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Synthesis of azide-fluoro-dehydrocoelenterazine analog as a photoaffinity-labeling probe and photolysis of azide-fluoro-coelenterazine

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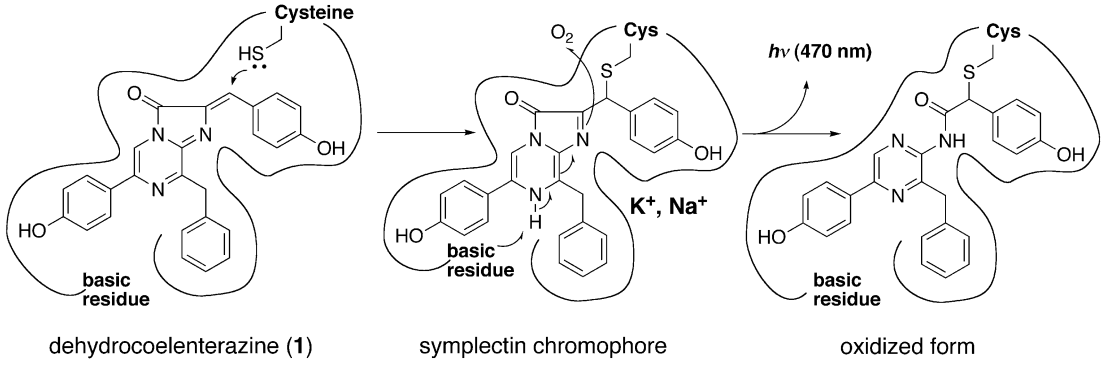
Abstract—A photosensitive azide-fluoro-dehydrocoelenterazine analog (Az-F-DCT) was synthesized, starting from 4-fluorophenylacetic acid, as a photoaffinity-labeling probe in order to analyze symplectin active site. To examine the photo-reactivity of Az-F-DCT, azide-fluoro-coelenterazine analog (Az-F-CT) was used as a potent symplectin chromophore model. Photolysis of Az-F-CT in 2,2,2-trifluoroethanol afforded nitrene intermediate to give an insertion product. The structure of this product was confirmed through spectroscopic analyses particularly by using a proton/deuterium (H/D) exchange experiments with ESI-Q-TOF-MS and -MS/MS measurement.

1. Introduction

Symplectin, the photoprotein of an oceanic luminous squid, TOBIIKA (*Symplectoteuthis oualaniensis*, L.),[1] has a covalently bound chromophore, and its structure has been known to be a conjugate addition product of dehydro-coelenterazine (DCT; **1**) to the protein through an active site cysteine.[2–6] In the presence of mono-valent ions (K^+, Na^+) and molecular oxygen, the chromophore is oxidized at pH 7.8 (optimum) to convert into an oxidized product while emitting a blue light (470 nm) as shown in Scheme 1.

We now focus on the molecular mechanism of symplectin bioluminescent process, especially on the dynamism of symplectin active site surrounding the chromophore. Photoaffinity labeling is the best method for such a research purpose, and has been established with many successful reports.[7,8] Though, there are many photosensitive functional groups such as diazirine and benzophenone,[9] we selected azide group as a photosensitive functional group, since, the introduction of azide into aromatic ring is not so difficult and the size of azide is relatively small to minimize a steric repulsion in the active site. Therefore, we decided to introduce an azide group into DCT.

Azide group always decomposes by light irradiation to give



Scheme 1. Postulated bioluminescent mechanism of symplectin photoprotein.

Keywords: Coelenterazine; Deuteriation MS; Exchangeable protons; Azide-fluoro-phenyl; Photoaffinity probe.
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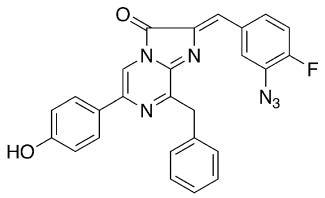
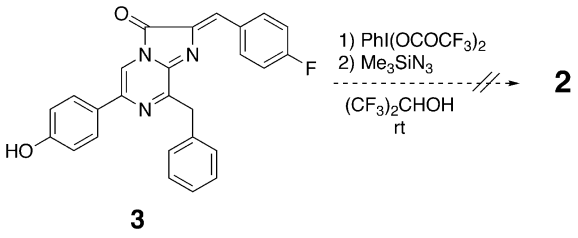


Figure 1. Structure of azide-fluoro-dehydrocoelenterazine analog (**2**).



Scheme 2. Trial for one-pot introduction of azide into 2′-fluoro-DCT analog (**3**) by using Kita's method.

a nitrene intermediate, which after follows a ring expansion to afford an electrophilic azepine intermediate. Actually, Niwa and Ohashi et al. reported the isolation of azepine derivative by photolysis of an azide-coelenteramide analog in MeOH containing Et_2NH.[10] The ring expansion is also useful if some nucleophilic residues exist near the azepine intermediate. However, in order to label the active site of symplectin protein efficiently even if there were no nucleophilic residues, we considered it better to avoid the ring expansion for the successful photoaffinity labeling. Instead of using azepine intermediate, we planned to use nitrene intermediate itself for labeling the symplectin active site. Platz et al. reported that fluorine atom adjacent to nitrene stabilized its singlet state.[11] Furthermore, we have demonstrated that the fluorinated DCT analogs are very active substances for symplectin bioluminescence.[12] Therefore, we planned to synthesize the azide-fluoro-dehydrocoelenterazine analog (Az-F-DCT; **2**) as a photoaffinity-labeling probe to analyze symplectin active site (Fig. 1). In this report, we here, describe the 14-step synthesis of **2** by starting from 4-fluorophenylacetic acid and photolysis of azide-fluoro-coelenterazine analog (Az-F-CT; **15**), which is the final oxidation precursor for **2** having the same oxidation state as the symplectin chromophore, in 2,2,2-trifluoroethanol.

2. Results and discussion

2.1. Synthesis of azide-fluoro-dehydrocoelenterazine analog (Az-F-DCT)

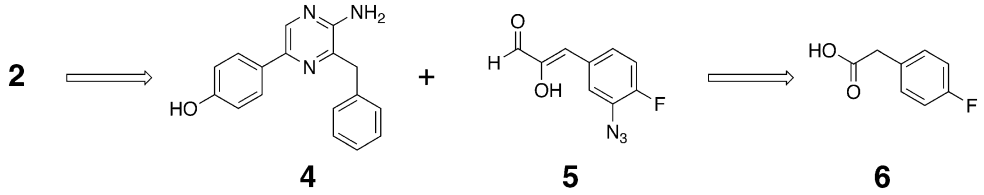
One-pot introduction of azide into DCT is an ideal strategy for the preparation of probe 2. For such a reason, Kita's method for the introduction of azide into an aromatic ring in one-pot is very attractive for us.[13] However, we could not prepare Az-F-DCT (**2**) from 2′-fluoro-DCT analog (**3**) selectively by using Kita's method (Scheme 2).

We then changed the strategy for the introduction of azide into 2′-fluoro-DCT analog (**3**); thus, we planned to prepare the probe 2 through using the conventional synthetic method established by Kishi, Inoue, and Goto et al.[14–17] As shown in Scheme 3, Az-F-DCT (**2**) would be derived from the condensation of coelenteramine (**4**)[15] and 3-(3-azide-4-fluorophenyl)-2-oxopropanal (**5**). The ketoaldehyde (**5**) would be prepared starting from 4-fluorophenylacetic acid (**6**).

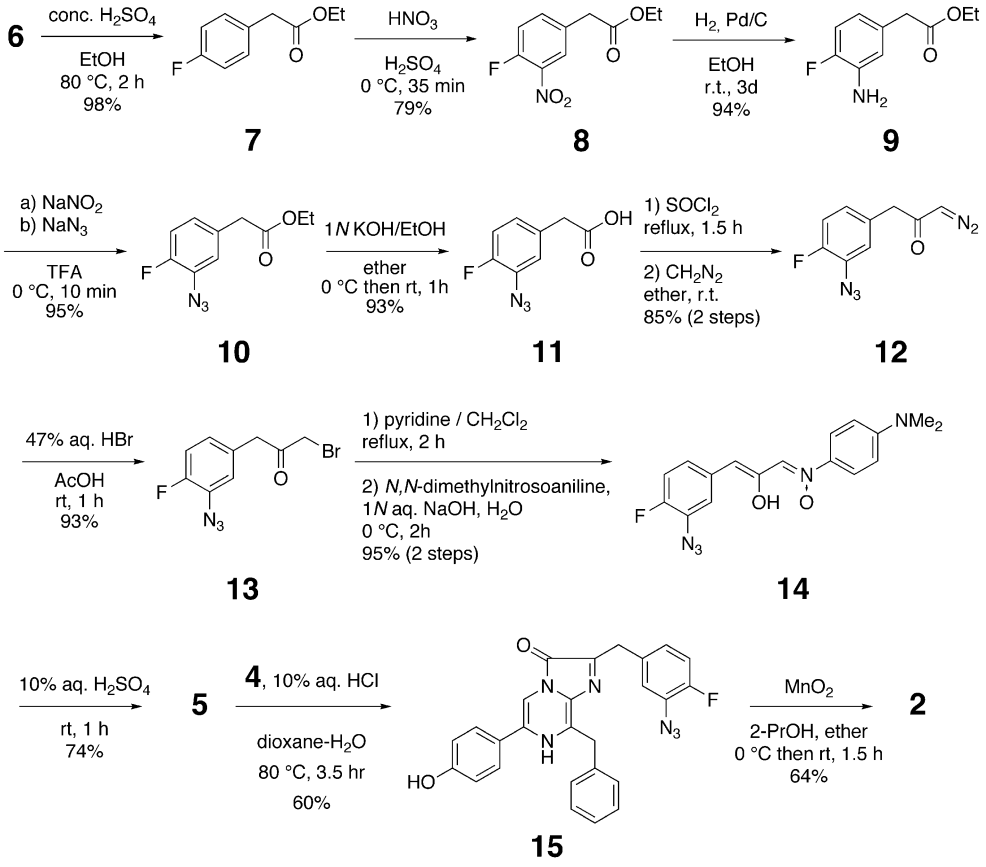
Esterification of 4-fluorophenylacetic acid (**6**) in EtOH afforded the ethyl ester (**7**) in 98% yield, followed by nitration of **7** provided ethyl-4-fluoro-3-nitrophenylacetate (**8**) in 79% yield as shown in Scheme 4 first row. Hydrogenolysis of the nitro group of **8** was facilitated by Pd/C catalyst to give ethyl-(3-amino-4-fluorophenyl)acetate (**9**) and the subsequent azidation[18] afforded ethyl-(3-azide-4-fluorophenyl)acetate (**10**). Saponification of the ester (**10**) afforded (3-azide-4-fluorophenyl)acetic acid (**11**) in 93% yield, which was further, converted to acid chloride. It was further, treated with diazomethane to give homologation productin (**12**) 85% (2 steps) (Scheme 4, second row). Bromination of this diazoketone (**12**) with HBr in acetic acid provided bromoketone (**13**) in 93% yield. After converted to its pyridinium salt, it was condensed with *N*,*N*-dimethyl-nitrosoaniline to give *N*-(*N*,*N*-dimethyl-aminophenyl)-3-(3-azide-4-fluorophenyl)-2-oxopropanimine oxide (**14**: nitrone) in 95% (2 steps) (Scheme 4, third row). Hydrolysis of the nitrone (**14**) in aqueous H_2SO_4 afforded 3-(3-azide-4-fluorophenyl)-2-oxopropanal (**5**) in 74% yield. Condensation of the ketoaldehyde (**5**) with coelenteramine (**4**) in a mixture of H_2O and dioxane in the presence of aqueous HCl provided azide-fluoro-coelenterazine analog (**15**: Az-F-CT) in 60% yield. Finally, oxidation of Az-F-CT (**15**) with manganese(IV) oxide in a mixture of ether and 2-PrOH afforded azide-fluoro-dehydrocoelenterazine analog (**2**: Az-F-DCT) in 64% yield (Scheme 4). Az-F-DCT (**2**) is a powerful probe to analyze symplectin active site by using photoaffinity-labeling, furthermore, Az-F-CT (**15**) will be also useful for the photoaffinity-labeling of aequorin photoprotein, which is the famous substance for jellyfish (*Aequoria aequoria*) bioluminescence.[19]

2.2. Photolysis of Az-F-coelenterazine analog

Although, we synthesized Az-F-DCT (**2**) as described in the previous section, the structure of symplectin chromophore is a reduced form of **2**. Thus, Az-F-CT (**15**) has the same



Scheme 3. Retro-synthesis of **2** from coelenteramine (**4**) and 3-(3-azide-4-fluorophenyl)-2-oxopropanal (**5**), and of **5** from 4-fluorophenylacetic acid (**6**).

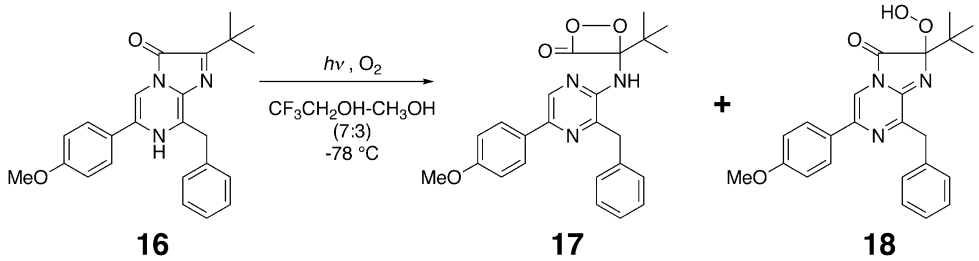


Scheme 4. Synthetic route for Az-F-DCT (**2**) starting from 4-fluorophenylacetic acid (**6**).

oxidation state with symplectin chromophore. To test the photo-reactivity of Az-F-DCT (**2**) in symplectin active site, we examined the photo-reactivity of Az-F-CT (**15**) as a symplectin chromophore model.

During the analysis of chemiluminescent mechanism of coelenterazine analog (**16**), we have reported that photo-oxygenation of the analog (**16**) in 2,2,2-trifluoroethanol (TFE)/methanol (7:3) afforded 2-peroxide (**17**) and dioxetanone (**18**) intermediates effectively as shown in Scheme 5.[20,21]

Since, luminous intermediates (**17**, **18**) were accumulated only when used (TFE) as solvent along with affording high light yield of luminescence,[20,21] we thought trifluoromethyl group might have some stabilization effect on the excited state of the coelenterazine analog (**16**) to give luminous intermediates in high yield. Therefore, we selected TFE as the solvent for light irradiation of **15** to decompose azide with anticipating the efficient production of a nitrene intermediate. The photolysis with a high-pressure mercury lamp was monitored with a nano-LC-Q-TOF-MS with our pre-packed-gradient (PPG) program.[22] The nano-LC chromatogram was shown in Figure 2 monitoring at 254 nm absorption. Az-F-CT (**15**) was almost consumed only remained 4% at 25.0 min after 15 min irradiation, and three new peaks were observed at 20.2, 20.5, 24.6 min in the chromatogram (Fig. 2). Then, these peaks were analyzed with ESI-Q-TOF-MS and -MS/MS measurements. The molecular weight of peak **A** (20.2 min, 33% yield) was *m/z* 441 $(M+H)^+$, which was assigned as reduced amine (**19**)[23] as shown in Figure 3. The peak at 20.5 min (24% yield) was proved to be coelenteramine (**4**) with molecular ion at *m/z* 278 $(M+H)^+$. The peak **B** at 24.6 min was a solvent insertion product with molecular ion at *m/z* 539 $(M+H)^+$. There were three possible structures for **B** as shown in Figure 3; O–H inserted product (**20**), C–H inserted product (**21**), or ring-expanded azepine derivative (**22**).



Scheme 5. Photooxygenation of coelenterazine analog (**16**) afforded 2-peroxide (**17**) and dioxetanone (**18**) intermediates.[20,21]

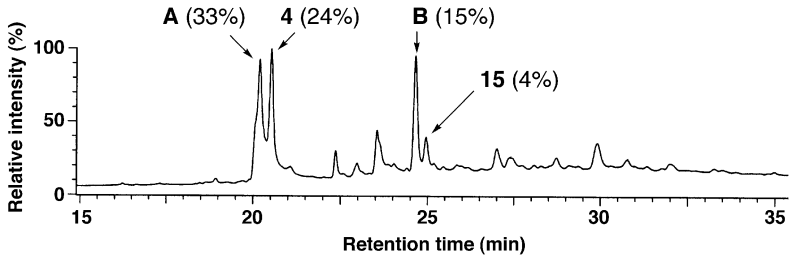


Figure 2. PPG-nano-LC chromatogram of photo-irradiated products of **15** in TFE (detection at UV 254 nm absorption). Values in parentheses show yields estimated by integration of peak areas.

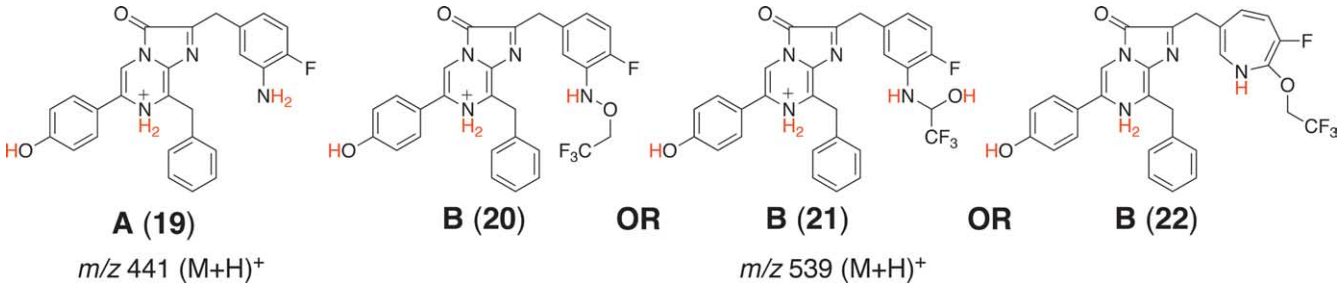


Figure 3. Assignment of the peaks with ESI-Q-TOF-MS analysis, and three possible structures for the solvent insertion product (**B**) (**20**, **21**, or **22**).

For the characterization of structure of peak **B**, proton/deuterium (H/D) exchange experiments were carried out with ESI-Q-TOF-MS and -MS/MS measurement,[24,25] on the basis of different numbers in the estimated exchangeable protons of **20** and **21**. Comparing the respective MS spectrum with both proton and deuterium measurement, the structure of peak **B** would be assigned from the fact of four exchangeable protons (**20**) or five (**21**).

The samples for proton/deuterium (H/D) exchange experiments were prepared as such that photo-irradiated sample of **15** was completely dried in vacuo, then redissolved with

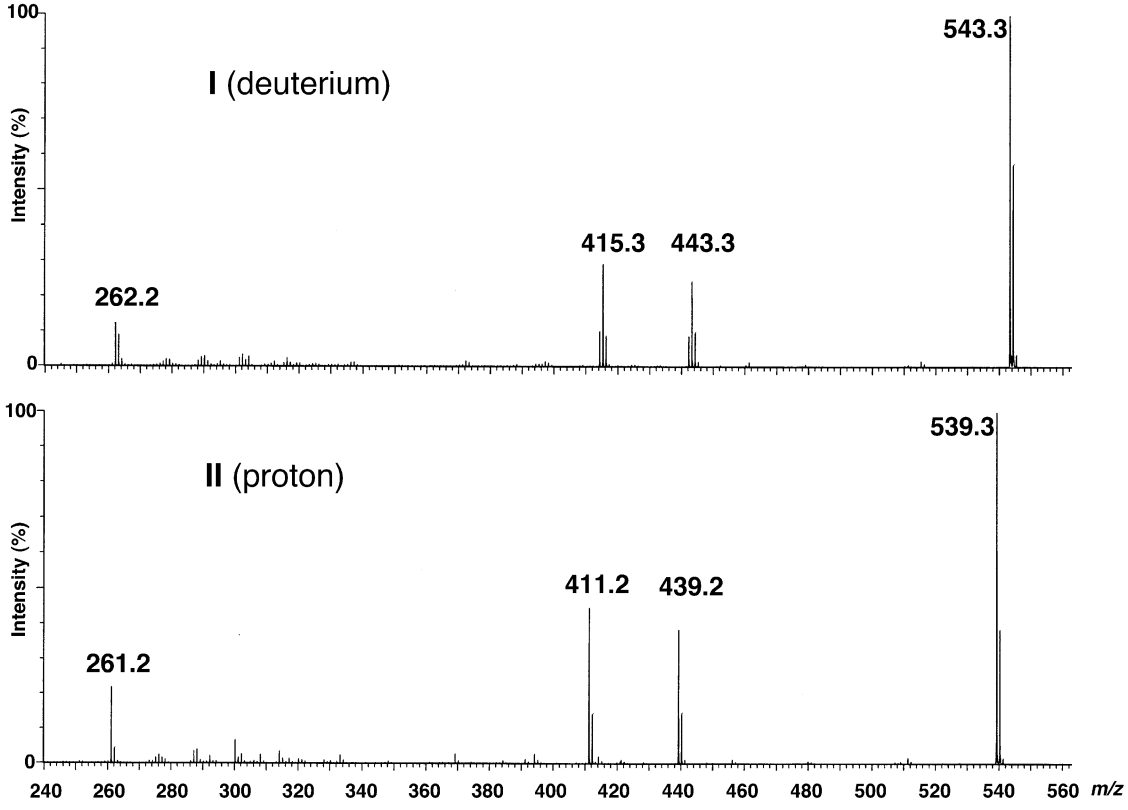


Figure 4. MS/MS spectra of solvent insertion product **B**: (I) MS/MS spectrum of deuteriated **B** precursor *m/z* 543; (II) MS/MS spectrum of natural abundant **B** precursor *m/z* 539.

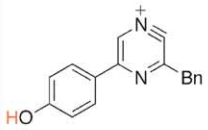
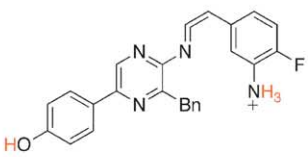
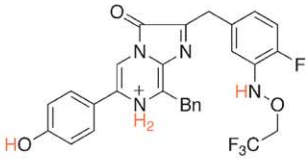
Structure				
m/z in D solvent	262	415	443	543
m/z in H solvent	261	411	439	539
Dn*	1	4	4	4

Figure 5. Assignment of precursor and fragment ions shown in MS/MS spectra (Fig. 4). *Dn: numbers of the exchangeable protons.

99% CH_3OD:1% CH_3COOD for ESI-Q-TOF-MS with deuterium measurement or with 99% CH_3OH:1% CH_3-COOH for MS with proton measurement. The resultant spectra are shown in Figure 4 (I: deuterium measurement, II: proton measurement). Pseudo molecular ion of peak **B** was observed at 539 $(M+H)^+$, which was employed as the precursor ion for MS/MS measurement (sample cone 60 V, collision 15–40 V), and the result is shown in Figure 4II. The product ions from *m/z* 539 precursor are seen at *m/z* 439, 411, and 261 in protonic solvent (Fig. 4II), while those from *m/z* 543 are also varied to appear at *m/z* 443, 415, and 262 in deuterium solvent as shown in Figure 4I.

Figure 5 summarizes the assignment of these fragment structures. For the validation of the above product ions generated during the MS/MS collision process, deuteriation of all the exchangeable protons, as shown in Figure 4I, made mass increase of mass numbers *m/z* 539 to *m/z* 543. The increased mass units (4, 4, 4, and 1) to the product ions also provide strong support for their structures in detail as shown in Figure 5. It has been concluded that the structure of solvent insertion product (**B**) is now concluded to be O–H inserted product (**20**) from the facts that four exchangeable protons of **B** was not for **21** (five exchangeable protons) but **20**.

Concerning the ring-expanded azepine derivative (**22**), from the results of MS and MS/MS measured in proton/deuterium, we excluded the possibility that the solvent adduct was azepine derivative (**22**).[26]

Through these experiments, we demonstrated that photolysis of compound **15** in TFE produced a nitrene intermediate to give the solvent O–H inserted adduct (**20**). We designed Az-F-DCT (**2**) to suppress ring-expansion of nitrene to give azepine intermediate up on photolysis. By using Az-F-CT (**15**) as symplectin chromophore model, no ring expansion was observed during photolysis as we expected.[23] These results enable us to utilize singlet nitrene itself for the photo-affinity labeling of symplectin active site. We also demonstrated that proton/deuterium (H/D) exchange experiments are very powerful method when coupled with LC-Q-TOF-MS and -MS/MS to investigate organic reaction. Photo-affinity labeling of semi-synthetic symplectin by using Az-F-DCT (**2**), and analysis of the photo-labeled site with nano-LC-Q-TOF-MS and -MS/MS equipped with PPG program[22] is now underway in our group.

3. Conclusion

Synthesis of photosensitive azide-fluoro-dehydrocoelenterazine analog (Az-F-DCT; **2**) has been accomplished starting from 4-fluorophenylacetic acid (**6**). Az-F-DCT (**2**) must be a powerful photoaffinity-labeling probe for the analysis of symplectin active site. For the examination of the photoreactivity of Az-F-DCT (**2**), the azide-fluoro-coelenterazine analog (Az-F-CT; **15**) was used as a symplectin chromophore model, due to the fact that chromophore structure in symplectin bound form of Az-F-DCT (**2**) should have the same chromophoric structure as Az-F-CT (**15**). Photoirradiation into a solution of Az-F-CT (**15**) in 2,2,2-trifluoroethanol (TFE) afforded a nitrene intermediate to give solvent O–H insertion product (**20**); this product (**20**) has been characterized from proton/deuterium (H/D) exchange data in ESI-Q-TOF-MS and -MS/MS spectra.

4. Experimental

4.1. General

All melting points were measured on a Yanaco MP-S3 and uncorrected. UV spectra were obtained on a JASCO U-best 50 spectrometer. IR spectra were recorded on a PERKIN ELMER Paragon 1000 FT-IR spectrophotometer. Proton NMR spectra were recorded on a JEOL GSX 270 for 270 MHz, or on a JEOL JNML-500 for 500 MHz, or on a Bruker AMX-600 for 600 MHz. Chemical shifts (δ) are given in parts per million relative to tetramethylsilane (δ 0.00), CD_3OD (δ 3.30), or DMSO-d_6 (δ 2.49) as internal standard. Coupling constants (J) are given in Hz. Proton decoupled carbon NMR spectra were recorded on a JEOL GSX 270 for 67.8 Hz, or on a JEOL JNML-500 for 125.7 Hz, 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), CD_3OD (δ 49.0), or DMSO-d_6 (δ 45.0) as

internal standard. Coupling constants (J) are given in Hz. Fluorine NMR spectra were recorded on a JEOL A-400 for 376 MHz. Chemical shifts are (δ) given in parts per million relative to 1,1,1-trifluorotoluene (δ 0.00) as external standard. Low-resolution EI MS and FAB MS were measured with a JEOL JMS-700. High-resolution (HR) MS were measured with a JEOL JMS-700. ESI mass spectra were measured with a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray type ESI sources. Mr. S. Kitamura in Analytical Laboratory of this school performed the combustion elemental analyses. Fluorescence spectra were measured with a JASCO FP-777 spectrometer. All the 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 Silica Gel 60 (spherical) 40–50 mm [KANTO CHEMICAL CO., INC].

4.1.1. Ethyl-(4-fluorophenyl)acetate (7). A solution (6.0 ml) of 4-fluorophenylacetic acid (**6**) (3.06 g, 19.9 mmol) in 6.0 ml of EtOH was refluxed at 80 °C for 2.5 h in the presence of 98% H_2SO_4 (0.84 ml) under Ar atmosphere. The solution was then poured into water at 0 °C, and the resultant solution was extracted with ether. The organic layer was washed with water and brine, and then was passed through a Na_2SO_4–SiO_2–Na_2SO_4 column. Evaporation of the effluent afforded Ethyl-(4-fluorophenyl)-acetate (**1**) in 98%. The product **7** was used for the following reaction without any purification. For the spectroscopic analyses, compound **7** was recrystallized with hexane to afforded a colorless crystalline. Mp 29.0–30.0 °C. IR (KBr) ν_{max} 1736, 1511, 1224 cm^{-1}. ^1H NMR (270 MHz, $CDCl_3$) δ 1.23 (3H, t, J=7.3 Hz, CH_3), 3.57 (2H, s, ArCH_2), 4.14 (2H, q, J=7.3 Hz, OCH_2), 6.99 (2H, t, J=8.8 Hz, Ar-3H), 7.24 (2H, dd, J=8.8, 5.1 Hz, Ar-2H) ppm. ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 14.2, 40.5, 60.9, 115.2 (d, $^2J_{C-F}$=22 Hz), 129.7, 130.6 (d, $^3J_{C-F}$=8 Hz), 161.8 (d, $^1J_{C-F}$=244 Hz), 171.2 ppm. ^{19}F NMR (376 MHz, $CDCl_3$) δ −53.1 ppm. EI-MS m/z 182 (M^+), 154, 109. HRMS (EI) calcd for $C_{10}H_{11}O_2F$: 182.0743, found 182.0719 (M^+). Anal. Calcd for $C_{10}H_{11}O_2F$: C, 65.92; H, 6.09; N, 0.00. Found: C, 65.91; H, 6.14; N, 0.27.

4.1.2. Ethyl-(4-fluoro-3-nitrophenyl)acetate (8). To a solution of **7** (3.54 g, 19.5 mmol) in 14 ml of H_2SO_4 at 0 °C was added 69% HNO_3 (1.4 ml) dropwise over 5 min. After stirring for 35 min at 0 °C, the solution was diluted with AcOEt at 0 °C, then the resultant solution was poured into a cold water. Water layer was extracted with AcOEt. The combined organic layer was washed with water, brine then dried over Na_2SO_4. After removing solvent in vacuo, the residue was purified by silica gel column chromatography (1:2 ether/hexane) to give 3.5 g (79%) of Ethyl-(4-fluoro-3-nitrophenyl)acetate (**8**) as pale yellow oil. IR (KBr) ν_{max} 1737, 1540, 1352, 1254, 1178 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 1.28 (3H, t, J=7.1 Hz, CH_3), 3.68 (2H, s, ArCH_2), 4.19 (2H, q, J=7.1 Hz, OCH_2), 7.26 (1H, dd, J=10.8, 8.6 Hz, Ar-4H), 7.57 (1H, ddd, J=8.6, 4.2, 2.4 Hz, Ar-5H), 8.00 (1H, dd, J=7.9, 2.3 Hz, Ar-2H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.1, 39.9, 61.4, 118.4 (d, $^2J_{C-F}$=21 Hz), 126.8, 131.1, 134.7 (d, $^2J_{C-F}$=7 Hz), 136.4 (d, $^3J_{C-F}$=8 Hz), 154.7 (d, $^1J_{C-F}$=263 Hz), 170.1 ppm. ^{19}F NMR (376 MHz, $CDCl_3$) δ −49.8 ppm. EI-MS m/z 227 (M^+). HRMS (EI) calcd for $C_{10}H_{10}O_4NF$: 227.0594, found 227.0619 (M^+).

4.1.3. Ethyl-(3-amino-4-fluorophenyl)acetate (9). To a solution of **8** (3.5 g, 15.4 mmol) in 150 ml of EtOH was added 10% Pd/C (175 mg) at rt in a round flask. The flask was plugged with hydrogen at atmospheric pressure. After stirred at rt for 2 days, Pd/C (175 mg) was further, added to the mixture. Then the stirring was continued one more day at rt under hydrogen atmosphere. The mixture was filtered through a Celite pad. After removing solvent in vacuo, the residue was purified by silica gel column chromatography (2:3 ether/hexane) to give 2.87 g (94%) of Ethyl-(2-amino-4-fluorophenyl)acetate (**9**) as orange oil. IR (KBr) ν_{max} 3375, 1731, 1633, 1519, 1445, 1303, 1207, 1161 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 1.25 (3H, t, J=7.1 Hz, CH_3), 3.47 (2H, s, ArCH_2), 3.70 (2H, br s, NH_2), 4.14 (2H, q, J=7.1 Hz, OCH_2), 6.58 (1H, ddd, J=8.3, 4.5, 2.0 Hz, Ar-6H), 6.70 (1H, dd, J=8.6, 2.0 Hz, Ar-2H), 6.90 (1H, dd, J=11.0, 8.3 Hz, Ar-5H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.1, 40.7, 60.8, 115.1 (d, $^2J_{C-F}$=18 Hz), 117.6 (d, $^3J_{C-F}$=3 Hz), 119.3 (d, $^3J_{C-F}$=6 Hz), 130.3 (d, $^4J_{C-F}$=4 Hz), 134.4 (d, $^2J_{C-F}$=14 Hz), 150.9 (d, $^1J_{C-F}$=236 Hz), 171.6 ppm. ^{19}F NMR (376 MHz, $CDCl_3$) δ −75.1 ppm. EI-MS m/z 197 (M^+), 124. HRMS (EI) calcd for $C_{10}H_{12}O_2NF$: 197.0852, found 197.0871 (M^+).

4.1.4. Ethyl-(2-azide-4-fluorophenyl)acetate (10). To a solution of **9** (2.87 g, 14.6 mmol) in 30 ml of trifluoroacetic acid (TFA) at 0 °C was added $NaNO_2$ (2.01 g, 29.2 ml) followed by NaN_3 (2.85 g, 43.8 mmol). After stirring for 10 min at 0 °C, the solution was diluted with ether at 0 °C, then the resultant solution was poured into a cold water. Water layer was extracted with ether. The combined organic layer was washed with water, brine then dried over Na_2SO_4. After removing solvent in vacuo, the residue was purified by silica gel column chromatography (1:2 ether/hexane) to give 3.09 g (95%) of Ethyl-(2-azide-4-fluorophenyl)acetate (**10**) as yellow oil. IR (KBr) ν_{max} 2125, 1736, 1515, 1427, 1320, 1227, 1160 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 1.26 (3H, t, J=7.1 Hz, CH_3), 3.56 (2H, s, ArCH_2), 4.16 (2H, q, J=7.1 Hz, OCH_2), 6.97–7.07 (3H, m, Ar-H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.1, 40.3, 61.1, 116.6 (d, $^2J_{C-F}$=19 Hz), 121.7, 126.6 (d, $^3J_{C-F}$=6 Hz), 127.8 (d, $^2J_{C-F}$=12 Hz), 131.0, 154.0 (d, $^1J_{C-F}$=248 Hz), 171.0 ppm. ^{19}F NMR (376 MHz, $CDCl_3$) δ −66.0 ppm. EI-MS m/z 223 (M^+). HRMS (EI) calcd for $C_{10}H_{10}O_2N_3F$: 223.0757, found 223.0771 (M^+). Anal. Calcd for $C_{10}H_{10}O_2N_3F$: C, 53.81; H, 4.52; N, 18.83. Found: C, 53.81; H, 4.71; N, 18.53.

4.1.5. 2-Azide-4-fluorophenylacetic acid (11). To a solution of **10** (3.09 g, 13.9 mmol) in 10 ml of ether was added 10 ml of 2 N KOH/EtOH at 0 °C. After stirring at 0 °C for 15 min, the reaction was warmed to rt and stirred for 1 h. Then the solution was cooled to 0 °C and was acidified with 1 N aqueous HCl until pH 1–2. The resultant solution was extracted with AcOEt. The organic layer was washed with water then dried over Na_2SO_4. Concentration in vacuo yielded 2.50 g (93%) of 2-Azide-4-fluorophenyl-acetic acid (**11**). The product **11** was used for the following reaction without any purification. For the spectroscopic

analyses, compound **11** was recrystallized with ether–hexane to afforded an orange crystalline. Mp 48.5–49.5 °C. IR (KBr) ν_{max} 2130, 1718, 1707, 1518, 1239 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 3.60 (2H, s, ArCH_2), 6.97–7.07 (2H, m, Ar-*H*), 7.05 (1H, br t, J=9.6 Hz, Ar-*H*) ppm. ^{13}C NMR (125 MHz, $CDCl_3$) δ 40.0, 116.8 (d, $^2J_{C-F}$=19 Hz), 122.0, 126.7 (d, $^3J_{C-F}$=7 Hz), 128.0 (d, $^2J_{C-F}$=12 Hz), 130.1 (d, $^4J_{C-F}$=4 Hz), 154.2 (d, $^1J_{C-F}$=248 Hz), 177.3 ppm. ^{19}F NMR (376 MHz, $CDCl_3$) δ −65.3 ppm. EI-MS *m/z* 195 (M^+), 167 (M^+-N_2). HRMS (EI) calcd for $C_8H_6O_2N_3F$: 195.0444, found 195.0449 (M^+). Anal. Calcd for $C_8H_6O_2N_3F$: C, 49.24; H, 3.10; N, 21.53. Found: C, 49.25; H, 3.31; N, 21.35.

4.1.6. (3-Azide-4-fluorophenyl)methyl diazomethyl ketone (12). A solution of **11** (2.50 g, 12.8 mmol) in 8.0 ml of thionylchloride was refluxed 1.5 h. Generating vapors (SO_2 and HCl) were trapped with saturated aqueous $NaHCO_3$. Concentration of the reaction mixture in vacuo equipped a trap cold by liquid N_2 gave a crude oil of acyl chloride of **5**. The acyl chloride was purified with Kugelrohr (145 °C) in vacuo, and was immediately used for the following reaction. To the solution of acyl chloride (2.54 g) of **5** in 60 ml of ether was added a ether solution of diazomethane until acylchloride was perfectly consumed at 0 °C. After removing solvent in vacuo, the residue was purified by silica gel column chromatography (2:1 ether/hexane) to give 2.23 g (85%, two steps) of (3-Azide-4-fluorophenyl)methyl diazomethyl ketone (**12**) as a lemon colored crystalline. Mp 34.8–35.5 °C. IR (KBr) ν_{max} 2092, 1614, 1512, 1426, 1112 cm^{-1}. ^1H NMR (270 MHz, $CDCl_3$) δ 3.56 (2H, s, ArCH_2), 5.18 (1H, s, C*H*N_2), 6.92–7.10 (3H, m, Ar-*H*) ppm. ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 46.7, 55.1, 116.9 (d, $^2J_{C-F}$=19 Hz), 121.8 (d, $^3J_{C-H}$=1 Hz), 126.5 (d, $^3J_{C-F}$=7 Hz), 128.1 (d, $^2J_{C-F}$=11 Hz), 131.3, 154.0 (d, $^1J_{C-F}$=248 Hz), 191.2 ppm. ^{19}F NMR (376 MHz, $CDCl_3$) δ −65.6 ppm. EI-MS *m/z* 219 (M^+), 163 (M^+-2N_2). HRMS (EI) calcd for $C_9H_6ON_5F$: 219.0556, found 219.0540 (M^+). Anal. Calcd for $C_9H_6ON_5F$: C, 49.32; H, 2.76; N, 31.95. Found: C, 49.36; H, 2.67; N, 31.61.

4.1.7. (3-Azide-4-fluorophenyl)methyl bromomethyl ketone (13). To a solution of **12** (2.10 g, 9.59 mmol) in 12 ml of AcOH at 0 °C was added 47% aqueous HBr (2.6 ml, 14.9 mmol) dropwise slowly. The reaction was warmed to rt and stirred for 1 h. Then the solution was poured into water washing with H_2O and ether. The mixture was neutralized with Na_2CO_3 to pH 4, then with $NaHCO_3$ to pH 7. The solution was extracted with ether. The organic layer was washed with water, brine, then dried over Na_2SO_4. Concentration in vacuo afforded crude compound. Recrystallization of the crude compound with ether yielded 2.44 g (93%) of (3-azide-4-fluorophenyl)methyl bromomethyl ketone (**13**) as a pale yellow needle. Mp 74.6–75.6 °C. IR (KBr) ν_{max} 2128, 1736, 1513, 1240 cm^{-1}. ^1H NMR (270 MHz, $CDCl_3$) δ 3.92 (4H, s, ArCH_2, CH_2Br), 6.90–6.96 (2H, m, Ar-*H*), 7.07 (1H, br t, J=9.5 Hz, Ar-*H*) ppm. ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 33.4, 45.4, 117.0 (d, $^2J_{C-F}$=28 Hz), 122.0 (d, $^3J_{C-H}$=2 Hz), 126.7 (d, $^3J_{C-F}$=7 Hz), 128.0, 129.9 (d, $^2J_{C-F}$=7 Hz), 154.1 (d, $^1J_{C-F}$=249 Hz), 198.5 ppm. ^{19}F NMR (376 MHz, $CDCl_3$) δ −65.1 ppm. EI-MS *m/z* 273 (M^+), 271 (M^+), 245 (M^+-N_2), 243 (M^+-N_2). HRMS (EI) calcd for $C_9H_7ON_3F^{79}Br$: 270.9757, found 270.9755 (M^+). Anal. Calcd for $C_9H_7ON_3FBr$: C, 39.73; H, 2.59; N, 15.44. Found: C, 39.84; H, 2.46; N, 15.56.

4.1.8. *N*-(*N*,*N*-Dimethylaminophenyl)-3-(3-azide-4-fluorophenyl)-2-oxopropanimine oxide (14). A solution of **13** (2.44 g, 8.97 mmol) in 30 ml of CH_2Cl_2 was refluxed at 70 °C in the presence of 3.0 ml of pyridine under Ar atmosphere for 2 h. Concentration of the solution in vacuo afforded a pyridinium salt as a foamy solid. To a solution of the pyridinium salt in 90 ml of H_2O at 0 °C was added *N*,*N*-dimetyl-4-nitrosoaniline (1.4 g, 9.42 mmol) followed by 9 ml of 1 N aqueous NaOH. The resultant suspension was warmed to rt and stirred for 2 h at rt with sonicating occasionally. Filtration of the suspension yielded 2.19 g (95%, two steps) of *N*-(*N*,*N*-dimethylaminophenyl)-3-(3-azide-4-fluorophenyl)-2-oxopropanimine oxide (**14**). The product **14** was used for the following reaction without any purification. IR (KBr) ν_{max} 2121, 1605, 1490, 1170 cm^{-1}. ^1H NMR (270 MHz, $CDCl_3$) δ 3.03 (6H, s, N*Me*), 5.45, (1H, s, ArC*H*), 6.66 (2H, d, J=9.2 Hz, *ortho*-aniline), 7.02 (1H, dd, J=10.6, 8.6 Hz, Ar-*5H*), 7.42 (1H, ddd, J=8.6, 5.0, 2.3 Hz, Ar-*6H*), 7.50 (1H, s, C*H*=NO), 7.57 (2H, d, J=9.2 Hz, *meta*-aniline), 7.62 (1H, dd, J=8.2, 2.3 Hz, Ar-*2H*), 13.77 (1H, br s, O*H*) ppm. ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 40.3, 110.7, 111.2, 116.5 (d, $^2J_{C-F}$=19 Hz), 120.9, 121.5, 122.3, 126.4 (d, $^2J_{C-F}$=7 Hz), 131.0, 133.3 (d, $^3J_{C-F}$=5 Hz), 134.1, 149.4 (d, $^3J_{C-F}$=2 Hz), 151.4, 153.1 (d, $^1J_{C-F}$=250 Hz) ppm. ^{19}F NMR (376 MHz, $CDCl_3$) δ −64.7 ppm. EI-MS *m/z* 341 (M^+), 325 (M^+-16). HRMS (EI) calcd for $C_{17}H_{16}O_2N_5F$: 341.1288, found 341.1238 (M^+).

4.1.9. 3-(3-Azide-4-fluorophenyl)-2-oxopropanal (5). Compound **14** (700 mg, 2.05 mmol) was suspended into 10% aqueous H_2SO_4 (40 ml) at 0 °C. The suspension was warmed to rt and stirred for 1 h with sonicating occasionally. The suspension was extracted with ether. The organic layer was washed with water and brine, and then was passed through a Na_2SO_4–SiO_2–Na_2SO_4 column. Removing solvent in vacuo, yielded 314 mg (74%) of 3-(3-azide-4-fluorophenyl)-2-oxopropanal (**5**) as a dark yellow amorphous. The product **5** was used for the following reaction without any purification. For the spectroscopic analyses, compound **5** was recrystallized with ether–hexane to afforded a pale yellow amorphous. Mp 102.0–104.0 °C (decomposed). IR (KBr) ν_{max} 3329, 2137, 1672, 1650, 1409 cm^{-1}. ^1H NMR (270 MHz, $CDCl_3$) δ 6.09 (1H, d J=0.9 Hz, ArC*H*), 6.47 (1H, s, O*H*), 7.12 (1H, dd, J=10.5, 8.4 Hz, Ar-*5H*), 7.51 (1H, ddd, J=8.4, 4.6, 2.1 Hz, Ar-*6H*), 7.66 (1H, dd, J=8.1, 2.1 Hz, Ar-*2H*), 9.25 (1H, d, J=0.9 Hz, CO*H*) ppm. ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 116.9 (d, $^2J_{C-F}$=20 Hz), 120.2, 122.5, 127.9 (d, $^3J_{C-F}$=7 Hz), 128.2, 130.7, 148.6 (d, $^3J_{C-F}$=3 Hz), 154.7 (d, $^1J_{C-F}$=253 Hz), 187.8 ppm. ^{19}F NMR (376 MHz, $CDCl_3$) δ −60.8 ppm. EI-MS *m/z* 207 (M^+), 179 (M^+-28), 163 (M^+-N_2–CHO). HRMS (EI) calcd for $C_9H_6O_2N_3F$: 207.0444, found 207.0436 (M^+). Anal. Calcd for $C_9H_6O_2N_3F$: C, 52.18; H, 2.92; N, 20.28. Found: C, 52.17; H, 2.66; N, 20.37.

4.1.10. 8-Benzyl-2-(3-azide-4-fluorophenylmethyl)-6-(4-hydroxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazine-3-one (15) (Az-F-CT). To a degassed solution of

coelenteramine (**4**) (214 mg, 0.77 mmol) and ketoaldehyde (**5**) (223 mg, 1.08 mmol) in 6.0 ml of 20% water/dioxane was added 1.0 ml of 10% aqueous HCl, and was stirred under argon atmosphere at rt for 5 min. Then the reaction was warmed to 80 °C and stirred for 3.5 h. After cooled, the solution was poured into water at 0 °C. The resultant mixture was extracted with AcOEt. The organic layer was washed with water, brine, and dried over anhydrous Na_2SO_4. After removing solvent in vacuo, the residue was purified by silica gel column chromatography (4% MeOH in CH_2Cl_2) to give 215 mg (60%) of 8-benzyl-2-(3-azide-4-fluorophenylmethyl)-6-(4-hydroxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazine-3-one (**15**) as a brown solid. For the spectroscopic analyses, compound **15** was recrystallized with ether–MeOH to afforded a yellow powder. Mp 140–143 °C (decomposed). UV (MeOH) λ_{max} (log ε) 438 (3.97) nm. FL (MeOH) Em. 526.5 nm (Ex. 350 nm). IR (KBr) ν_{max} 3100 (br), 2357, 2349, 2121, 1564, 1548, 1513 cm^{-1}. ^1H NMR (CD_3OD, 600 MHz), δ 4.14 (2H, s, CH_2Ph), 4.41 (2H, s, CH_2Ph), 6.88 (3H, ddd, J=9.6, 8.4, 4.8 Hz, Ar-*H*), 7.07 (1H, dd, J=10.8, 8.4 Hz, F-Ar-*H*), 7.13 (1H, m, Ar-*H*), 7.19 (1H, br d, J=8.4 Hz, Ar-*H*), 7.23 (1H, t, J=7.2 Hz, Ar-*H*), 7.30 (2H, t, J=7.5 Hz, Ar-*H*), 7.38 (2H, d, J=7.8 Hz, Ar-*H*), 7.45 (2H, br s, Ar-*H*) ppm. ^{13}C NMR (CD_3OD, 150 MHz), δ 49.9, 108.0, 116.8, 117.4 (d, $^2J_{C-F}$=20 Hz), 122.5, 127.4 (d, $^3J_{C-F}$=6 Hz), 128.2, 128.7 (d, $^2J_{C-F}$=11 Hz), 129.3, 129.6, 129.7, 137.1, 137.7, 154.8 (d, $^1J_{C-F}$=247 Hz), 160.1 ppm. ^{19}F NMR (376 MHz, CD_3OD) δ −68.9 ppm. FAB-MS (NBA) *m*/*z* 467 (MH^+). HRMS (FAB/NBA) calcd for $C_{26}H_{20}N_6O_2F$: 467.1632, found 467.1663 (MH^+). Anal. Calcd for $C_{26}H_{19}N_6O_2F$: C, 66.95; H, 4.11; N, 18.02. Found: C, 66.95; H, 4.37; N, 17.72.

4.1.11. 8-Benzyl-2-(3-azide-4-fluorobenzylidene)-6-(4-hydroxyphenyl)-2,3-dyhydroimidazo[1,2-*a*]pyrazine-3-one (2) (Az-F-DCT). To a solution of coelenterazine analog (**15**) (70 mg, 0.15 mmol) in a mixture of diethyl ether (175 ml) and EtOH (35 ml) was added 85% manganese(II) oxide (700 mg, 6.83 mmol) at 0 °C under Ar atmosphere. After stirring for 1 h, the reaction was warmed to rt and stirred for 1.5 h. The mixture was filtered through a Celite pad, then was concentrated in vacuo. Recrystallization of the residue with 2-PrOH/ether yielded 45 mg (64%) of 8-benzyl-2-(3-azide-4-fluorobenzylidene)-6-(4-hydroxyphenyl)-2,3-dyhydroimidazo[1,2-*a*]pyrazine-3-one (**2**) as a purple amorphous. Mp 154–156 °C (decomposed). UV (2-PrOH) λ_{max} (log ε) 541 (3.55), 371 (3.93), 281 (4.11) nm. IR (KBr) ν_{max} 2359, 2133, 1721, 1591, 1503 cm^{-1}. ^1H NMR (DMSO-d_6, 600 MHz) δ 4.29 (2H, s, CH_2Ph), 6.81 (2H, d, J=8.8 Hz, Ar-*H*), 7.24 (1H, t, J=7.3 Hz, Ar-*H*), 7.35 (2H, t, J=7.7 Hz, Ar-*H*), 7.50 (3H, dd, J=13.2, 5.4 Hz, Ar-*H*), 7.53 (1H, s, 5-*H*), 7.78 (2H, d, J=8.6 Hz, Ar-*H*), 7.94 (1H, s, Ar-*H*), 8.10–8.12 (1H, m, Ar-*H*), 8.62 (1H, d, J=8.4 Hz, Ar-*H*), 9.66 (1H, br s, O*H*) ppm. ^{13}C NMR (DMSO-d_6, 150 MHz) δ 49.3, 108.0, 116.8, 117.4 ($^2J_{C-F}$=20 Hz), 127.4 ($^3J_{C-F}$=6 Hz), 128.1, 128.7 ($^2J_{C-F}$=11 Hz), 129.3, 129.6, 129.7, 137.1, 137.7, 154.8 ($^1J_{C-F}$=247 Hz), 160.1 ppm. ^{19}F NMR (376 MHz, CD_3OD) δ −58.4 ppm. FAB-MS (NBA) *m*/*z* 465 (MH^+). HRMS (FAB/NBA) calcd for $C_{26}H_{18}N_6O_2F$: 465.1475, found 465.1501 (MH^+). Anal. Calcd for $C_{26}H_{17}N_6O_2F$: C, 67.24; H, 3.69; N, 18.09. Found: C, 67.02; H, 3.80; N, 18.16.

4.1.12. Photolysis of Az-F-CT (15): general procedure. To a solution of Az-F-CT (**15**) (2.0 mM) in 2,2,2-trifluoroethanol in a NMR tubing was irradiated light with a high-pressure mercury lamp (100-W high pressure Hg lamp) for 15 min at rt with Ar bubbling. For nano-LC-Q-TOF-MS measurement, 1 μl of the sample was injected. For proton/deuterium (H/D) exchange measurement, 2 μl of the sample was dried in vacuo, then redissolved with 99% CH_3OD:1% CH_3COOD (200 μl) for ESI-Q-TOF-MS with deuterium measurement or with 99% CH_3OH:1% CH_3COOH for MS (200 μl) with proton measurement. The resultant solutions were injected into ion-source with a mechanical syringe. TFE insertion product **20**. HRMS (FAB/NBA) calcd for $C_{28}H_{23}N_4O_3F_4$: 539.1706, found 539.1710 (MH^+).

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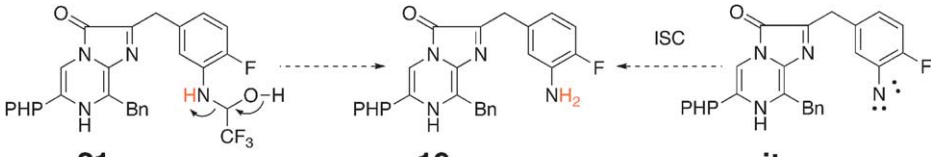

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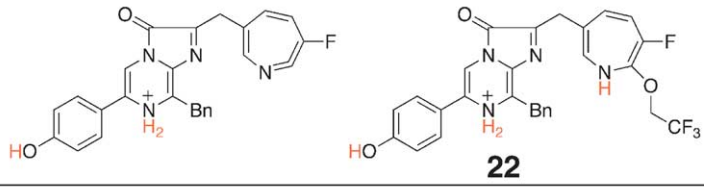
23. In deuterium ESI-MS measurement, the molecular weight of **19** (*m*/*z* 441) increased to plus 5 mass units to give molecular ion at *m*/*z* 446. The exchangeable 5 protons strongly support our assignment. We also suppose it possible that **19** might be produced from C–H inserted product (**21**) by hydrolyzed aminal moiety, or be directly produced from nitrene intermediate through intersystem crossing.[11] No ring expansion product to benzazepine type intermediate was observed.[26] This may be responsible for the fluorine atom, which prevents carbon migration to the nitrene due to its electronegativity.



m/z in D solvent		
calculated	442	543
observed	443	543
m/z in H solvent		
calculated	439	539
observed	439	539

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26. We considered the possibility for the ring expansion of the nitrene intermediate. If ring expansion happened, the resultant azepine derivative (**22**) also has four exchangeable protons. However, the initial fragmentation product observed at *m*/*z* 439 should have three exchangeable protons. If this is the case, *m*/*z* 442 should be observed in deuterium solvent. Thus, the peak at *m*/*z* 443 in Figure 4I was critical to exclude the possibility of ring expansion product (**22**).