeO).



eq. 1

1a R_1= H, R_2= H
1b R_1= Me, R_2= H
1c R_1= H, R_2= Me
1d R_1= Me, R_2= Me

21a R_1= H, R_2= H
21b R_1= Me, R_2= H
21c R_1= H, R_2= Me
21d R_1= Me, R_2= Me

22b R_1= Me, R_2= H

The equilibrium between compound **1**, DTT and **21** is monitored by means of UV-Vis spectra as shown in **Fig. 3**, where **A-D** are corresponding to the compounds **1a-d** (**21a-d**), respectively. Namely, the natural dehydrocoelenterazine (**1a**) clearly indicates in **A** that the increase of the absorbance is observed at around 600 nm corresponding to the free dehydrocoelenterazine. (Arrows indicates the direction of increasing pH.) Similar tendency is observed with **21c** in **C**. Those compounds having methyl ether on the right phenol group (**21b** and **21d**) does not show such absorbance, thus no elimination happens under these alkaline condition. As a consequence dehydrocoelenterazine analog having R_2= Me can provide stable adduct with SH compounds.

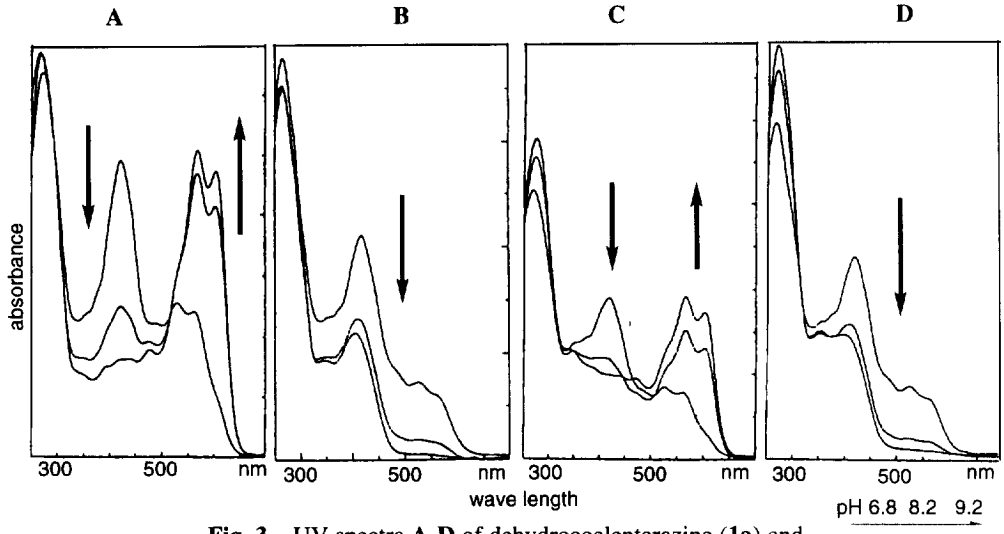


Fig. 3. UV spectra **A-D** of dehydrocoelenterazine (**1a**) and its analogs **1b-d** ⇌ **21a-d**, respectively,at various pH.

Dehydrooelenterazine mono methyl ether **1b*** (enriched 2' carbon with ^{13}C) is the best candidate to mimic the studies with photoprotein. Mixing of **1b*** and dithiothreitol (DTT) with a 1:10 molar ratio in a mixture solvent of methanol and dichloromethane (1:1) provided an adduct **21b*** at room temperature (**Fig. 4**). After stirring for 20 min the reaction mixture was acidified to pH 3.0 by addition of 1N HCl. Concentration of the DTT-adduct solution of **21b*** under reduced pressure gave yellow oil, and this residue

was analyzed with NMR to find two signals on ^{13}C spectra; thus δ 45.2 and 45.4 ppm. Proton NMR also showed two singlets at ca. 5.6 ppm, and C-H COSY spectrum made correlation of ^{1}H-NMR δ 5.67 and 5.62 ppm, respectively.

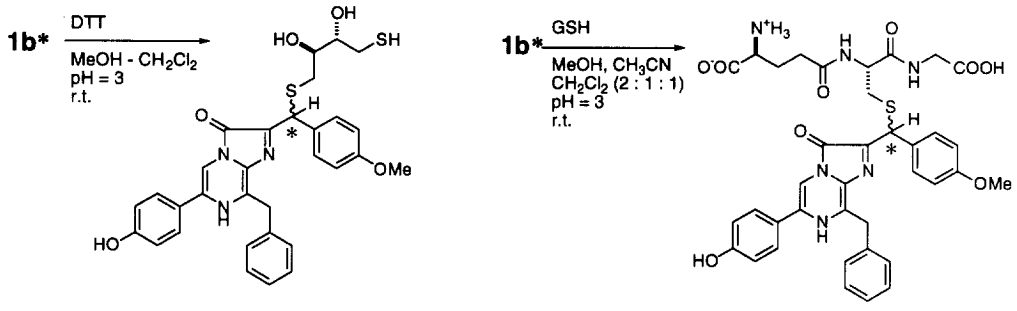


Fig. 4. Structures of DTT adduct and GSH adduct of ^{13}C-enriched dehydrocoelenterazine analog.

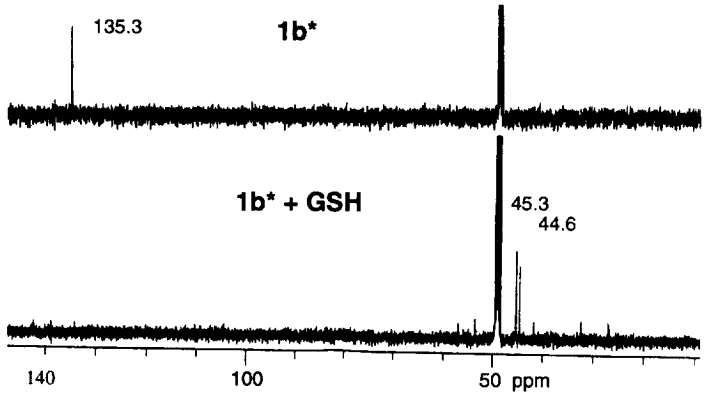


Fig. 5. ^{13}C-NMR of adduct **23*** of ^{13}C enriched dehydrocoelenterazine analog **1b*** and GSH.

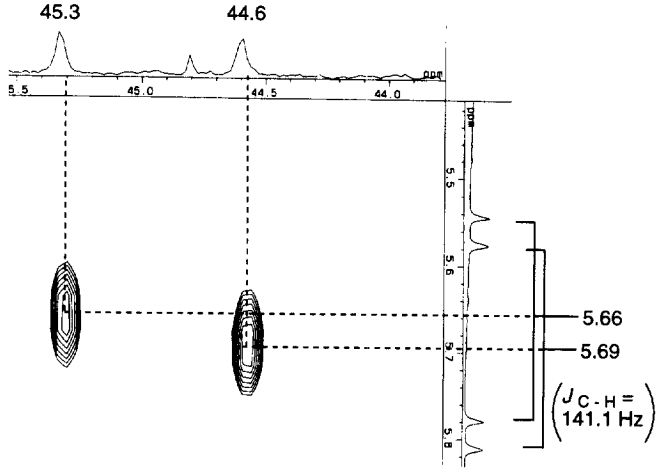


Fig. 6. C-H COSY of adduct **23*** of 2'-^{13}C-dehydrocoelenterazine analog **1b*** and GSH.

Similarly glutathion (GSH) was added to **1b*** with a 1:5 molar ratio in a mixture of methanol, acetonitrile and dichloromethane (2:1:1) at room temperature for 50 min, and acidification to pH 3 afforded **23***, that gave a diastereomer as shown in **Fig. 5** and **6**: δ 44.6 and 45.3 ppm and ^{1}H-NMR δ 5.69 and 5.66 ppm, respectively. Chemiluminescence of DTT adduct **21b** emits light (520 nm) in DMSO under basic condition (containing 10 % of 1 *N* ^{t}BuOK/^{t}BuOH). The product was separated by silica gel chromatography and analyzed to give m/z = 577 (using natural abundance ^{13}C) by FAB-MS spectrum. In its ^{13}C NMR spectrum two sets of signals at δ 42.5, 43.2 ppm may be derived from the two diastereoisomers of coelenteramide analog (**22b***) (eq. 1). In the bioluminescence of *Symplectoteuthis oualaniensis*, the molecular mechanism may be similar to the above mimic studies, while the natural dehydrocoelenterazine (**1a**) exhibits efficient bioluminescence presumably the local pH around phenol and dihydroimidazopyrazinone groups should be controlled by the protein surface. Further evidence of the molecular mechanism should be studied directly using the photoprotein. Structural and cloning studies on this photoprotein are now under investigation.

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9 Gassman, P. G.; Talley, J. J. *Tetrahedron Lett.* **1978**, 3773-3776. Trimethylsilyl cyanide was prepared according to the method described in ref. # 5a.

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12 Selected data for ^{13}C-enriched dehydrocoelenterazine mono methyl ether **1b***: dark purple powder: Mp 198-199 °C (decomp). UV (MeOH), λ_{max} (log ε) 526 (3.89), 407 (4.20), 272 (4.18) nm. ^{1}H-NMR (CD_3OD, 500 MHz), δ 8.45 (s, 1H), 8.24 (dd, *J* = 8.8, 4.9 Hz, 2H), 7.64 (s, 1H), 7.61 (d, *J* = 8.8 Hz, 2H), 7.44 (d, *J* = 7.4 Hz, 2H), 7.38 (d, $J_{^{13}C\text{-}H}$ = 155.8 Hz, 1H), 7.22 (t, *J* = 7.8 Hz, 2H), 7.12 (t, *J* = 7.8 Hz, 1H), 6.97 (d, *J* = 8.8 Hz, 2H), 6.74 (d, *J* = 8.8 Hz, 2H), 4.26 (s, 2H), 3.81 (s, 3H) ppm. ^{13}C-NMR (CD_3OD, 125.7 MHz), δ *135.3 ppm. MS (FAB) *m/z* 437 (M^+).