Pergamon

Tetrahedron Letters 42 (2001) 2997–3000

TETRAHEDRON LETTERS

Chemi- and bioluminescence of coelenterazine analogues with a conjugated group at the C-8 position

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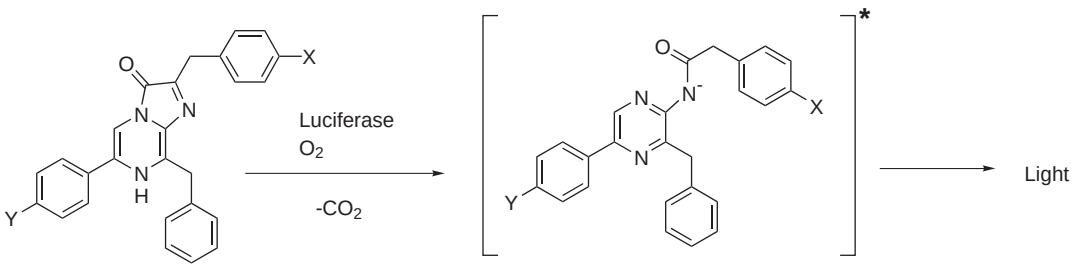
Received 18 January 2001; revised 21 February 2001; accepted 23 February 2001

Abstract—The chemiliminescent compound coelenterazine is involved in various bioluminescence reactions as the substrates, causing the luminescence with an emission peak in the range of 450–475 nm. Anticipating the introduction of a bathochromicshift of the luminescence, several new coelenterazine analogues that have conjugated olefins or aromatic groups at the 8-position of imidazopyrazinone ring were synthesized. In the chemiluminescence reaction, the emission spectra of a majority of the compounds synthesized showed a bathochromic shift, giving an emission peak in the range of 520–580 nm. In the bioluminescence catalyzed by *Oplophorus* luciferase, the bisthienyl analogue of coelenterazine emitted a moderate intensity of luminescence (5% of coelenterazine) with an emission maximum at 528 nm, which was the longest wavelength of all the analogues tested.

Coelenterazine **1** is well known as a chromophoric compound of aequorin and also as the luciferins of various bioluminescent marine organisms such as the sea pansy *Renilla reniformis* and the deep-sea shrimp *Oplophorus gracilirostris*. The bioluminescence of coelenterazine is highly efficient, thus the luciferases involved may be usable as reporter proteins. The emission maxima of the bioluminescence of coelenterazine have been reported in the range 450–475 nm, showing slight differences by the luciferase species used.[1] The amide anion **2** is believed to be the light emitter (see Fig. 1).[2] We have recently reported that an imidazopyrazinone having chlorostyryl functionality at the 8-position displayed a large bathochromic shift in the chemiluminescence in neutral condition,[3] showing that the color of bioluminescence can be spectrally shifted by the introduction of a conjugated group at 8-position. In the present study we synthesized several new coelen-



coelenterazine **1a:** X=Y=OH
2-deoxycoelenterazine **1b**: X=H, Y=OH
bisdeoxycoelenterazine **1c**: X=Y=H

2

Figure 1. The light emitters involved in the bioluminescence of coelenterazine (**1a**), 2-deoxy-coelenterazine (**1b**) and bisdeoxycoelenterazine (**1c**).

Keywords: coelenterazine; bathochromic shift; *Oplophorus* luciferase; chemiluminescence; bioluminescence.

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0040-4039/01/$ - see front matter
PII: S0040-4039(01)00340-9

terazine analogues having a conjugated group at the 8-postion by the use of Pd-cross coupling reactions,[4] then examined the luminescence characteristics of the products.

Introduction of a conjugated chromophore at the 8-position of coelenterazine was easily achieved by the method previously reported, using 2-amino-3,5-dibromopyrazine and tin reagents, as shown in Fig. 2.[4] This cross coupling reaction, which gave yields of 52–74%, is regioselective due to the chelation of an amino group and the electron deficiency at the 3-position. The second cross coupling reactions at the 5-position were performed using an excess of tin reagents. The derivatives having a benzyl substituent at 2-position[5,6] were synthesized by the condensation of 2-ketoaldehyde with 3,5-disubstitued-2-aminopyrazine.

Coelenterazine analogues synthesized showed chemiluminescence in polar aprotic solvents. In chemiluminescence, the analogue **3a** having a styryl group showed the largest bathochromic shift under neutral conditions, giving a peak at 580 nm. A comparison of the analogue **3c** (peak at 519 nm) and the analogue **4a** (peak at 520 nm) suggests that the electron rich function group may not enhance the bathochromic effect. Two analogues **4b** and **4c** having hetero rings showed slightly larger shifts than the analogue **4a** having aromatic rings. The chemiluminescence spectra of **4a–c** were superimposable with the fluorescence spectra of these compounds after chemiluminescence reaction (Table 1), showing that their light emitters are amide anion. The chemiluminescence spectra of the analogues **3a–c** in the presence of alkali did not match the fluorescence spectra of the spent solution after chemiluminescence, suggesting that the fluorescence is emitted probably from the pyrazine-*N*-anions of the amide compounds produced by a chemiluminescence reaction.[7]

Bioluminescence properties of these analogues were investigated using recombinant *Renilla* luciferase,[1] *Oplophorus* luciferase[1] and apoaequorin.[1] In the presence of *Renilla* luciferase, the luminescence of the analogues having a 6-phenyl group, **4a–c** and **1c**, were poor.[8] The Ca-triggered luminescence of the aequorins that were regenerated with analogues **4a–c** were also very weak,[8] although the aequorin regenerated with **3c** emitted a total light corresponding to 5.5% of that regenerated with coelenterazine, with an emission maximum at 438 nm accompanying two very weak peaks at 545 and 610 nm.

Oplophorus luciferase was previously found to catalyze the luminescence of bisdeoxycoelenterazine **1c** with high efficiency.[8] The luminescence of the new coelenterazine analogues was considerably more efficient with *Oplophorus* luciferase than with *Renilla* luciferase or apoaequorin. For example, the analogue **3b** having one conjugated double bond at the 8-postion emitted luminescence at 482 nm, which was moderately strong in both the intensity and the total light. The luminescence of the analogue **4a** having an aromatic group at the 8-position was similar to that of the analogue **3b**, whereas the analogue **3c** that also has an aromatic group at the 8-position emitted only feeble luminescence at 476 nm, although the total light emitted from this compound was close to those emitted from the analogues **4a** and **3b**. The analogues **3c** and **4a**, both with an aromatic group at the 8-position, showed their chemiluminescence maxima at the wavelengths considerably longer than their bioluminescence maxima, for reasons which are unclear. A significant bathochromic

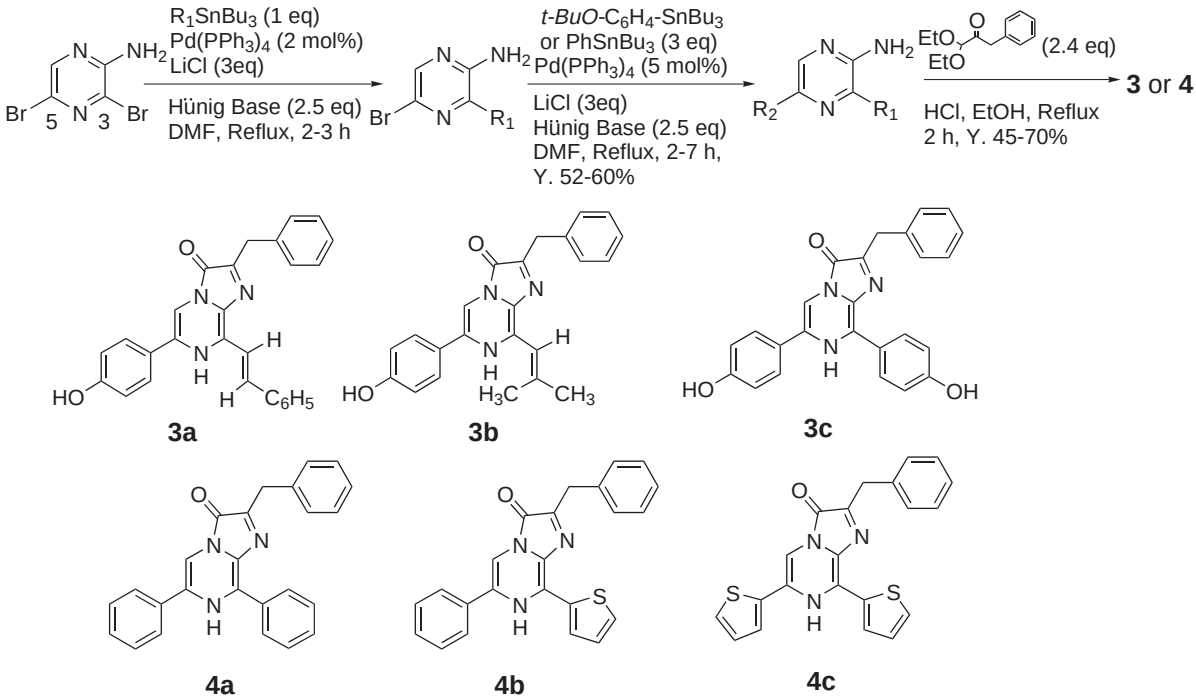

Figure 2. Synthesis of coelenterazine analogues **3** and **4** by Pd-mediated cross couplings.

Table 1. Bioluminescence, chemiluminescence and fluorescence of coelenterazine analogues

	1a	1b	3a	3b	3c	1c	4a	4b	4c
Aequorin[a]									
Total light (%)	100	82	0.4	1.5	5.5	0.018	ND	0	ND
Emission max. (nm)	465[f]	464[f]	ND	455	438 (1):545 (0.2):610 (0.06)	450[g]	ND	ND	ND
Oplophorus luciferase[b]									
Initial intensity (%)	100	97	0.001	16	0.43	79	9.4	0.057	0.18
Total light (%)	100	75	0.007	31	20	66	26	3	5.5
Emission max. (nm)	452[f]	457[f]	ND	482	476 (1):545 (0.2)	448[g]	483	510	528
Renilla luciferase[c]									
Initial intensity (%)	100[f]	57[f]	ND	0.12	0.017	0.32[g]	0.0025	ND	0.0026
Chemiluminescence[d]									
Emission max. (nm)	465[h]	466	580	461	519	453	520	525	534
Fluorescence[e]									
Emission max. in neutral (nm)	411[h]	412	540	425	420	405	448	423	430
Emission max. with base (nm)	524[h]	526	612	531	541	453	520	525	534

[a] Regenerated from 10 μg analogues and recombinant apoaequorin (0.5 mg) in 0.5 ml of 10 mM Tris–HCl/2 mM EDTA/5 mM 2-mercaptoethanol, pH 7.5 for overnight, then luminescence was measured by adding 10 μl of the solution to 3 ml of 10 mM calcium acetate.
[b] Analogue (0.24 nmol) was added to *Oplophorus* luciferase (10 μg) in 3 ml of 50 mM NaCl/15 mM Tris–HCl, pH 8.3.
[c] Analogue (0.24 nmol) was added to recombinant *R.* luciferase in 3 ml of 0.1 M NaCl/25 mM Tris–HCl, pH 7.5.
[d] 10 mM ethanol solution of analogue (30 μl) was added to DMSO (2 ml) and chemiluminescence was triggered by the addition of 0.1 M acetate buffer pH 6.5 (100 μl).
[e] Fluorescence was measured with the spent solution of chemiluminescence under neutral condition or with addition of 1 M KOH.
[f] Data from Ref. 1.
[g] Data from Ref. 8.
[h] Data from Ref. 9. ND: not determined due to low light intensity.

shift was found with the low intensity luminescence of analogue **4b** having a compact thienyl group at the 8-position, and a further red-shift was observed with the bisthienyl compound **4c** that showed an emission maximum at 528 nm.

It has been reported that *Oplophorus* luciferase could be a candidate for useful reporter protein because of its favorable properties.[1] The present study shows that, in the bioluminescence of coelenterazines catalyzed by *Oplophorus* luciferase, a conjugated group can induce a bathochromic shift. Efforts are in progress to improve the efficiency of the coelenterazine–luciferase bioluminescence reactions for practical applications.

Acknowledgements

We acknowledge financial support from Grants-in-Aid for Scientific Research in priority areas (A) from the Ministry of Education, Sciences, Sports and Culture, Japan and from Naito Foundation. We would also like to give thanks for the JSPS scholarship which was awarded to C. Wu.

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5. Compound **3a**: brown solid; mp 137–138°C; ^{1}H NMR (400 MHz, 9:1 $CDCl_3$–CD_3OD 12 M HCl): δ 4.35 (2H, s), 6.97 (2H, d, $J=8$ Hz), 7.95 (2H, d, $J=8$ Hz), 7.27 (1H, d, $J=16$ Hz), 7.76 (1H, d, $J=16$ Hz), 7.28–7.40 (4H, m), 7.45–7.48 (4H, m), 7.78–7.80 (2H, m), 8.50 (1H, s); HR-FABMS, m/z 420.1648 (calcd for $C_{27}H_{22}N_3O_2$ 420.1633). Compound **3b**: brown solid; mp 145–146°C; ^{1}H NMR (400 MHz, 9:1 $CDCl_3$–CD_3OD 12 M HCl): δ 2.13 (3H, s), 2.37 (3H, s), 4.27 (2H, s), 6.73 (1H, s), 8.40 (1H, s), 7.20–7.40 (5H, m), 7.80 (2H, d, $J=8$ Hz), 6.87 (2H, d, $J=8$ Hz); HR-FDMS, m/z 371.1663 (calcd for $C_{23}H_{21}N_3O_2$ 371.1633). Compound **3c**: red solid; mp 129–131°C; ^{1}H NMR (400 MHz, 9:1 $CDCl_3$–CD_3OD): δ 8.15 (1H, s), 4.36 (2H, s), 7.20–7.40 (5H, m), 7.62 (2H, d, $J=8$ Hz), 6.98 (2H, d, $J=8$ Hz), 6.85 (2H, d, $J=8$ Hz), 7.86 (2H, d, $J=8$ Hz); HR-FABMS, m/z 410.1440 (calcd for $C_{25}H_{19}N_3O_3$ 410.1426).
6. Compound **4a**: brown solid; mp 101–102°C; ^{1}H NMR (300 MHz, CD_3OD): δ 4.15 (2H, s), 7.90 (1H, s), 7.14–7.58 (9H, s), 7.84 (2H, d, $J=6$ Hz), 7.47 (2H, d, $J=6$ Hz), 8.06 (2H, d, $J=6$ Hz); HR-FABMS, m/z 378.1542 (calcd for $C_{25}H_{20}N_3O$ 378.1542). Compound **4b**: brown solid; mp 152–154°C; ^{1}H NMR (300 MHz, DMSO): δ 4.12 (2H, s), 8.50 (1H, s), 7.16–7.53 (9H, s), 7.30–7.35 (5H, s), 7.80 (1H, d, $J=6$ Hz), 8.69 (2H, d, $J=6$ Hz), 8.11 (1H, s, $J=6$ Hz); HR-EIMS, m/z 383.1075 (calcd for $C_{23}H_{17}N_3OS$ 383.1058). Compound **4c**: brown solid; mp 122–124°C; ^{1}H

NMR (300 MHz, CD_3OD 12 M HCl): δ 4.45 (2H, s), 8.70 (1H, s), 7.18–7.20 (2H, m), 7.30–7.35 (5H, s), 7.61 (1H, s, $J=6$ Hz), 7.86–7.90 (2H, m), 8.11 (1H, d, $J=6$ Hz); HR-FABMS, m/z 390.0728 (calcd for $C_{12}H_{16}N_3OS_2$ 390.0696).

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