DOI: 10.1002/asia.201100089

Dynamic Chirality Determines Critical Roles for Bioluminescence in Symplectin–Dehydrocoelenterazine System

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Dedicated to Professor Eun Lee on the occasion of his retirement and 65th birthday

Abstract: Symplectin is a photoprotein containing the dehydrocoelenterazine (DCL) chromophore, which links to a cysteine residue through a covalent bond with the emission of blue light. This study focuses on the stereochemical process of the emerging stereogenic centers. Two isomeric fluorinated DCL analogs (2,4-diF- and 2,6-diF-DCL) were employed owing to their different bioluminescence activities, these being 200% and 20% compared to natural DCL, respectively. Each of these diF-DCLs was found to exchange with the natural DCL in symplectin at pH 6.0. The emerging stereogenic carbons were racemic at the binding sites. Changing the pH of this storage form to the protein's optimum solubility pH (pH 7.8) resulted in 2,4-diF-DCL-bound symplectin luminescence, and the spent solutions were then analyzed and coelenteramide-390-CGLK-peptide and coelenteramine were detected after a peptidase digestion. The same analysis of the 2,6-diF-DCL-bound symplectin, on the other hand, afforded coelenteramine only but no coelenteramide. When the racemic storage diF-DCLs moved to the active site at pH 7.8, a change in the chirality with the 390-Cys residue resulted. Model experiments using L-cysteine-containing CGLK-peptide supported two diastereoisomers from each diF-DCL. The significant difference in the luminescence from these two chromophores is attributed to a plausible mechanism including the dynamically variable stereogenic center emerging at the storage and then the active site on the symplectin. It is concluded that such dynamic chirality plays a significant role in the symplectoteuthis bioluminescence.

Keywords: chirality · circular dichroism · coelenterazine · L-cysteine · proteins

Introduction

Developments in structural biology have revealed the details of protein–substrate interactions through X-ray crystallographic analysis of the co-crystals. As an example of X-ray structural analysis, Shimomura has reported that the coelenterazine (**1**) substrate changes to its hydroperoxide having a 2-(*S*)-configuration in the photoprotein aequorin system.[1] It further changes in the presence of Ca^{2+} ions to a dioxetanone intermediate,[2] and then produces luminescence.[3–6] The coelenterazine hydroperoxide selectively oxidizes the cysteine-bound dithiothreitol moieties nearby in this protein molecule.[7] The hydroperoxide in aequorin remains stable before adding calcium ions, but usually the peroxide and dioxetanone are very unstable without the protein and decompose spontaneously with emission of light. The luminous peroxides were chemically synthesized by Usami and Isobe and co-workers through photooxygenation of the coelenterazine analogs at −78 °C in trifluoroethanol solvent. [8,9] They

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/asia.201100089.

characterized the unstable structures using the 100% ^{13}C-enriched samples by means of low-temperature ^{13}C NMR and FT-IR, which provided direct assignments for the short lifetime dioxetanone and hydroperoxide structures.[8,9] Many marine bioluminescence systems, particularly in *Aequorin*,[3] *Oplophorus*,[10,11] *Watasenia*,[12–14] *Renilla*,[15] *Pholasin*,[16,17] and *Obelin*,[18] use coelenterazine **1** (CL; Figure 1) as the luminous substrate for light emission in different photoproteins.[19]

Symplectoteuthis oualaniensis (Tobi-Ika in Japanese) is collected in Okinawa, and was used in this study. It is an oceanic squid emitting blue light from the photogenic organ.

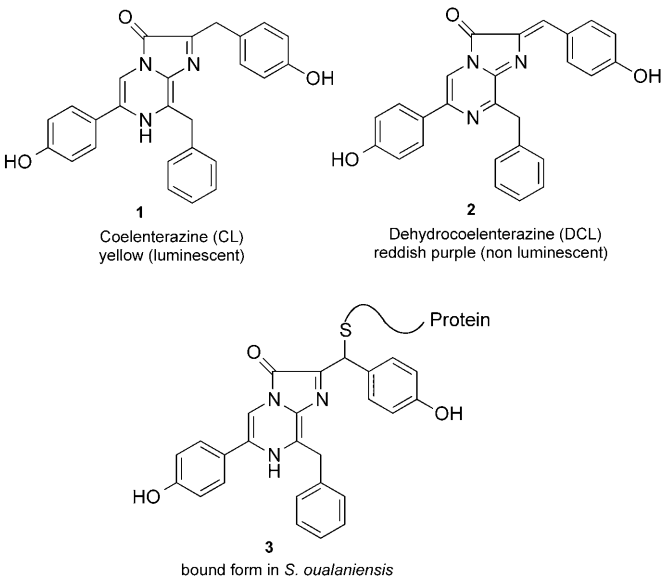

Figure 1. Chromophores in symplectin bioluminescence.

Abstract in Japanese:

海洋生物発光物質セレンテラジンは、沖縄県水域で捕獲されるトビイカ発光器中では、酸化型デヒドロ体 DCL がシステイン残基と共有結合し緑色蛍光を示す貯蔵型となる。発光時には、基質は活性中心の 390-Cys に移動して青色発光する。発光前の発光タンパク質シンプレクチンに、2 種の電子吸引性基を持つフッ素化類縁体を pH 6 で添加すると、双方とも天然発色団と交換する。2,4-diF-DCL と交換後のタンパク質を至適 pH7.8 とすると天然 DCL の 2 倍ほどの発光を示して、セレンテラミドとなる。一方、2,6-diF-DCL では pH7.8 でも阻害的で発光しないが、基質の消費反応は進行して直接セレンテラミンとなる。構造の酷似したこれら 2 種の発光基質は、いずれも強い化学発光（タンパク質を用いない強塩基条件）を示すものの、シンプレクチン中で展開されるこの重要な差異は、反応中の立体過程の差によるものと結論した。発生する不斉中心を LC-CD で観測すると、貯蔵時にはラセミ型であるが、動的に短時間生ずる発光直前の活性中心での不斉炭素原子は、2,4-diF-DCL と 2,6-diF-DCL とでは逆となると結論した。

In 1981, Tsuji found its membrane-bound photoprotein responded to monovalent cations, such as K^+ or Na^+, acting as a trigger.[20] In 1993, Takahashi and Isobe reported that this squid used dehydrocoelenterazine (DCL) **2** (Figure 1) as a substrate instead of coelenterazine.[21,22] The *Symplectoteuthis* bioluminescence system is the first example using oxidized coelenterazine DCL by covalently binding to the photoprotein. Kuse recently reported a second example in *Pholasin*.[23,24]

In 2002, Fujii, Isobe, and co-workers elucidated that a 60 kDa photoprotein, named symplectin,[25] is responsible for the luminescence of *S. oualaniensis*. Symplectin contains 11 cysteine residues among 501 amino acids, and allows DCL **2** binding through a covalent thioether bond as **3** (Figure 1). Binding-model studies, using 100% ^{13}C-enriched DCL and dithiothreitol (DTT), were also published in 1998 to reveal that the bound form of the chromophore exists in equilibrium with its dissociated form.[26] Studies using a luminous mono-fluorinated DCL analog, and subsequent detection of the CGLK-chromo-peptide after trypsin digestion, led us to conclude that the active site-thiol belonged to 390-Cys.[27] Symplectin is not soluble in plain water, but it is only soluble in a buffer at pH between 5–9 (optimum 7.8) containing KCl salt of 0.6 M or higher concentration.

Results and Discussion

Cysteine Analysis of Symplectin

The amino acid sequence of symplectin was reported in 2008.[27] The S−S/SH analysis of symplectin was further implemented about the eleven cysteine residues according to an iodoacetamide protocol. The modified protein was digested by the endopeptidase Lysine-C at pH 8 for 4 hours, and then the mixture was subjected to capillary liquid chromatography-mass spectrometry (LC-MS) analysis (see details in the Supporting Information, sections S4 and S13), and the results are shown in Figure 2. The five cysteine residues exist in free SH form locating at 196-, 339-, 344-, 345-, and 390-Cys, while three pairs of cystines are in S−S form at 92/110, 129/137, and 380/385. Other modifications after Lysine-C endopeptidase digestion using the capillary LC-MS method are also shown in Figure 2. As was previously reported for a sulfur-containing photoprotein, two methionines (181 and 484) were found in the oxide form.[28]

Preparation of Difluorinated Dehydrocoelenterazine Analogs

The symplectin luminescence system incorporates chromophore DCL to its aposymplectin by Michael addition to form the photoprotein with a thioether covalent bond (compound **3** in Figure 1). In this case, the equilibrium of addition/elimination depends on the pH and electronic nature of the substituent of the chromophore; namely, the phenyl ring at the 2a-position of the chromophore.

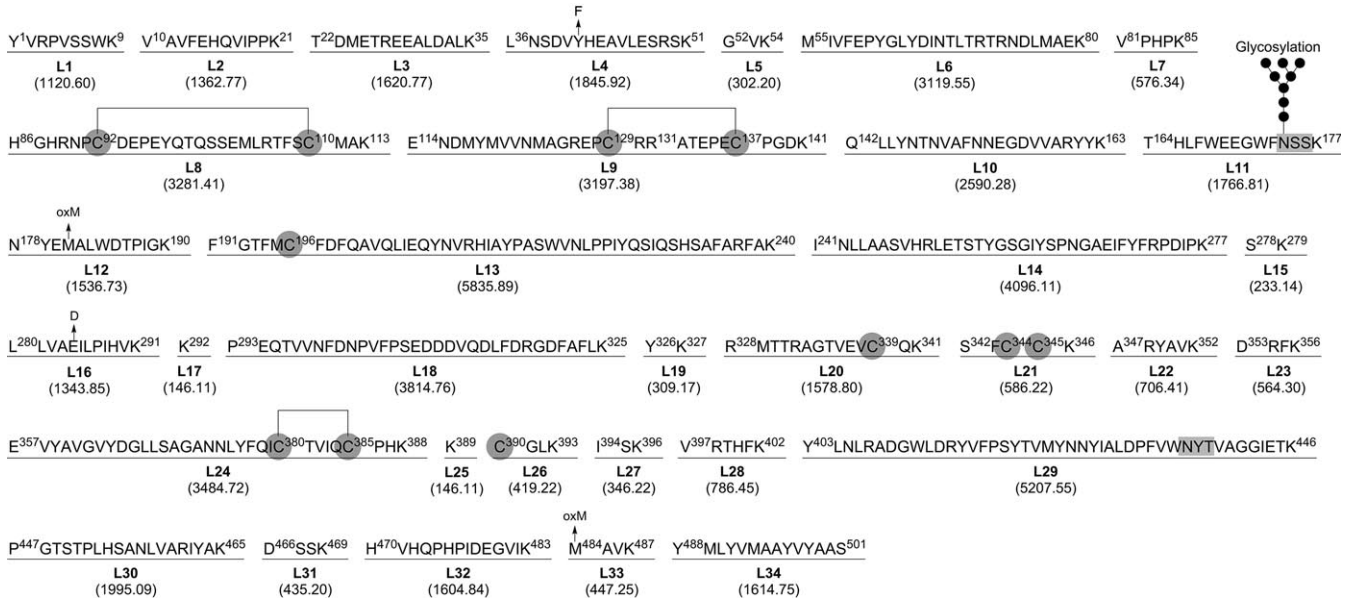

Figure 2. Amino acid sequence and cysteine/cystine analysis.

An *ortho*- or *para*-fluoro analog recorded good results in the active site studies.[27] We became interested in more electron-deficient analogs to serve as substrates for this study. Among several compounds we have prepared in preliminary experiments, it was suggested that the 2,4-difluoro-dehydrocoelenterazine (2,4-DiF-DCL, **4**, Figure 3) analog indicated a much higher bioluminescence activity than DCL. So here we describe the synthesis of **4** and its position isomer 2,6-diF-DCL (**5**, Figure 3). The preparation of **4** and **5** was achieved from difluorophenylacetic acids **8a** and **8b** in eight steps in 14.6% and 25.5% overall yield, respectively, as shown in Scheme 1, and the experimental details are shown in the Supporting Information (sections S5–S13).

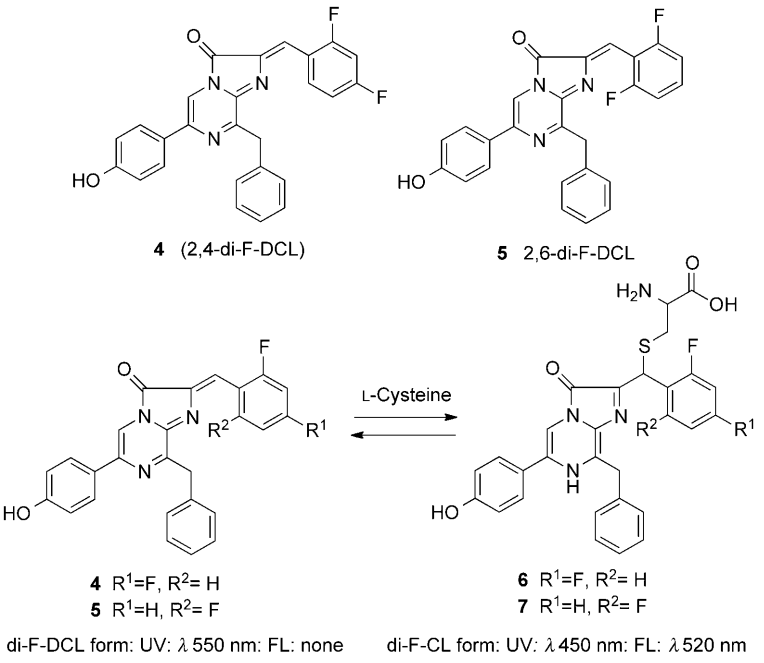

Figure 3. Two difluorinated dehydrocoelenterazine analogs, which behave differently on symplectin for luminescence.

Equilibrium of Dehydrocoelenterazine Analogs 2,4- and 2,6-DiF-DCL with L-Cysteine

In Figures 4–6, the UV/Vis spectra of **4** and **5** are compared with those from their respective adducts **6** and **7** (Figure 3), when mixed with varying amounts of L-cysteine (from 0 to10 equiv, as indicated in the figures) at pH 3.2, 6.0, and 8.0, respectively. These pH values were selected from previous results with natural DCL **2**; thus, pH 3: adducts being stable, pH 6.0: slow equilibration, and pH 8: protein's optimum pH is 7.8.[22] The arrows indicate an increase or decrease of the absorbance peaks. All of the free DCL derivatives **2**, **4**, and **5**, are reddish in color (abs = 550 nm) and non-fluorescent. On the other hand, all the adducts **3**, **6**, and **7** changed to yellow in color (abs = 450 nm) and emit a green fluorescence (GF) at 520 nm.

Under these conditions using a methanol-buffer solvent, 2,6-

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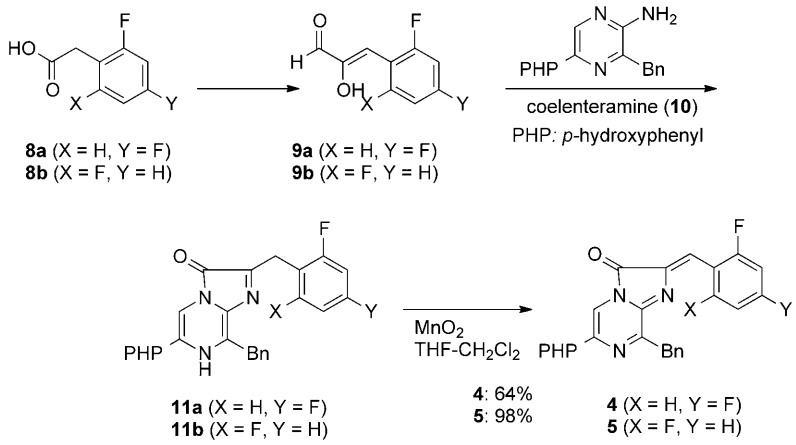


Scheme 1. Synthetic route for di-fluoro-DCLs (**4** and **5**).

Incorporation and Luminescence of DiF-DCL with Aposymplectin

The capacity and rate of the binding of the aposymplectin to **4** or **5** were different as well when monitored by the increase in green fluorescence at 520 nm at pH 5.6 (Figure S1 in the Supporting Information). The increase in fluorescence upon binding to **5** was faster and greater than that observed with **4** (Figure S1 in the Supporting Information). After these binding experiments, the pH of the final mixture was raised from 5.6 to 7.8, and the green fluorescence (GF) intensity decreased over 10 minutes (see Figures S2–S3 in the Supporting Information). At the same time, the bioluminescence was observed, and the light yields were integrated for 5 minutes. The experiment with **4** emitted 180% more light than that with natural DCL (**2**), while **5** emitted less light than

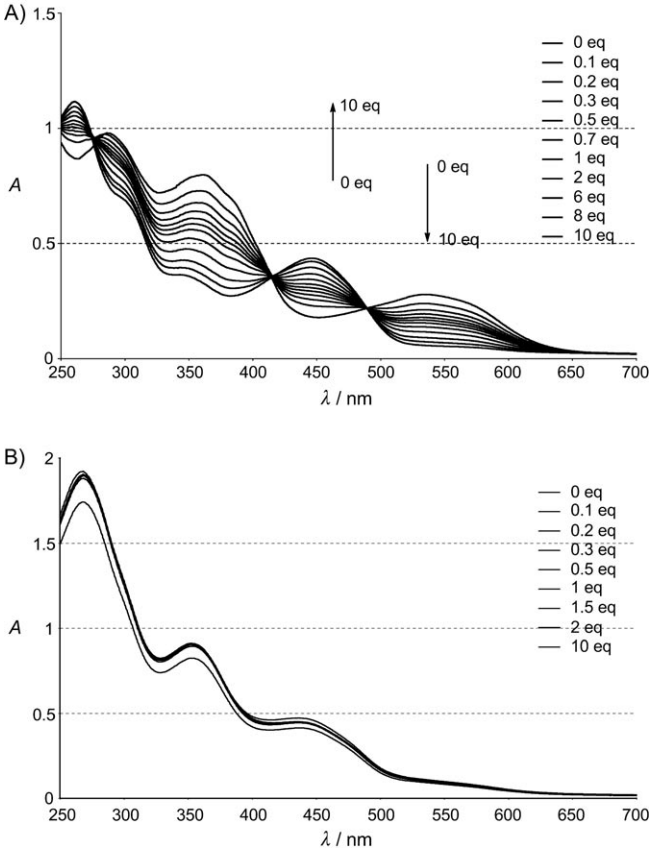


Figure 4. UV/Vis spectra of diF-DCL analogs in the presence of various amounts of L-cysteine at pH 3.2; A) 2,4-diF-DCL **4** and **6**; B) 2,6-diF-DCL **5** and **7**. Buffer components are 17.6 mM KCl, 10 mM citric acid in 68% MeOH/H_2O.

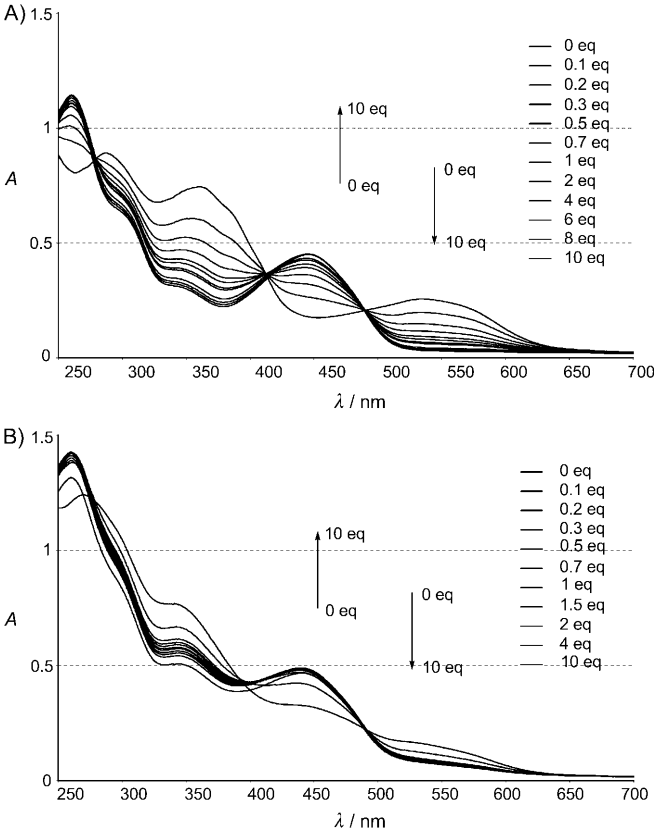


Figure 5. UV/Vis spectra of diF-DCL analogs in the presence of various amounts of L-cysteine at pH 6.0; A) 2,4-diF-DCL **4** and **6**; B) 2,6-diF-DCL **5** and **7**. Buffer components are 10 mM KCl, 12 mM AcOH/12 mM AcONa in 68% MeOH/H_2O.

diF-DCL tends to exist as the adduct form **7** rather than in equilibrium with **5**. The 2,4-diF-DCL may be insoluble at pH 8.0 without cysteine.

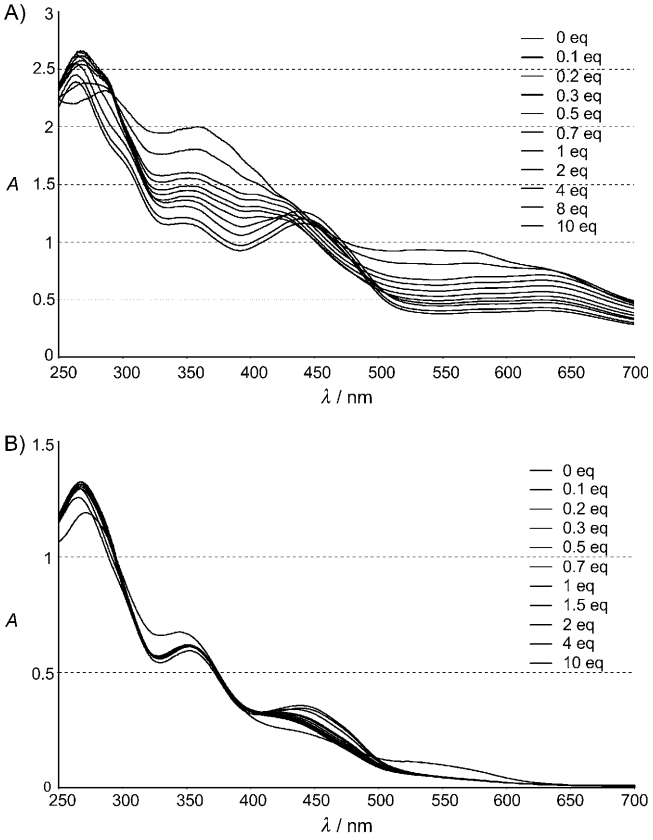


Figure 6. UV/Vis spectra of diF-DCL analogs in the presence of various amounts of L-cysteine at pH 8.0; A) 2,4-diF-DCL **4** and **6**; B) 2,6-diF-DCL **5** and **7**. Buffer components are 2 mM Tris, 2 mM Tris-HCl in 68% MeOH/H_2O.

the control (Figure 7). In both cases, the GF decreases within 10 minutes at pH 7.8. While the absolute fluorescence value of the control GF varies, owing to different amounts of natural DCL (**2**) remaining in the dimethyl sulfoxide "(DMSO)"-experiment in each aposymplectin preparation, the trends in the binding of these three compounds in each set of experiments are the same and are highly reproducible. The lack of luminescence produced upon the incubation of

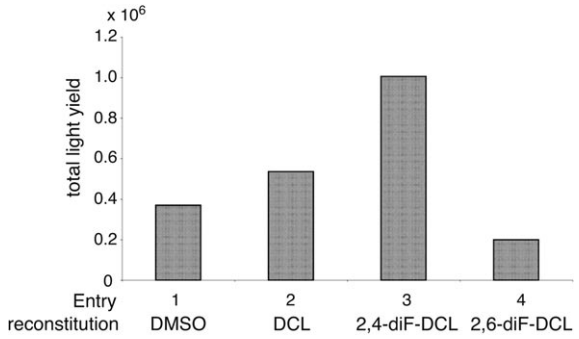


Figure 7. Bioluminescence ability after reconstituted symplectin with natural DCL **2**, 2,4-diF-DCL **4**, and 2,6-diF-DCL **5**. The total light yield is in arbitrary units, but comparable with Figure 9 and Figure 14.

aposymplectin with **5** suggests not only that **5** produced a non-luminescent adduct, but also its inhibitory action in the photoprotein. Although approximately 40% of the light yield is still observed, this might arise from the DCL remaining in the apoprotein or 2,6-diF-DCL (**5**) itself.

Molecular Shape Difference of DCL

With respect to the decrease of 520 nm fluorescence of photoprotein symplectin in accordance with time after changing the buffer from pH 5.6 to pH 7.8, both the 2,4-diF-DCL and 2,6-diF-DCL lose GF almost at the same rate (Figure S2 in the Supporting Information). One of the factors making the difference between the binding and bioluminescence abilities of **4** and **5** might be a result of the different physicochemical properties of these two chromophores. Figure 8 indicates two kinds of dipole–dipole interactions between the two isomeric fluorine atoms and the carbonyl group, thus, **4** (2,4-diF, b+a) is assumed to be smaller than **5** (2,6-diF, c+a). In addition, *ortho*-diF of the latter has a higher steric interaction with the imidazolone nitrogen atom. Therefore the conformation of these two aromatic ring planes may be twisted differently. In fact, the dihedral angles of energy-optimized analogs were calculated to be 37.8° for **4** and 55.9° for **5** (Figure 8), while that of natural DCL **2** is 39.5°. Only 2,6-diF-DCL shows a different molecular shape from natural DCL. This difference could be caused by the different binding to the active site in symplectin.

Double Incubation of DCL Analogs to Symplectin

The results of the luminescent assay (Figure 7) and conformational analysis (Figure 8) led us to design the following double incubation assay using systematic combinations of the chromophores. Namely, aposymplectin was first incubated with one chromophore for 20 minutes at pH 5.6 (1st reconstitution), then, with a second or the same chromophore for another 20 minutes (2nd reconstitution). The integrated amounts of light emission at pH 7.8 after each of these double incubations are shown in Figure 9.

As shown in Figure 9, the double incubation experiments with DCL (**2**) and 2,4-diF-DCL (**4**) either in the first and/or second round (Figure 9, entries 3, 5, 7) always yielded more than 200% light amounts relative to the control (Figure 9, entries 1, 2). In contrast, using 2,6-diF-DCL (**5**) in either of the double incubations (Figure 9, entries 4, 6, 8, 9, and 10) resulted in very little light yields (compared with the 20–45% in Figure 9, entry 2). This implies that 2,6-diF-DCL (**5**) serves as an inhibitor and does not exhibit a bioluminescence ability. On the contrary, **5** shows approximately a two times higher chemiluminescence ability than **4**, when each of them was bound to a model peptide, glutathione (γGlu-Cys-Gly) and was subjected to *t*BuOK in DMSO. Both of them were converted into coelenteramide analogs (**18** and **19**; X = S-(γGlu)Cys(Gly))[26,29] (data not shown). Thus the difference between **4** and **5** in the bioluminescence with

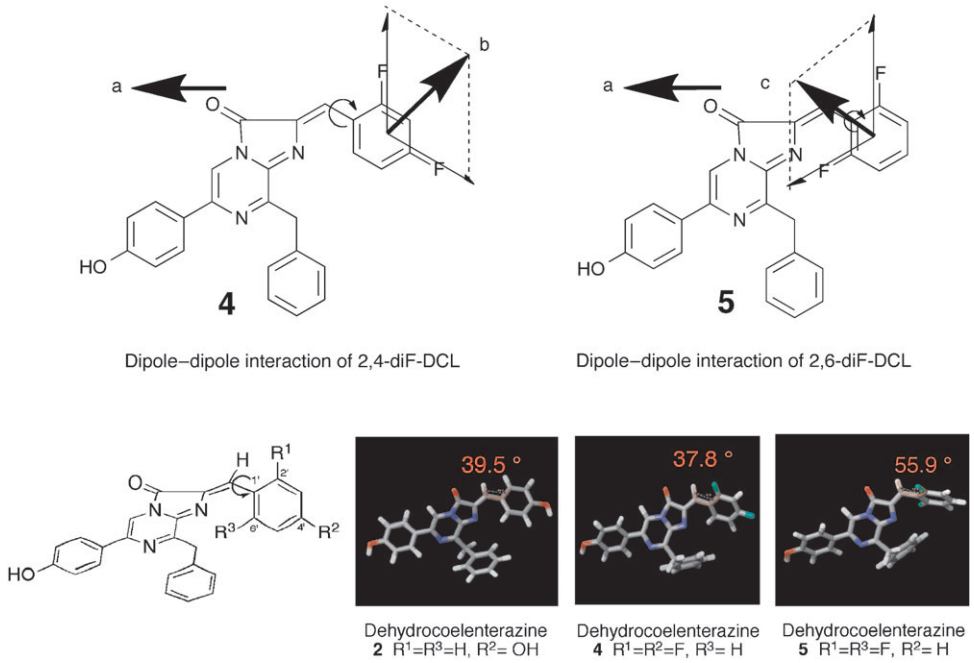


Figure 8. Dipole–dipole interaction of **4** and **5** with the two aromatic planes twisted by 38° and 56°, respectively.

symplectin is assumed to be attributed to their interacting or binding modes with symplectin.

Product Analysis of the DCL Analogs after the Bioluminescence

Both of the isomeric diF-DCL analogs **4** and **5** incorporated to aposymplectin at pH 5.6 (Figure S1 in the Supporting Information) and were similarly consumed at pH 7.8 (Figure S3 in the Supporting Information), while **4** shows bioluminescence but **5** does not (Figures 7 and 9). Next, we checked the products after the pH 7.8 condition in order to answer the question whether the non-luminescent 2,6-diF-DCL analog would provide the corresponding coelenteramide analog **19** or not. After the luminescent conditions, both of the spent solutions from the experiments relating to Figures 7 and 9 showed blue fluorescence. Of the possible products, as shown in Scheme 2, the analogs coelenteramide **17**, **18**, and **19** should show similar fluorescence maxima; thus, FL_{max} at around 420 nm and coelenteramine **23** at FL_{max} 410 nm. However, the differentiation of these molecules was not so

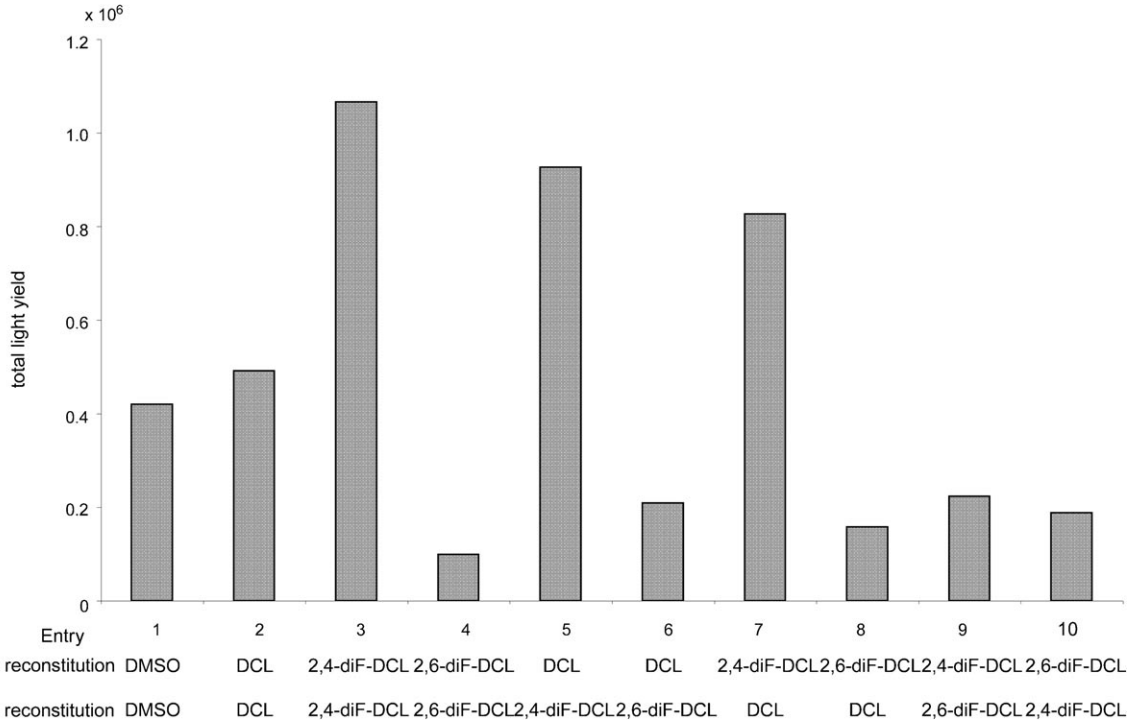
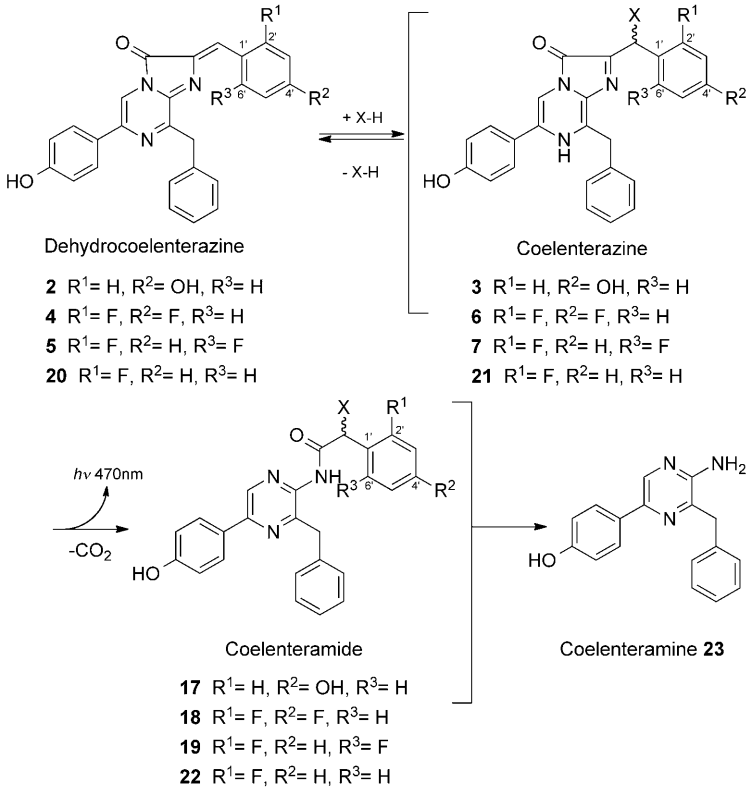


Figure 9. Bioluminescence at pH 7.8 of samples from doubly reconstituted symplectin with DCL and/or diF-DCL analogs at pH 5.6 for 20 min in each incubation.



Scheme 2. Structures of dehydrocoelenterazine (DCL) and its analogs and their possible changes on protein surface.

easy owing to the similar fluorescence at pH 7.8. Fortunately, coelenteramide showed a fluorescence-shift at alkaline pH in DMSO solvent to around 530 nm. This FL-shift method was, in fact, applied to the spent solutions from **4** and **5** by addition of 1 N NaOH and DMSO and the difference in fluorescence spectra was measured (for details, see Figures S4 and S5 in the Supporting Information). It was concluded that **4** showed a significant alkaline shift but none was observed for **5**. Thus, **5** would directly yield **23**, and would never provide coelenteramide **19**.

Further product analysis of **18** but not **19** was performed by capillary LC-MS as follows. Each of the spent solutions from symplectin and diF-DCL analogs **4** and **5** were digested with endopeptidase Lys-C at pH 8 for 4 hours or overnight. The resultant hydrolysates were analyzed with a house-assembled non-split-flow capillary LC-ESI-IT-MS (electrospray ionization-ion trap-mass spectrometry) experiment equipped with a capillary FL-detector, and the results are shown in Figure 10. From the digested peptide mixture derived from **4**, we observed 2,4-diF-coelenteramide-S-CGLK **18**, (FL 420 nm, X = S-Cys^{390}-Gly^{391}-Leu^{392}-Lys^{393}; *m*/*z*: 849.3, at retention time (r.t.) = 46.6 min) together with coelenteramine **23** (*m*/*z*: 278.2, r.t. = 50.6 min). These fragment ions and the assignments are illustrated in Figure 10. These experiments confirmed our previous results that the active site cystine being 390-Cys with mono-fluoro-DCL **20** and **22**.[27] On the other hand, no analogous chromo-peptide peak corresponding to **19** was found from the experiments derived from **5** either by FL or MS, but coelenteramine **23** was clearly detected as the only fluorescent product.

LC-CD-UV Analysis of the DiF-DCL Analogs with L-Cysteine and CGLK-Peptides

We now became interested in the absolute configuration that emerged on the chromophore as a result of the temporary stereogenic carbon atom attached to an S-atom from L-cysteine or a cysteine residue of symplectin. In our previous work, DCL was proven to bind at the 390-Cys of symplectin as the active center for the bioluminescence.[27] Judging from the reconstitution experiments (Figures 7 and 9), this binding seems to be more dynamic in that the chromophores exchange in the photoprotein. The methodology for determining the absolute configurations[30] usually requires exciton coupling, X-ray crystallography, or the ^{1}H NMR anisotropy effect. However, these methods are very limited for cases where the temporary chirality changes dynamically taking place on the protein surface need to be determined, as in the current case. None of these methods are applicable to pico-mol scale experiments. In addition, the current case includes the exchanging of chromophores on account of the addition–elimination equilibrium at the binding site and moving from storage site to active site by dynamically changing the chirality. In the current research, we focused on the significant role of the stereochemistry of the chromophore determining the luminescent activity (Scheme 2).

As model experiments for chirality studies, DCL **4** was converted to the L-cysteine-containing CGLK peptide (X = −S-Cys-Gly-Leu-Lys in Scheme 2) by addition of 1.2 equivalents of the peptide and the diastereomeric mixture **6** (X = −SCH_2CH_2CGLK) was immediately analyzed by LC-CD equipped with a reversed phase ODS column (4.6 mmϕ × 25 cm) under acidic conditions (35 % CH_3CN/H_2O containing 0.1 % TFA, approximately pH 3) using a flow-cell UV-CD detector. The results are shown in Figure 11, where the first elution peak 1 at 20.2 minutes shows negative CD chromatographed at 310 nm (A, C), while the second elution peak 2 at 22.9 minutes shows a positive signal. From the UV data of Figure 11 B, D, the same amount of two diastereomers were monitored. The flow-stop-scanning of CD and

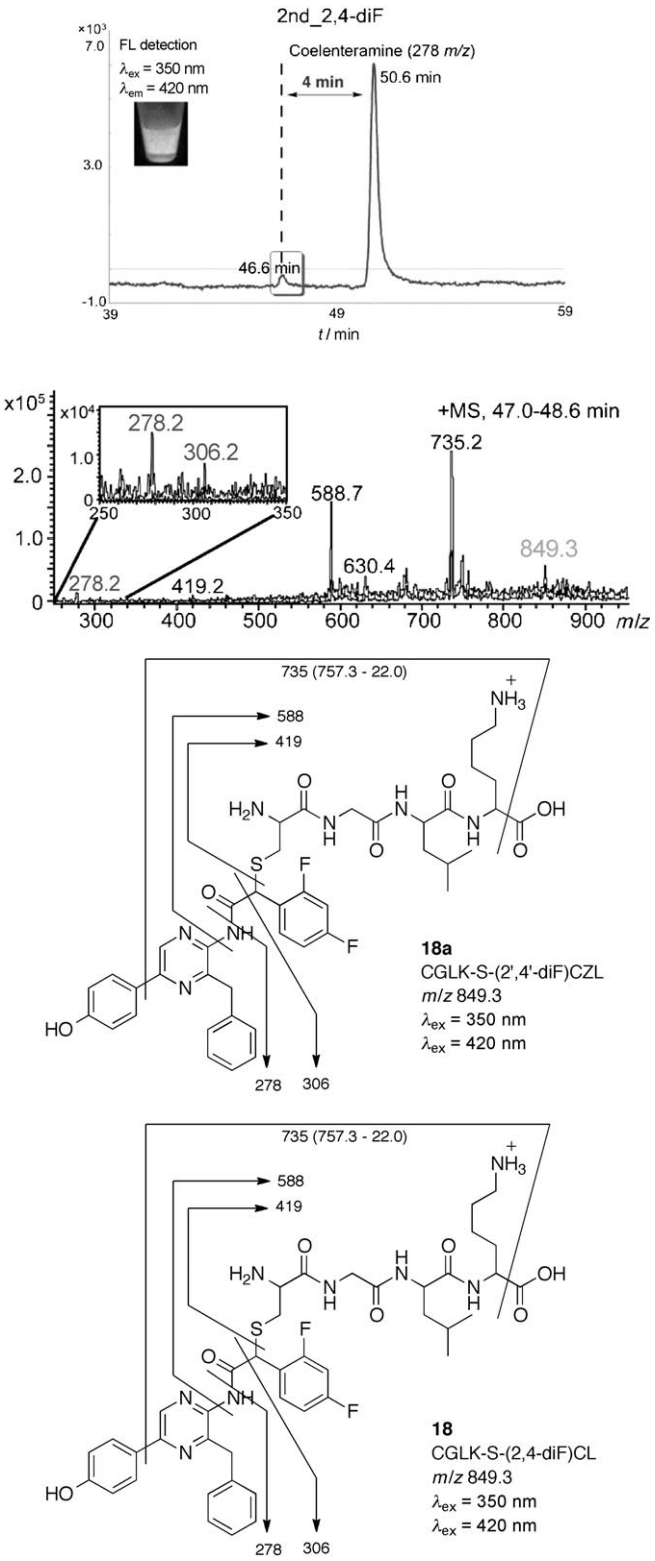


Figure 10. Product analysis of the 2,4-diF-DCL after bioluminescence and symplectin endopeptidase Lys-C digest, using capillary HPLC-ESI-IT-MS.

UV spectra at each peak top are also seen in Figure 11 C, in which the Cotton effect is seen around 310 nm and 250 nm with negative first and positive second Cotton effect on elution peak 1. The reversely positive first and negative second Cotton effect is seen on the elution peak 2. These Cotton effects correspond to the emerging stereogenic carbons on chromophore **6**. Similarly those spectra of **7** (X = −S-CGLK), which were prepared from 2,6-diF-DCL **5**, are illustrated in Figure 12 A–D. Simpler examples from L-cysteine adducts of **2**, **4**, and **5** are shown in the Supporting Information (Figure S8–S10, respectively). The delta epsilon values Δε of these CD data were calculated to be 5–8, and are tabulated in Table S1 in the Supporting Information.

All of these samples (**3**, **6**, and **7**) showed a similar tendency in the chromatograms and CD spectra. Among the three adducts **3**, **6**, and **7**, 2,6-diF-DCL/L-cysteine adduct **7** seems the most stable judging from the higher peak height ratios of elution peak 2 to peak 1. The different stability may be attributed to the electron-withdrawing nature by fluorine atoms as discussed above. So the CD data was accurately measured with 2,6-diF-DCL/L-Cys adduct using 2,6-diF-CL (**7**, X = H) having $\varepsilon = 16900$. The small values of delta epsilon Δε from the CD spectra and Cotton peak found at 310 nm and around 260 nm are so small that the absolute configuration is unable to be predicted by using the exciton chirality method. It is, however, suggested that the same dominant stereogenic center is observed when the chromophore binds with the L-cysteine residue of an amino acid or peptide model as summarized in Table S1 in the Supporting Information. It is clear that the chirality comes from the stereogenic carbon carrying the sulfur atom, so determination of this chirality requires further comparison with authentic sample preparations in a future work.

Molecular Mechanic Calculation of 2,4-DiF-DCL- and 2,6-DiF-DCL-Cysteine Adducts

In order to compare the molecular shape difference of DCL analogs, as shown in Figure 8, here we have performed molecular mechanic calculations of diF-DCL analogs and L-cysteine adducts for determination of the global minimum conformation. To make the calculation easier, we simplified the substituents to CH_3 groups instead of CH_2Ph and the CH_3−SH (S−Me) instead of cysteine adduct as the 2,4-diF-DCL model compound **24** (*2R*)- and 2,6-diF-DCL model **25** (*2S*)-configuration of the isomeric diF-DCL chromophores **4** and **5**, respectively (Figure 13).

The calculations above are focusing on the stereogenic center. Full geometry optimizations and relaxed potential energy surface scans were performed by means of hybrid density functional theory B3LYP with 6-31G(d) basis sets using the Gaussian 09 program package.[31,32] The conformational analysis for the torsional energy curve was computed by varying C1-C2-C3-C4 dihedral angle θ around the C2−C3 bond (as shown in the Supporting Information, Figures S11–S12) stepwise by 7.5° for (*R*)-2,4-DCL **24** and (*S*)-2,6-DCL **25** from the global minima. To obtain local minima and transition states on the scanned curve, the structural points close to the top and the bottom in the scanned tor-

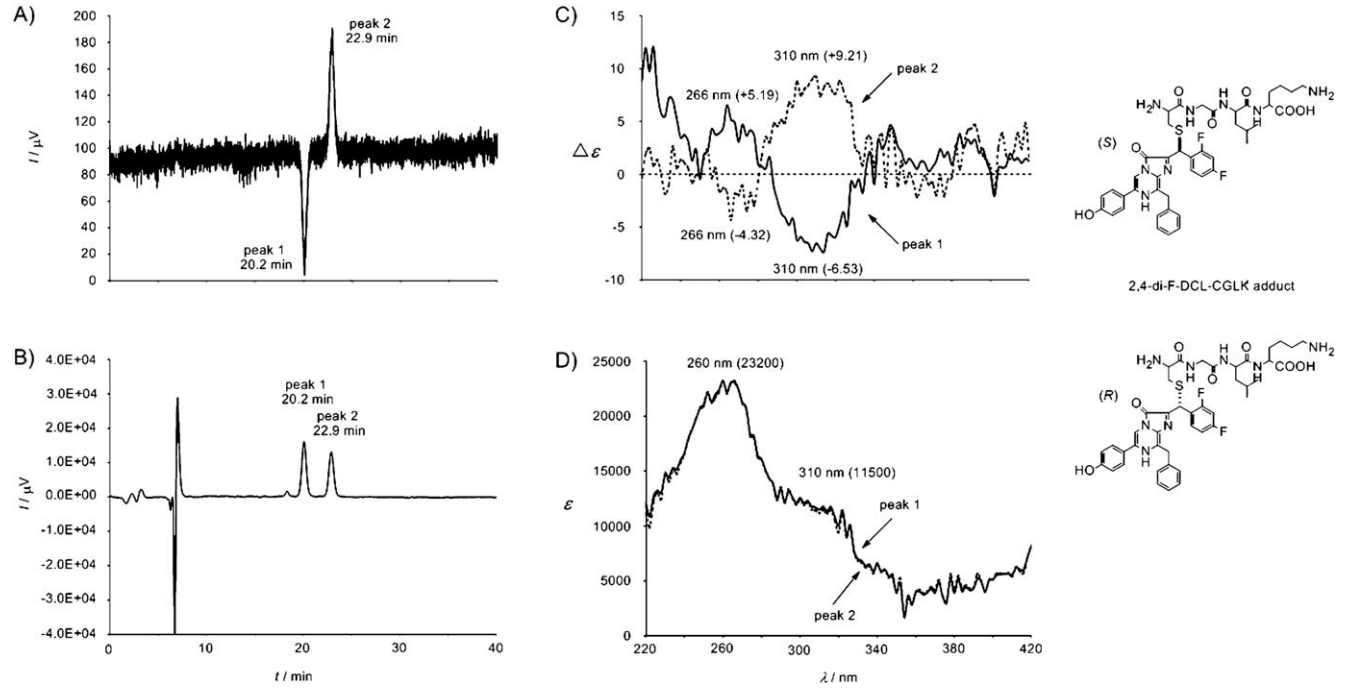


Figure 11. LC-CD analysis of authentic chromopeptide, CGLK-2,4-diF-DCL-(L)-cysteine diastereomeric adducts; A) CD chromatogram at $\lambda = 310$ nm, B) UV chromatogram $\lambda = 310$ nm, C) CD spectra, and D) UV spectra. Chromatogram and spectra were separated with ODS-5 column, 4.6 mm-i.d. × 250 mm using 35% CH_3CN/H_2O containing 0.1% TFA as a mobile phase at 0.5 mL min^{-1}. The sample was applied in the amount of 2 μL at 0.2 mM (0.4 nmol).

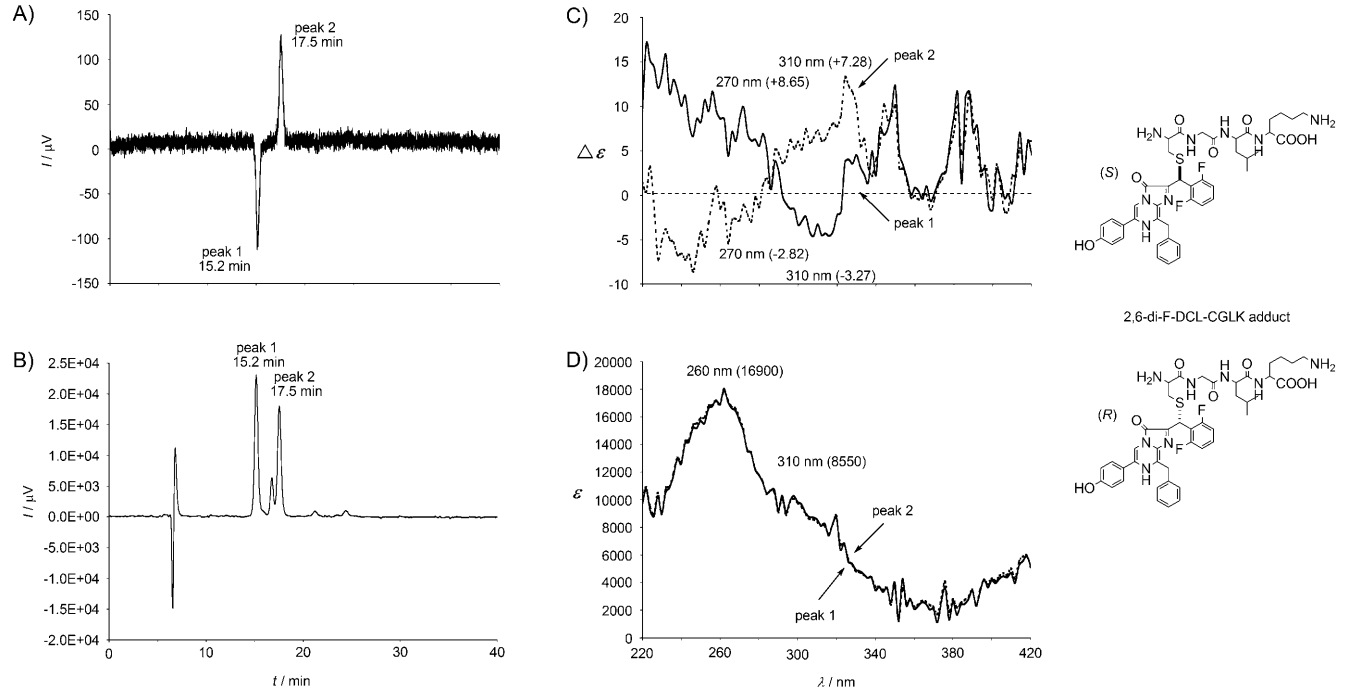


Figure 12. LC-CD analysis of chromopeptide, CGLK-2,6-diF-DCL-(L)-cysteine diastereomeric adducts; A) CD chromatogram at $\lambda = 310$ nm, B) UV chromatogram $\lambda = 310$ nm, C) CD spectra, and D) UV spectra. Chromatogram and spectra were separated with ODS-5 column, 4.6 mm-i.d. × 250 mm using 35% CH_3CN/H_2O containing 0.1% TFA as a mobile phase at 0.5 mL min^{-1}. The sample was applied in the amount of 3 μL at 0.5 mM (1.5 nmol).

sional curve were further fully optimized. Frequency calculations were employed to verify stationary points to be minima with all real frequencies and transition states with only one imaginary frequency. The conformation around the

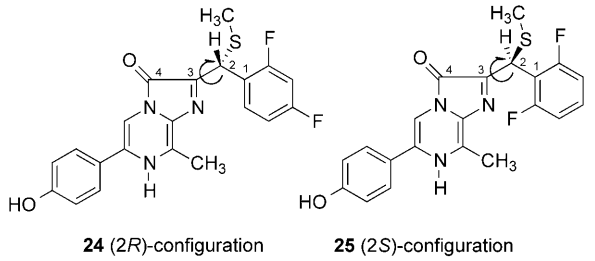


Figure 13. Chemical representations of the simplified structures of the diF-DCL analogs and L-cysteine adducts for determination of the global minimum conformation. See the Supporting Information, Figure S13 for more details on the conformational calculations.

stereogenic carbon of **6** and **7** (both X = L-Cys) are concluded to be similar having the heterocyclic aromatic ring and di-fluorophenyl ring located in a dihedral angle with C2′(*R*)-configuration as negative as −59° and +153° twisting by approximately 60° counter-clockwise; while C2′(*S*)-configuration as negative as −158° twisting by approximately +20° clockwise. These calculations, however, do not lead to a conclusion for the absolute configuration. It should be mentioned that the rotational restriction of the 2,6-diF-DCL-Cys adduct **7** is only one conformation compared with **6** having two conformations in about 1:1 population.

LC-CD Analysis of Symplectin and DCL and its Analogs

To compare these results from L-Cys adducts to the symplectin-bound chromophore, we prepared symplectin samples in a similar fashion as for the experiments relating to Figure 9. In this case, the samples were incubated only for a very short time (2 min) and then subjected to the corresponding bioluminescence measurements. The results are summarized in Figure 14.

The same set of 2 minute experiments were implemented before injecting into the HPLC equipped with a very short GPC column (TSK-gel guard column SWXL 40 mm × 6.0 mm i.d. for separating the free chromophore) and the same CD/UV/FL detectors as previous cases to detect approximately 1.5 nmol, estimated from Figures 11 and 12. The results are shown in Figure 15. The absorption and FL intensities suggested that even though nearly all equal amounts of DCL **2** analogs **4** and **5** are injected (Figure 15 entries 2–5), the CD detector at 310 nm only shows an increase of the negative signal of 30% with 2,4-diF-DCL **4** (Figure 15, entry 2). This negative CD cotton signal clearly showed the similarity of the result from peak 1 with L-cysteine containing the peptide model experiments.

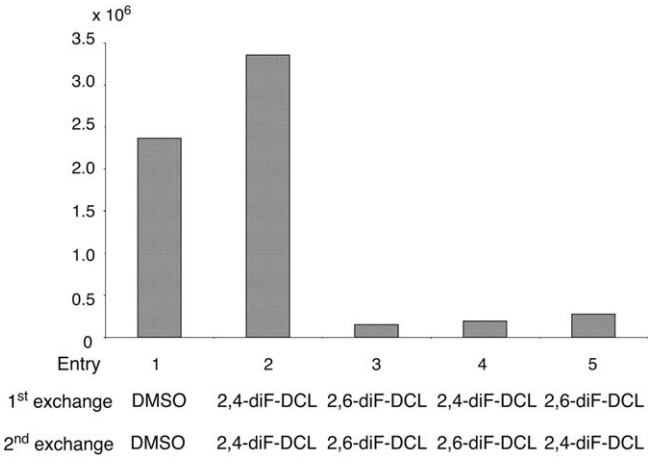


Figure 14. Bioluminescence activity of symplectin re-exchanged with DCL and diF-DCL analogs after each 2 min incubation. A test of DMSO corresponds to the control for the activity of the symplectin solution.

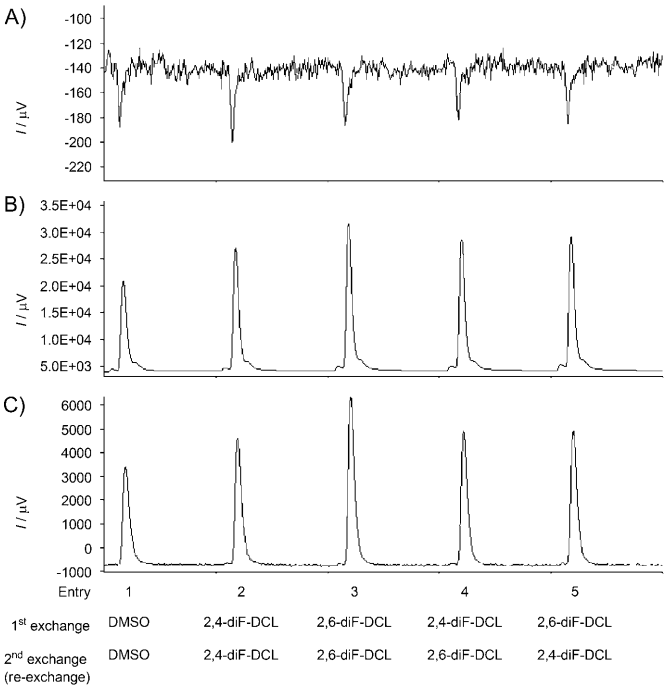
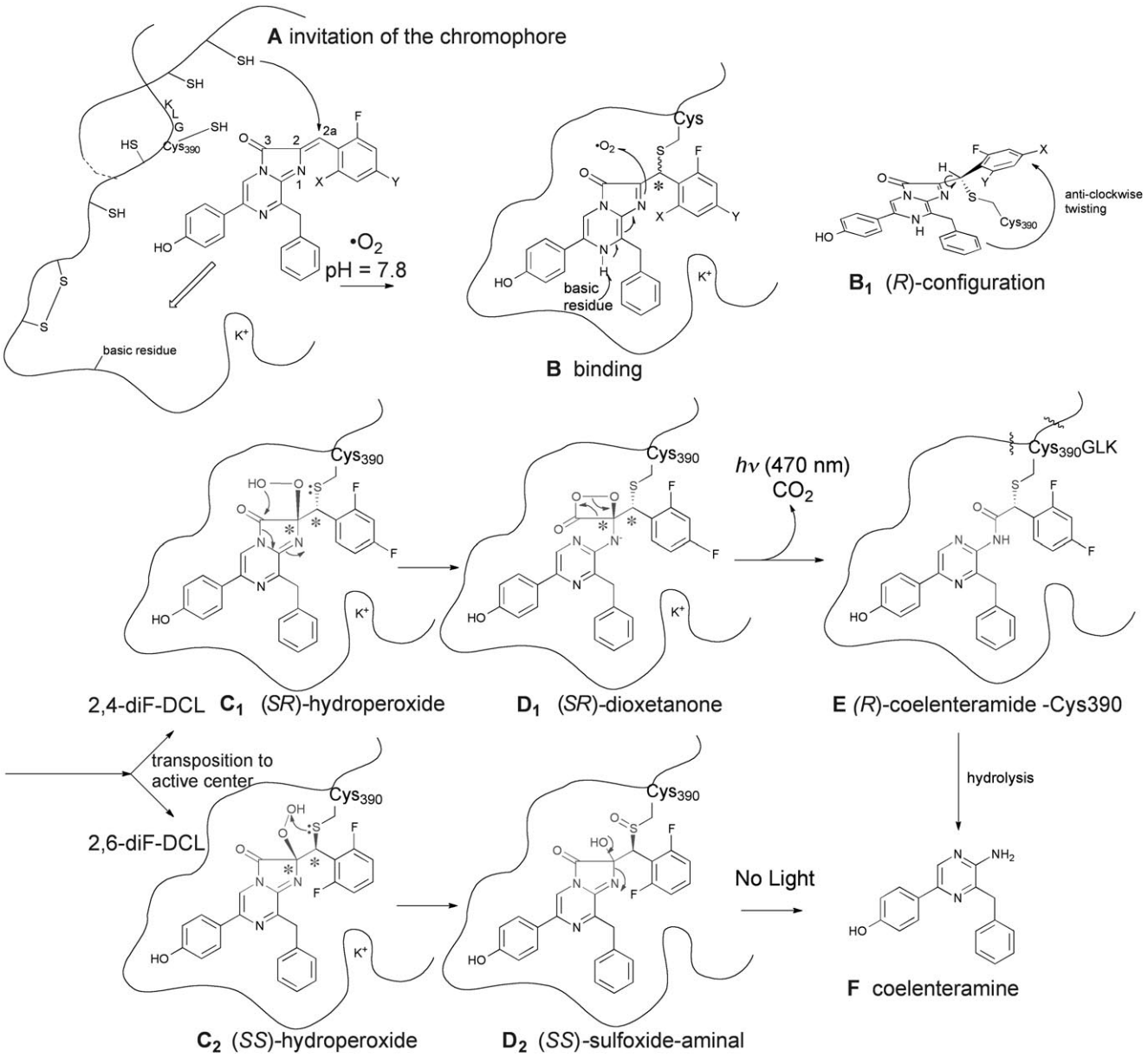


Figure 15. LC-CD-UV-FL analysis of symplectin exchanged and re-exchanged with DCL analogs for 2 min at pH 7.8; A) CD chromatogram at $\lambda = 310$ nm at pH 6, B) UV chromatogram at $\lambda = 310$ nm, and C) FL chromatogram at 520 nm.

Conclusions

When the two isomeric diF-DCL analogs were added to aposymplectin, a significant increase in green fluorescence (GF) intensity was observed at 520 nm at pH 5.6 (see the Supporting Information, Figure S1), and GF decreased by increasing the pH to 7.8 (see the Supporting Information, Figure S3) by a similar degree for both the 2,4- and 2,6-diF-DCL samples (see the Supporting Information, Figure S1). The LC-CD signal increase was observed only in the 2,4-diF case (Figure 15). As summarized in Scheme 3, this implies that the DCLs are bound with Cys–SH residues at the binding sites (**A**) largely as a racemic mixture at pH 5.6–6.0. At pH 7.8, the chromophores are then transferred from the storage site cysteines to the active site 390-Cys to form the intermediate (**B**). The chromophore 2,4-diF-DCL binds largely in one stereochemistry (either *R*- or *S*-configuration,



Scheme 3. Postulated mechanism of *Symplectoteuthis* bioluminescence.

e.g., **B_1**) and then it is further oxidized to the hydroperoxides by photoprotein and active oxygen. In this oxidation, it is likely to result in the peroxi-carbon atom in either one of the *R*- or *S*-configurations.[9,33]

On the contrary, 2,6-diF-DCL binds more tightly with Cys−SH, and moves to the active center resulting presumably in a different configuration (e.g., *S*-**B**), and then forms the hydroperoxide as well. The stereoisomer in this case is hypothesized to be a diastereoisomer (e.g., *SS*, **C_2**), which is further reacted intramolecularly between the hydroperoxide and the sulfide (of 390-cysteine) to give sulfoxide-aminal (**D_2**) and then further to an imino-acylamide.[9,21] Subsequently, it is directly hydrolyzed without luminescence to aminopyrazine (**F**=**23**) without going through coelenteramide, and the photoprotein cannot accept the next substrate anymore owing to the damage of 390-Cys−SH. This damage might make the second run of the photoprotein inefficient, so it looks like inhibition. The chirality at the binding site is racemic judging from the lack of increase of the CD but there is an increase in green fluorescence (see the Supporting Information, Figure S1), and then the chromophores move to the active site at pH 7.8 and stay for a short time at a different diastereoisomer before the next oxidation step (**B** to **C**). Thus, such dynamism of the chirality in **B**–**E** plays a crucial role for determining the bioluminescence ability although the starting material and final product have no stereogenic center. Other photoproteins might include such a temporary and dynamic chirality mechanism, the studies of which are still in progress[34,35] as well as to be continued including new bioanalytical and substrate design.[34]

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Experimental Section

The Experimental Section for this work can be found in the Supporting Information.

Acknowledgements

The authors are indebted to Nagoya University, MEXT-Japan, and JSPS, as well as National Tsing Hua University and National Science Council of Taiwan for financial support. Technical support by JACSO Co. Ltd. and Toyo-Soda Co. Ltd. are also acknowledged. The authors are also grateful to Japanese government scholarship (Monbukagakusho) for V.K. to perform her PhD thesis work in the foreign student program.

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Received: January 31, 2011
Published online: June 7, 2011

