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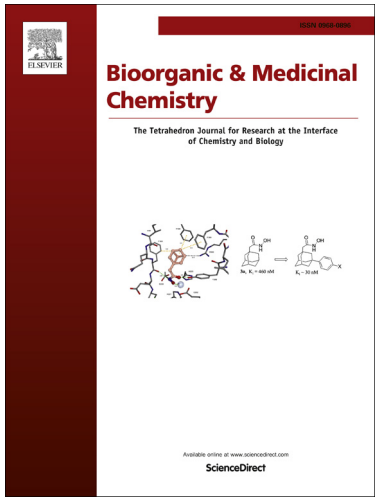


Molecular Mechanism of *Symplectoteuthis* Bioluminescence---Part 4: Chromophore Exchange and Oxidation of the Cysteine Residue

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## Graphical Abstract

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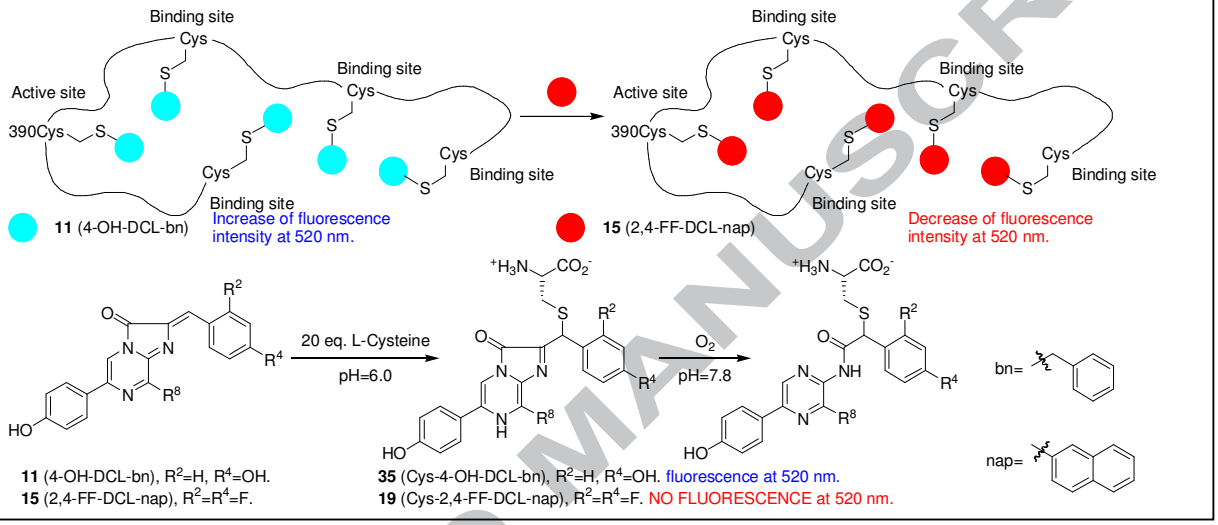

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# Molecular Mechanism of *Symplectoteuthis* Bioluminescence---Part 4: Chromophore Exchange and Oxidation of the Cysteine Residue

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ABSTRACT

*Symplectin* is one of the few photoproteins, which forms covalent bonds with the dehydro-coelenterazine (DCL) at the binding sites and the active site. This binding takes place through the SH's of the cysteine residues via conjugate addition reaction. This photoprotein contains the chromophore molecules at the binding cites first, and then moves to the active cite Cys-390 for the luminescence. The current study focuses on these dynamic aspects of the chromophore using the natural photoprotein by analyzing the fluorescence changing of the DCL chromophores analogs with 8-(4'-methoxyphenyl)- or 8-(2'-naphthyl)-group and 2-(2',4'-difluorophenyl)-group. Exchanges of these chromophores were monitored the fluorescence at slightly acidic media and also from the luminescence function observed at the optimum pH 7.8. The non-fluorescent naphthyl analogs was even proven to make the covalent bond formation at pH 6.0 and evidently to obtain the corresponding luminescent product amide by liquid chromatographic detection from the spent solutions.



## 1. Introduction

This article is the continuation of Part 3 of our previous report on *Symplectoteuthis* bioluminescence.[1]

Light emission from living cells has been commonly used in chemical biology research as one of the important tools for specific visualization of the target molecules on the basis of molecular-binding in living cells. Coelenterazine is the most typical chromophore in marine bioluminescent systems, and recently 2-hydroperoxycoelenterazine is the key feature in the calcium dependent photoproteins such as aequorin and obelin. These studies have made the molecular mechanism of the bioluminescence by means of structural biology using X-ray analysis of the co-crystallized photoproteins with the substrates.[2] We have been working on the coelenterazine-containing photoprotein *symplectin*, the luminescence of which is triggered by monovalent ions such as $Na^+$ and $K^+$.[3] This photoprotein *symplectin* works in an oceanic squid, *Symplectoteuthis oualaniensis* (*Japanese name, TOBI-IKA*) collected in the sea around Okinawa, Japan,[4] and obtainable in a water-insoluble fraction but only soluble with high salt-concentration, e.g. KCl >0.6 mol/L.[4] In 2008, the amino acid sequence of *symplectin* was elucidated with its 501 AA containing 11 cysteine residues (including 3 Cys-S-S-Cys). The current study was carried out using the purified photoprotein from the squid since gene-manipulated preparation of this protein has not been reported. Isobe et al assigned that the 390-Cys worked as the active site of *symplectin* by means of LC-MS-detection and the following experiments.[5] Namely, the chromo-peptide fragment, *chrom-S-390C-G-L-393K*, was observed by using the mono-fluorinated dehydro-coelenterazine analog; thus, a peak corresponding to MW 843.55 as the evidence of the coelenterazine analog being bound to the active site before the luminescence. Another peak equivalent to MW 831.53 was observed after the luminescence from the tryptic digest as the coelenteramide analog.[5] This detection system includes a house-modified capillary-HPLC connected to ESI-Q-TOF/MS on the samples from the photoprotein, which was reconstructed with synthetic chromophore and then hydrolyzed with proteolytic enzyme such as Trypsin or Lys-C.[5] Further proof of the active site was implemented using di-fluorinated coelenterazine, which binds to the same 390-Cys residue more tightly. It gave the molecular ions of the chromopeptide after luminescence at m/z 849.3 as well as the fragment ions (m/z 735, 588, 419, 306 and 278) from the capillary HPLC-ESI-Ion Trap MS to show the coelenteramide substructure.[1]

Shimomura states that many marine bioluminescent animals give light by using coelenterazine or similar compounds as the substrate having common chromophore.[6] The oxidation mechanism and peroxy intermediates of these chromophores show the common substructures as the hydroperoxide **5** and peroxylactone (dioxetanone) **6**,[6] as has been demonstrated by Isobe and co-workers to prove the more detailed mechanism in a direct manner using 100%-$^{13}$C-enriched luminous coelenterazine analogs at some specific carbon atoms and analyses through low-temperature $^{13}$C-NMR of the short-life time peroxidic intermediates (**Figure 1**).[7] The presence of 2-hydroperoxycoelenterazine in aequorin and obelin has been reported recently.[2, 8]

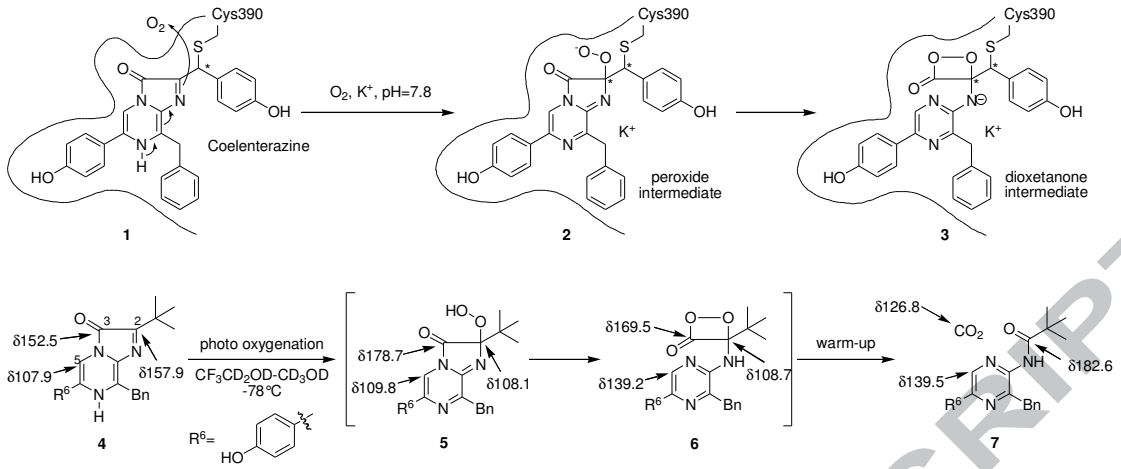



**Figure 1**. The possible bioluminescence intermediates **1** and **2** of *symplectin* (top) were proved through synthesizing a *t*-butyl model **4**, having 100%-$^{13}$C enriched coelenterazine and the corresponding hydroperoxide **5** and peroxy lactone **6** and assigning the structures by low-temp NMR (bottom).[7]

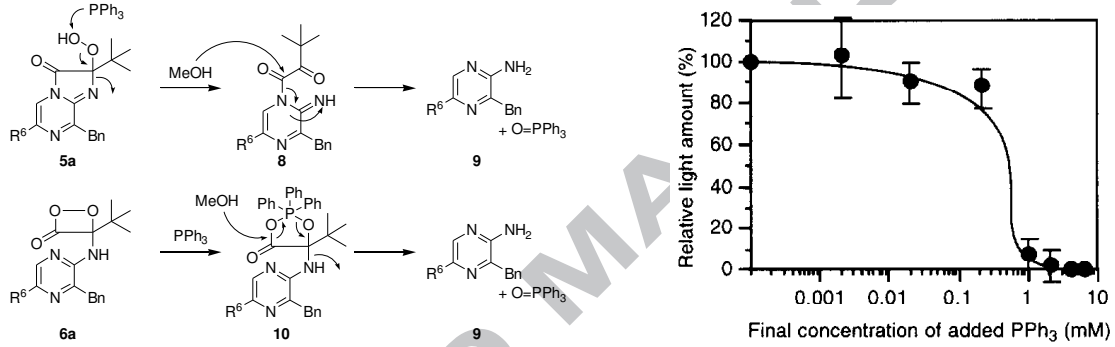



**Figure 2.** Titrative reduction of the peroxide **5a** and dioxetanone **6a** were effected by triphenyl phosphine at -78 °C to diminish the light emission (Usami and Isobe.[6]).

In this case, those reactive intermediates were prepared *via* low-temperature photo-oxygenation in $CF_3CD_2OD$ solvent. The NMR at -78 °C proved the structures of 2-hydroperoxy-coelenterazine analog **5** and dioxetanone **6** with concomitant measurements of luminescent spectra from these intermediates under different media. Namely, **5** and **6** provided significant luminescence spectra derived not only from neutral media but also even from acidic media by simply allowing the temperature at ca -50 °C or 0 °C, respectively. This provided new tools elucidating the excited-molecular species, which is strong contrast to the fact that previous chemi-luminescence had been reported only under strongly basic conditions in dipolar aprotic solvents such as DMSO. Titrative reduction of the peroxides **5** and **6** at -78 °C with triphenyl phosphine or diphenyl sulfide took place in the stoichiometry-dependent manner with diminishing the amount of luminescence; (triphenyl phosphine was as example as shown in **Figure 2**).[7] The products in the reduction were amino-pyrazines **9** and triphenylphosphine oxide or diphenyl sulfoxide. So the bioluminescence mechanism should include the same chemical principle on the enzymic surface. Recently, Isobe et al. further reported the fact that two different bioluminescence systems including sulfur atom of DTT (dithiothreitol) attached at the cysteine residues of the photoprotein *aequorin*.[5,9] Involvement of the sulfur atom of Cys residues provided new tools for the studies of bioluminescence, which is described in the current study.

Kongjinda and Isobe[1] reported that two di-fluoro-dehydrocoelenterazine regio-isomers (FF-DCL-bn: deep red color, non-fluorescent) **12** and **13** showed quite different bioluminescence behavior in *symplectin* photoprotein from that of natural chromophore **11** (4-OH-DCL-bn). Namely, 2,4-difluoro-analog **12** (2,4-FF-DCL-bn) provides larger amount of the luminescent light than the natural chromophore **11**, while 2,6-difluoro-analog **13** (2,6-FF-DCL-bn) gives smaller amount (1/3~1/4) of light from **11**.[9] Both of these two chromophores **12** and **13**, however, showed similar behavior such as increase of the green fluorescence at 520 nm during the incubation to *apo-symplectin* at pH 6.0 in accordance with the binding as a conjugate addition manner of the SH group of Cys to these chromophores (**Figure 5**). When the pH of the DCL-analog-incubated photoproteins was changed to pH 7.8, both analogs show decreasing of the green fluorescent intensities. Namely, the non-bioluminescent species from **13** was consumed as well like the luminescent one **12**. This suggested some different pathways might take place for each of them on the protein surface when subjected to the luminescence conditions.

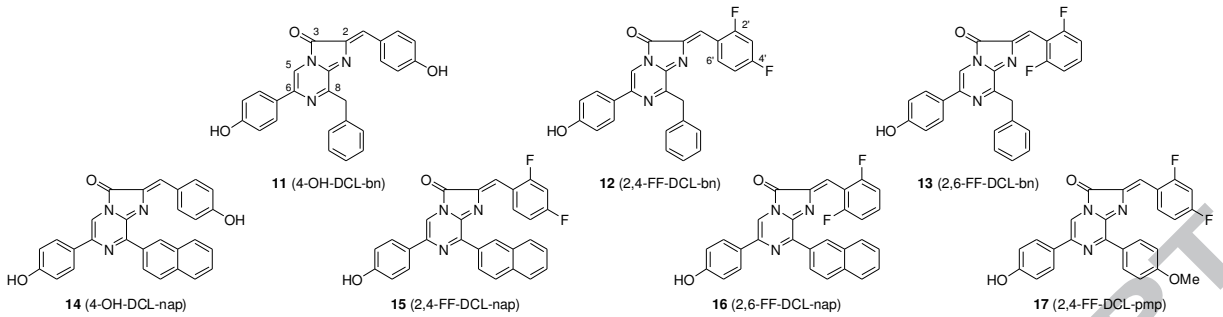



**Figure 3.** Natural (**11**, 4-OH-DCL-bn) and unnatural (**12**-**17**) chromophores applied to *apo-symplectin* photoprotein.

The synthetic route of the coelenterazine-chromophores[10] was redesigned to avoid using hazardous diazo-reagents as reported by Kishi et al.[11] and the improved version reported by Kondo et al.[12] Alternative routes on the basis of Pd-mediated cross coupling[13] was also developed by Makarasen and Isobe,[14] and Wong and Isobe.[15] Chou, one of the authors of this paper, has published a new synthetic route from aminopyrazine, which was suitable for the synthesis of the current study.[16] In this paper, we prepared such chromophores **11**-**17** (see **Figure 3**) via the new synthetic route that have different fluorescence maxima and intensities emerging after binding with the cysteine residues. These compounds led us to have the first experimental achievement of the chromophore-exchange on the photoprotein, *symplectin*, from originally bound-natural chromophore **11** to the new difluoro-derivatives **12, 13**, **15,** and **16**. Of particular interests, **15** (2,4-FF-DCL-nap) and **16** (2,6-FF-DCL-nap) derivatives did not exhibit strong fluorescence at 520 nm. We have confirmed these observations with *symplectin* by using various sample preparations which yield different amounts of *apo-symplectin*.

## 2. Results and Discussions

### *2.1. Preparation of the symplectin solution*

A partially purified (SDS-PAGE single band) sample solution of *symplectin* was prepared on the basis of its limited solubility upon KCl-concentration according to our previously reported method with slight modification.[1] Every *symplectin*-preparation was prepared from the frozen-photogenic organ by cutting as a round disk by using a 1.5 cm-diameter borer to give a disk-weight of 50 mg (±2 mg). The disk was homogenized with a glass-homogenizer using the basic buffer (containing 0.4M KCl, pH=7.8 , 1000 μL) and washed by washing buffer A (containing 0.4M KCl with DTT, pH=5.6, 1000 μL) once and washing buffer B (containing 0.4M KCl without DTT, pH=5.6, 1000 μL) twice. The residue was extracted with the extracting buffer A (containing 0.6M KCl, pH=5.6, 1000 μL). Each photoprotein preparation was kept in extracting buffer A at pH 6.0 which contained sucrose without DTT at 0 °C in an ice bath placed in a refrigerator ca. 4 °C. All of them were used within 18 hrs for the following experiments.

### *2.2. Bioluminescence activity*

Each given sample was placed in a short test tube in a dark small house equipped within a photon counter and the light amount was measured by repeating three times and averaged. First, bioluminescence light amount from each aliquot (40 μL) of *symplectin* solution was measured (without incubation with any additional synthetic chromophore) under a pH 7.8 environment by simply adding the luminescence buffer (400 μL) and catalase solution for providing active oxygen species. The light amounts were integrated for 5 min, and the resulting values were compared as the light yields, which were almost identical (±□3 %) from the same sample-preparation obtained from the same individual squid organ (data not shown). Incubation of each three different chromophore-DCL-analog **11**, **12**, and **13** in DMSO solution (2 mM, 4 μL) with the *symplectin*-solution (40 μL) was implemented at 0 °C for 20 min, followed by luminescence measurement by mixing the luminescence buffer and catalase solution at 25 °C. Each chromophore was incubated with three different *symplectin*-solutions P-1, P-2 or P-3 prepared from the organs of different squids. (In fact, 10 *symplectin*-solutions were prepared from the organs of 10 different squids, and typical three examples (photoprotein **P-1**, **P-2** and **P-3**) are illustrated in **Figure 4**.)

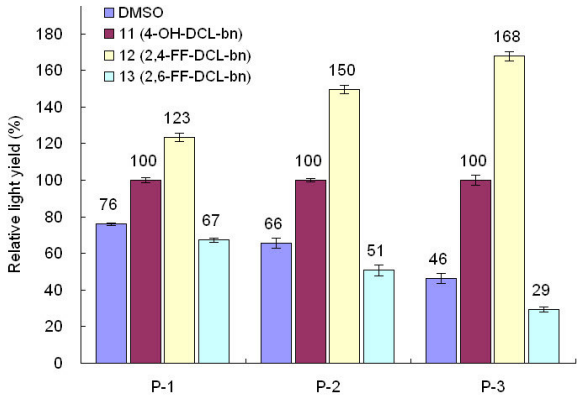
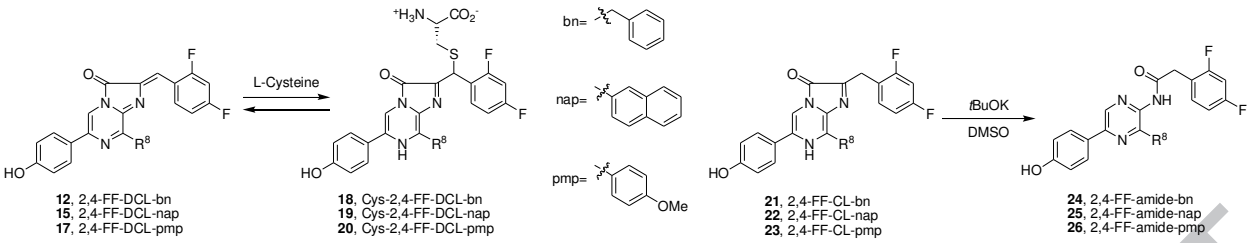



**Figure 4.** Typical results of the light amounts from three different *symplectin*/*apo-symplectin* photoprotein (P-1, P-2 and P-3) after incubating with different chromophores; thus, DMSO (containing no chromophore), and containing 4-OH-DCL (**11**), 2,4-FF-DCL (**12**), or 2,6-FF-DCL (**13**) solution; first in buffer pH 5.6 for 20 min and then the light amounts were counted by changing the pH to 7.8 and averaged from three measurements.

In **Figure 4**, the control experiments by adding only DMSO solvent afforded different amounts of light (76%, 66% and 46%) with the photoprotein **P-1**, **P-2**, and **P-3**, respectively (see **Figure 4**/DMSO). These results suggest that the photoprotein contains luminous chromophore in different amounts in the photoprotein, which is considered to remain as storage form but in different quantities. In fact, the incubation with the natural chromophore **11** (4-OH-DCL-bn) afforded the results of the same light-production in these three photoprotein preparations P-1, -2, -3, so these values are plotted as 100% as the full capacity of *symplectin* with the natural chromophore. Namely, the ratios of *apo-symplectin*:*holo-symplectin* can be determined on each individual squid as [(100-DMSO)/DMSO], thus, concluding that photoprotein **P-1** has 24%, and **P-2** has 35% and **P-3** has 54% of the apo-protein, respectively.

A.

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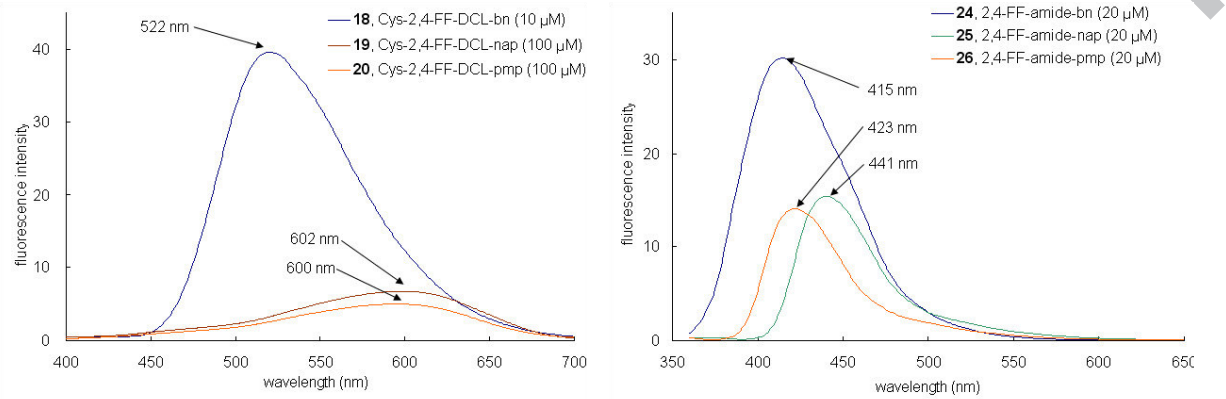



**Figure 5.** (A) Synthetic analogs showing different property in UV absorbance (**12**, **15**, and **17**) and fluorescence (**18**, **19**, and **20**). (B) Fluorescence spectra of the three chromophores **18** (10 μM), **19** (100 μM), and **20** (100 μM), and three coelenteramides, which are the luminescence product as known as oxyluciferin, **24** (20 μM), **25** (20 μM), and **26** (20 μM).

The yellow bars and blue bars in **Figure 4** represent the light amounts observed after incubation with **12** (2,4-difluoro-dehydrocoelenterazine; 2,4-FF-DCL-bn), and with **13** (2,6-difluoro-dehydrocoelenterazine; 2,6-FF-DCL-bn). The results of the light yields from the chromophore **12** were increased by +23%, +50% and +68% with P-1, P-2 and P-3, respectively. Meanwhile, the corresponding amounts from **13** decreased by -33%, -49% and -72%. These results (the increase or decrease figures) proposed a new question whether it might be simply related to the amount of *apo-symplectin* due to those figures being somewhat similar; thus, 24%, 35% and 54%. What is the reason by increase or decrease of the luminescence activity under the same operation with different chromophores **12** and **13**. Therefore, we have designed some experiments to observe in more details (1) whether simple intake of the chromophore takes place at the vacant binding sites or (2) whether any chromophore exchange takes place from natural DCL with unnatural di-FF-DCL. To urge such experiments onward, we synthesized three naphthyl chromophores **14**, **15** and **16** as well as a methoxyphenyl chromophore **17** displacing the benzyl group at the 8-position of the imidazopyrazinone nucleus. In fact, the 8-β-naphtyl and 8-(*p*-OMe)-phenyl derivatives did not show fluorescence at 520 nm as the 8-benzyl derivatives.

### *2.3. Dynamic Profile of the Chromophore Exchange by using 2,4-diF-DCL-naphtyl*

The different fluorescent nature between the green fluorescent coelenterazine and non-fluorescent dehydrocoelenterazine is striking, although UV-Vis spectra behaves similar but different wave length as CL is pale yellow color but DCL shows deep red color (**Figure 5**). The weak fluorescence of **19** (Cys-2,4-FF-DCL-nap, 602 nm) and **20** (Cys-2,4-FF-DCL-pmp, 600 nm) are much different than **18** (Cys-2,4-FF-DCL-bn, 522 nm). However, 8-β-naphtyl derivatives were selected as a better analog than 8-(*p*-OMe)-phenyl derivatives in the following experiments, because the resulted bioluminescence product **25** (2,4-FF-amine-nap) has the fluorescence peak at 441 nm away from that of **24** (2,4-FF-amide-bn) showing at 415 nm. Informatively, the fluorescence of **26** (2,4-FF-amide-pmp) has the similar peat at 423 nm as that of **24**. A conjugate addition of a cysteine-SH residue to DCL can readily demonstrate *in vitro* as shown in **Figure 6**, where red color of DCL changes to pale yellow color of CL by stepwise addition of cysteine into the chromophores. Such equilibria with these difluoro DCL derivatives were measured to provide the dissociation constants as $K_{D50}$= 2.8x10$^{-4}$ M for **12**, as $K_{D50}$= 1.3x10$^{-3}$ M for **15**, and $K_{D50}$= 5.3x10$^{-4}$ M for **17**, with clear isosbestic points at 410 nm and 500 nm showing direct equilibria between these chromophores and Cysteines as in **Figure 6**. A decreasing $K_{D50}$ value of the naphthyl analog **19** may be attributed to a repulsive interaction of larger and non-flexible naphthyl group than the benzyl group of the cysteine adducts **18**. And $K_{D50}$ values of **18** and **20** were similar. Cysteine adducts **19** and **20** should, however, showed enough binding affinity to investigate the reaction with *apo-symplectin*.

Similar equilibria may be observed through changing the fluorescent intensity at 520 nm when the synthetic chromophores, eg. **12** (2,4-FF-DCL-bn) and **15** (2,4-FF-DCL-nap), were incubated to the (*apo*)-*symplectin* preparations. In case of the 8-benzyl DCL derivatives **12**, such an exchange highly exceeds the natural coelenterazine **11** due to the presence of 2 electron-withdrawing F-atoms.

In order to confirm the fact that incorporation of naphthyl-DCL analog **15** indeed takes place into *apo-symplectin*, the other DCL and its analogs (shown in **Figure 7**) were incubated, one by one, to *apo-symplectin* at pH 5.6 and raised to pH 7.8 to compare the bioluminescence activities. The relative light yields are illustrated in **Figure 7**.

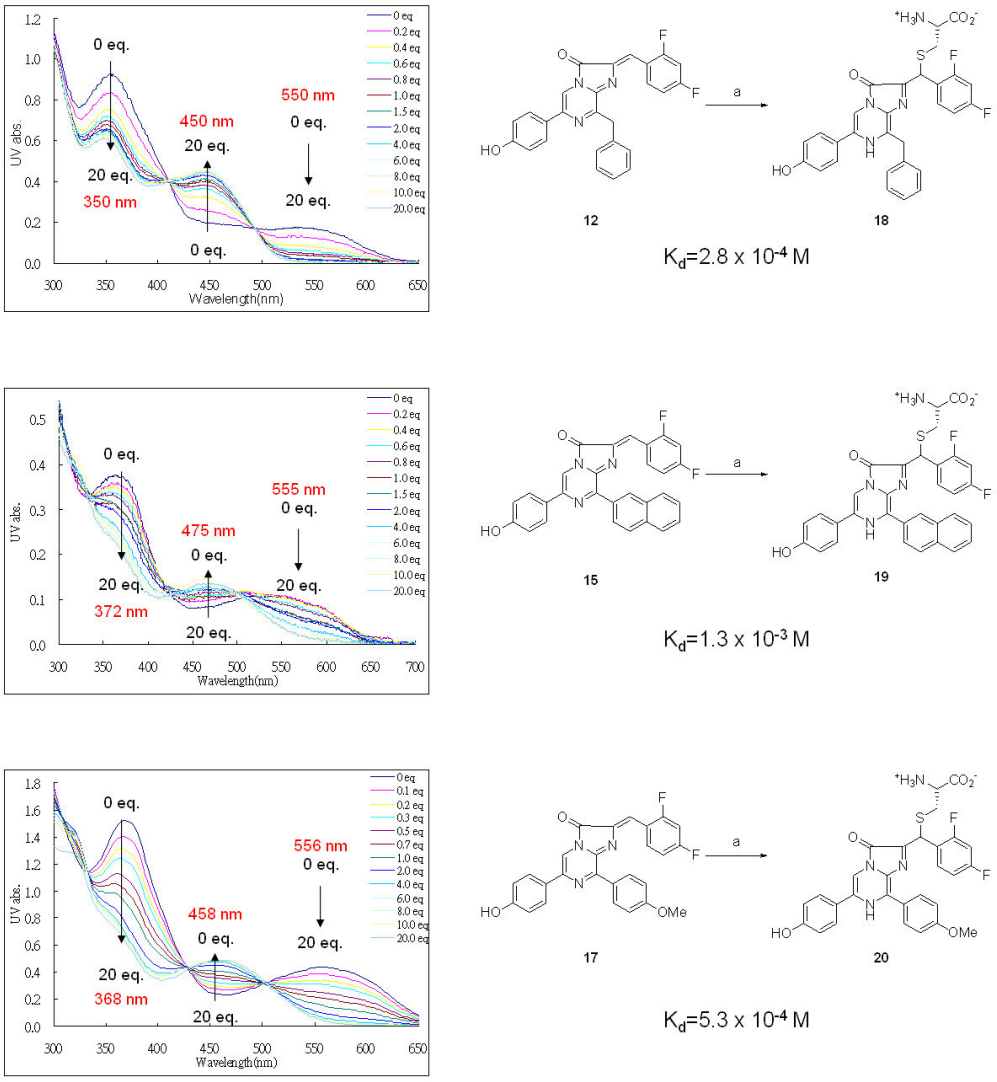



**Figure 6.** UV absorbance spectrum of the equilibrium DCL and analog with Cysteine. The DCL samples were dissolved in degassed DMSO, and then mixed with pH 6 buffer (10 mM KCl, 12 mM AcOH, 12 mM NaOAc in 68% MeOH/$H_2O$. The volume ratio of DMSO to buffer is 1 : 2.) in the UV cell by shaking. a) x eq. L-Cysteine, pH 6 buffer.

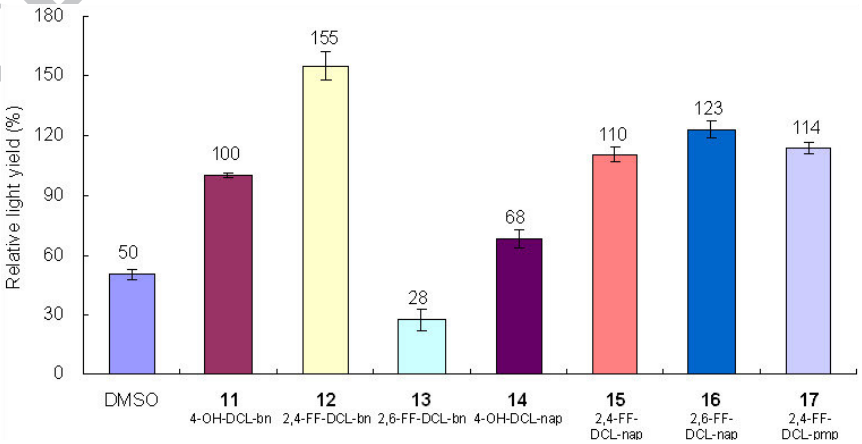



**Figure 7.** The bioluminescence activity of DCL and analogs. DMSO and chromophore were incubated with the *apo-symplectin* which was extracted from the same squid for 20 mins, and then measured luminescent light yield.

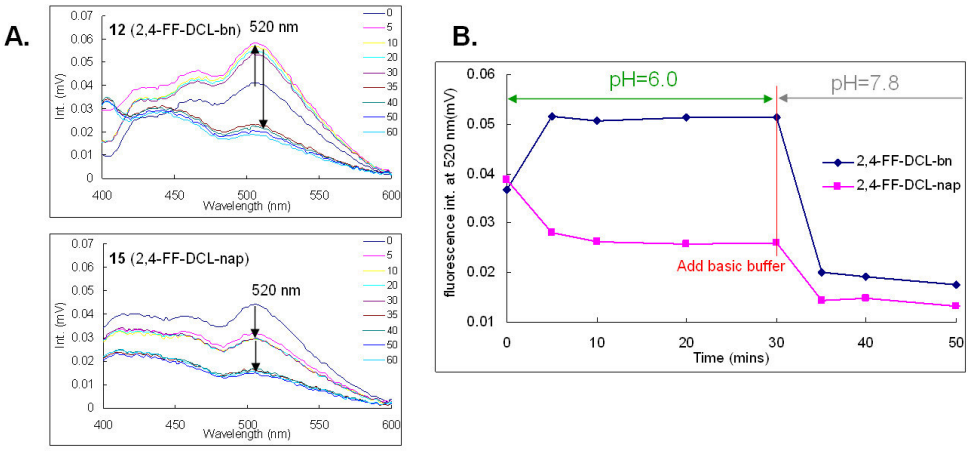



**Figure 8.** The fluorescence change of *symplectin* with DCL and analogs incubation (A). The plotting at 520 nm of fluorescence change (B).

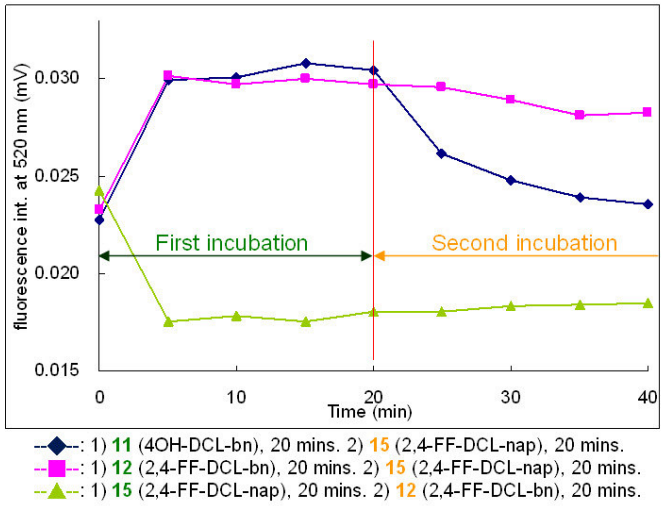



**Figure 9.** The fluorescence change at 520 nm of the double incubation with 2 different DCL analogs. The **12** (2,4-FF-DCL-bn) and **15** (2,4-FF-DCL-nap) have similar binding affinity in *symplectin* because the fluorescence intensity at 520 nm remained in similar level while the competitive experiment.

The synthetic naphthyl chromophores **15** (2,4-FF-DCL-nap) and **16** (2,6-FF-DCL-nap) showed higher light yield than **11** (4-OH-DCL-bn), but **14** (4-OH-DCL-nap) didn't. **17** (2,4-FF-DCL-pmp) also had similar function as **15** (2,4-FF-DCL-nap). According to the results, the synthetic analog **15** (2,4-FF-DCL-nap) has similar binding affinity and bioluminescence as **11** (4-OH-DCL-bn).

The time-dependent fluorescence spectrum-changes are shown in **Figure 8**. This measurement was implemented by sucking the reconstructed symplectin-solution into a flow cell equipped with a fluorometer at the 5-10 min intervals from 0 min to 30 min after starting the incubation. The *increase of the FL intensity at 520 nm* with **12** (2,4-FF-DCL-bn) was due to the binding to a cysteine residue of symplectin to form an apparently-reduced coelenterazine chromophore with the green fluorescence. However, the *decrease of the FL intensity at 520 nm* after the incubation with **15** (2,4-FF-DCL-nap) was due to the fact that the cysteine adduct (the same chromophore as **19**) has only 1% intensity (compared to **18**)(see **Figure 5B**) to result in the decreasing-intensity when natural 4-OH-DCL-bn on symplectin was displaced by **15** (2,4-FF-DCL-nap).

After 30 min, the reconstructed symplectin was treated with basic buffer (pH=11) to change to pH 7.8. So these base treated solutions should consume the chromophore (*pseudo*-reduced coelenterazines) to form coelenteramides with concomitant light emission so both solutions showed the decrease at 520 nm (**Figure 8B**). It is worth to mention that the fluorescence didn't become 0 even the chromophores were consumed by adding of basic buffer. The possible reason was due to the coelenteramide which has 10% of fluorescence intensity at 520 nm compared to (*pseudo*-reduced chromophore) coelenterazine.

A competitive experiment is shown in **Figure 9**. It is observed that **15** (2,4-FF-DCL-nap) binds stronger with cysteine residue of *symplectin* than **11** the natural 4-OH-DCL-bn. This is concluded from the observation of decreasing 520 nm intensity by incubation with **15** (2,4-FF-DCL-nap). This is consistent from the $K_{D50}$ values between the reconstructed *symplectin* preparations with **11** (4-OH-DCL-bn) (see **Figure 7**).

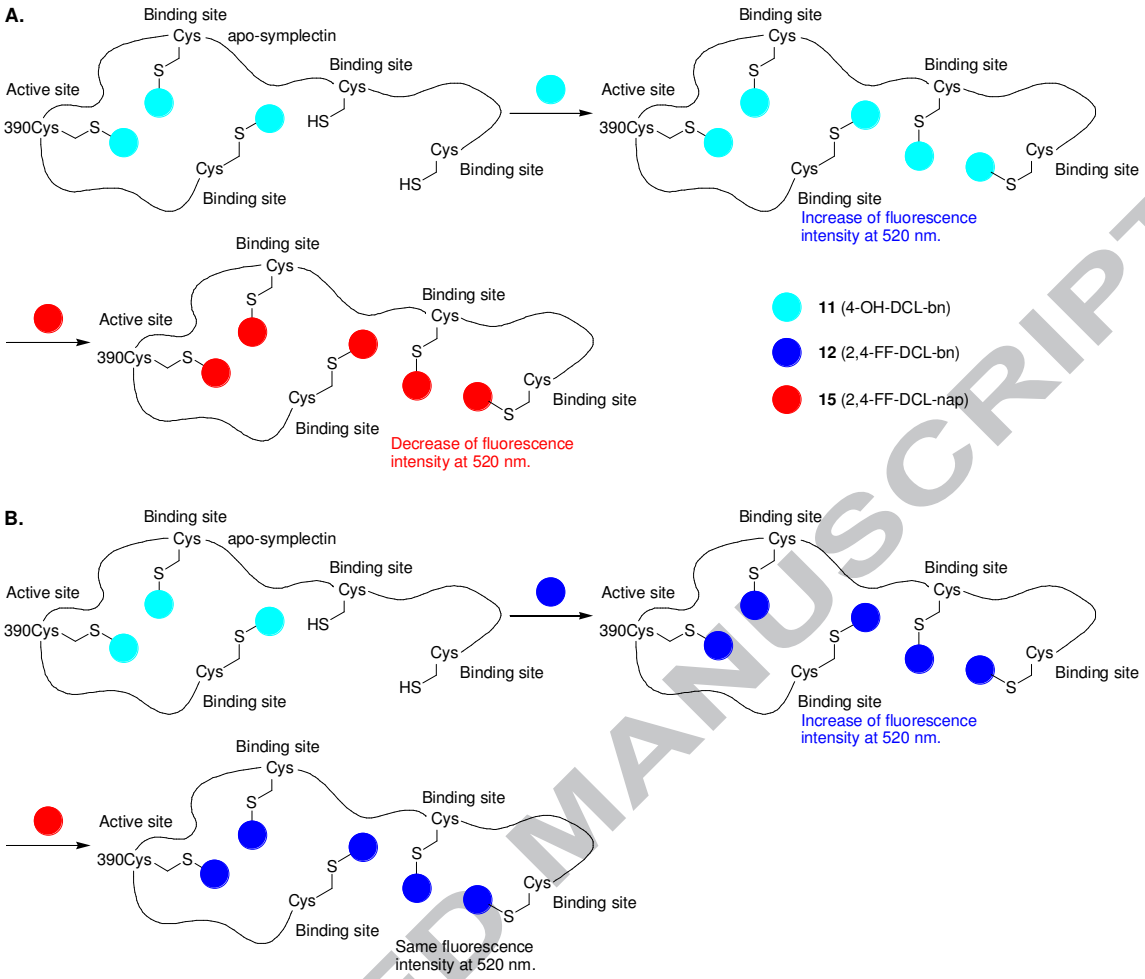



**Figure 10**. The possible exchange happened in *symplectin* with double incubation by 2 different DCL analogs.
(A) The chromophores in **11** (4-OH-DCL-bn) incubated *symplectin* were easy exchanged by **15** (2,4-FF-DCL-nap) and the fluorescence intensity at 520 nm is decreasing as blue line(--◆--) as shown in **Figure 9**.
(B) The pink line (--■--) in **Figure 9** shows the **12** (2,4-FF-DCL) incubation and followed by **15** (2,4-FF-DCL-nap).

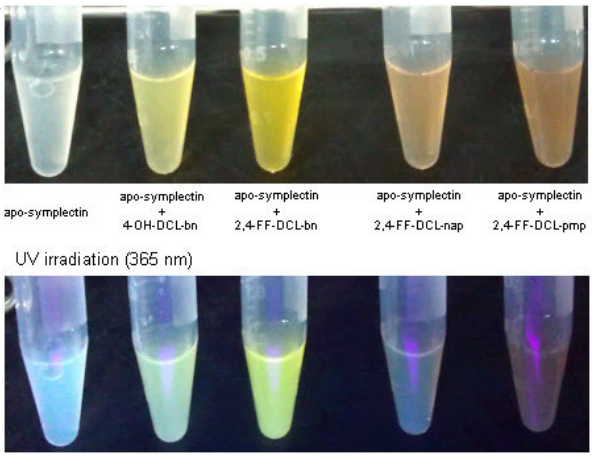



**Figure 11.** Color and fluorescence change of *symplectin* with different incubation. The **11** (4-OH-DCL-bn) or **12** (2,4-FF-DCL-bn) chromophore incubated *symplectin* showed the fluorescence under UV irradiation, but **15** (2,4-FF-DCL-nap) or **17** (2,4-FF-DCL-pmp) didn't.

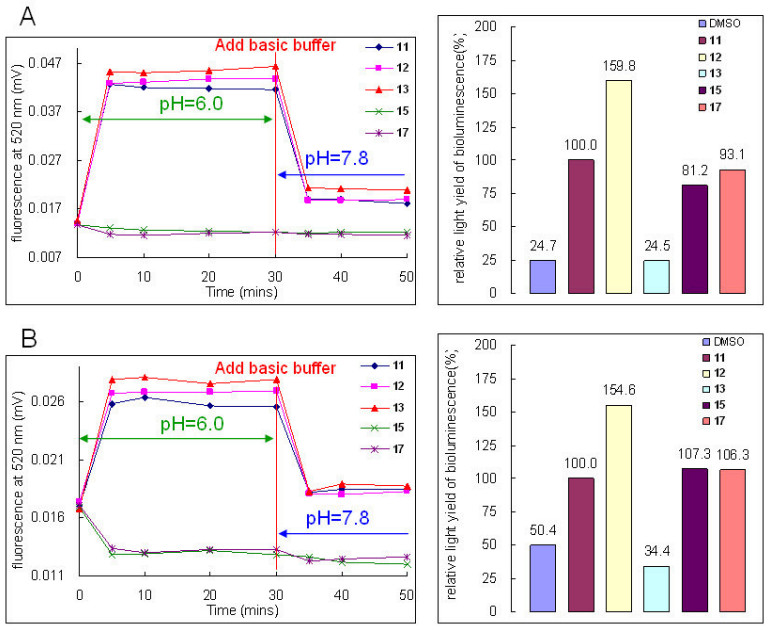



**Figure 12.** Fluorescence intensity-changes by different chromophores incubated with two *apo-symplectin* A and B (retaining more natural chromophore). And the corresponding bioluminescence light amounts in pH 7.8.

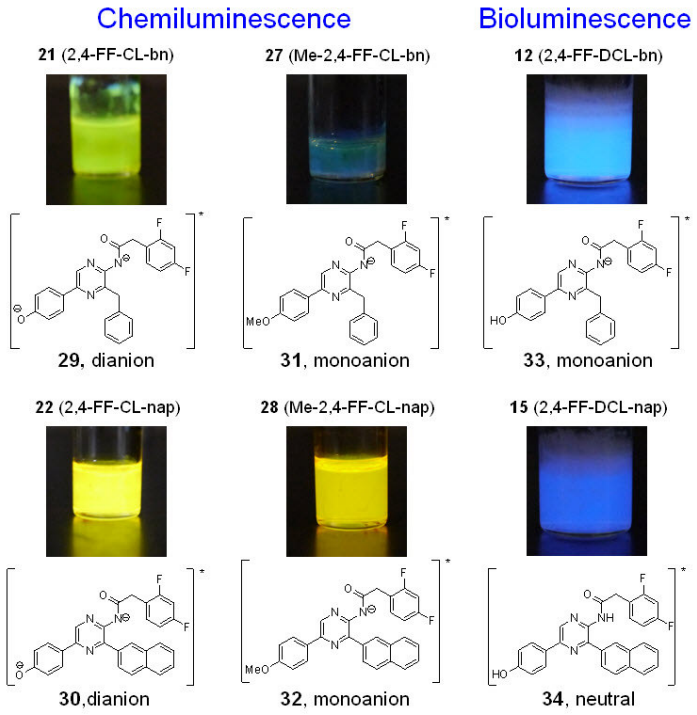



**Figure 13**. Color difference in chemiluminescence and bioluminescence of **12** (2,4-FF-DCL-bn) and **15** (2,4-FF-DCL-nap).

The relative binding affinity maybe similar strength between **12** (2,4-FF-DCL-bn) and **15** (2,4-FF-DCL-nap) in symplectin judging from the intensities at 520 nm remained similarly after the 2nd incubation. Based on these results, **15** (2,4-FF-DCL-nap) is recognized by *symplectin* with stronger binding affinity than natural chromophore, and also shows bioluminescence. Such a difference in the fluorescence intensities at 520 nm provides good evidence for the chromophore-exchange to take place in *symplectin* (see **Figure 10**).

To confirm the result, the naked eye observations are shown in **Figure 11** by monitoring the fluorescence of the reconstructed *symplectin* under UV light (365 nm) with various synthetic chromophores for 20 min-incubation. There is obvious difference in the colors between before and after the incubation with **12** (2,4-FF-DCL-bn) or **15** (2,4-FF-DCL-nap). Only the incubation of **11** (4-OH-DCL-bn) and **12** (2,4-FF-DCL-bn) to *symplectin* shows the fluorescence at 520 nm, but the ones from **15** (2,4-FF-DCL-nap) and **17** (2,4-FF-DCL-pmp) do not.

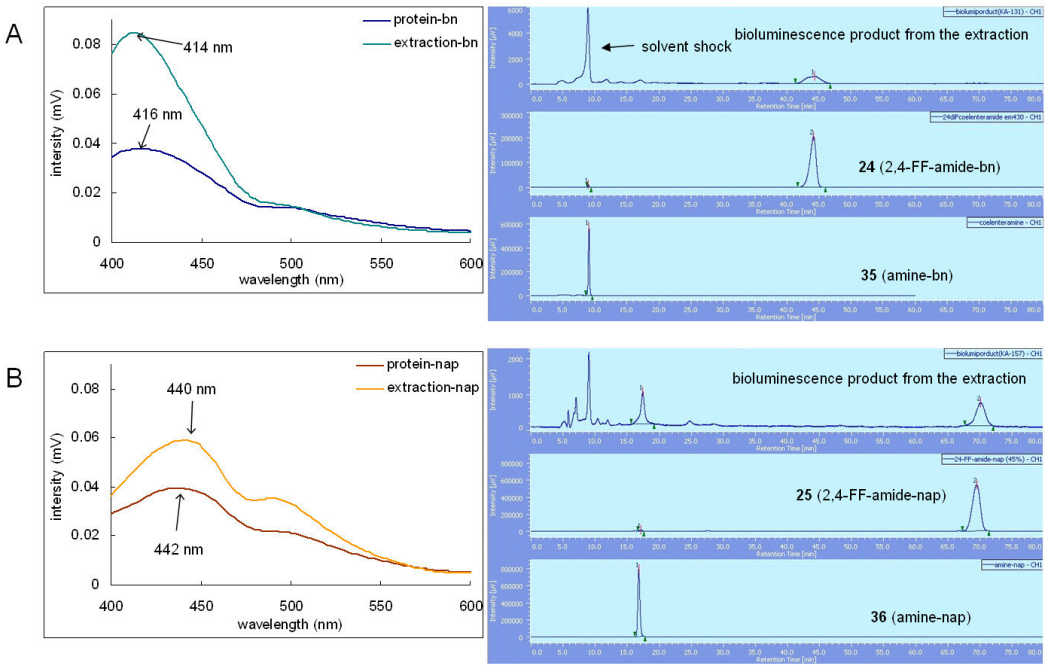



**Figure 14.** Fluorescence spectra and chromatograms and of bioluminescence product, which were extracted from the *symplectin* after the incubation with **12** (2,4-FF-DCL-bn) (**A**) or **15** (2,4-FF-DCL-nap) (**B**).

The remaining **11** (4-OH-DCL-bn) in *symplectin* solution could affect the light yield of bioluminescence. The larger rest amount of 4-OH-DCL-bn led the change of fluorescence at 520 nm more obviously (**Figure 12B**). To prove the luminescence is indeed resulted from **15** (2,4-FF-DCL-nap), the bioluminescence was recorded by digital camera. For the incubation of **12** (2,4-FF-DCL-bn), it is obviously that the mono-anion intermediate is the light emitter in the bioluminescence compared to chemiluminescence (**Figure 13**). For the incubation of **15** (2,4-FF-DCL-nap), the bioluminescence was different to the mono-anion or dianion intermediate in chemiluminescence. It is possible that **15** (2,4-FF-DCL-nap) would form neutral intermediate to result in the bioluminescence of *symplectin*. The anion structures of natural coelenterazine and coelenteramide are discussed by Shimomura and Teranishi.[8]

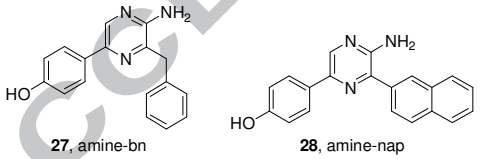



**Figure 15.** Structure of **35** (amine-bn) and **36** (amine-nap)

Because of the fact that no direct evidence of **15** (2,4-FF-DCL-nap) results in light emission with *symplectin,* we try to search for the luminescence product instead. The spent solution of *nap-symplectin* solution was extracted with dichloromethane, and then analyzed by HPLC (**Figure 14**). Comparison of the chromatograms (retention time and the fluorescence intensity) of the related peaks of luminescence product coelenteramide **24** and **25**, and coelenteramine **35** and **36** (the structure was shown in **Figure 15**), it is obvious to find coelenteramide **24** (2,4-FF-amide-bn) was found in the extraction from the incubation of **12** (2,4-FF-DCL-bn) to symplectin, while the correlated peaks were found coelenteramide **25** (2,4-FF-amide-nap) at 70 min and **36** (amine-nap) at 17 min, respectively, from the **15** (2,4-FF-DCL-nap) incubated *symplectin*.

## 3. Conclusions

The fluorescence change by the incubation to symplectin provided us a good evidence to prove the chromophore, which bound to the binding sites of symplectin, can exchange with the outcome synthetic chromophore depended on the relative binding affinity to cysteine residue. Because **19** (Cys-2,4-FF-DCL-nap) only shows weak fluorescence at 600 nm, this characteristic nature makes the observation much easier to confirm the decreasing of the fluorescence at 520 nm, which is represented by coelenterazine. Those results indicate the effect of fluorinated modification on the coelenterazine lead to different bioluminescence activity. And also the ratios of *apo-symplectin* : *holo-symplectin* can be determined, such as 35% of *apo-symplectin* in the protein solution and 65% of *holo-symplectin* in **Figure 8**, and 91% of *apo-symplectin* in the protein solution and 9% of *holo-symplectin* in **Figure 12A**.

The result is also provided us that the light yield of **12** (2,4-FF-DCL-bn) and **13** (2,6-FF-DCL-bn) in symplectin is due to the directly reaction between the chromophore and *symplectin*. And the decrease of light yield from **13**-(2,6-FF-DCL-bn)-incubated *symplectin* may be caused by the intermolecular oxidation on Cysteine390 while the *pseudo*-reduced coelenterazine **2** was formed.

## 4. Experimental

*Instruments:* Fluorescence spectra were measured with a JASCO FP-2020+ spectrometer and Hitachi F-4500 spectrometer. Bioluminescence was obtained on a Hamamastu Photonic Multi-channel Analyzer, PMA-11 by simultaneous integration of light with different wavelengths (300-600 nm) without scanning against the wavelength. Total amounts of the luminescent light were acquired with Gene Light GL-200S to obtain the relative light yield. Centrifugation was performed by using a Hettich Mikro 120 centrifuge machine. Fluorescence spectra were recorded and analyzed by Spectra Manager version 2.07.02. $^{1}$H NMR spectra were measured at Varian MR-400 (400 MHz) and Varian Mercury-400 (400 MHz). $^{1}$H NMR chemical shifts are referenced to the $CHCl_3$ singlet (7.24 ppm) and the center of DMSO quintet (3.30 ppm). Data are reported as follows; chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, qn = quintet, sep =septet, br = broadened, m = multiplet), coupling constant(s), assignment, and integration, respectively. $^{13}$C NMR spectra were measured at Bruker NMR DMX-600 (125 MHz), Varian MR-400 (100 MHz) and Varian Mercury-400 (100 MHz). $^{13}$C NMR chemical shifts are referenced to the center of the CDCl3 triplet (77.0 ppm) and the center of DMSO septet (39.5 ppm). High resolution mass spectra (HRMS) were obtained by Finnigan/ Thermo Quest MAT 95XL mass spectrometer in NCHU, Varian 901-MS/ TQ- FT mass spectrometer and MAT-95XL HRMS in NTHU. High resolution mass spectra (HRMS) are reported in m/z.

*Chemicals:* Potassium chloride (KCl), Potassium dihydrogenphosphate ($KH_2PO_4$), Dipotassuium hydrogenphosphate ($K_2HPO_4$), Ethylenediaminetetraacetic acid (EDTA), and 30% Hydrogen Peroxide ($H_2O_2$) were purchased from SHOWA. 99% L-Cysteine and sucrose were purchased from ACROS. Dithiothreitol was purchased from Baker Analyzed. Dimethyl sulfoxide (DMSO) was purchased from Baker. Catalase (Catalase from bovine liver, 2000-5000 units/mg protein) was purchased from Sigma-Aldrich.

*UV absorbance measurement of Cys-DCL adducts:* DMSO and pH 6 buffer (10 mM KCl, 12 mM AcOH, 12 mM NaOAc in 68% MeOH/$H_2O$) were degassed by Ar purging and then stored under Ar atmosphere. The sample was dissolved in degassed DMSO, and then mixed with pH 6 buffer (the volume ratio of DMSO to buffer is 1 : 2.) in the UV cell or fluorescence cell. For UV measurements, the cysteine solution was added in dropwise to the cell, mixed by shaking. The UV spectra were measured with different amount of L-cysteine.

### *4.1. Synthesis of coelenterazine, coelenteramide, coelenteramine and analogs*

Coupound **11**-**17**, **24**, **27**, and **28** were reported in previous study[1,16,17].

#### *4.1.1. General method to synthesize coelenteramide*

2,4-difluorophenylacetic acid (320 mg, 1.86 mmol) dissolve in thionyl chloride (1.3 mL) was heated to reflux for 2 h. Vapors ($SO_2$ and HCl) generated were trapped with saturated $NaHCO_{3(aq)}$. The solution was cooled to room temperature, evaporated under reduced pressure (distillation in large scale), and dried under vacuo. Crude 2,4-difluorophenylacetyl chloride 2-15 was obtained as a colorless oil. To a solution of coelenteramine (0.186 mmol) in anhydrous $CH_2Cl_2$ (0.4 mL) and pyridine (1 mL) was added solution of 2,4-difluorophenylacetyl chloride in $CH_2Cl_2$ (0.6 mL) slowly at 0 ˚C and stirred for 5 h. Then the solution was poured into sat. $Na_2HCO_{3(aq)}$ (80 mL), extracted with $CH_2Cl_2$. The combined organic layer was washed with brine, dried over $MgSO_4$ and then concentrated under reduced pressureto obtain diacyl compound. The diacyl compound was dissolved in MeOH/dioxane (15 mL/22 mL) and 1N NaOH(aq) (5.6 mL) was added. After stirring at rt for 1.5 h, the reaction was stopped by adding 1N $HCl_{(aq)}$ (5.6 mL). Water (50 mL) was added and the solution was extracted with EtOAc (3 x 20 mL). The combined organic layer was washed with brine, dried over $MgSO_4$ and then concentrated under reduced pressure. The residue was purified by silica column chromatography with EtOAc in hexane. The product is a mixture of coelenteramide and bis-acylated compound. It was then recrystallized by dissolving in hot acetone and adding 50% EtOAc in hexane to afford amide 2-21 only as a white crystal.

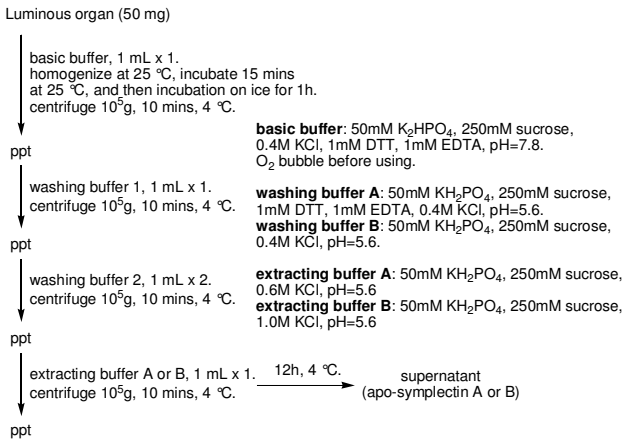
##### *4.1.1.1. N-(3-benzyl-5-(4-hydroxyphenyl)pyrazin-2-yl)-2-(2,4-difluorophenyl)acetamide (**24**)*

Yield: 50%; $^{1}$H NMR (500 MHz, DMSO-d6): δ 3.76 (2H, s), 4.11 (2H, s), 7.04-7.08 (3H, m), 7.16-7.27 (6H, m), 7.43 (1H, q, J= 8.3 Hz), 8.03 (2H, d, J= 8.7 Hz), 8.88 (1H, s), 10.54 (1H, s) ppm. $^{13}$C NMR (125 MHz, DMSO-d6): δ 103.6 (t, J= 26 Hz), 111.2 (dd, J= 21, 3 Hz), 114.4, 118.9 (dd, J= 16, 3 Hz), 126.3, 128.0, 128.3, 129.0, 133.1 (dd, J= 9, 6 Hz), 137.2, 138.3, 143.5, 148.3, 150.5, 160.6, 160.7 (dd, J= 246, 12 Hz), 161.4 (dd, J=246, 12 Hz), 168.9 ppm. $^{19}$F NMR (470 MHz, DMSO-d6): -112.8, -112.5 ppm. HRMS (APCI): calcd for $C_{26}H_{22}F_2N_3O_2$ ($M+H^+$) 432.1445; found 432.1674.

##### *4.1.1.2. 2-(2,4-difluorophenyl)-N-(5-(4-hydroxyphenyl)-3-(naphthalen-2-yl)pyrazin-2-yl)acetamide (**25**)*

Yield: 46%. $^{1}$H NMR (500 MHz, DMSO-d6): δ3.85 (2H, s),4.16 (2H, s), 7.43 (1H, q, J= 8.5 Hz), 7.56-7.58 (2H, m), 7.91-8.01 (5H, m), 8.07 (2H, d, J= 8.4 Hz), 8.98 (1H, s), 10.64 (1H, s) ppm. $^{13}$C NMR (125 MHz, DMSO-d6): δ 103.7 (t, J= 26 Hz), 111.2 (dd, J= 21, 3 Hz), 114.4, 118.9 (dd, J= 16, 3 Hz), 125.1, 125.7, 126.2, 126.3, 128.3, 128.5, 128.7, 133.1 (dd, J= 9, 6 Hz), 132.3, 134.6, 138.3, 143.5, 148.3, 150.5, 160.6 (dd, J= 245, 13 Hz), 161.7 (dd, J= 245, 13 Hz), 167.9 ppm. ppm. $^{19}$F NMR (470 MHz, DMSO-d6): -112.6, -112.8 ppm. HRMS (APCI): calcd for $C_{28}H_{19}F_2N_3O_2$ ($M+H^+$) 468.1447; found 468.1578.

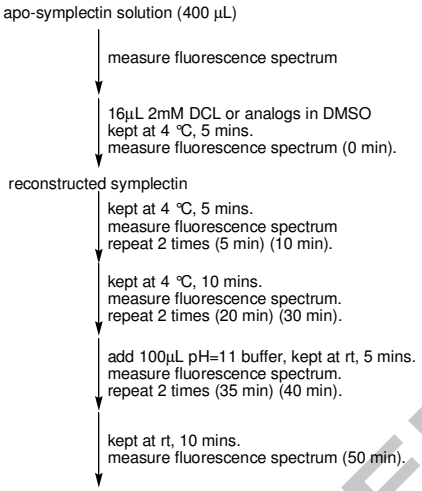
### *4.2. Preparation of apo-symplectin solution*




The yellow luminous organ of a squid *Symplectoteuthis oualaniensis (kept frozen at -78 °C) was cut in each* 50 mg using a borer into a round disk of 15 mm diameter.

For each experimental preparation one disk was homogenized with 1 mL basic buffer (50 mM potassium phosphate, 250 mM sucrose, 1 mM DTT, 1 mM EDTA, pH 8.0, the buffer being saturated with $O_2$ by bubbling before use) at 25 °C for 15 min. This operation allowed consumption of the remaining natural chromophore in the homogenate. Each homogenate was then cooled to 4 °C for 1 h and centrifuged at 10,100 G at 4 °C for 10 min to yield the photoprotein in precipitation. This precipitate was washed once with washing buffer A (50 mM $KH_2PO_4$, 250 mM sucrose, 1 mM DTT, 1 mM EDTA, 0.4 M KCl, pH= 5.6), and twice with washing buffer B (50 mM $KH_2PO_4$, 250 mM sucrose, 0.4 M KCl, pH= 5.6). The photoprotein was extracted from the washed precipitates with extracting buffer A (50 mM $KH_2PO_4$, 250mM sucrose, 0.6 M KCl, pH= 5.6) or extracting buffer B (50 mM $KH_2PO_4$, 250 mM sucrose, 1.0 M KCl, pH=5.6) to obtain an apo-Symplectin *solution A* with buffer A, or *solution B* with buffer B, respectively. The apo-Symplectin A and B was kept at 4 °C for 12 h, and used for the following experiments.

*4.3. Measurements of fluorescence spectra:*




Each reconstructed symplectin preparation was prepared in a similar way by addition of 16 µL 2 mM DCL in DMSO into 400 µL apo-symplectin solution as above. The fluorescence spectra were measured with a JASCO FP-2020 spectro-fluorescence detector equiped with a flow cell (size, 20 µL capacity) by sucking the 50 µL sample solution into the dry cell with a microcyrinder. Each fluorescence spectrum was recorded from 0 min (before incubation), 5 min, 10 min, 15 min, 20 min, and 30 min. Then a 100 µL pH=11 buffer (50 mM $K_2HPO_4$, 0.6 M KCl, 1 mM EDTA, pH= 11) was add to the reconstructed symplectin solution to change the pH to 7.8 in 30 min, and the fluorescence spectra were recorded at 35 min, 40 min, and 50 min.

*4.4. Fluorescence measurement of reconstructed symplectin*

The reconstructed symplectin was prepared by the addition of 16 µL 2 mM DCL in DMSO to 400 µL apo-symplectin preparation. The reconstructed symplectin solution was sucked into the flow cell of JASCO FP-2020 spectrometer. The fluorescence spectra were recorded and analyzed by a Spectra Manager software-version 2.07.02. Before the each measurement, the extracting buffer A (50 mM $KH_2PO_4$, 250mM sucrose, 0.6 M KCl, pH=6.0) was sucked into the flow cell to wash the remained photoprotein in the cell.

*4.4.1. Fluorescence spectrum of **single incubation** at pH=6.0.*

In these chromophore exchange experiments, apo-symplectin solutions were prepared from the same squid in the incubation step. And each sample was sucked into the flow cell to record the spectrum as 0 min. Each photoprotein solution was drained back to an eppendorf tubing and incubated with a chromophore either of 2,4-FF-DCL-bn, 2,4-FF-DCL-nap, or necessary DCL as a control such as 4-OH-DCL-bn. The repeated measurements were applied after 5, 10, and 20, 30 minutes to compare the fluorescence change with different incubation time.

*4.4.2. Fluorescence spectrum of **double incubation** at pH=6.0.*

In the incubation and comparison step the relative binding affinity of synthetic DCL to symplectin, apo-symplectin solutions were prepared from the same squid, and operated as the same as above. Thus obtained reconstructed-symplectin solutions were further incubated with DCL or analogs for comparison and sucked into the flow cell to record the spectra and plotted at 520 nm against these operations to analyze the relative binding affinity.

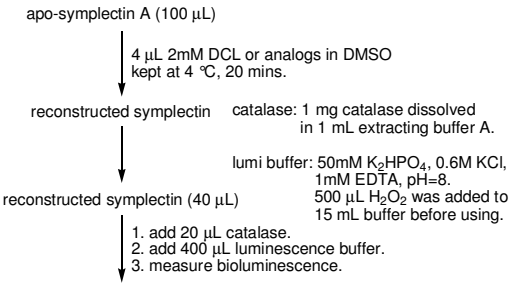
*4.4.3. Fluorescence spectra of consumption of the chromophores.*

To obtain the fluorescence change with the chromophores consumption, the reconstructed symplectin (as prepared in B) was treated with 100 µl basic buffer (50mM $K_2HPO_4$, 0.6M KCl, 1mM EDTA, pH=11.) to adjust the pH value to 7.8. This operation leads symplectin to give bioluminescence (reported by Vorawan et al; and photograph Fig. 7 and 10.). The fluorescence spectra were recorded at 5, 10, 20 min, and recorded as 35, 40, 50 min.

*4.4.4. Comparison*

To compare the fluorescence change, the intensity at 520 nm of each spectrum was plotted into the diagram intensity at 520 nm via time.

*4.5. Measurements of integrated light amount from*




*bioluminescence*

The reconstructed symplectin was prepared by addition of 4 µL synthetic chromophore solution (2 mM DCL in DMSO) to 100 µL apo-Symplectin *solution A* at 4 °C, and kept for 20 min for incubation. The bioluminescence, each total integrated light was measured by simultaneous mixing of the following three solutions; thus, (1) 20 µL catalase solution (1 mg catalase dissolved in 1 mL extracting buffer A), (2) 400 µL luminescence buffer (50 mM $K_2HPO_4$, 0.6 M KCl, 1 mM EDTA, pH= 8), and (3) 500 µL $H_2O_2$ was added to 15 mL buffer before using). This mixture was added to a 40 µL reconstructed symplectin solution, which was placed in a dark cell equipped with a photo counter. The light amounts were integrated for 5 min from each sample preparation with a Gene Light GL-200S photo counter. Relative integrated-light amounts were compared each other.

*4.6. Chemiluminescence measurement*

To a 0.6 mL 2 mM solution of coelenterazine analogs in anhydrous DMSO was added 1 *N* *t*-BuOK in *t*-BuOH. The chemiluminescence was recorded by a digital camera or a Hamamastu Photonic Multi-channel Analyzer, PMA-11 by simultaneous integration of light with different wavelengths (300-600 nm) without scanning against the wavelength.

*4.7. Analysis of bioluminescence product*

The bioluminescence product was extracted by $CH_2Cl_2$ from the symplectin solution which was finished bioluminescence, concentrated by vacuum, and then dissolved in MeOH for HPLC analysis. The HPLC was purchased from Jasco. The pump is Jasco PU-2080. The UV detector is UV-2075+, and the fluorescence detector is FP-2020+. The sample was analyzed by 45% MeCN containing 0.1% TFA in ODS-HG-5 column (4.6 x 250 mm) with 0.5 mL/min flow rate. The UV detector was set for 350 nm absorbance. The fluorescence detector is recorded 520 nm (em) which is with 350 nm excited irradiation.

*4.8. Fluorescence measurement of synthetic compound*

DMSO and pH 6 buffer (10 mM KCl, 12 mM AcOH, 12 mM NaOAc in 68% MeOH/$H_2O$) were degassed by Ar purging and then stored under Ar atmosphere. The sample was dissolved in degassed DMSO, and then mixed with pH 6 buffer (the volume ratio of DMSO to buffer is 1 : 2.) in a UV cell or a fluorescence cell. The final concentration was 2 μM for coelenteramine, 10 μM for coelenteramide. For fluorescence measurements, the cysteine solution was added to cell, mixed by shaking. The fluorescence spectrum was measured as soon as possible after mixing to avoid the air oxidation to result coelenteramine.

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