

Chapter 2

Synthetic Bioluminescent Coelenterazine Derivatives

Ryo Nishihara, Daniel Citterio, and Koji Suzuki

Abstract

The development of coelenterazine (CTZ) derivatives resulting in superior optical characteristics is an efficient method to extend the range of its possible applications. Here, we describe the synthesis of three C-6 substituted CTZ derivatives retaining the recognition by *Renilla* luciferase (RLuc) and its derivatives. The novel derivatives are useful as bright blue-shifted CTZ derivatives, which can be used as an alternative to hitherto reported compound DeepBlueC™.

Key words Bioluminescence, Coelenterazine (CTZ), *Renilla* Luciferase (RLuc), DeepBlueC, Luciferin, Luciferase, Chemiluminescence

1 Introduction

Bioluminescence is emitted by an enzymatic oxidation reaction involving a bioluminescent substrate (luciferin) and an enzyme (luciferase). Firefly luciferin, which emits light at a relatively long wavelength ($\lambda_{\text{max}} = 560 \text{ nm}$) in the presence of Mg^{2+} and ATP as cofactors, is widely used in bioassays. However, the cofactors potentially lead to complex assay protocols in bioanalysis [1]. In contrast, marine luciferases such as *Renilla* luciferase (RLuc) generate cofactor-free bioluminescence with native coelenterazine (nCTZ). There is a lot of interest in developing new CTZ derivatives [2–7]. However, the design of novel CTZ derivatives resulting in enhanced optical intensity with prolonged bioluminescence is challenging, because the detailed enzymatic recognition mechanism of the RLuc/CTZ reaction is still mostly unknown [8–10]. In fact, most of the reported CTZ analogs fail to emit bioluminescence, since their structural modifications prevent their enzymatic recognition.

As the bioluminescence capacity of CTZ is due to its imidazopyrazinone backbone, precedent studies have focused on the effect of substitution at the C-2, C-5, C-6, and C-8 positions of the backbone [2, 3, 7, 11, 12]. Although the substitution effect at

the C-2 position on enzymatic recognition is relatively low, most of the CTZ analogs substituted at C-2 position cannot show bioluminescence properties superior to those of native CTZ. In contrast to the C-2 position, the substitution at the C-8 position resulted in negligibly low bioluminescence in combination with RLuc [2, 13]. Formation of a bridge between C-5 and C-6 positions leads to more planar and rigid molecular structures and sacrifices chemical stability [14]. Although the C-6 position is an alternative site for substitution, most of the studies have focused on the chemiluminescence properties [5, 6, 15, 16].

In this protocol, we introduce the creation of efficient CTZ derivatives optimized for RLuc and its derivatives, which is the most widely used marine luciferase [17].

2 Materials

2.1 Components for Synthesis of CTZ

All solvents and routine reagents for organic synthesis can be purchased from commercial suppliers.

1. 2-Amino-3,5-dibromopyrazine (starting material; store at 5 °C).
2. Benzylmagnesium chloride solution 2.0 M in THF (Grignard reagent).
3. Zinc chloride.
4. Tetrakis(triphenylphosphine)palladium(0) (store at –30 °C).
5. Trans-2-phenylvinylboronic acid.
6. Trans-2-(4-methoxyphenyl)vinylboronic acid (Aldrich Chemical).
7. Trans-(2-([1,1'-biphenyl]-4-yl)vinyl)boronic acid (Aldrich Chemical).
8. 1.0 M Boron tribromide dichloromethane solution (Lewis acid).
9. 4-Hydroxybenzaldehyde (starting material).
10. *tert*-Butyldimethylchlorosilane.
11. Triethylamine.
12. Sodium tetrahydridoborate (reducing reagent).
13. Methanesulfonyl chloride.
14. Magnesium turnings.
15. Ethyl diethoxyacetate.

2.2 Components for Chemiluminescence Assay

All solvents for spectrometry can be purchased from commercial suppliers.

1. Native CTZ (nCTZ, Biotium) (store at –30 °C) (*see Note 1*).
2. DeepBlueC™ (Biotium) (store at –30 °C).

2.3 Components for Bioluminescence Assay

1. pcDNA3.1(+) (Invitrogen) encoding wild-type Renilla luciferase (pGL4.75) (Promega, Madison, WI, USA).
2. pcDNA3.1(+) (Invitrogen) encoding RLuc variants (RLuc8 and RLuc8.6-535) (Gambhir lab., Stanford Univ.).
3. Native CTZ (nCTZ, NanoLight Technologies, Pinetop, AZ, USA).
4. TransIT-LT1 transfection reagent (Takara, Osaka, Japan).
5. Lysis buffer (E291A) (Promega, Madison, WI, USA).
6. Hanks' balanced salt solution (HBSS).

3 Methods

3.1 General Procedure for Synthesis

1. Carry out all moisture-sensitive reactions under an atmosphere of argon.
2. The composition of mixed solvents is given by the volume ratio (v/v).
3. Record ^1H -NMR and ^{13}C -NMR spectra on an ECA-500 (JEOL Ltd.) or ECA-600 (JEOL Ltd.) spectrometer at room temperature.
4. The measurement for ^1H -NMR is performed at 500 MHz.
5. The measurement of ^{13}C -NMR is performed at 125 MHz or 150 MHz.
6. All chemical shifts are relative to an internal standard of tetramethylsilane ($\delta=0.0$ ppm) or solvent residual peaks (CDCl_3 : $\delta=7.26$ ppm, CD_3OD : $\delta=3.31$ ppm, $\text{DMSO}-d_6$: $\delta=2.50$ ppm for ^1H ; CDCl_3 : $\delta=77.16$ ppm, CD_3OD : $\delta=49.00$ ppm, $\text{DMSO}-d_6$: $\delta=39.52$ ppm for ^{13}C), and coupling constants are given in Hz.
7. Conduct flash chromatography separation using a YFLC-Al-560 chromatograph (Yamazen Co. Ltd.).
8. Perform HPLC purification on a reversed-phase column, Inertsil ODS-3 (30×50 mm) (GL Sciences Inc.), fitted on an LC-918 recycling preparative HPLC system (Japan Analytical Industry Co. Ltd.).
9. Record high-resolution MS spectra (HR-MS) on a Waters LCT premier XE with MeOH as the eluent.

3.1.1 Synthesis of 3-Benzyl-5-bromopyrazin-2-amine (See Fig. 1 Compound (2))

1. Dissolve zinc chloride (1.62 g, 11.9 mmol, 3.0 eq.) in Et_2O (12 mL) and THF (27 mL) (see Note 2).
2. Add 2.0 M benzylmagnesium chloride solution (4.4 mL, 8.94 mmol, 2.2 eq.) slowly into the solution at room temperature under argon (see Note 3).
3. Add 3,5-dibromopyrazin-2-amine (1.0 g, 3.96 mmol, 1 eq.) dissolved in THF (5 mL) into the solution (see Note 4).

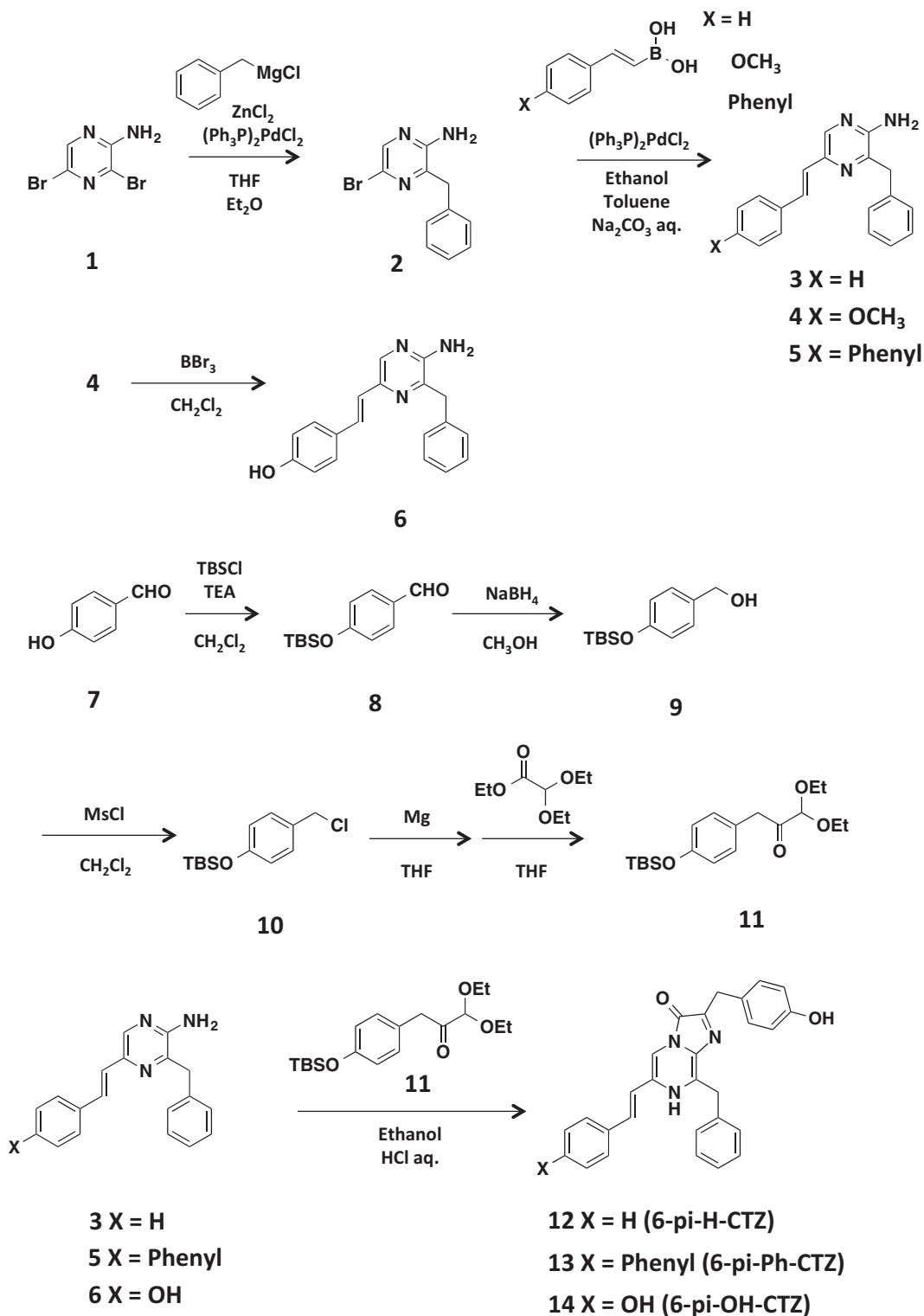


Fig. 1 Synthesis scheme for 6-pi-H-CTZ, 6-pi-Ph-CTZ, and 6-pi-OH-CTZ Compound (**2**), (**3**), (**8**), (**9**), (**10**), and (**11**) were synthesized according to reported procedure [18–20]

4. After vacuum deaeration, add a catalytic amount of tetrakis(triphenylphosphine)palladium(0) into the solution, deaerate the mixture again and stir for 23 h at room temperature (*see Note 5*).
5. Filter the solution through a Celite pad to remove the palladium catalyst, and evaporate to remove most of solvent.
6. Extract the residue with ethyl acetate, and wash the yellow organic phase with water and brine, dry over Na_2SO_4 , and evaporate.
7. Purify the resulting residue by flash chromatography (silica gel, eluent composition: *n*-hexane/ethyl acetate = 80/20 to 70/30), affording 3-benzyl-5-bromopyrazin-2-amine (**2**) as yellow liquid (0.73 g, 69%).

^1H -NMR (500 MHz, CDCl_3): δ (ppm) = 8.04 (s, 1H), 7.21–7.38 (m, 5H), 4.37 (s, 2H), 4.08 (s, 2H).

3.1.2 General Procedure for Preparations of Compounds (3) to (5)

1. Dissolve 3-benzyl-5-bromopyrazine-2-amine (**2**) (200 mg, 0.76 mmol, 1 eq.) and (*E*)-styrylboronic acid derivatives (1.22 mmol, 1.6 eq.) in toluene (16 mL) and stir at room temperature.
2. Add ethanol (2.4 mL) and 1 M Na_2CO_3 aq. (6 mL) into the reaction mixture.
3. After vacuum deaeration, add a catalytic amount of tetrakis(triphenylphosphine)palladium(0) into the solution, deaerate the mixture again, and stir for 12 h at 100 °C.
4. After cooling to room temperature, filter the solution through a Celite pad to remove the palladium catalyst.
5. Extract the solution with ethyl acetate, and wash the brown organic phase with water and brine, dry over Na_2SO_4 , and evaporate.
6. Purify the resulting residue by flash chromatography (silica gel, eluent: *n*-hexane/ethyl acetate).

3.1.3 Synthesis of (*E*)-3-Benzyl-5-styrylpyrazin-2-amine (See Fig. 1 Compound (3))

Yield 67% (yellow solid compound). Eluent composition: *n*-hexane/ethyl acetate = 67/33 to 50/50. ^1H -NMR (500 MHz, CDCl_3): δ (ppm) = 7.94 (s, 1H), 7.53 (d, J = 7.7 Hz, 2H), 7.47 (d, J = 16.0 Hz, 1H), 7.23–7.36 (m, 8H), 7.06 (d, J = 16.0 Hz, 1H), 4.49 (s, 2H), 4.12 (s, 2H). ^{13}C -NMR (125 MHz, CDCl_3): δ (ppm) = 41.5, 124.9, 126.8, 127.2, 127.9, 128.6, 128.7, 129.1, 136.7, 137.2, 139.6, 141.1, 141.3, 151.8. HR-MS: m/z calcd for $\text{C}_{19}\text{H}_{17}\text{N}_3$: 287.1422, found: 288.1501 [$\text{M} + \text{H}$] $^+$.

3.1.4 Synthesis of (*E*)-3-Benzyl-5-(4-methoxystyryl)pyrazin-2-amine (See Fig. 1 Compound (4))

Yield 50% (yellow solid compound). Eluent composition: *n*-hexane/ethyl acetate = 80/20 to 50/50. ^1H -NMR (500 MHz, CDCl_3): δ (ppm) = 7.99 (s, 1H), 7.47 (dd, J = 8.6 Hz, 16.0 Hz, 2H), 7.24–7.33 (m, 5H), 6.96 (d, J = 16.0 Hz, 1H), 6.90 (d, J = 14.6 Hz, 2H), 4.42 (s, 2H), 4.15 (s, 2H), 3.82 (s, 3H). ^{13}C -

NMR (125 MHz, CDCl_3): δ (ppm)=41.4, 55.4, 114.2, 122.8, 127.1, 128.1, 128.6, 129.1, 129.3, 130.0, 136.8, 139.2, 141.0, 141.7, 151.6, 159.5. HR-MS: m/z calcd for $\text{C}_{20}\text{H}_{19}\text{N}_3\text{O}$: 317.1528, found: 318.1606 $[\text{M}+\text{H}]^+$.

3.1.5 Synthesis of
(E)-5-(2-([1,1'-Biphenyl]-4-yl)vinyl)-3-benzylpyrazin-2-amine
(See Fig. 1 Compound (5))

Yield 24% (yellow solid compound). Eluent composition: n-hexane/ethyl acetate=80/20 to 50/50. ^1H -NMR (500 MHz, CDCl_3): δ (ppm)=8.03 (s, 1H), 7.63–7.59 (m, 7H), 7.54 (d, $J=16.0$ Hz, 1H), 7.43 (t, $J=15.5$, 2H), 7.34–7.31 (m, 3H), 7.27–7.24 (m, 3H), 7.14 (d, $J=16.0$ Hz, 1H), 4.45 (s, 2H), 4.16 (s, 2H). ^{13}C -NMR (125 MHz, CDCl_3): δ (ppm)=41.4, 124.8, 127.0, 127.2, 127.3, 127.4, 128.6, 128.9, 129.1, 136.3, 136.6, 139.6, 140.5, 140.7, 141.1, 141.3, 151.8. HR-MS: m/z calcd for $\text{C}_{25}\text{H}_{21}\text{N}_3$: 363.1735, found: 364.1814 $[\text{M}+\text{H}]^+$.

3.1.6 Synthesis of
(E)-4-(2-(5-Amino-6-benzylpyrazin-2-yl)vinyl)phenol (See Fig. 1 Compound (6))

1. Dissolve (*E*)-3-benzyl-5-(4-methoxystyryl)pyrazin-2-amine (**4**) (202 mg, 0.64 mmol, 1 eq.) in dichloromethane (12 mL) (see **Note 6**).
2. Add 1.0 M boron tribromide dichloromethane solution (12 mL) slowly into the solution at 0 °C, followed by stirring for 13 h at 40 °C (see **Note 7**).
3. After cooling to room temperature, add saturated NaHCO_3 aq. into the reaction mixture to neutralize, and evaporate to remove most of the solvent.
4. Extract the residue with ethyl acetate, and wash the yellow organic phase with water and brine, dry over Na_2SO_4 , and evaporate.
5. Purify the resulting residue by flash chromatography (silica gel, eluent composition: n-hexane/ethyl acetate=50/50), affording (*E*)-4-(2-(5-amino-6-benzylpyrazin-2-yl)vinyl)phenol (**6**) as a yellow solid (42.7 mg, 22%).

^1H -NMR (500 MHz, CD_3OD): δ (ppm)=7.93 (s, 1H), 7.37 (d, $J=8.6$ Hz, 2H), 7.38–7.21 (m, 6H), 6.93 (d, $J=16.0$ Hz, 1H), 6.77 (d, $J=8.6$ Hz, 2H), 4.11 (s, 2H). ^{13}C -NMR (125 MHz, $\text{DMSO}-d_6$): δ (ppm)=39.2, 116.1, 122.6, 126.7, 127.7, 128.3, 128.7, 128.8, 129.3, 138.7, 139.3, 139.5, 141.1, 152.9, 157.7. HR-MS: m/z calcd for $\text{C}_{19}\text{H}_{17}\text{N}_3\text{O}$: 303.1372, found: 304.1450 $[\text{M}+\text{H}]^+$.

3.1.7 Synthesis of
4-(tert-Butyldimethylsilyloxy)benzaldehyde (See Fig. 1 Compound (8))

1. Dissolve 4-hydroxybenzaldehyde (**7**) (10.0 g, 82.3 mmol, 1 eq.) and *tert*-butyldimethylsilyl chloride (13.6 g, 90.8 mmol, 1.1 eq.) in dichloromethane (400 mL).
2. Add triethylamine (15 mL) into the solution at 0 °C, followed by stirring for 18 h at room temperature.
3. Evaporate to remove a part of the solvent and wash the transparent organic phase with water and brine, dry over Na_2SO_4 , and evaporate.

4. Purify the resulting residue by flash chromatography (silica gel, eluent composition: n-hexane/ethyl acetate=90/10), affording 4-((*tert*-butyldimethylsilyl)oxy)benzaldehyde (**8**) as a water-clear viscous oil (18.5 g, 95 %).

¹H-NMR (500 MHz, CDCl₃): δ (ppm)=9.89 (s, 1H), 7.99 (d, J =8.7 Hz, 2H), 6.95 (d, J =8.7 Hz, 2H), 1.00 (s, 9H), 0.25 (s, 6H).

3.1.8 Synthesis of 4-((*tert*-Butyldimethylsilyl)oxy)phenyl)methanol (See Fig. 1 Compound (9))

1. Dissolve 4-((*tert*-butyldimethylsilyl)oxy)benzaldehyde (**8**) (18.9 g, 79.9 mmol, 1 eq.) in methanol (300 mL).
2. Add sodium borohydride (3.7 g, 97.5 mmol, 1.2 eq.) into the solution, followed by stirring for 30 min at room temperature.
3. Evaporate to remove most of the solvent.
4. Extract the residue with dichloromethane, and wash the transparent organic phase with water and brine, dry over Na₂SO₄, and evaporate, affording 4-((*tert*-butyldimethylsilyl)oxy)phenyl)methanol (**9**) as a water-clear viscous oil (21.1 g, 99 %).

¹H-NMR (500 MHz, CDCl₃): δ (ppm)=7.24 (d, J =8.4 Hz, 2H), 6.83 (d, J =8.4 Hz, 2H), 4.61 (s, 2H), 0.95 (s, 9H), 0.20 (s, 6H).

3.1.9 Synthesis of *tert*-Butyl(4-(chloromethyl)phenoxy)dimethylsilane (See Fig. 1 Compound (10)) (see Note 8)

1. Dissolve 4-((*tert*-butyldimethylsilyl)oxy)phenyl)methanol (**9**) (7.0 g, 29.3 mmol, 1.0 eq.) and triethylamine (8.0 mL, 58.6 mmol, 2.0 eq.) in dichloromethane (150 mL).
2. Add methylsulfonyl chloride (3.4 g, 44.6 mmol, 1.5 eq.) dissolved in dichloromethane (50 mL) into the solution at 0 °C, followed by stirring for 3 h at room temperature.
3. Evaporate to remove a part of the solvent and wash the transparent organic phase with water and brine, dry over Na₂SO₄, and evaporate.
4. Purify the resulting residue by flash chromatography (silica gel, eluent composition: n-hexane/ethyl acetate=95/5), affording *tert*-butyl(4-(chloromethyl)phenoxy)dimethylsilane (**10**) as a water-clear viscous oil (5.09 g, 68 %).

¹H-NMR (500 MHz, CDCl₃): δ (ppm)=7.24 (d, J =8.6 Hz, 2H), 6.81 (d, J =8.6 Hz, 2H), 4.55 (s, 2H), 0.98 (s, 9H), 0.20 (s, 6H).

3.1.10 Synthesis of 3-(4-((*tert*-Butyldimethylsilyl)oxy)phenyl)-1,1-diethoxypropane-2-one (See Fig. 1 Compound (11))

1. Add magnesium turnings (774.5 mg, 31.9 mmol, 2 eq.) into THF (10 mL), followed by additional THF (10 mL) and a catalytic amount of 1,2-dibromoethane (0.4 mL, 0.004 mmol) under argon to activate magnesium turnings.
2. Add *tert*-butyl(4-(chloromethyl)phenoxy)dimethylsilane (**10**) (4.0 g, 15.6 mmol, 1.0 eq.) dissolved in THF (40 mL) slowly into the solution, followed by stirring for 1 h at 50 °C (see **Note 9**).

3. After cooling to room temperature, the Grignard reagent is obtained as dark gray solution.
5. Add ethyl diethoxyacetate (3.2 mL, 17.9 mmol, 0.9 eq.) and THF (30 mL) into a separate reaction flask, followed by stirring at -78°C (acetone / dry ice).
4. Add the Grignard reagent slowly into the solution under argon, followed by stirring for 2 h at -78°C .
5. Add water into the solution to quench, followed by warming to room temperature and evaporating to remove most of the solvent.
6. Extract the residue with ethyl acetate, and wash the transparent organic phase with water and brine, dry over Na_2SO_4 , and evaporate.
7. Purify the resulting residue by silica gel chromatography (eluent composition: n-hexane/ethyl acetate = 95/5 to 90/10), affording 3-(4-((*tert*-butyldimethylsilyl)oxy)phenyl)-1,1-diethoxypropane-2-one (**11**) as a water-clear viscous oil (2.5 g, 47%).

$^1\text{H-NMR}$ (500 MHz, CDCl_3): δ (ppm) = 7.08–7.06 (m, 2H), 6.79–6.76 (m, 2H), 4.62 (s, 1H), 3.80 (s, 2H), 3.73–3.63 (m, 2H), 3.58–3.48 (m, 2H), 1.24 (t, J = 7.1 Hz, 6H), 0.97 (s, 9H), 0.18 (s, 6H).

3.1.11 General

Procedure for Preparation of Compounds (12) to (14)

1. Dissolve corresponding coelenteramines (compounds (**3**), (**5**), or (**6**)) (0.10 mmol, 1 eq.) and ketoacetal (**11**) (76.62 mg, 0.21 mmol, 2 eq.) in ethanol (2.0 mL) and H_2O (0.2 mL) and stir at room temperature (see **Note 10**).
2. Cool the solution to 0°C and add HCl (0.1 mL) under nitrogen flow.
3. Once the solution reached room temperature, heat it and stir for 10 h at 70°C .
4. Evaporate the solvent under vacuum and purify the crude compound by semi-preparative reversed-phase HPLC.

3.1.12 Synthesis

of (E)-8-Benzyl-2-(4-hydroxybenzyl)-6-styrylimidazo[1,2-a]pyrazin-3(7H)-one (See Fig. 1 Compound (12)) (6-*pi*-H-CTZ)

Yield 22 % (yellow solid compound). Eluent composition: MeCN/ H_2O = 50/50 with 0.1 % formic acid.

$^1\text{H-NMR}$ (500 MHz, CD_3OD , CDCl_3): δ (ppm) = 7.59 (s, 1H), 7.37–7.21 (m, 10H), 7.15 (d, J = 8.6 Hz, 2H), 6.96 (t, J = 16.3 Hz, 1H), 6.69 (d, J = 8.6 Hz, 2H), 4.40 (s, 2H), 4.04 (s, 2H). $^{13}\text{C-NMR}$ (150 MHz, CD_3OD , CDCl_3): δ (ppm) = 33.8, 115.9, 127.4, 127.9, 129.3, 129.3, 129.4, 129.5, 130.5, 156.3. HR-MS: m/z calcd for $\text{C}_{28}\text{H}_{23}\text{N}_3\text{O}_2$: 433.1790, found: 432.1712 $[\text{M-H}]^-$.

3.1.13 Synthesis of (E)-6-(2-([1,1'-Biphenyl]-4-yl)vinyl)-8-benzyl-2-(4-hydroxybenzyl)imidazo[1,2-a]pyrazin-3(7H)-one (See Fig. 1 Compound (13)) (6-*pi*-Ph-CTZ)

Yield 5 % (yellow solid compound). Eluent composition; CH₃OH/H₂O = 20/1 with 0.1 % formic acid.

¹H-NMR (500 MHz, CD₃OD): δ (ppm) = 7.62–7.57 (m, 7H), 7.43–7.22 (m, 9H), 7.15 (d, J = 8.59 Hz, 2H), 7.05 (d, J = 15.4 Hz, 1H), 6.69 (d, J = 8.59 Hz, 2H), 4.39 (s, 2H), 4.03 (s, 2H). ¹³C-NMR (150 MHz, CD₃OD, CDCl₃): δ (ppm) = 21.4, 116.2, 127.8, 128.3, 128.5, 129.7, 129.9, 130.7, 130.8, 131.3, 136.6, 138.2, 141.7, 142.4, 156.9. HR-MS: m/z calcd for C₃₄H₂₇N₃O₂: 509.2103, found: 509.2025 [M-H]⁻.

3.1.14 Synthesis of (E)-8-Benzyl-2-(4-hydroxybenzyl)-6-(4-hydroxystyryl)imidazo[1,2-a]pyrazin-3(7H)-one (See Fig. 1 Compound (14)) (6-*pi*-OH-CTZ)

Yield 23 % (yellow solid compound). Eluent composition: MeCN/H₂O = 33/67 with 0.1 % formic acid.

¹H-NMR (500 MHz, CD₃OD, CDCl₃): δ (ppm) = 7.52 (s, 1H), 7.41–7.21 (m, 10H), 6.82 (d, J = 8.6 Hz, 2H), 6.82 (d, J = 8.6 Hz, 2H), 6.75 (d, J = 8.6 Hz, 2H), 6.62 (d, J = 16.9 Hz, 1H), 4.37 (s, 2H), 4.09 (s, 2H). ¹³C-NMR (150 MHz, CD₃OD, CDCl₃): δ (ppm) = 33.6, 33.8, 108.6, 115.9, 116.4, 127.9, 128.9, 129.3, 129.4, 130.5, 137.3, 149.9, 153.7, 156.2, 158.8. HR-MS: m/z calcd for C₂₈H₂₃N₃O₃: 449.1739, found: 448.1661 [M-H]⁻.

3.2 Chemiluminescence Assay of CTZ Derivatives

1. Add a CH₃OH solution of the respective CTZ derivative (1 mM, 200 μ L) to a quartz cell and set in a SREX Fluorolog-3 fluorescence spectrophotometer (Model FL-3-11, Horiba Jobin Yvon, Kyoto, Japan).
2. Measure the chemiluminescence spectra at a scan rate of 1200 nm/min after injecting 2 mL of DMSO (see **Note 11**). Figure 1 shows the chemiluminescence spectra of native CTZ and the CTZ derivatives (see Fig. 2).

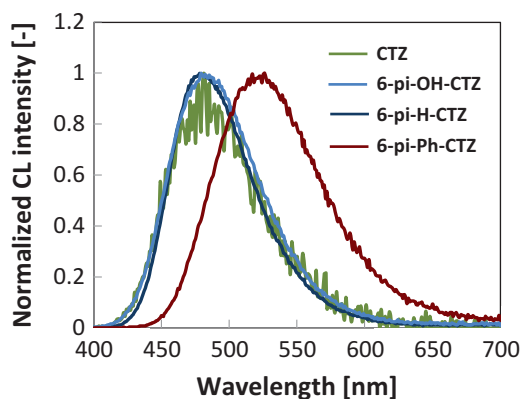


Fig. 2 Chemiluminescence spectra. The chemiluminescence reaction is triggered by addition of DMSO to stock solution of MeOH. The spectra are normalized to 1 at the peak emission

3.3 Bioluminescence Assay of CTZ Derivatives

3.3.1 General Procedure for Bioluminescence Assay

1. To determine the bioluminescence properties of the novel CTZ derivatives, transfect plasmids encoding wild-type Renilla luciferase (RLuc), and variants RLuc8 and RLuc8.6 separately into COS-7 cells cultured in a 24-well plate using a TransIT-LT1 transfection reagent (Takara, Osaka, Japan).
2. Incubate the cells for 48 h and treat with a lysis buffer (E291A) (Promega, Madison, WI, USA) according to the manufacturer's protocol.

3.3.2 Bioluminescence Intensities

1. To measure bioluminescence intensities, mix an aliquot of the cell lysate (1 μ L) with Hanks' balanced salt solution (HBSS) (50 μ L) containing 2 μ M native CTZ or the respective CTZ derivative in Röhren polystyrene tubes (Sarstedt, Nümbrecht, Germany).
2. Measure the bioluminescence intensities of native CTZ and the derivatives immediately for the first 1 s with a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany) (*see* Fig. 3).

3.3.3 Kinetic Profiles of Bioluminescence

1. To measure the kinetic profiles of bioluminescence, continue signal monitoring for 600 s after mixing.
2. The reaction conditions are identical to those described in the previous section (*see* Table 1).

3.3.4 Bioluminescence Spectra

1. For the recording of bioluminescence spectra, mix an aliquot of the lysate (100 μ L) with HBSS (400 μ L) containing 20 μ M native CTZ or CTZ derivative in a quartz cell, and measure the mixture with a F-7000 spectrophotometer (Hitachi, Tokyo, Japan) at a scan rate of 2400 nm/min.

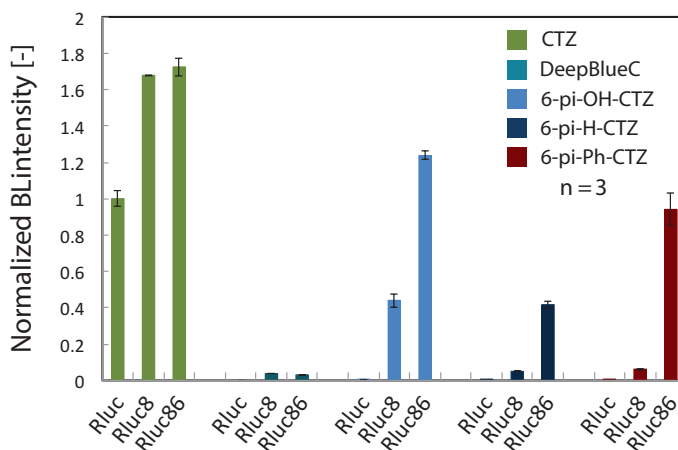


Fig. 3 Bioluminescence intensity. The intensities are normalized to intensity of native CTZ in combination with RLuc. C-6 substituted CTZ derivatives show drastically robust bioluminescence compared to that of DeepBlueC™ in combination with RLuc8. DeepBlueC™/RLuc8 pair is applied as bioluminescence resonance energy transfer (BRET) research

Table 1 Kinetic profile of native CTZ, CTZ derivatives (6-pi-X-CTZ: X = OH, H, Phenyl) and DeepBlueC™

	Native CTZ	OH	H	Phenyl	DeepBlueC™
BL half-life time (RLuc8) [s]	433	544	>600	213	342
BL half-life time (RLuc8.6) [s]	313	463	325	182	59

The kinetic profiles are obtained in 1 s intervals for 600 s

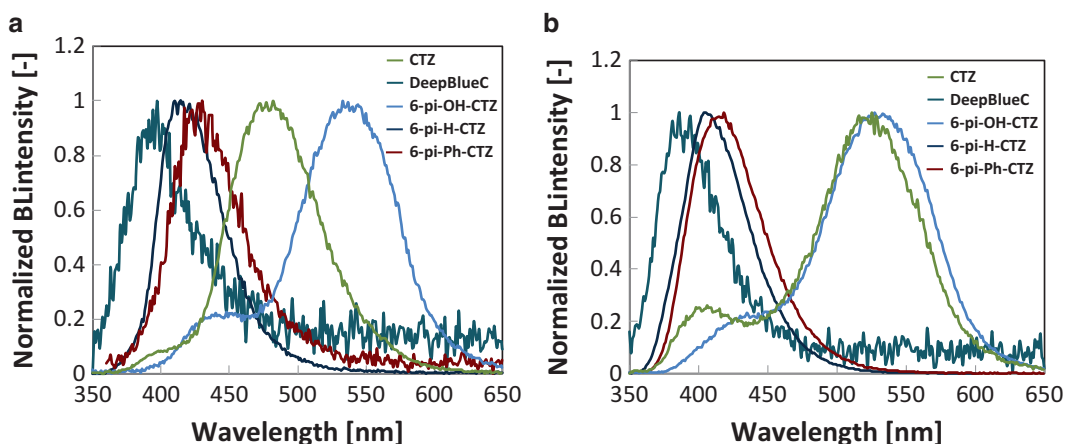


Fig. 4 Bioluminescence spectra obtained with (a) RLuc8 and (b) RLuc8.6. The spectra are normalized to 1 at the peak. All CTZ derivatives except for 6-pi-OH-CTZ show an approximately 40 nm blue-shifted emission compared to native CTZ/RLuc pair (480 nm). These blue-shifted spectra are in a wavelength range similar to that of DeepBlueC™

2. Determine the wavelengths of maximal bioluminescence intensities (λ_{\max}) using the instrument software (FL Solutions ver. 2.1) (*see* Fig. 4).

4 Notes

1. CTZ should be stored at $-30\text{ }^{\circ}\text{C}$ and protected from light. In addition, it should be stored in the solid state, because this is more stable than the liquid state. If CTZ is stored as ethanol or methanol stock solution, the stability is enhanced by addition of a trace of HCl [21].
2. To efficiently prepare the organozinc reagent, zinc chloride needs to be sufficiently dried before addition of benzylmagnesium chloride.
3. Benzylmagnesium chloride should be used and stored under inert gas. We find that it is best to prepare this fresh.
4. 3,5-Dibromopyrazin-2-amine (**1**) needs to be dried enough before adding.

5. Tetrakis(triphenylphosphine)palladium(0) is unstable under aerobic conditions. The reaction mixture needs to be deaerated before and after adding of palladium catalyst.
6. (*E*)-3-Benzyl-5-(4-methoxystyryl)pyrazin-2-amine (**4**) needs to be sufficiently dried before adding.
7. Boron tribromide is decomposed by water. The reaction system needs to be sufficiently dried before adding of boron tribromide.
8. 3-(4-((*tert*-Butyldimethylsilyl)oxy)phenyl)-1,1-ethoxypropane-2-one (**11**) can be synthesized from *tert*-butyl[4-(bromomethyl)phenoxy]dimethylsilane, which is the bromo compound corresponding to compound (**10**). However, the stability of *tert*-butyl[4-(bromomethyl)phenoxy]dimethylsilane is lower than compound (**10**) [19].
9. *tert*-Butyl(4-(chloromethyl)phenoxy)dimethylsilane (**10**) is delivered dropwise into the reaction mixture immediately after magnesium turnings start to activate.
10. CTZ is very unstable under aerobic conditions. Therefore, after dissolving the coelenteramine and the ketoacetal in solvents, the solution should be deaerated.
11. In DMSO, CTZ is decomposed while showing chemiluminescence emission. After injection of DMSO, the measurement was started immediately.

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