Solvent and substituent effects on the fluorescent properties of coelenteramide analogues

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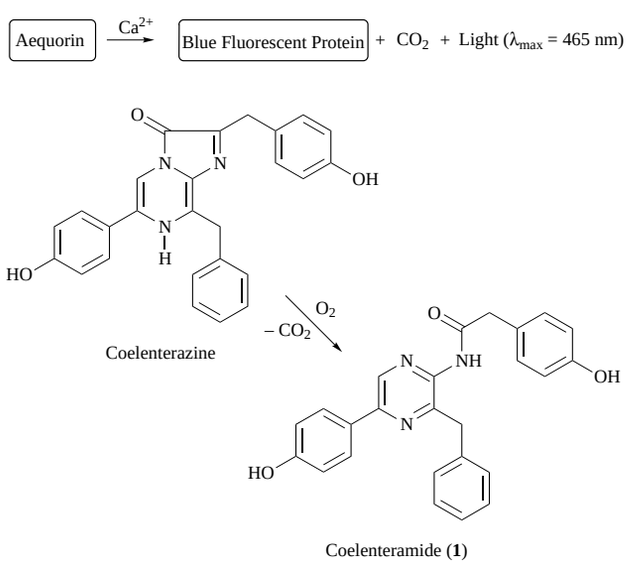
Coelenteramide 1 is the light emitter in aequorin bioluminescence. To establish the fluorescent character of 1, the fluorescence properties of 1 and a series of its analogues, 3a–f, possessing a substituent R [= CF_3, F, H, OCH_3, OH, $N(CH_3)_2$] at the *para*-position on the 5-phenyl group have been investigated in solvents of various polarity. The fluorescence emission maxima of 1 and 3d–f, possessing an electron-donating group R [= OCH_3, OH, $N(CH_3)_2$] shift to lower energy with increasing solvent polarity, while those of the analogues 3a–c (R = CF_3, F, H) are independent of the solvent polarity. The linear correlation between the fluorescence maxima of 1 and 3d–f and the solvent polarity scales can be explained by formation of the singlet excited state with a charge-transfer (CT) character. The quantum yields of CT fluorescence of 1 and 3d–f have been found to be higher than those of 3a–c. These results indicate that the solvatochromic fluorescence of 1 originates from the CT excited state and the existence of an electron donating hydroxy group on the 5-phenyl group is essential for determining a wavelength and a high fluorescence quantum yield of aequorin bioluminescence.

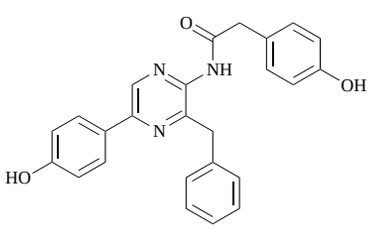
Introduction

A photoprotein, aequorin (AQ) isolated from the jellyfish *Aequorea victoria*, emits blue light by a reaction with calcium ions.[1] AQ is composed of apoaequorin (an apoprotein), coelenterazine and a molecular oxygen.[2] The binding of calcium ions to AQ triggers the transformation of AQ into an excited state of a blue fluorescent protein (BFP), resulting in emission of blue light (λ_{max} = 465 nm). BFP consists of coelenteramide **1** bound to apoaequorin noncovalently. Coelenteramide **1** is an oxidation product from coelenterazine (Scheme 1). The singlet excited state of **1** in BFP is the light emitter in the bioluminescent reaction.[3] Coelenteramide **1** has two phenolic hydroxy groups and an amide group, and can exist as the ionic structures **II** and **III** as well as the neutral structure **I**(**1**) under appropriate

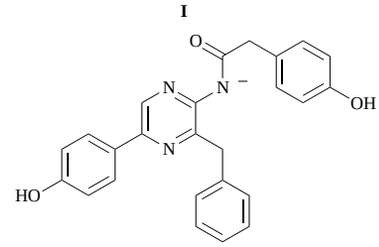


Scheme 1

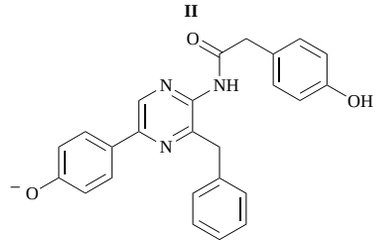
conditions.[4,5] From the results of studies on a chemiluminescence reaction of coelenterazine derivatives in an aprotic solvent, it has been suggested that the structure of the excited coelenteramide **1** in aequorin bioluminescence is the amide anion **II**.[6] However, we proposed that the emitting structure is the phenolate anion **III** on the basis of studies on the fluorescence properties of a semi-synthetic BFP regenerated



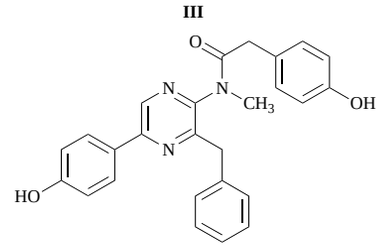
I



II



III



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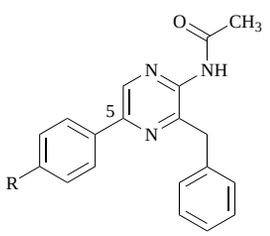
from apoaequorin and the *N*-methylcoelenteramide analogue **2**.[5] To confirm this hypothesis, we have been studying systematically the fluorescent properties of **1** and its derivatives under various conditions. For the neutral structure **I**, Shimomura has shown that the fluorescence maxima of **1** is changed with a change in solvent polarity,[7] in a similar manner to the fluorescence spectra of *Cypridina* (*Vargula*) oxyluciferin.[8] This paper deals with our studies of the solvent and substituent effects on the fluorescence character of the neutral structure, **1**, by comparison with the fluorescence spectra of coelenteramide analogues **3a**–**f,** possessing a substituent at the *para*-position on the 5-phenyl group. From these results, it is clear that the solvent-dependent fluorescence of the neutral structure **I** has charge transfer (CT) character in the singlet excited state.



3a : R = CF_3
3b : R = F
3c : R = H
3d : R = OCH_3
3e : R = OH
3f : R = $N(CH_3)_2$

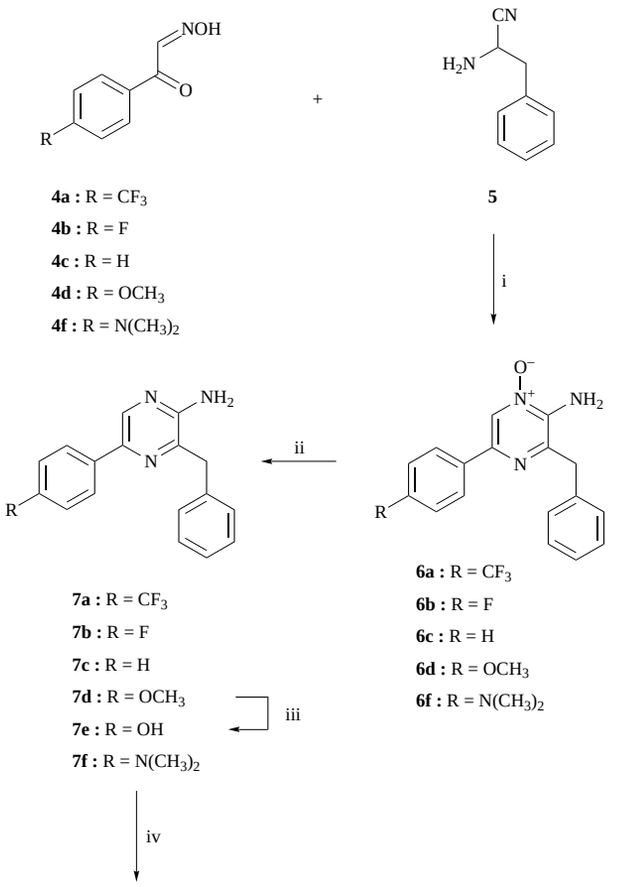
Results and discussion

Coelenteramide **1** was prepared by acylation of coelenteramine **7e** using a previously reported procedure.[3,9] Coelenteramide analogues **3a**–**f** were synthesized by acetylation of the corresponding coelenteramine analogues **7a**–**f**, which were prepared by the coupling of oximino ketone **4a**–**d**, **f** and α-amino nitrile **5** followed by reduction with Raney Ni, as illustrated in Scheme 2.[9–12]

The UV–VIS absorption spectra of **1** and **3a**–**f** in various solvents are summarized in Table 1. Each compound showed several bands in the region of 250–370 nm. The lowest energy bands of **1** and **3a**–**f** were virtually independent of solvent polarity, suggesting that the lowest energy transition does not have a charge transfer (CT) character.

In contrast to the absorption spectra, the fluorescence spectra of **1** were dependent on solvent polarity as shown in Fig. 1. The fluorescence spectra of selected coelenteramide analogues **3a**, **3d** and **3f** in various solvents are also depicted in Fig. 1. The fluorescence maxima of **1** and **3a**–**f** are summarized in Table 2, accompanied by the quantum yields. Coelenteramide **1** showed a bathochromic shift of the fluorescence maxima with increasing solvent polarity. This result reproduces the fluorescent character of **1** reported by Shimomura.[7] Similarly, the fluorescence maxima of **3d**–**f**, which possess an electron donating substituent R [R = OCH_3, OH, $N(CH_3)_2$], showed a solvent-dependent bathochromic shift, while **3a**–**c** showed negligible solvent dependency. The fluorescence maxima of **3e** matched exactly those of **1**. The solvatochromic fluorescence of **1** and **3d**–**f** is a typical characteristic of fluorescent compounds which consist of an electron-donating moiety and an electron-accepting moiety, such as 2(arylamino)naphthalene-6-sulfonates (ANS)[13] and *N*-phenyl-4-methylenepiperidine derivatives.[14] These donor–acceptor compounds have a CT state, labeled $S_{1,ct}$, as the lowest excited state, whose stability is dependent on solvent polarity.

In order to obtain a quantitative evaluation of the solvent effect, the fluorescence emission energies E_F of **1**, **3d** and **3f**

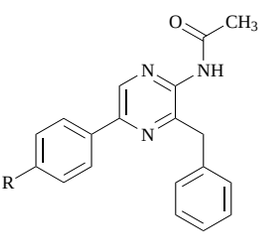


4a : R = CF_3
4b : R = F
4c : R = H
4d : R = OCH_3
4f : R = $N(CH_3)_2$

5

6a : R = CF_3
6b : R = F
6c : R = H
6d : R = OCH_3
6f : R = $N(CH_3)_2$

7a : R = CF_3
7b : R = F
7c : R = H
7d : R = OCH_3
7e : R = OH
7f : R = $N(CH_3)_2$



3a : R = CF_3
3b : R = F
3c : R = H
3d : R = OCH_3
3e : R = OH
3f : R = $N(CH_3)_2$

Scheme 2 *Reagents and conditions:* (i) $TiCl_4$, pyridine, 80–85 °C, 2–3 h; (ii) Raney–Ni (W-2), EtOH, reflux, 1.5 h; (iii) pyridinium chloride, 200 °C, 40 min; (iv) AcCl, pyridine, $CHCl_3$, room temp., 30 min

were correlated with the $E_T(30)$ solvent polarity scale, which is used to evaluate the solvatochromism of electronic transitions with CT character,[15] as shown in Fig. 2. For all three compounds, a good proportional relationship between E_F and $E_T(30)$ was observed. The regression lines of **1** and **3d**–**f** support the proposal that their excited states are CT states. The slopes for **1** and **3d**–**f** were estimated as 0.31, 0.24, 0.28 and 0.63, respectively. The slope value increases with an increasing electron-donating ability of the substituent R. In particular, the large gradient of **3f** indicates that the CT excited state of **3f** is highly polar, similar to that of 2-[(2,6-dimethylphenyl)-amino]naphthalene-6-sulfonate, whose slope was reported as 0.62 obtained from the E_F–$E_T(30)$ plot in a dioxane–water solvent system.[16]

In contrast to **1** and **3d**–**f**, analogues **3a**–**c** showed no solvatochromic fluorescence. To evaluate the substituent effect, the fluorescence emission energies E_F of **3a**–**f** in cyclohexane, chloroform and propan-2-ol were plotted as a function of the

Table 1 Electronic absorption data of coelenteramide **1** and its analogues **3a–f** in various solvents

Solvents	Absorption maximum/nm ($\varepsilon/10^4$ dm^3 mol^{-1} cm^{-1})						
	1	**3a**	**3b**	**3c**	**3d**	**3e**	**3f**
Cyclohexane	332 (1.70)	319 (1.41)	321 (1.48)	321 (1.26)	330 (1.61)	333 (1.63)	355 (2.11)
	291 (1.80)	292 (1.52)	287 (1.57)	288 (1.27)	294 (1.78)	295 (1.84)	323 (1.99)
	277 (1.80)	266 (1.26)	260 (1.71)	262 (1.40)	275 (1.74)	277 (1.75)	266 (0.91)
Benzene	333 (1.63)	320 (1.39)	323 (1.25)	322 (1.31)	332 (1.63)	334 (1.61)	364 (2.15)
	294 (1.66)	292 (1.44)	288 (1.29)	292 (1.24)	295 (1.71)	295 (1.72)	331 (1.90)
	279 (1.69)				278 (1.60)	280 (1.59)	
Diglyme	333 (1.68)	318 (1.36)	321 (1.43)	320 (1.24)	331 (1.62)	333 (1.64)	360 (2.06)
	294 (1.78)	304 (sh, 1.37)	286 (1.42)	291 (1.19)	294 (1.70)	295 (1.78)	328 (1.87)
	277 (1.81)	292 (1.46)	258 (1.66)	260 (1.35)	274 (1.60)	276 (1.67)	266 (0.84)
		262 (1.21)					
Chloroform	333 (1.59)	319 (1.38)	322 (1.49)	321 (1.27)	332 (1.60)	333 (1.63)	365 (1.96)
	294 (1.61)	294 (1.39)	287 (1.44)	291 (1.16)	295 (1.61)	295 (1.69)	331 (1.79)
	278 (1.67)	262 (1.34)	260 (1.71)	261 (1.43)	278 (1.57)	277 (1.65)	268 (0.91)
DMSO	336 (1.63)	320 (sh, 1.26)	321 (1.34)	321 (1.20)	333 (1.55)	336 (1.63)	366 (2.01)
	295 (1.73)	305 (sh, 1.32)	288 (1.33)	294 (1.15)	295 (1.56)	297 (1.74)	333 (1.81)
	280 (1.75)	295 (1.37)	264 (1.39)	261 (1.31)	275 (1.49)	279 (1.60)	272 (0.80)
		262 (1.17)					
Acetonitrile	329 (1.57)	316 (sh, 1.29)	317 (1.38)	316 (1.17)	328 (1.53)	329 (1.55)	359 (2.02)
	292 (1.66)	303 (sh, 1.35)	285 (1.41)	291 (1.14)	294 (1.55)	293 (1.66)	331 (1.85)
	274 (1.72)	292 (1.40)	255 (1.70)	256 (1.38)	274 (1.53)	273 (1.62)	266 (0.89)
		257 (1.28)					
Propan-2-ol	333 (1.67)	315 (sh, 1.28)	317 (1.41)	317 (1.21)	329 (1.59)	333 (1.65)	361 (1.99)
	294 (1.77)	301 (sh, 1.39)	285 (1.43)	292 (1.20)	294 (1.64)	295 (1.77)	329 (1.85)
	278 (1.75)	293 (1.46)	256 (1.68)	257 (1.40)	274 (1.52)	277 (1.60)	267 (0.92)
		258 (1.27)					
Methanol	332 (1.61)	314 (sh, 1.30)	316 (1.40)	316 (1.18)	330 (1.57)	332 (1.61)	363 (1.96)
	293 (1.67)	301 (sh, 1.41)	285 (1.37)	292 (1.13)	329 (1.58)	293 (1.67)	330 (1.79)
	277 (1.69)	293 (1.46)	255 (1.68)	257 (1.37)	275 (1.50)	277 (1.56)	267 (0.92)
		255 (1.34)					

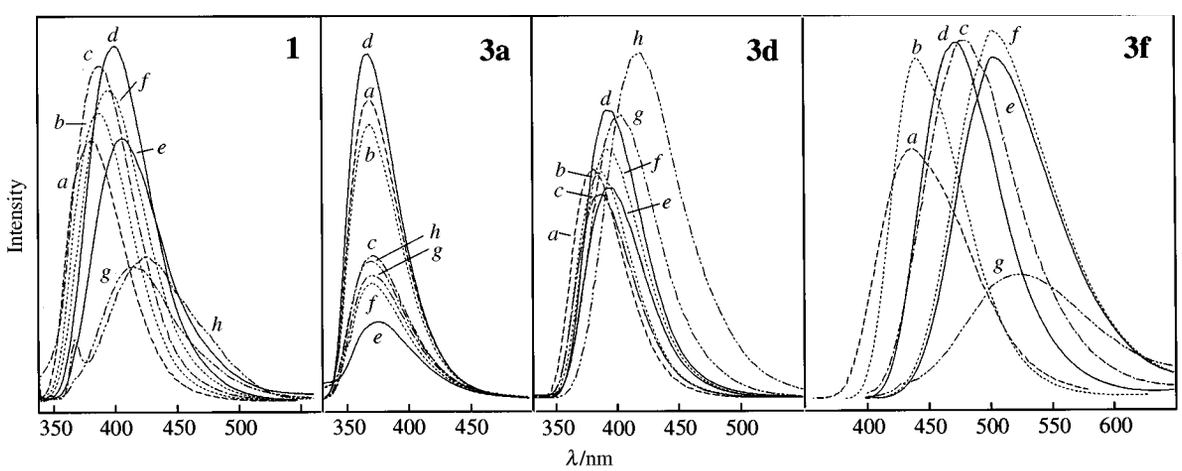


Fig. 1 Fluorescence spectra of coelenteramide **1** and its selected analogues **3a**, **3d** and **3f** in solvents of different polarities: (*a*) in cyclohexane, (*b*) in benzene, (*c*) in diglyme, (*d*) in chloroform, (*e*) in DMSO, (*f*) in acetonitrile, (*g*) in propan-2-ol and (*h*) in methanol

Hammett σ_p substituent constant of a substituent R,[17] as shown in Fig. 3.[16] For each solvent two lines were plotted. The high slope lines are made by **3d–f**, whose excited states are CT states, $S_{1,ct}$, and the horizontal lines are made by **3a–c**. The lowest excited states of **3a–c**, whose stability is insensitive to a solvent polarity, are assigned as the locally excited states S_1. The polarity of the S_1 state of **3a–c** is modestly different from that of the ground state.

The difference in character between S_1 and $S_{1,ct}$ in the coelenteramide analogues was also distinguished by the fluorescence quantum yields Φ_F compiled in Table 2. The Φ_F values of **3a–c** are smaller than 0.15 and are insensitive to solvent polarity. On the other hand, the Φ_F values of **1** and **3d–f** are larger than 0.28 in an aprotic solvent. In propan-2-ol and methanol, **3d** maintained its high Φ_F values, while the Φ_F values of **1**, **3e** and **3f** were smaller than those in the aprotic solvents. This result suggests that a specific interaction between the solvating alcohol and a hydroxy group (or dimethylamino group) of **1** and **3e** (or **3f**) quenches the emission process from the $S_{1,ct}$ state. The decrease of the Φ_F value of **1**, **3e** and **3f** in a protic solvent is a characteristic similar to those of the CT fluorescent compounds, such as the ANS derivatives[18] and the *N*-phenyl-4-methylenepiperidines.[14] One explanation of this quenching process was given by Kosower, who elucidated that the fluorescence quenching of an ANS derivative in polar protic solvents results from the promotion of nonradiative electron-transfer by the solvent.[18]

In conclusion, solvatochromic fluorescence of coelenteramide **1** and its analogues **3d–f** is emitted from the CT excited state $S_{1,ct}$. On the other hand, fluorescence of **3a–c** from the S_1 state is insensitive to solvent polarity. The $S_{1,ct}$ state is stabilized by the electron donating character of the *para*-substituted phenyl group at the 5-position of coelenteramide **1**. The electron donation from R [R = OCH_3, OH and $N(CH_3)_2$] is enough to stabilize the $S_{1,ct}$ state more than the S_1 state. The CT fluorescence of **1** and **3d–f** influences the value ($\Phi_F > 0.28$) of the

Table 2 Fluorescence data of coelenteramide **1** and its analogues **3a–f** in various solvents

Solvents	Fluorescence maximum/nm (quantum yield)						
	1	**3a**	**3b**	**3c**	**3d**	**3e**	**3f**
Cyclohexane	383 (0.25)	367 (0.067)	370 (0.074)	369 (0.014)	380 (0.30)	387 (0.28)	438 (0.38)
Benzene	389 (0.32)	369 (0.061)	376 (0.13)	369 (0.10)	387 (0.30)	390 (0.33)	441 (0.54)
Diglyme	389 (0.33)	372 (0.039)	370 (0.046)	370 (0.067)	391 (0.30)	390 (0.30)	481 (0.33)
Chloroform	403 (0.37)	367 (0.080)	371 (0.11)	370 (0.15)	392 (0.44)	403 (0.38)	473 (0.40)
DMSO	409 (0.30)	376 (0.025)	374 (0.044)	372 (0.063)	396 (0.36)	407 (0.30)	505 (0.35)
Acetonitrile	397 (0.30)	370 (0.023)	370 (0.043)	370 (0.062)	391 (0.33)	396 (0.29)	503 (0.33)
Propan-2-ol	418 (0.13)	369 (0.032)	370 (0.054)	369 (0.076)	403 (0.44)	417 (0.11)	525 (0.08)
Methanol	428 (0.02)	371 (0.027)	374 (0.061)	372 (0.084)	420 (0.52)	426 (0.02)	n.d.[a]

[a] Fluorescence could not be detected.

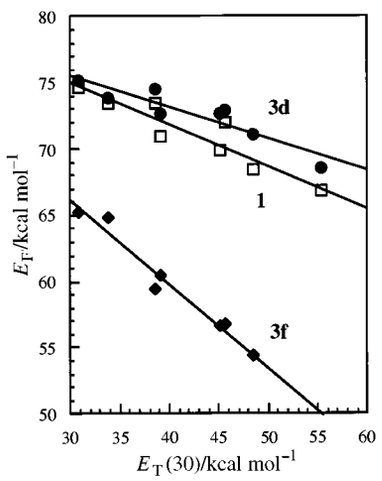


Fig. 2 Plots of fluorescence emission energies E_F (in kcal mol^{-1}) for **1** (□), **3d** (●) and **3f** (◆) against the solvent polarity parameter $E_{T(30)}$ (in kcal mol^{-1})

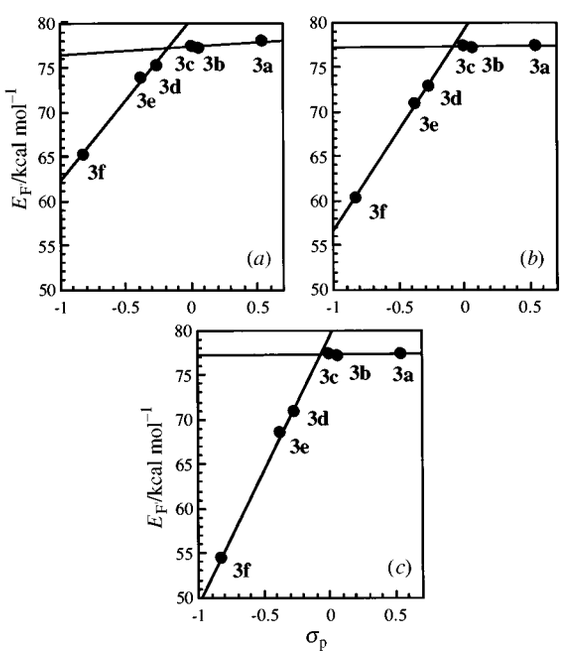
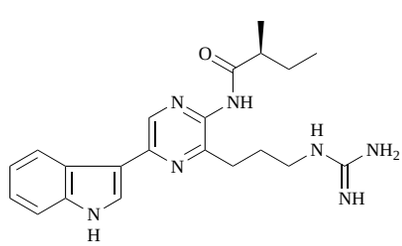


Fig. 3 Plots of fluorescence emission energies E_F (in kcal mol^{-1}) for **3a–f** against Hammett substituent constant (*a*) in cyclohexane, (*b*) in chloroform and (*c*) in propan-2-ol

fluorescence quantum yield in an aprotic solvent. Therefore, in aequorin bioluminescence the CT fluorescent character of coelenteramide **1** may have an important role in determining the emission wavelength and the value of the quantum yield. We proposed a hypothesis that blue light emission (λ_{max} = 465 nm) in aequorin bioluminescence originates from the $S_{1,ct}$ state of the phenolate anion **III**.[5] Because the Hammett σ_p values of O^- ($\sigma_p = -0.81$) and $N(CH_3)_2$ ($\sigma_p = -0.83$) are similar, the fluorescent behaviour of **III** is predicted using that of coelenteramide analogue **3f** [R = $N(CH_3)_2$]. The wavelength region of the CT fluorescence of **3f** contains the bioluminescence maximum, supporting our hypothesis. Further, we need to evaluate the influence of the ionic character of **III** on the fluorescence properties. To clarify this problem, characterization of the fluorescence of the anion species of **1** is now in progress.

To design a coelenterazine derivative with highly efficient bio- or chemi-luminescence, a CT fluorescent coelenteramide analogue with high fluorescence quantum yield should be considered as the light emitter. Because the efficiency of a bio- and chemi-luminescence reaction is a product of three efficiencies, which are (*i*) the fraction of reacting molecules that pursue the correct chemical pathway (Φ_r), (*ii*) the efficiency of chemical generation of a singlet excited state (Φ_s) and (*iii*) the fluorescence quantum yield of the light emitter (Φ_{fl}), it is also necessary to design for increasing Φ_r and Φ_s accompanied by Φ_{fl}. On this basis, we have been investigating substituent effects on the chemiluminescence behaviour of a 7*H*-imidazo[1,2-*a*]pyrazin-3-one derivative and the results will be reported elsewhere.

Our results are also useful as a standard scale of the substituent effect on the 5-position on the fluorescence emission of amidopyrazine derivatives. *Cypridina* oxyluciferin **8** possessing



8

an indol-3-yl group at the 5-position, for instance, shows a fluorescence maximum at 420 nm in diglyme.[6c,8,19] This maximum of **8** is longer than that of **3e** (R = OH, λ_{max} = 390 nm) and is shorter than that of **3f** [R = $N(CH_3)_2$, λ_{max} = 481 nm]. Therefore, the order of the electron donating ability is estimated as *p*-dimethylaminophenyl > indol-3-yl > hydroxyphenyl. This order matches the order of the ionization potentials of these groups.[20] Using the table of Hammett σ_p values,[17] it is predicted that the fluorescence maximum of **8** matches that of an acetamidopyrazine derivative possessing *p*-hydrazinophenyl group at the 5-position.

Experimental

All melting points were measured on a Yamato Model MP-21 apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a 270 MHz JEOL JNM-GX270 spectrometer. Chemical shifts (δ) are reported in ppm using as an internal standard tetramethylsilane or an undeuteriated solvent in the deuteriated

solvent used. Coupling constants (J) are given in Hz. IR spectra were obtained using a JASCO IR-810 spectrophotometer. High and low resolution electron impact (EI) mass spectra were measured on a Hitachi Model M-80B mass spectrometer with a Hitachi M-0101 data system. The ionization energy and the accelerating voltage were 70 eV and 3 kV, respectively.

UV–VIS absorption and fluorescence spectrometry

Spectral grade solvents were used for the measurement of UV–VIS absorption and fluorescence spectra except diglyme. Diglyme was passed through an alumina column, dried over CaH_2 and purified by distillation under reduced pressure. UV–VIS absorption spectra were obtained using a Hitachi Model 320 spectrophotometer. Fluorescence spectra were measured with a Hitachi Model F-4010 fluorescence spectrophotometer (excitation bandpass, 5 nm; emission bandpass, 5 nm; response, 2 s; scan speed, 60 nm min^{-1}) and corrected according to manufacturer's instructions. A solution of coelenteramide **1** or its analogues **3a**–**f** for fluorescence measurement was prepared by mixing a stock solution (100 μl) of 2.0×10^{-5} mol dm^{-3} **1** or **3a**–**f** in diglyme and a solvent (2.0 ml) within a quartz cuvette at room temperature. The fluorescence quantum yields were determined using quinine sulfate in 0.1 M H_2SO_4 ($\Phi_F = 0.55$, excitation wavelength: 355 nm, room temperature) as a standard.

Synthesis of coelenteramide 1, 2-acetamidopyrazine 3e, 2-aminopyrazine 1-oxide 6d and 2-aminopyrazines 7d–f

1, **3e**, **6d** and **7d**–**f** were synthesized by a previously reported procedure.[3,9–12]

Synthesis of 2-amino-3-benzyl-5-(4-trifluoromethylphenyl)-pyrazine 1-oxide 6a

To a solution of *p*-trifluoromethyl-2-hydroxyiminoacetophenone **4a** (1.99 g, 9.36 mmol) and 2-amino-3-phenylpropionitrile hydrochloride **5** (2.35 g, 13.8 mmol) in dried pyridine (35 ml) was added dropwise $TiCl_4$ (0.5 ml, 4.56 mmol) in an ice bath under N_2 and the mixture was heated at 82–83 °C for 3 h. After cooling to room temperature, the reaction mixture was filtered through Celite. The filtrate was concentrated under reduced pressure and the black residue was partitioned between CH_2Cl_2 and water. The organic phase was washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (1:1 AcOEt–$CHCl_3$) and recrystallization from methanol, affording **6a** (1.15 g, 33%) as brownish plates; mp 155–157 °C; $\delta_H(CDCl_3)$ 4.30 (s, 2 H), 5.60 (s, 2 H), 7.29–7.37 (m, 5 H), 7.74 (d, *J* 8.4, 2 H), 8.20 (d, *J* 9.2, 2 H) and 8.55 (s, 1 H); ν_{max}(KBr)/cm^{-1} 3439, 3282, 3136, 1612, 1482, 1324 and 1116; *m/z* (P-EI) (relative intensity) 345 (M^+, 8), 329 (100) and 91 (29).

Synthesis of coelenteramine 1-oxide derivatives 6b and c

Coelenteramine 1-oxide derivatives **6b** and **c** were prepared by an analogous procedure to that for **6a**.

2-Amino-3-benzyl-5-(4-fluorophenyl)pyrazine 1-oxide 6b. 48% Yield, tan needles (from methanol); mp 163.5–164.5 °C; $\delta_H(CDCl_3)$ 4.26 (s, 2 H), 5.40 (s, 2 H), 7.13–7.37 (m, 7 H), 7.84–7.89 (m, 2 H) and 8.39 (s, 1 H); ν_{max}(KBr)/cm^{-1} 3310, 3086, 1611, 1599, 1509, 1477, 1343 and 1227; *m/z* (P-EI) (relative intensity) 296 (21), 295 (M^+, 100), 278 (80) and 91 (48).

2-Amino-3-benzyl-5-phenylpyrazine 1-oxide 6c. 38% Yield, slightly yellow needles (from methanol); mp 164.5–165.5 °C, $\delta_H(CDCl_3)$ 4.27 (s, 2 H), 5.36 (s, 2 H), 7.30–7.52 (m, 8 H), 7.88–7.91 (m, 2 H) and 8.44 (s, 1 H); ν_{max}(KBr)/cm^{-1} 3406, 3266, 3108, 3062, 1618, 1585, 1570, 1479, 1351 and 1128; *m/z* (P-EI) (relative intensity) 278 (21), 277 (M^+, 100), 261 (42), 260 (99), 233 (34) and 91 (26).

Synthesis of 2-amino-3-benzyl-5-(4-trifluoromethylphenyl)-pyrazine 7a

A mixture of the aminopyrazine 1-oxide **6a** (354 mg, 1.02 mmol), EtOH and Raney Ni (W-2, 6.31 g) was heated to reflux under H_2 atmosphere for 1.5 h. After cooling to room temperature, the reaction mixture was filtered through Celite and the Celite bed was washed thoroughly with EtOH. The filtrate was concentrated *in vacuo* and purified by recrystallization from methanol to give **7a** (280 mg, 83%) as tan needles; mp 154.5–156 °C; $\delta_H(CDCl_3)$ 4.21 (s, 2 H), 4.53 (s, 2 H), 7.28–7.37 (m, 5 H), 7.71 (d, *J* 8.4, 2 H), 8.06 (d, *J* 8.1, 2 H) and 8.44 (s, 1 H); ν_{max}(KBr)/cm^{-1} 3482, 3294, 3142, 1637, 1463, 1327 and 1112; *m/z* (P-EI) (relative intensity) 329 (M^+, 100) and 91 (24) [HRMS (positive EI) Calc. for $C_{18}H_{14}F_3N_3$ 329.1141. Found 329.1126].

Synthesis of coelenteramine analogues 7b and c

Coelenteramine analogues **7b** and **c** were prepared by an analogous procedure to that for **7a**.

2-Amino-3-benzyl-5-(4-fluorophenyl)pyrazine 7b. 72% Yield, pale yellow needles (from methanol); mp 129.5–130 °C; $\delta_H(CDCl_3)$ 4.20 (s, 2 H), 4.66 (s, 2 H), 7.12–7.34 (m, 7 H), 7.89–7.94 (m, 2 H) and 8.31 (s, 1 H); ν_{max}(KBr)/cm^{-1} 3480, 3301, 3142, 1628 and 1461; *m/z* (P-EI) (relative intensity) 280 (18), 279 (M^+, 100) and 278 (54) [HRMS (positive EI) Calc. for $C_{17}H_{14}FN_3$ 279.1172. Found 279.1166].

2-Amino-3-benzyl-5-phenylpyrazine 7c. 77% Yield, pale yellow needles (from methanol); mp 138–139 °C; $\delta_H(CDCl_3)$ 4.24 (s, 2 H), 4.54 (s, 2 H), 7.26–7.49 (m, 8 H) and 7.93–7.95 (m, 2 H); ν_{max}(KBr)/cm^{-1} 3484, 3288, 3118, 1634 and 1447; *m/z* (P-EI) (relative intensity) 261 (M^+, 100) and 260 (68) [HRMS (positive EI) Calc. for $C_{17}H_{15}N_3$ 261.1267. Found 261.1264].

Synthesis of 2-acetamido-3-benzyl-5-(4-trifluoromethylphenyl)-pyrazine 3a

To a solution of **7a** (103 mg, 0.31 mmol) and anhydrous pyridine (0.65 ml, 8.04 mmol) in anhydrous $CHCl_3$ (2.0 ml), acetyl chloride (150 mg, 2.31 mmol) was added at 0 °C, giving a colorless precipitate. After stirring for 30 min at room temperature, the reaction was quenched by the addition of saturated aqueous $NaHCO_3$, and extracted three times with $CHCl_3$. The organic layer was dried over $MgSO_4$, concentrated, and purified by recrystallization from methanol, affording **3a** (88.8 mg, 74%) as colorless needles; mp 241–242 °C; $\delta_H([^2H_6]DMSO)$ 2.08 (s, 3 H), 4.22 (s, 2 H), 7.19–7.31 (m, 5 H), 7.88 (d, *J* 8.4, 2H), 8.30 (d, *J* 7.9, 2 H), 9.05 (s, 1 H) and 10.38 (s, 1 H); ν_{max}(KBr)/cm^{-1} 3442, 3234, 1672, 1493, 1333 and 1117; *m/z* (P-EI) (relative intensity) 372 (24), 371 (M^+, 100), 329 (74), 328 (85), 91 (32), 77 (13), 65 (10) and 43 (93) [HRMS (positive EI) Calc. for $C_{20}H_{16}F_3N_3O$ 371.1246. Found 371.1231].

Synthesis of coelenteramide analogues 3b–f

Coelenteramide analogues **3b–f** were prepared by an analogous procedure to that for **3a**.

2-Acetamido-3-benzyl-5-(4-fluorophenyl)pyrazine 3b. 60% Yield, colorless needles (from methanol); mp 218–218.5 °C; $\delta_H([^2H_6]DMSO)$ 2.06 (s, 3 H), 4.18 (s, 2 H), 7.18–7.39 (m, 5 H), 8.11–8.16 (m, 2 H), 8.95 (s, 1 H) and 10.32 (s, 1 H); ν_{max}(KBr)/cm^{-1} 3444, 3256, 1668 and 1499; *m/z* (P-EI) (relative intensity) 322 (22), 321 (M^+, 100), 279 (78), 278 (77), 134 (19), 120 (23), 91 (20), 77 (10), 65 (6) and 43 (43) [HRMS (positive EI) Calc. for $C_{19}H_{16}FN_3O$ 321.1278. Found 321.1286].

2-Acetamido-3-benzyl-5-phenylpyrazine 3c. 66% Yield, colorless needles (from methanol); mp 206.5–207 °C; $\delta_H([^2H_6]$-DMSO) 2.07 (s, 2 H), 4.19 (s, 2 H), 7.19–7.31 (m, 5 H), 7.44–7.55 (m, 3 H), 8.06–8.10 (m, 2 H), 8.94 (s, 1 H) and 10.29 (s, 1 H); ν_{max}(KBr)/cm^{-1} 3432, 3278, 1668, 1505 and 1492; *m/z* (P-EI) (relative intensity) 304 (24), 303 (M^+, 100), 261 (73), 260 (86), 91 (31), 77 (23), 65 (10) and 43 (79) [HRMS (positive EI) Calc. for $C_{19}H_{17}N_3O$ 303.1372. Found 303.1381].

2-Acetamido-3-benzyl-5-(4-methoxyphenyl)pyrazine 3d. 41% Yield, colorless needles (from methanol); mp 150.5–152 °C; $\delta_H([^2H_6]DMSO)$ 2.05 (s, 2 H), 3.82 (s, 3 H), 4.16 (s, 2 H), 7.06 (d, *J* 8.9, 2 H), 7.16–7.30 (m, 5 H), 8.04 (d, *J* 8.9, 2 H), 8.87 (s, 1 H) and 10.24 (s, 1 H); ν_{max}(KBr)/cm^{-1} 3450, 3256, 1670, 1496

and 1174; *m/z* (P-EI) (relative intensity) 334 (24), 333 (M^+, 100), 291 (88), 290 (54), 91 (25), 77 (14) and 43 (64) [HRMS (positive EI) Calc. for $C_{20}H_{19}N_3O_2$ 333.1478. Found 333.1479].

2-Acetamido-3-benzyl-5-(4-dimethylaminophenyl)pyrazine 3f. 45% Yield, pale yellow needles (from methanol); mp 211.5–212.5 °C; δ_H([$^2H_6$]DMSO) 2.04 (s, 3 H), 2.97 (s, 6 H), 4.11 (s, 2 H), 6.79 (d, *J* 8.9, 2 H), 7.19–7.30 (m, 5 H), 7.93 (d, *J* 8.9, 2 H) and 10.17 (s, 1 H); ν_{max}(KBr)/cm^{-1} 3438, 3248, 1662, 1609, 1529, 1496, 1439 and 1363; *m/z* (P-EI) (relative intensity) 347 (25), 346 (M^+, 100), 305 (22), 304 (93), 303 (26) and 43 (52) [HRMS (positive EI) Calc. for $C_{21}H_{22}N_4O$ 346.1795. Found 346.1778].

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