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^{19}F-Dehydrocoelenterazine as probe to investigate the active site of symplectin

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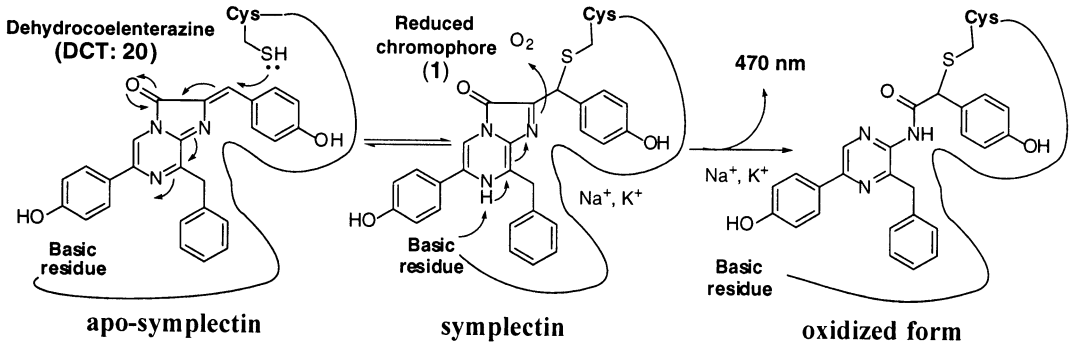
Abstract—Fluorinated dehydrocoelenterazines (F-DCTs) were synthesized to study molecular mechanisms of symplectin; a photoprotein of luminous squid *Symplectoteuthis oualaniensis* L. F-DCTs reacted with dithiothreitol and glutathione under neutral conditions to give the stable chromophores as symplectin model. Reconstructed symplectin was also obtained by addition of F-DCTs into apo-symplectin, and showed bioluminescence to emit 50–65% amount of light as natural symplectin. The structure of the chromophores was determined by ^{19}F NMR, Q-TOF-MS, and MS/MS analyses. Sequencing of the chromopeptides of symplectin models prepared from F-DCTs and thiol compounds was accomplished by ESI-Q-TOF-MS/MS analysis.

1. Introduction[1]

Many luminous lives possess photoprotein as a device for light emission. Tobiika, a luminous oceanic squid, is quite unique in the use of dehydrocoelenterazine (**20**) as an organic substrate of a photoprotein (symplectin: the photoprotein of a luminous squid *Symplectoteuthis oualaniensis* L.[2]), and for the existence of the photoprotein as adduct form of **20**. Among many luminous lives, tobiika is so far the only one to use **20** as the organic substrate. Our studies on the bioluminescent mechanism of this squid have indicated to us the following bioluminescent mechanism: Michael addition of a sulfhydryl group of apo-symplectin to a dehydrocoelenterazine (DCT: **20**) is the initial step in the symplectin bioluminescence to give a pseudo-reduced chromophore (**1**) (Scheme 1).[3,4] Structural changes of symplectin by mono-cations (Na^+, K^+)[5] initiate the oxidation of this chromophore with oxygen to emit blue light (470 nm) and to give the oxidized form. Our interest is now focused on the active site of symplectin, especially on the cysteine residue to make a covalent bond with DCT. In this report, we describe the synthesis of fluorinated DCT (F-DCT) to make a covalent bond between a cysteine residue and DCT tighter to enable spectroscopic analysis (NMR and MS) of the chromopeptide under mild conditions. By studying a model bioluminescence prepared from F-DCT and thiols, we demonstrate that F-DCT is the best probe to investigate the active site of symplectin.



Scheme 1. Bioluminescent mechanism of *S. oualaniensis*.

Keywords: symplectin; fluorine; dehydrocoelenterazine; bioluminescence.
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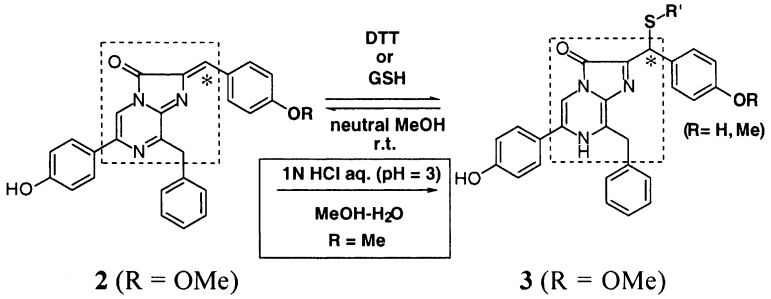


Figure 1. Equilibrium between DCT and thiol adduct.

2. Results and discussion

2.1. Stability of chromophore under neutral conditions

In our previous papers, we reported the preparation of a symplectin model (**3**) from ^{13}C-labeled DCT analog (**2**) with thiol compounds such as dithiothreitol (DTT: **14**) and glutathione (GSH: **15**) under acidic conditions (pH 3) (asterisk indicates ^{13}C-labeled position).[6,7] However, the symplectin model dissociated in equilibrium into DCT and DTT under neutral conditions (physiological condition) (Fig. 1).

This equilibrium between DCT and thiol adduct stops under acidic conditions, so that the thiol adducts can be separated, but those systems are under equilibrium at the optimum pH (7.8). To overcome the unstableness of these thiol adducts under neutral conditions, we planned to introduce a fluorine atom into the DCT C-ring to make the covalent bond between DCT and sulfhydryl residue tighter (Fig. 2). We postulated that the strong electron withdrawing nature of fluorine atom in inductive effect would stabilize the symplectin model even at pH 7.8. Three kinds of fluorine-labeled DCTs (F-DCT: **13a**–**c** in Fig. 2) were synthesized to study the regio-isomeric effect on the fluorine-labeled position.[8]

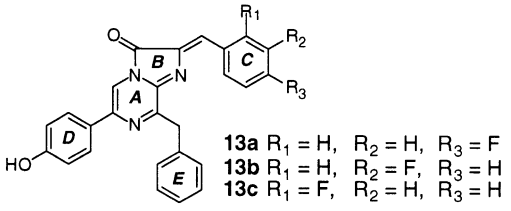
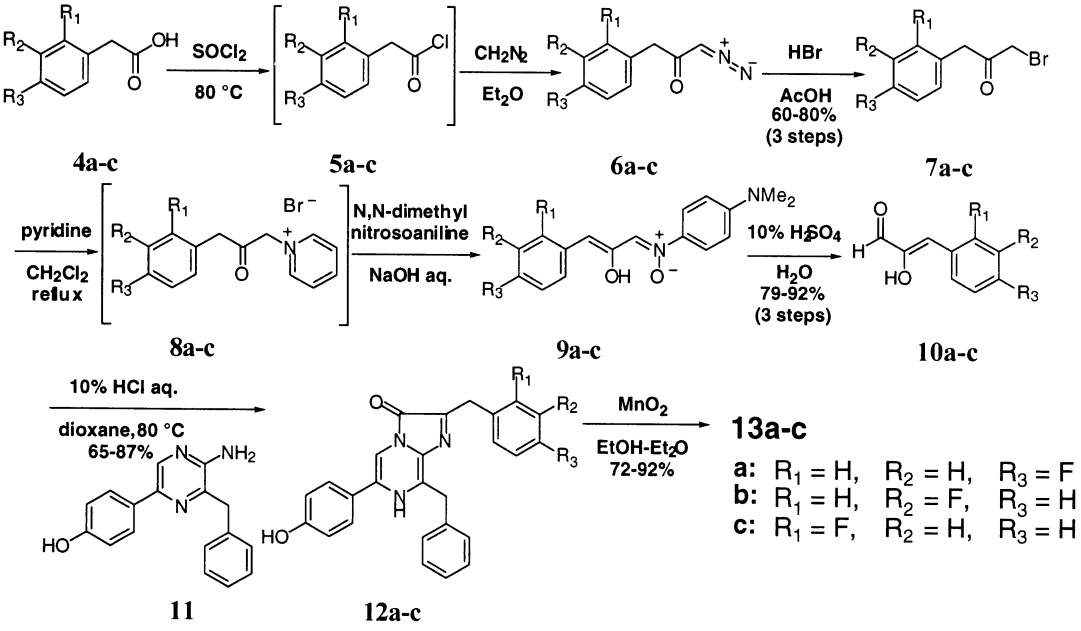


Figure 2. Structure of F-labeled DCT.

2.2. Synthesis of F-DCT from *F*-phenylacetic acid

Scheme 2 shows the synthetic schemes of F-DCT (**13a**–**c**). Commercially available fluorophenylacetic acid (**4a**–**c**) was converted to α-bromoketone (**7a**–**c**) in three steps (60–80% yield). After deriving as a pyridinium salt (**8a**–**c**), condensation with a *N*,*N*-dimethylnitrosoaniline gave a nitron compound (**9a**–**c**). Then, hydrolysis of **9a**–**c** with sulfuric acid afforded a ketoaldehyde (**10a**–**c**) (79–92% yield from **7a**–**c**). Fluoro-coelenterazine[9] (**12a**–**c**) was obtained by the condensation of **10a**–**c** with coelenteramine (**11**)[10] at 80°C in a mixture of 10% HCl and dioxane in 65–87% yield. Finally, **12a**–**c** oxidized to ^{19}F-labeled dehydrocoelenterazine (**13a**–**c**) with MnO_2 in 74–77% yield. The purity of



Scheme 2. Synthetic route toward fluorine labeled dehydrocoelenterazine.

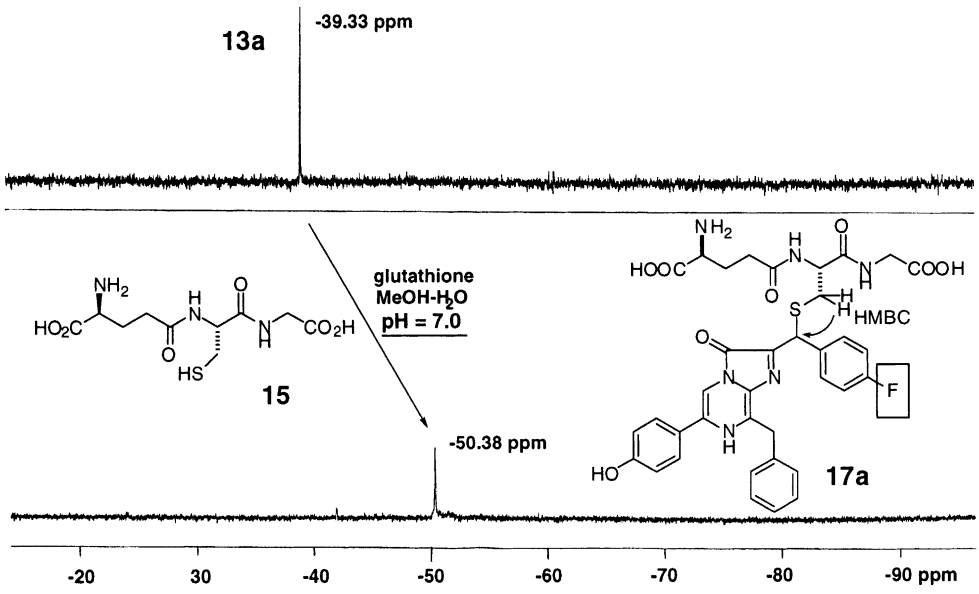


Figure 3. ^{19}F NMR spectra of symplectin model **17a** prepared from **13a** and GSH (**15**); 8 scans by 376 MHz in CD_3OD.

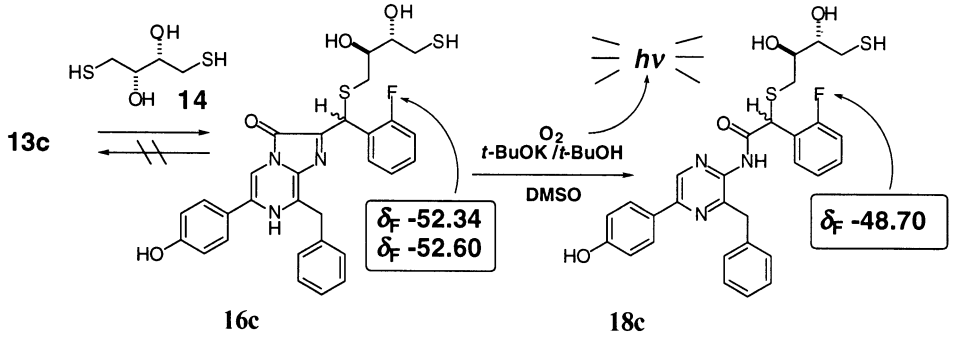
these synthetic F-DCTs (**13a–c**) was confirmed to be more than 97% by HPLC analysis.[11]

2.3. ^{19}F NMR of model bioluminescence by F-DCT and GSH

Three ^{19}F-labeled DCTs (**13a–c**) reacted with respective two kinds of thiol compounds (**14**, **15**) to afford the stable symplectin model (**16a–c**, **17a–c**) under neutral-physiological condition as we expected. ^{19}F NMR analysis of these symplectin models showed up-field shifts of the chemical shifts from −39.33 ppm for **13a** into −50.38 ppm for **17a** as shown in Fig. 3; −48.20 ppm for **17b** and −52.18, −52.45 ppm for two diastereoisomeric **17c** were also observed. Only the diastereomeric ^{19}F atom of **17c** was distinguished by NMR. Mixing of **16c** with *t*-BuOK in DMSO as one of model bioluminescence emitted light in the presence of oxygen to give amide compound **18c** which was observed with ^{19}F NMR spectroscopy (Scheme 3). Two diastereomeric −52.34 and −52.60 ppm for **16c** changed into −48.70 ppm for **18c**. The diastereomeric ^{19}F atom of **18c** was not separated on the NMR spectrum. In conclusion, we succeeded in monitoring the chemiluminescence of the symplectin model with ^{19}F NMR spectroscopy, and found that F-DCT is a suitable probe for studying the molecular mechanism of symplectin bioluminescence with ^{19}F NMR spectroscopy. No spectrum could be obtained in this neutral condition when **20** was used.

2.4. MS/MS analysis of ^{19}F-DCT–GSH adducts

Peptide sequencing of the symplectin model (**17a–c**) was performed by ESI-Q-TOF-MS/MS measurement[12] to confirm the precise structure, especially to find the peptide fragment having chromophoric DCT adduct. MS/MS analysis of the chromopeptide containing DCT is the strong support to determine the active site cysteine in amino acid sequence of symplectin, particularly the ions after luminescence (**21a**). Fig. 4 shows the results on analysis of symplectin model (**17a–c**) by using ESI-Q-TOF-MS and MS/MS measurement, and Fig. 5 shows the results for **21a**. In Fig. 4, the product ions of MS/MS of **17a–c** at *m/z* 656 and 602 were assigned to both b_2 and y''_2 for **17a–c**. Fragment ions appeared more clearly in **21a**; *m/z* 644 and 590 were assigned to both b_2 and y''_2, respectively, as shown in Fig. 5. We also noted that **17c** gave the largest intensity of mass peaks in MS and MS/MS spectra. Although the reasons for these high intensities of **17c** could not be explained yet, *ortho*-F-labeled DCT (**13c**) was the most suitable probe to detect and to map the chromo-peptide by using MS and MS/MS measurements.



Scheme 3. Model bioluminescence of symplectin analog **16c**.

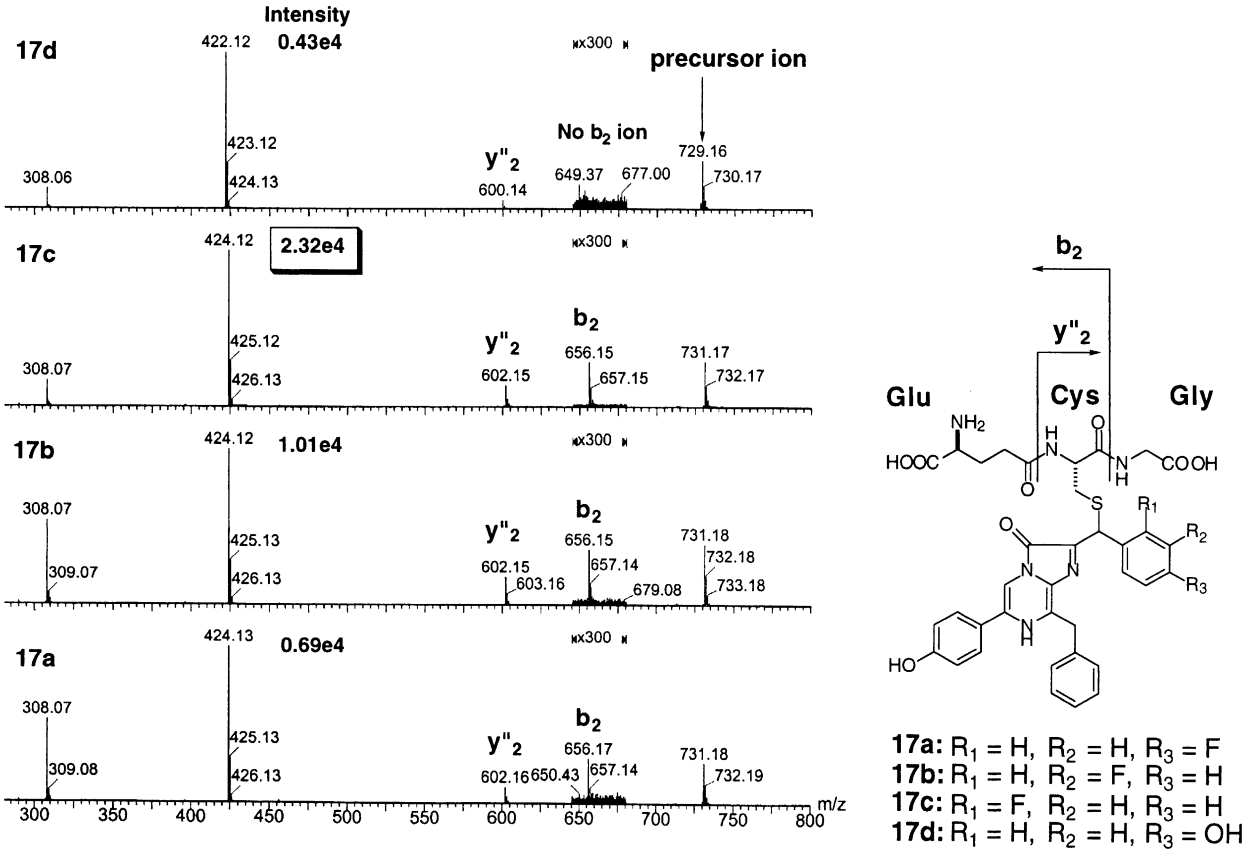


Figure 4. MS/MS spectra of symplectin model (**17a–d**) and assignment of fragment ions.

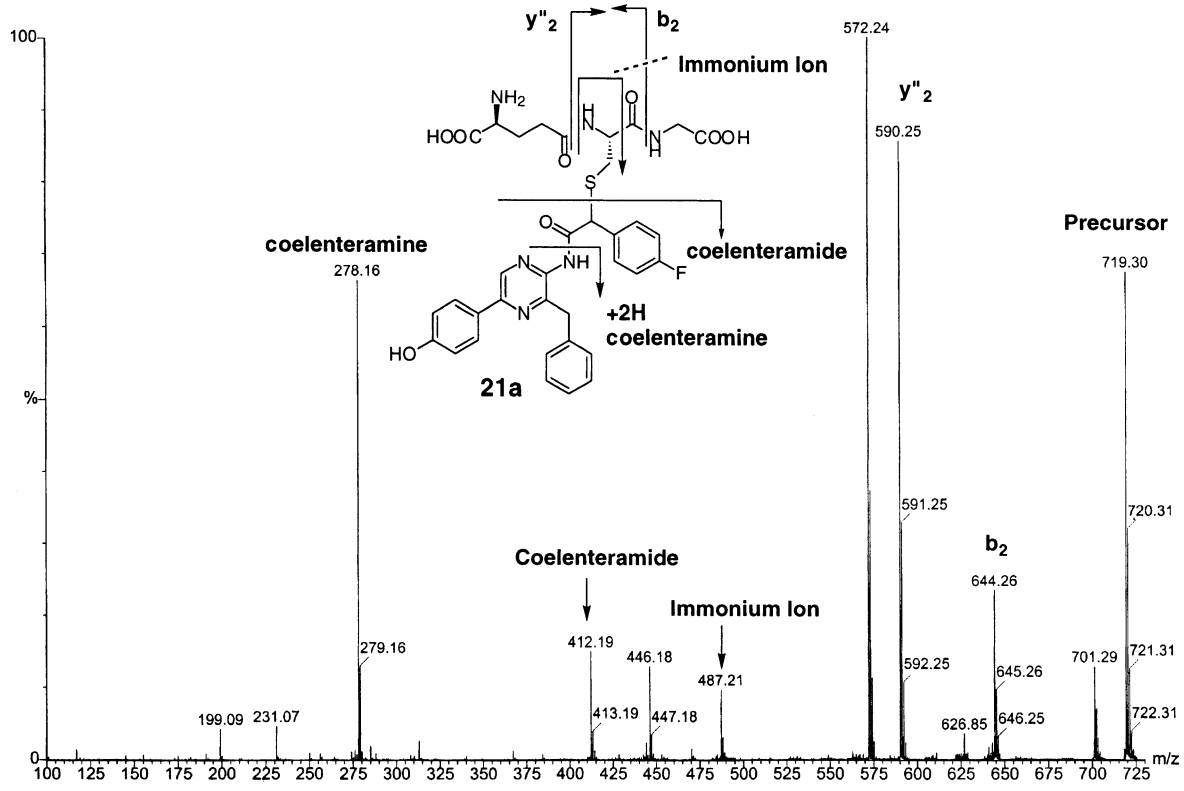
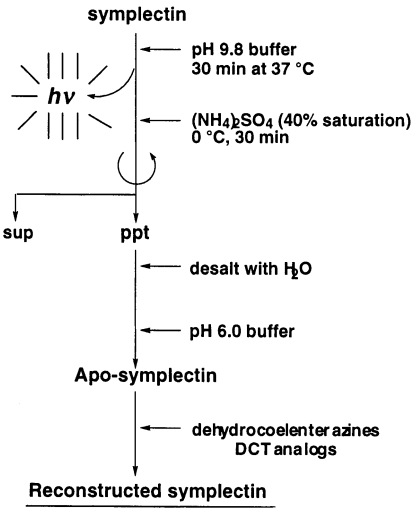


Figure 5. MS/MS spectra of (**21a**) after model bioluminescence and assignment of fragment ions.



Scheme 4. Preparation of apo-symplectin.

2.5. Preparation of apo-symplectin from symplectin[13]

Our interests were then focused upon the bioluminescent ability of reconstructed symplectins prepared from synthetic F-DCTs (**13a**–**c**) and apo-symplectin. To compare the exact bioluminescence of these reconstructed symplectins, we had to establish a method for the preparation of an apo-symplectin. First of all, it was necessary to remove the natural DCT which was contained as natural organic substance in symplectin. If DCT remained in apo-symplectin, the background level in bioluminescence profiles should be too large to correctly compare the bioluminescence ability of reconstructed symplectins. In these experiments, we used symplectin extracted from luminous organ which contained more than 90% purity of symplectin, therefore we performed comparison experiments for the luminescent abilities of these reconstructed symplectins.

Therefore, we thought that consuming the naturally-contained DCT of symplectin was a solution to get apo-symplectin. This idea was attained as follows: first, we initiated bioluminescence by addition of pH 9.8 buffer into an extracted symplectin solution in the presence of oxygen to consume the natural DCT. Then the resulting symplectin was precipitated by ammonium sulfate (40% saturation) to remove both oxidized DCT and remaining organic substances into a supernatant fraction by centrifugation. Finally, since the ammonium pellet existed as almost pure apo-symplectin, the pellet was washed with water to desalt such as ammonium sulfate and was dissolved in pH 6.0 buffer to afford apo-symplectin solution. By using these operations, we succeeded reproducibly in getting apo-symplectin.

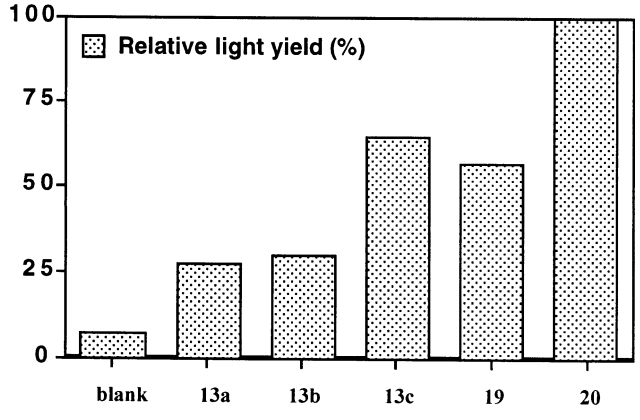
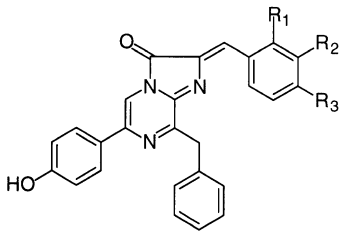


Figure 6. Bioluminescent profiles of reconstructed symplectin from various DCTs.



13a R_1 = H, R_2 = H, R_3 = F
13b R_1 = H, R_2 = F, R_3 = H
13c R_1 = F, R_2 = H, R_3 = H
19 R_1 = H, R_2 = H, R_3 = OMe
20 R_1 = H, R_2 = H, R_3 = OH

Figure 7. Structures of DCT analogs used for bioluminescent assay.

2.6. Bioluminescence profiles of re-constructed symplectin (OH, OMe, F(*o*-,*m*-,*p*-))

To the apo-symplectin solution, synthetic DCTs were pre-incubated to reactivate symplectin at pH 6.0 in 0.6 M KCl buffer at room temperature. After 30 min, bioluminescence was profiled by changing pH to 7.8 to measure the total light yield for 3 min with a lumiphotometer. Scheme 4 shows protocols of the preparation of apo-symplectin and of these reactivated symplectins. Fig. 6 shows the summary of bioluminescent profiles for the five reconstructed symplectins. DCT analogs used for this bioluminescence assay are shown in Fig. 7. We found that *ortho*-F-labeled DCT (**13c**) gave strongest bioluminescence in the series of F-DCTs and was consistent with the results obtained with ^{19}F NMR spectroscopy and by ESI-Q-TOF-MS and MS/MS measurements.

3. Conclusions

Three fluorinated dehydrocoelenterazines (F-DCTs) were synthesized to study molecular mechanisms of symplectin. F-DCTs reacted with dithiothreitol (DTT) and glutathione (GSH) even under neutral-physiological condition to afford the stable chromopeptides as symplectin models. The structure of these chromopeptides was determined by ^{19}F NMR, ESI-Q-TOF-MS, and MS/MS analyses. Sequencing of the chromopeptides was accomplished by using symplectin models prepared from three F-DCTs and GSH by ESI-Q-TOF-MS/MS measurements. Three reconstructed symplectins were also obtained by addition of F-DCTs into apo-symplectin, and showed more efficient bioluminescence compared with two other DCT analogs. In the series of F-DCT, *ortho*-F-labeled DCT (**13c**) was the most suitable probe to study symplectin bioluminescence. Determination of the real symplectin chromopeptide by a combination of F-DCT and ESI-Q-TOF-MS, MS/MS analysis is now underway in our group.

4. Experimental

4.1. General

UV spectra were obtained on a JASCO U-best 50 spectrometer. Proton NMR spectra were recorded on a JEOL GSX 270 for 270 MHz, a JEOL JNML-500 for 500 MHz or a Bruker AMX-600 for 600 MHz. Chemical shifts (δ) are given in parts per million relative to tetramethylsilane (δ 0.00) as internal standard and coupling constants (J) in Hz. Carbon NMR were recorded on a JEOL GSX 270 for 67.8 MHz or a JEOL JNML-500 for 125.7 MHz or on a Bruker AMX-600 for 150.9 MHz. Chemical shifts are (δ) given in parts per million relative to $CDCl_3$ (δ 77.0) or CD_3OD (δ 49.0) or DMSO-d_6 (δ 45.0) as internal standard. Fluorine NMR spectra were recorded on a Bruker ARX-400 for 376 MHz. Chemical shifts are (δ) given in parts per million relative to 1,1,1-trifluorotoluene (δ 0.00) as internal standard. Low-resolution EI mass spectra and FAB mass spectra were measured with a JEOL JMS-700. High-resolution (HR) mass spectra were measured with a JEOL JMS-700. Fluorescence spectra were measured with a JASCO FP-777 spectrometer. Light yields of bioluminescence of reconstructed symplectins were determined with Labo Science TD-4000 Lumiphotometer by integrating total light emission.

Dichloromethane (CH_2Cl_2) was distilled from calcium hydride. 1,4-Dioxane was distilled from sodium metal in the presence of sodium benzophenone ketyl as indicator. Pyridine was dried over NaOH pellets and used without distillation. The other solvents were of reagent grade.

Analytical thin-layer chromatography (TLC) was conducted on precoated TLC plates: silica gel 60 F-254 (E. Merck (Art 5715) Darmstadt, Germany), layer thickness 0.25 mm. Silica gel column chromatography utilized Merck (silica gel 60: 70–230 mesh).

The HPLC analyses for nano-LC-Q-TOF-MS spectrometer were carried out using modified house made system based on a JASCO PU-980 pump systems equipped with a JASCO UV-970 UV/vis detector and a JASCO 807-IT integrator. An ODS column of 0.3×150 mm (Develosil ODS-UG-5, Nomura Chemicals Co., Ltd) was eluted with acetonitrile/water containing 0.1% of trifluoroacetic acid (TFA) at a flow-rate of 5 μl/min at room temperature.

4.1.1. 1-Bromo-3-(4-fluorophenyl)-propan-2-one (7a). The 4-fluorophenylacetic acid **4a** (1.0 g, 6.5 mmol) was refluxed (2 h) with $SOCl_2$ (3.0 ml). After cooling, thionyl chloride was removed under reduced pressure, and the resultant liquid was distilled with a Kugerlohr distillation (100°C, 3 mmHg) to afford the corresponding acid chloride **5a**. The acid chloride **5a** was then dissolved in 30 ml of ether and to this solution was added dropwise another solution of diazomethane (prepared according to the general procedure) at −78°C. After the addition of 25 ml of diazomethane, the reaction mixture was allowed to warm to 0°C and stirred. The solution was evaporated under reduced pressure after the disappearance of the spot of the starting material on TLC to give a crude compound **6a** as yellow oil. When 4 ml of 47% HBr at 0°C was added dropwise to a solution of the crude diazoketone **6a** in 6 ml of acetic acid, nitrogen gas evolved vigorously at first. After being stirred for 10 min, the solution was allowed to come to room temperature and stirred for 10 min. To this solution was added 10 ml of water at 0°C to give white precipitate. The reaction mixture was neutralized with sat. $NaHCO_3$ and extracted with ethyl acetate (×3). The combined organic layers were washed successively with water (×2) and brine (×1). The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to dryness. The residue was chromatographed on silica gel with ethyl acetate/*n*-hexane (1/2) to give 890 mg of **7a** as pale lemon colored oil in 60% yield (3 steps). Compound **6a**: ^{1}H NMR ($CDCl_3$, 500 MHz), δ 3.58 (2H, s), 5.15 (1H, s), 7.03 (2H, t, J=8.5 Hz), 7.20 (2H, dd, J=8.8, 5.4 Hz) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz), δ 47.0, 54.9, 115.7 (d, J_{C-F}=21 Hz), 130.3, 130.8 (d, J_{C-F}=8.3 Hz), 162.0 (d, J_{C-F}=247 Hz), 192.4 ppm. EI-MS m/z 178 (M^+), 150 (M^+-N_2). HRMS (EI) calcd for $C_9H_7ON_2F$ 178.0542, found 178.0508 (M^+), for C_9H_7OF 150.0481, found 150.0464 (M^+-N_2). Compound **7a**: ^{1}H NMR ($CDCl_3$, 270 MHz), δ 3.91 (4H, s), 7.02 (2H, dd, J=8.8, 5.4 Hz), 7.18 (2H, t, J=8.8 Hz) ppm. ^{13}C NMR ($CDCl_3$, 67.8 MHz), δ 33.5, 45.5, 115.6 (d, J_{C-F}=21 Hz), 128.7, 131.0, 162.0 (d, J_{C-F}=245 Hz), 199.1 ppm. EI-MS m/z 232/230 (M^+), 137, 109.

4.1.2. 1-Bromo-3-(3-fluorophenyl)-propan-2-one (7b). 7b was synthesized according to the same procedures described in **7a**. **7b** (1.2 g) in 80% yield (3 steps) from **4b** (1.0 g, 6.5 mmol). Compound **6b**: ^{1}H NMR ($CDCl_3$, 500 MHz), δ 3.64 (2H, s), 5.28 (1H, s), 6.90–7.06 (2H, m), 7.26–7.31 (2H, m) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz), δ 40.5, 55.0, 114.3 (d, J_{C-F}=21.1 Hz), 116.5 (d, J_{C-F}=22.0 Hz), 125.0 (d, J_{C-F}=2.8 Hz), 130.5 (d, J_{C-F}=8.2 Hz), 135.1 (d, J_{C-F}=8.3 Hz), 162.7 (d, J_{C-F}=245 Hz), 199.4 ppm. EI-MS m/z 178 (M^+), 150 (M^+-N_2). HRMS (EI) calcd for $C_9H_7ON_2F$ 178.0542, found 178.0563 (M^+), for C_9H_7OF 150.0481, found 150.0452 (M^+-N_2). Compound **7b**: ^{1}H NMR ($CDCl_3$, 500 MHz), δ 3.89 (2H, s), 3.91 (2H, s), 7.00–6.98 (2H, m), 7.36–7.28 (2H, m) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz), δ 35.5, 47.7, 114.4 (d, J_{C-F}=20.9 Hz), 116.6 (d, J_{C-F}=21.9 Hz), 125.2 (d, J_{C-F}=2.8 Hz), 130.4 (d, J_{C-F}=8.1 Hz), 135.1 (d, J_{C-F}=8.3 Hz), 163.0 (d, J_{C-F}=245 Hz), 199.4 ppm. EI-MS m/z 232/230 (M^+), 137, 109.

4.1.3. 1-Bromo-3-(2-fluorophenyl)-propan-2-one (7c). 7c was synthesized according to the same procedures described in **7a**. **7c** (1.0 g, 6.5 mmol) in 67% yield (3 steps) from **4c** (1.0 g). Compound **6c**: ^{1}H NMR ($CDCl_3$, 500 MHz), δ 3.65 (2H, s), 5.23 (1H, s), 7.07 (1H, t, J=8.4 Hz), 7.12 (1H, dt, J=7.5, 1.0 Hz), 7.27 (2H, t, J=7.6 Hz)) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz), δ 40.9, 54.6, 115.5 (d, J_{C-F}=22.0 Hz), 124.4, 129.3, 129.5, 131.6, 160.8 (d, J_{C-F}=246 Hz), 191.4 ppm. EI-MS m/z 178 (M^+), 150 (M^+-N_2). HRMS (EI) calcd for $C_9H_7ON_2F$ 178.0542, found 178.0515 (M^+), for C_9H_7OF 150.0481, found 150.0452 (M^+-N_2). Compound **7c**: ^{1}H NMR ($CDCl_3$, 270 MHz), δ 3.98 (4H, s), 7.34–7.02 (4H, m) ppm. ^{13}C NMR ($CDCl_3$, 67.8 MHz), δ 33.7, 40.0, 115.5 (d, J_{C-F}=21.1 Hz), 120.6 (d, J_{C-F}=16.1 Hz), 124.4, 129.5, 131.6, 160.9 (d, J_{C-F}=243 Hz), 198.3 ppm. EI-MS m/z 232/230 (M^+), 137, 109.

4.1.4. 3-(4-Fluorophenyl)-2-hydroxy-2-propen-1-al (10a). A solution of 1-Bromo-3-(4-fluorophenyl)-propan-2-one

(**7a**) (880 mg, 3.8 mmol) in 12 ml of CH_2Cl_2 was heated at 70°C for 3 h with pyridine (0.90 ml). Evaporation of the reaction mixture under reduced pressure to dryness afforded the pyridinium salt **8a** as highly viscous oil. To a solution of the pyridinium salt **8a** and *N*,*N*-dimethyl-*p*-nitrosoaniline (572 mg, 3.81 mmol) in 38 ml of water was added dropwise 3.8 ml of 1N NaOH at 0°C. After being stirred for 10 min at 0°C, the reaction mixture was allowed to come to room temperature and stirred for 90 min. Filtration of the reaction mixture gave the crude compound **9a**. A suspension of the *p*-fluoronitron **9a** in 65 ml of 10% H_2SO_4 was stirred for 90 min at room temperature. Then the suspension was extracted with ether (×3) and the organic layer was washed successively with water (×2) and brine (×1). The organic layer was dried over anhydrous Na_2SO_4 and concentrated to give 503 mg of **10a** as lemon colored oil in 79% (3 steps). Compound **9a**: ^{1}H NMR ($CDCl_3$, 500 MHz), δ 3.03 (6H, s), 5.53 (1H, s), 6.67 (2H, d, J=9.3 Hz), 7.02 (2H, t, J=8.3 Hz), 7.51 (1H, s), 7.58 (2H, d, J=9.3 Hz), 7.77 (2H, dd, J=8.8, 5.9 Hz) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz), δ 40.3, 111.3, 112.1, 115.4, 121.6, 130.7, 131.6, 132.4, 134.4, 148.8, 151.6, 162.0 (d, J_{C-F}=250 Hz) ppm. EI-MS m/z 301 (M^+), 163. Compound **10a**: ^{1}H NMR ($CDCl_3$, 500 MHz), δ 6.14 (1H, s), 7.10 (2H, d, J=9.5 Hz), 7.84 (2H, dd, J=8.5, 5.5 Hz) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz), δ 115.8, 116.0, 121.6, 129.9, 132.4, 132.5, 148.4, 132.4, 132.5, 148.4, 163.1 (d, J_{C-F}=250 Hz), 188.2 ppm. EI-MS m/z 166 (M^+), 109, 57.

4.1.5. 3-(3-Fluorophenyl)-2-hydroxy-2-propen-1-al (10b). **10b** was synthesized according to the same procedures described in **10a**. **10b** (583 mg) in 92% yield (3 steps) from **7b** (1.20 g, 5.19 mmol) and *N*,*N*-dimethyl-*p*-nitrosoaniline (779 mg, 5.19 mmol). Compound **9b**: ^{1}H NMR ($CDCl_3$, 500 MHz), δ 3.06 (6H, s), 4.50 (2H, s), 6.65 (2H, d, J=9.0 Hz), 7.01–7.27 (3H, m), 7.43 (1H, d, J=9.3 Hz), 7.51 (1H, s), 7.63 (2H, d, J=9.0 Hz), 7.72 (1H, d, J=10.7 Hz) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz), δ 40.2, 44.0, 111.0, 111.3, 115.1 (d, J_{C-F}=21.1 Hz), 121.6, 121.8 (d, J_{C-F}=16.5 Hz), 122.8, 124.0, 128.8 (d, J_{C-F}=8.2 Hz), 130.3, 131.8,136.7, 152.4, 161.3 (d, J_{C-F}=247 Hz), 192.2 ppm. EI-MS m/z 300 (M^+). HRMS (EI) calcd for $C_{17}H_{17}O_2N_2F$ 300.1274, found 300.1260 (MH^+). Compound **10b**: ^{1}H NMR ($CDCl_3$, 500 MHz), δ 6.13 (1H, s), 6.67 (1H, brs), 7.07–7.03 (1H, d, m), 7.39–7.35 (1H, m), 7.53 (1H, d, J=7.3 Hz), 7.69 (1H, d, J=7.4 Hz), 9.26 (1H, s) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz), δ 116.2 (d, J_{C-F}=21.9 Hz), 116.8 (d, J_{C-F}=21.9 Hz), 121.0 (d, J_{C-F}=2.8 Hz), 126.2 (d, J_{C-F}=2.8 Hz), 130.1 (d, J_{C-F}=8.1 Hz), 135.5 (d, J_{C-F}=8.3 Hz), 162.8 (d, J_{C-F}=244 Hz), 188.1 ppm. EI-MS m/z 166 (M^+), 137, 109, 57. HRMS (EI) calcd for $C_9H_7O_2F$ 166.0430, found 166.0421. Anal. calcd for $C_9H_7O_2F$; C, 66.06; H, 4.25; N, 0.00%. Found C, 66.07; H, 4.34; N, 0.15%.

4.1.6. 3-(2-Fluorophenyl)-2-hydroxy-2-propen-1-al (10c). **10c** was synthesized according to the same procedures described in **10a**. **10c** (503 mg) in 79% yield (3 steps) from **7c** (880 mg, 3.81 mmol) and *N*,*N*-dimethyl-*p*-nitrosoaniline (572 mg, 3.81 mmol). Compound **9c**: ^{1}H NMR ($CDCl_3$, 500 MHz), δ 3.03 (6H, s), 5.51 (1H, s), 6.66 (2H, d, J=9.3 Hz), 6.88 (1H, td, J=8.3, 2.5 Hz), 7.27–7.24 (1H, m), 7.43 (1H, d, J=9.3 Hz), 7.51 (1H, s), 7.58 (2H, d, J=9.3 Hz), 7.61 (2H, d, J=10.7 Hz) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz), δ 40.2, 111.3, 111.7, 113.5 (d, J_{C-F}=21.8 Hz), 115.4 (d, J_{C-F}=22.8 Hz), 121.7,124.7, 129.5 (d, J_{C-F}=8.3 Hz), 131.3, 134.3,138.1 (d, J_{C-F}=8.1 Hz), 150.1, 151.7, 162.8 (d, J_{C-F}=242 Hz) ppm. EI-MS m/z 301 (M^+), 163. Compound **10c**: ^{1}H NMR ($CDCl_3$, 500 MHz), δ 6.50 (1H, s), 6.72 (1H, s), 7.10 (1H, d, J=9.6 Hz), 7.21 (1H, t, J= 7.1 Hz), 7.35–7.31 (1H, m), 8.30 (1H, td, J=7.8, 2.0 Hz), 9.29 (1H, s) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz), δ 113.1 (d, J_{C-F}=7.3 Hz), 115.2 (d, J_{C-F}=21.9 Hz), 121.7 (d, J_{C-F}= 11.9 Hz), 124.4 (d, J_{C-F}=3.6 Hz), 130.9 (d, J_{C-F}=9.1 Hz), 131.4, 149.3, 160.3 (d, J_{C-F}=251 Hz), 188.2 ppm. EI-MS m/z 166 (M^+), 109.

4.1.7. 2-(4-Fluorophenylmethyl)-6-(4-hydroxyphenyl)-8-benzyl-3,7-dihydroimidazo[1,2-*a*]pyrazine-3-one (12a). A solution of the coelenteramine **11** (198 mg, 0.71 mmol) and 3-(4-fluorophenyl)-2-hydroxy-2-propen-1-al (**10a**) (298 mg, 1.78 mmol) in 8.4 ml of 20% water in dioxane was degassed. To this solution was added 1.2 ml of 10% HCl and stirred under argon atmosphere at room temperature for 5 min, then at 80°C for 2 h. After cooling, to this solution was added 30 ml of water at 0°C. The reaction mixture was extracted with ethyl acetate (×3). The combined organic layers were washed successively with brine (×1). The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to dry. The residue was chromatographed on silica gel with 10% MeOH/CH_2Cl_2 to give 264 mg of **12a** as yellow amorphous powder in 87% yield. Compound **12a**: UV (MeOH/H_2O=1/2) λ_{max} (log ε), 433 (0.76), 259 (1.99) nm. ^{1}H NMR (CD_3OD, 500 MHz), δ 4.11 (2H, s), 4.36 (2H, s), 6.83 (2H, d, J=8.8 Hz), 6.95 (2H, t, J=8.3 Hz), 7.19 (1H, t, J= 7.3 Hz), 7.26 (2H, t, J=6.8 Hz), 7.31 (2H, brt, J= 6.8 Hz), 7.35 (2H, d, J=7.4 Hz), 7.40 (2H, d, J=8.3 Hz) ppm. ^{13}C NMR (CD_3OD, 125 MHz), δ 33.4, 34.9, 108.0, 116.0 (d, J_{C-F}=21 Hz), 116.9, 128.0, 128.2, 129.4, 129.7, 129.7, 131.5 (d, J_{C-F}=7.4 Hz), 135.8, 138.0, 160.2, 163.0 (d, J_{C-F}=241 Hz) ppm. FAB-MS (NBA) m/z 426 (MH^+). HRMS (FAB/NBA) calcd for $C_{26}H_{21}O_2N_3F$ 426.1618, found 426.1621 (MH^+).

4.1.8. 2-(3-Fluorophenylmethyl)-6-(4-hydroxyphenyl)-8-benzyl-3,7-dihydroimidazo[1,2-*a*]pyrazine-3-one (12b). **12b** was synthesized according to the same procedures described in **12a**. **12b** (300 mg) in 85% yield from **10b** (347 mg, 2.08 mmol) and **11** (230 mg, 0.83 mmol). Compound **12b**: UV (MeOH/H_2O=1/2) λ_{max} (log ε), 433 (0.92), 258 (2.35) nm. ^{1}H NMR (CD_3OD, 500 MHz), δ 4.17 (2H, s), 4.36 (2H, s), 6.85 (2H, d, J=8.8 Hz), 6.90–6.84 (1H, m), 7.09 (1H, dd, J=10.1, 2.0 Hz), 7.13 (1H, t, J= 7.3 Hz), 7.21 (1H, m), 7.27 (1H, t, J=7.4 Hz), 7.28–7.24 (2H, m), 7.37 (2H, d, J=6.9 Hz), 7.43 (2H, d, J=8.3 Hz), 7.56 (1H, brs) ppm. ^{13}C NMR (CD_3OD, 125 MHz), δ 33.9, 34.9, 108.1, 114.1 (d, J_{C-F}=20.6 Hz), 116.6 (d, J_{C-F}= 21.5 Hz), 116.9.0, 125.7, 128.2, 129.4, 129.7, 130.1 (d, J_{C-F}=8.3 Hz), 138.0, 142.7 (d, J_{C-F}=8.3 Hz), 160.3, 164.3 (d, J_{C-F}=242 Hz) ppm. FAB-MS (NBA) m/z 426 (MH^+). HRMS (FAB/NBA) calcd for $C_{26}H_{21}O_2N_3F$ 426.1618, found 426.1654 (MH^+).

4.1.9. 2-(2-Fluorophenylmethyl)-6-(4-hydroxyphenyl)-8-benzyl-3,7-dihydroimidazo[1,2-*a*]pyrazine-3-one (12c).

12c was synthesized according to the same procedures described in **12a**. **12c** (184 mg) in 65% yield from **10c** (300 mg, 1.80 mmol) and **11** (200 mg, 0.72 mmol). Compound **12c**: UV (MeOH/H_2O=1/2) λ_{max} (log ε), 433 (0.66), 259 (1.79) nm. ^{1}H NMR (CD_3OD, 500 MHz), δ 4.20 (2H, s), 4.36 (2H, s), 6.83 (2H, d, J=8.8 Hz), 7.07–7.04 (2H, m), 7.21–7.19 (5H, m), 7.36 (2H, d, J=7.3 Hz), 7.44 (2H, d, J=8.8 Hz), 7.56 (1H, brs) ppm. ^{13}C NMR (CD_3OD, 125 MHz), δ 34.9, 40.6, 107.9, 116.1 (d, J_{C-F}=21.9 Hz), 116.9.0, 125.2, 126.7 (d, J_{C-F}=17.4 Hz), 128.2, 129.4, 129.7, 129.8, 132.1 (d, J_{C-F}=4.6 Hz), 138.0, 160.2, 162.3 (d, J_{C-F}=244 Hz) ppm. FAB-MS (NBA) m/z 426 (MH^+). HRMS (FAB/NBA) calcd for $C_{26}H_{21}O_2N_3F$ 426.1618, found 426.1638 (MH^+).

4.1.10. 4-Fluorodehydrocoelenterazine (13a). Manganese (II) oxide (1.2 g, 1.3 mmol) was added to the solution of 4-fluorocoelenterazine **12a** (123 mg, 0.29 mmol) in ether (150 ml) and ethanol (30 ml) at 0°C. The mixture was stirred at 0°C for 2 h. The solution was filtered through a pad of Celite and then concentrated under reduced pressure to dry. The residue was chromatographed on silica gel with 5% MeOH/CH_2Cl_2 to give 90 mg of **13a** as dark purple amorphous powder in 74% yield. Compound **13a**: UV (MeOH/H_2O=1/2) λ_{max} (log ε), 537 (0.28), 346 (1.14), 278 (1.75) nm. ^{1}H NMR (DMSO-d_6, 500 MHz), δ 4.28 (2H, s), 7.23 (1H, m), 7.33 (2H, t, J=7.6 Hz), 7.37 (2H, t, J=8.8 Hz), 7.47 (2H, d, J=9.8 Hz), 7.48 (1H, s), 7.73 (2H, d, J=8.8 Hz), 7.87 (1H, s), 8.43 (2H, m), 9.73 (1H, s) ppm. ^{13}C NMR (DMSO-d_6, 125 MHz), δ 109.4, 115.7, 116.4 (d, J_{C-F}=20.0 Hz), 126.1, 126.8, 128.5, 129.6, 130.9, 133.9, 135.9 (d, J_{C-F}=4.5 Hz), 136.7, 148.3, 157.5 (d, J_{C-F}=80.3 Hz), 166.7 ppm. FAB-MS (NBA) m/z 424 (MH^+). ^{19}F NMR (DMSO-d_6, 376 MHz), δ −39.33 ppm. HRMS (FAB/NBA) calcd for $C_{26}H_{19}O_2N_3F$ 424.1461, found 424.1454 (MH^+).

4.1.11. 3-Fluorodehydrocoelenterazine (13b). 13b was synthesized according to the same procedures described in **13a**. **13b** (54 mg) in 77% yield from **12b** (70 mg, 0.23 mmol) and manganese(II) oxide (1.23 g, 1.3 mmol). Compound **13b**: UV (MeOH/H_2O=1/2) λ_{max} (log ε), 537 (0.12), 351 (0.66), 271 (1.19) nm. ^{1}H NMR (DMSO-d_6, 500 MHz), δ 4.29 (2H, s), 6.81 (2H, d, J=8.8 Hz), 7.23 (1H, t, J=7.6 Hz), 7.33 (2H, t, J=7.6 Hz), 7.33 (1H, t, J=7.6 Hz), 7.48 (2H, d, J=6.8 Hz), 7.48 (1H, s), 7.55 (1H, m), 7.76 (2H, d, J=8.3 Hz), 7.94 (1H, s), 8.09 (1H, d, J=7.8 Hz), 8.31 (1H, d, J=11.0 Hz), 9.65 (1H, s) ppm. ^{13}C NMR (CD_3OD, 125 MHz), δ 109.3, 115.5, 118.0 (d, J_{C-F}=20.0 Hz), 118.5 (d, J_{C-F}=20.0 Hz), 125.8, 126.6, 126.6,128.3, 129.5, 129.7, 130.8, 133.9, 136.5, 139.7, 148.8, 157.3 (d, J_{C-F}=105 Hz), 166.5 ppm. ^{19}F NMR (DMSO-d_6, 376 MHz), δ −39.01 ppm. FAB-MS (NBA) m/z 424 (MH^+). HRMS (FAB/NBA) calcd for $C_{26}H_{19}O_2N_3F$ 424.1461, found 426.11452 (MH^+).

4.1.12. 2-Fluorodehydrocoelenterazine (13c). 13c was synthesized according to the same procedures described in **13a**. **13c** (107 mg) in 77% yield from **12c** (140 mg, 0.33 mmol) and manganese(II) oxide (1.23 g, 1.3 mmol). Compound **13c**: UV (MeOH/H_2O=1/2) λ_{max} (log ε), 546 (0.24), 353 (1.07), 280 (1.69) nm. ^{1}H NMR (DMSO-d_6, 500 MHz), δ 4.28 (2H, s), 6.80 (2H, d, J=8.3 Hz), 7.23 (1H, t, J=7.1 Hz), 7.35–7.31 (3H, m), 7.41 (1H, t, J=7.8 Hz), 7.43 (1H, s), 7.48 (2H, d, J=7.8 Hz), 7.55 (1H, brd, J=6.4 Hz), 7.74 (2H, d, J=8.3 Hz), 7.90 (1H, s), 8.94 (1H, t, J=7.6 Hz), 9.63 (1H, s) ppm. ^{13}C NMR (CD_3OD, 125 MHz), δ 109.3, 115.4, 120.3, 122.0, 125.2, 125.7, 125.8,126.5, 126.6,128.3, 129.5, 133.0, 133.7 (d, J_{C-F}=9.0 Hz), 134.0, 136.5, 139.8, 149.0 (d, J_{C-F}=4.5 Hz), 157.3 (d, J_{C-F}=112 Hz), 166.5 ppm. ^{19}F NMR (DMSO-d_6, 376 MHz), δ −36.4 ppm. FAB-MS (NBA) m/z 424 (MH^+). HRMS (FAB/NBA) calcd for $C_{26}H_{19}O_2N_3F$ 424.1461, found 426.1459 (MH^+). Anal. calcd for $C_{26}H_{19}O_2N_{3F}F$; C, 73.75; H, 4.28; N, 9.92%. Found C, 73.76; H, 4.33; N, 9.84%.

4.1.13. *p*-F-DTT adduct (16a). Dithiothreitol (**14**) (2.2 mg, 0.014 mmol) was added to a solution of dehydrocoelenterazine **13a** (6.0 mg, 0.014 mmol) in MeOH (1.0 ml) and CH_2Cl_2 (1.0 ml) at room temperature under argon atmosphere. After stirring for 2 h at room temperature, the reaction mixture was concentrated under reduced pressure to give a DTT adduct **16a** (8.2 mg) as yellow oil compound in quantitative yield. Compound **16a**: UV (MeOH/H_2O=1/2) λ_{max} (log ε), 445 (1.46), 259 (1.20) nm. ^{1}H NMR (CD_3OD, 600 MHz), δ 2.55–2.50 (1H, m), 2.69–2.65 (1H, m), 3.57–3.53 (2H, m), 3.74–3.70 (2H, m), 4.45 (2H, AB), 5.56 (1H, s), 6.88 (2H, d, J=8.3 Hz), 7.04 (2H, t, J=8.3 Hz), 7.24–7.22 (1H, m), 7.29 (2H, t, J=7.4 Hz), 7.43 (2H, d, J=8.8 Hz), 7.44 (2H, d, J=7.3 Hz), 7.55 (1H, s), 7.72 (2H, d, J=11.7, 2.0 Hz) ppm. ^{19}F NMR (DMSO-d_6, 376 MHz), δ −50.68 ppm. FAB-MS (NBA) m/z 578 (MH^+). HRMS (FAB/NBA) calcd for $C_{30}H_{29}O_4N_3FS_2$ 578.1584, found 578.1581 (MH^+).

4.1.14. *m*-F-DTT adduct (16b). 16b (8.2 mg) was synthesized from **13b** (6.0 mg, 0.014 mmol) and **14** (2.2 mg, 0.014 mmol) in quantitative yield according to the same procedures described in **16a**. Compound **13b**: UV (MeOH/H_2O=1/2) λ_{max} (log ε), 438 (0.55), 261 (1.60) nm. ^{1}H NMR (CD_3OD, 500 MHz), δ 2.50–2.55 (1H, m), 2.68–2.72 (1H, m), 3.52–3.56 (2H, m), 3.60–3.65 (2H, m), 4.45 (2H, AB), 5.58 (1H, s), 6.87 (2H, d, J=8.0 Hz), 6.96 (1H, t, J=8.0 Hz), 7.16–7.33 (5H, m), 7.37–7.58 (5H, m) ppm. ^{13}C NMR (CD_3OD, 125 MHz), δ 36.4, 36.6, 46.2, 58.3, 71.2, 75.3, 108.7, 108.8, 115.1 (d, J_{C-F}=21.9 Hz), 115.2 (d, J_{C-F}=21.0 Hz), 116.8, 117.0, 126.0, 128.3, 129.6, 129.8, 130.0, 130.9 (d, J_{C-F}=8.3 Hz), 137.9, 143.9 (d, J_{C-F}=6.4 Hz), 160.3, 164.2 (d, J_{C-F}=243 Hz) ppm. ^{19}F NMR (DMSO-d_6, 376 MHz), δ −48.50 ppm. FAB-MS (NBA) m/z 578 (MH^+). HRMS (FAB/NBA) calcd for $C_{30}H_{29}O_4N_3FS_2$ 578.1584, found 578.1559 (MH^+).

4.1.15. *o*-F-DTT adduct (16c). 16c (8.2 mg) was synthesized from **13c** (6.0 mg, 0.014 mmol) and **14** (2.2 mg, 0.014 mmol) in quantitative yield according to the same procedures described in **16a**. Compound **13c**: UV (MeOH/H_2O=1/2) λ_{max} (log ε), 440 (0.90), 260 (2.45) nm. ^{1}H NMR (CD_3OD, 500 MHz), δ 4.43 (2H, AB), 4.53 (1H, brs), 5.90 (1H, AB), 6.88 (2H, d, J=8.8 Hz), 7.02 (1H, d, J=8.8 Hz), 7.07 (1H, td, J=8.5, 1.5 Hz), 7.15–7.30 (5H, m), 7.43 (2H, d, J=8.8 Hz), 7.54 (1H, d, J=6.4 Hz), 8.04 (1H, td, J=7.8, 1.5 Hz) ppm. ^{13}C NMR (CD_3OD, 125 MHz), δ 36.5, 36.8, 71.8, 72.1, 75.2, 75.5, 101.9, 108.7, 115.5, 116.1 (d, J_{C-F}=22.8 Hz), 116.9, 125.3, 128.2, 129.5, 129.8, 130.0, 130.1,

130.2, 132.3, 138.0, 160.3 ppm. ^{19}F NMR (CD_3OD, 376 MHz), δ −52.34, −52.60 ppm. FAB-MS (NBA) *m/z* 578 (MH^+). HRMS (FAB/NBA) calcd for $C_{30}H_{29}O_4N_3FS_2$ 578.1584, found 578.1586 (MH^+).

4.1.16. *p*-F-GSH adduct (17a). A solution of gluthathione (**15**) (6.2 mg, 0.020 mmol) in water (0.1 ml) was added to a solution of dehydrocoelenterazine **13a** (7.0 mg, 0.017 mmol) in MeOH (1.0 ml) and CH_2Cl_2 (1.0 ml) at room temperature. After stirring for 3 h at room temperature, the reaction mixture was concentrated under reduced pressure to give a gluthathione adduct **17a** (13.2 mg) as yellow oil compound in quantitative yield. UV (MeOH/H_2O=1/2) λ_{max} (log ε), 443 (0.92), 260 (2.40) nm. ^{1}H NMR (CD_3OD, 600 MHz), δ 2.10–2.13 (2H, m), 2.51–2.52 (2H, m), 2.62–2.98 (2H, m), 3.64–3.66 (1H, m), 3.80–3.95 (2H, m), 4.50 (2H, AB), 4.56–4.80 (2H, m), 5.66 (1H, s), 6.88 (2H, d, *J*=7.5 Hz), 7.05 (2H, t, *J*=9.0 Hz), 7.21–7.33 (3H, m), 7.42–7.45 (4H, m), 7.60 (1H, s), 7.70–7.80 (2H, m) ppm. ^{19}F NMR (CD_3OD, 376 MHz), δ −50.68 ppm. FAB-MS (NBA) *m/z* 731 (MH^+). HRMS (FAB/NBA) calcd for $C_{30}H_{29}O_4N_3FS_2$ 731.2299, found 731.2263 (MH^+).

4.1.17. *m*-F-GSH adduct (17b). 17b (13.2 mg) was synthesized from **13b** (7.0 mg, 0.017 mmol) and **15** (6.2 mg, 0.020 mmol in quantitative yield according to the same procedures described in **17a**. Compound **17b**: UV (MeOH/H_2O=1/2) λ_{max} (log ε), 445 (0.88), 260 (2.28) nm. ^{1}H NMR (CD_3OD, 500 MHz), δ 2.11–2.13 (2H, m), 2.50–2.55 (2H, m), 2.77–3.10 (2H, m), 3.62–3.67 (1H, m), 3.81–3.91 (2H, m), 4.44 (2H, AB), 4.57–4.60 (2H, m), 5.93 (1H, AB), 6.88 (2H, d, *J*=8.3 Hz), 7.02 (2H, t, *J*=8.8 Hz), 7.07 (1H, brt), 7.15–7.30 (5H, m), 7.40–7.45 (3H, m), 7.58 (1H, brd), 8.04 (1H, brt) ppm. ^{19}F NMR (CD_3OD, 376 MHz), δ −48.20 ppm. FAB-MS (NBA) *m/z* 731 (MH^+). HRMS (FAB/NBA) calcd for $C_{30}H_{29}O_4N_3FS_2$ 731.2299, found 731.2307 (MH^+).

4.1.18. *o*-F-GSH adduct (17c). 17c (13.2 mg) was synthesized from **13c** (7.0 mg, 0.017 mmol) and **15** (6.2 mg, 0.020 mmol in quantitative yield according to the same procedures described in **17a**. Compound **17c**: UV (MeOH/H_2O=1/2) λ_{max} (log ε), 443 (0.85), 259 (2.14) nm. ^{1}H NMR (CD_3OD, 500 MHz), δ 2.11–2.13 (2H, m), 2.50–2.55 (2H, m), 2.77–3.10 (2H, m), 3.62–3.67 (1H, m), 3.81–3.91 (2H, m), 4.44 (2H, AB), 4.57–4.60 (2H, m), 5.93 (1H, AB), 6.88 (2H, d, *J*=8.3 Hz), 7.02 (2H, t, *J*=8.8 Hz), 7.07 (1H, brt), 7.15–7.30 (5H, m), 7.40–7.45 (3H, m), 7.58 (1H, brd), 8.04 (1H, brt) ppm. ^{19}F NMR (CD_3OD, 376 MHz), δ −52.18, −52.45 ppm. FAB-MS (NBA) *m/z* 731 (MH^+). HRMS (FAB/NBA) calcd for $C_{30}H_{29}O_4N_3FS_2$ 731.2299, found 731.2299 (MH^+).

4.2. Model bioluminescence of 16c

Compound **16c**: (5.0 mg, 8.7 μmol) was dissolved in DMSO (0.9 ml). To the solution, 0.10 ml of 1N *t*-BuOK/*t*-BuOH was added at room temperature with bubbling oxygen into the solution. The model bioluminescence of **16c** ceased in 15 min, then the resultant solution was poured into a saturated ammonium chloride solution. After extraction of the solution with AcOEt (×3), the resultant organic layer was washed with water and brine. After drying with Na_2SO_4, evaporation of the organic layer afforded **18c** (4.2 mg) as lemon colored oil in almost pure form. Finally, CD_3OD solution (0.6 ml) of **18c** was analyzed with ^{19}F NMR. ^{19}F NMR (CD_3OD, 376 MHz), δ −48.70 ppm.

4.3. Preparation of apo-photoprotein

To an extracted symplectin solution (1.0 ml), 0.14 ml of Tris buffer (50 mM Tris, 0.60 M KCl, 1.0 mM DTT; pH 9.8) was added to initiate bioluminescence. After incubating at 37°C for 30 min, ammonium sulfate (204 mg) was added to the symplectin solution. Incubation at 0°C for 30 min gave apo-symplectin precipitate. Then centrifugation of the solution separated supernatant from apo-symplectin pellet. After removing the supernatant by decantation, ammonium pellet was washed with water (0.70 ml×2), then was dissolved into 0.70 ml of phosphate buffer (50 mM $NaHPO_4$, 0.60 M KCl, 1.0 mM DTT; pH 6.0) to afford apo-symplectin solution.

4.4. Bioluminescence profiles of reconstructed symplectin

Dehydrocoelenterazines were dissolved in DMSO at 2.0 mM concentration. To an apo-symplectin solution (0.10 ml), each dehydrocoelenterazine solution (2.0 μl) was added to re-activate apo-symplectin to form reconstructed symplectin and was incubated at room temperature for 20 min. To the reconstructed symplectin (50 μl), 0.20 ml of Tris buffer (pH 9.8) was added to initiate bioluminescence. The resultant bioluminescence was recorded with lumiphotometer TD-4000 at room temperature for 3 min.

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11. HPLC condition: Develosil ODS-5 column (4.6×250 mm) eluted at a flow rate 0.5 ml/min, using 0.1% aqueous TFA containing linear gradients of acetonitrile: 0% (v/v) for 5 min; 90% after 35 min. The peaks of F-DCTs appeared at 48 min, detected by 350 nm absorption.
12. Q-TOF spec II spectrometer; Micro Mass Co. Ltd., Manchester, UK.
13. Manuscript for the preparation of symplectin will be reported soon.