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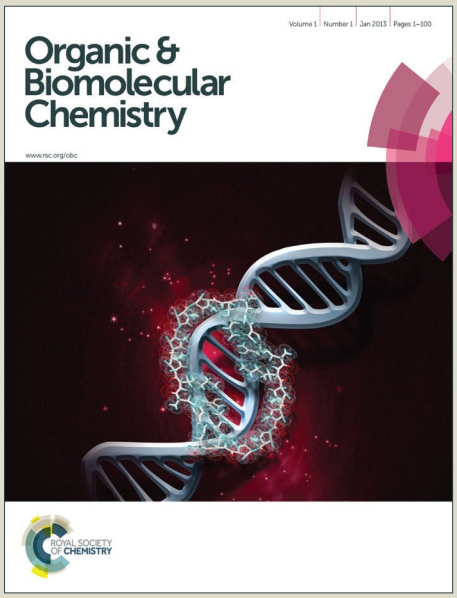

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FULL ARTICLE

Novel bioluminescent coelenterazine derivatives with imidazopyrazinone C-6 extended substitution for *Renilla* luciferase

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Two series (alkynes and triazoles) of novel coelenterazine analogs with imidazopyrazinone C-6 extended substitutions have been well designed and synthesized successfully for extension of bioluminescence substrates. After extensive evaluation, some compounds display excellent bioluminescence properties compared with DeepBlueC *in cellulo*, thus becoming potential molecules involved in bioluminescence techniques.

Introduction

Bioluminescence is a luminescent phenomenon that results from an enzyme-catalyzed reaction within a living organism, which widely exists in lots of organisms, such as bacteria, insects, and marine organisms[1-3]. In a general bioluminescence system, a molecule named luciferin is oxidized by the corresponding luciferase to produce the bioluminescence signal with co-factors or not. For example, coelenterazine (CTZ) is a universal substrate that can be recognized and catalyzed by various luciferases, such as *Renilla*, *Gaussia,* and *Oplophorus,* to yield bioluminescence[2, 4]. Coelenterazine – luciferase system is the simplest bioluminescence system, and the mechanism is biocatalysis by luciferase to yield strong peak emission around 480 nm without any cofactor[2, 5]. Coelenterazine was discovered and defined by the Cormier's and Johnson's groups[6-12]. So far, a mass of coelenterazine analogues have been prepared ever since it was chemically synthesized in 1975[13, 14], and the bioluminescent and chemiluminescent properties of these analogues have been well investigated as well. The modification at C-2, C-6 and C-8 positions of the imidazopyrazinone core is the most important to influence the bioluminescence intensity and maximum emission wavelength[15]. Some of CTZ analogues have been reported remarkably with their individual characteristics on bioluminescent intensity, peak wavelength, half decay, etc. Among them, coelenterazine 400a (DeepBlueC) is a case in point that was reported by Milton J. Cormier et al in the period of 1973-1979,[10, 16, 17] and therefore, it has been a common coelenterazine analogue that widely be used in the bioluminescent assay. Compared with coelenterazine, DeepBlueC does not have hydroxyl groups at the C-2 and C-6 position, which leads to easier synthesis and blue-shifted bioluminescence.

As mentioned above, alternative substituents at the C-6 position of the imidazopyrazinone core effects on bioluminescence properties. Most of the them are aromatic groups according to previously reported[15]. To further investigate the influence of substituents at the C-6 position of coelenterazine, we designed and synthesized two series (**A** and **T**, short for alkynes and triazoles) of substrates with imidazopyrazinone C-6 extended substitution based on DeepBlueC and investigated their bioluminescent properties with Renilla and Gaussia luciferase in vitro herein. As for alkynes series (**A**) of coelenterazine derivatives (Scheme 1), the introduction of alkyne substituents at the C-6 position leads to more rigid molecular structure. Besides, triazole series (**T**) compounds (Scheme 2) have the triazole substituents by taking the place of the benzene group of DeepBlueC. The synthesis was based on the classic synthesis of coelenterazine[18-20]. The key intermediates **1** and **3** were

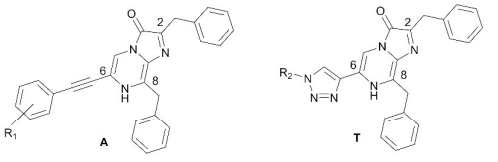

Figure 1. Structures of novel compounds.

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obtained according to the previous report[20]. As for alkynes series (A), key intermediates **2** were synthesized in good yield via Sonogashira coupling reaction[21]. When it comes to triazole series (**T**), the key intermediates **7** were produced by the click reaction[22].

Methods and Materials

Materials and instruments

All reagents and solvents available were used as received unless otherwise noted. All reactions were monitored by TLC with 0.25 mm silica gel plates (60GF-254). UV light, iodine stain, and ninhydrin were used to visualize the spots. Silica gel was utilized for column chromatography purification. ^{1}H NMR and ^{13}C-NMR were recorded on a Bruker DRX spectrometer at 300 or 400 MHz, δ in parts per million and J in hertz, using TMS as an internal standard. Mass spectra were performed by the analytical and the mass spectrometry facilities at Shandong University. HPLC tests were measured with Agilent Technologies 1260 liquid chromatography (Singapore City, Singapore). Water used for the fluorescence and bioluminescence studies was doubly distilled and further purified with a Mill-Q filtration system (Millipore, Watertown, MA, USA). Bioluminescence measurements were determined with an IVIS Kinetic (Caliper Life Sciences, USA) equipped with a cooled charge-coupled device (CCD) camera or Omega microplate reader (POLARstar Omega, Germany). Bioluminescence spectra were measured with F-2500 FL Spectrophotometer (HITACHI High Technologies Corporation, Tokyo, Japan). Fluorescence spectra were obtained with a Varioskan microplate spectrophotometer (Thermo Electron Corporation, Waltham, MA, USA). Recombinant *Renilla reniformis* luciferase was purchased from RayBiotech (Norcross, GA, USA). Recombinant *Gaussia* luciferase was purchased from NanoLight (Pinetop, AZ, USA). ES-2 cells (human ovarian cancers cell line) expressing *Reilla* luciferase (Rluc) were purchased from Shanghai BioDiagnosis Co., Ltd. Coelenterazine was purchased from Chemedir Biopharm-tech.Co., Ltd.

Organic synthesis

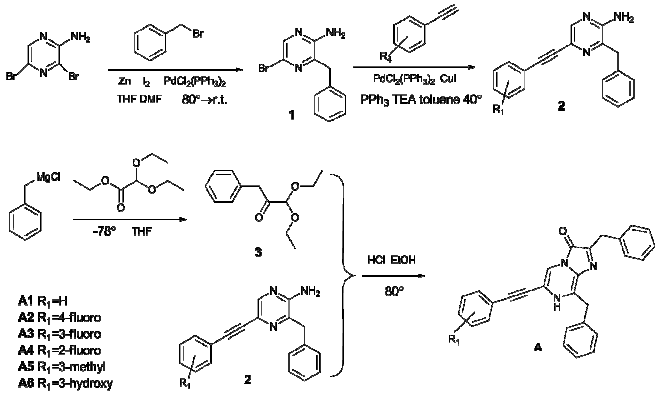
The preparation of 3-benzyl-5-bromo-2-amino-pyrazine (**1**)[20]:

Zn dust (235 mg, 3.6 mmol) and I_2 (12 mg) were suspended in fresh anhydrous THF under argon atmosphere and the mixture was stirred at room temperature until the brown color of I_2 disappeared. Then the anhydrous benzyl bromide was added by using a syringe and the reaction mixture was refluxed at 80 °C for 3 h. After insertion of Zn, the reaction mixture was cooled to room temperature. Then the suspension of 2-amino-3, 5-dibromopyrazine (506 mg, 2 mmol) and $PdCl_2(PPh_3)_2$ (70 mg, 0.1 mmol) in 7 mL of DMF was added. The reaction mixture was continuously stirred overnight. Then the mixture was filtered by celatom and the filtrate was collected and evaporated under vacuum. The collection was dissolved in and extracted by ethyl acetate and washed with saturated sodium chloride solution. After dried over anhydrous sodium sulfate and concentrated under reduced pressure, the crude product was further purified by chromatography on silica gel (PE/EtOAc 20:3) to give a viscous yellow solid (313 mg). Yield: 59%. ^{1}HNMR (400 MHz, DMSO-d_6): δ 7.65 (s, 1 H), 7.30~7.25 (m, 5 H), 6.56 (s, 1 H), 3.98(s, 2H). ESI-MS: m/z [M+H] + calcd for 264.01, 266. 01, found 264.2, 266.3。

The preparation of 2-amino-3-benzyl-5- phenylethynyl -pyrazine (**2-1**)[21]:

The suspension of $PdCl_2(PPh_3)_2$ (27 mg, 0.038 mmol), CuI (29 mg, 0.151 mmol) and PPh_3 (20 mg, 0.076 mmol) in 2 mL toluene was added to the solution of 3-benzyl-5-bromo-2-amino-pyrazine (200 mg, 0.76 mmol) in 3 mL toluene under argon atmosphere. Then phenylacetylene (232 mg, 2.27 mmol) and triethylamine (0.7 mL) were sequentially added. The mixture was stirred at 40 °C for 6 h. Then the mixture was filtered and the filtrate was collected and evaporated under vacuum. The collection was extracted by ethyl acetate and washed with saturated sodium chloride solution. After dried over anhydrous sodium sulfate and concentrated under reduced pressure, the crude product was further purified by chromatography on silica gel (PE/EtOAc 10:1) to give a yellow solid (165 mg). Yield: 77%. ^{1}HNMR (400 MHz, DMSO-d_6): δ8.09(s, 1H), 7.43~7.41(m, 6H), 7.29~7.28(m, 4H), 6.81(s, 2H), 4.04(s, 2H). ^{13}CNMR (100 MHz, DMSO-d_6): δ 153.21, 144.57, 141.87, 138.19, 129.29, 129.22, 129.13, 128.78, 126.75, 122.69, 88.96, 88.32, 38.83. ESI-HRMS: m/z $[M+H]^+$ calcd for 286.1344, found 286.1341.

2-Amino-3-benzyl-5-(4-fluorophenylethynyl)-pyrazine (**2-2**): yellow



Scheme 1: the synthesis route of alkynes series (A) coelenterazine analogs.

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solid; yield 50%; ^{1}HNMR (400 MHz, DMSO-d_6): δ8.09(s, 1H), 7.61~7.58(m, 2H), 7.48~7.43(m, 2H), 7.30~7.21(m, 5H). 6.82(s, 2H), 4.03(s, 2H). ^{13}CNMR (100 MHz, DMSO-d_6): δ 163.67, 161.21, 153.23, 144.59, 138.97, 134.85, 133.97, 129.29, 128.78, 126.75, 125.15, 119.11, 116.58, 116.36, 88.03, 87.90, 38.82. ESI-HRMS: m/z $[M+H]^+$ calcd for 304.1250, found 304.1244.

2-Amino-3-benzyl-5-(3-fluorophenylethynyl)-pyrazine (**2-3**): yellow solid; yield 45%; ^{1}HNMR (400 MHz, DMSO-d_6): δ8.11(s, 1H), 7.49~7.38(m, 3H), 7.32~7.19(m, 6H), 6.86(s, 2H), 4.04(s, 2H). ^{13}CNMR (100 MHz, DMSO-d_6): δ 163.57, 161.14, 153.36, 144.91, 141.96, 138.14, 131.37, 131.28, 129.29, 128.79, 128.06, 128.04, 126.77, 124.75, 124.73, 118.30, 118.08, 116.51, 116.30, 89.32, 87.74, 87.71, 38.80.ESI-HRMS: m/z $[M+H]^+$ calcd for 304.1250, found 304.1253.

2-Amino-3-benzyl-5-(2-fluorophenylethynyl)-pyrazine (**2-4**): yellow solid; yield 44%; ^{1}HNMR (400 MHz, DMSO-d_6): δ8.11(s, 1H), 7.64~7.60(td, J=7.53Hz, 1.6Hz, 1H), 7.48~7.46(m, 1H), 7.36~7.20(m, 7H), 6.88(s, 2H), 4.04(s, 2H). ^{13}CNMR (100 MHz, DMSO-d_6): δ 163.44, 160.96, 153.39, 144.80, 138.11, 133.79, 129.28, 128.79, 126.77, 125.32, 116.31, 116.10, 93.23, 82.25, 38.81. ESI-HRMS: m/z $[M+H]^+$ calcd for 304.1250, found 304.1242.

2-Amino-3-benzyl-5-(3-methylphenylethynyl)-pyrazine (**2-5**): yellow solid; yield 62%; ^{1}HNMR (400 MHz, DMSO-d_6): δ8.07(s, 1H), 7.36~7.19(m, 9H), 6.77(s, 2H), 4.03(s, 2H), 2.31(s, 3H). ^{13}CNMR (100 MHz, DMSO-d_6): δ 153.17, 144.48, 141.87, 138.54, 138.21, 132.10, 129.90, 129.29, 129.10, 128.78, 128.74, 126.75, 125.38, 125.54, 89.11, 88.04, 38.85, 21.18. ESI-HRMS: m/z $[M+h]^+$ calcd for 300.1501, found 300.1500.

2-Amino-3-benzyl-5-(3-hydroxyphenylethynyl)-pyrazine (**2-6**): yellow solid; yield 62%; ^{1}HNMR (400 MHz, DMSO-d_6): δ9.69(s, 1H), 8.07(s, 1H), 7.29~7.26(m, 3H), 7.22~7.18(t, J=8Hz, 2H), 6.95(d, J=8Hz, 1H), 6.87(s, 1H), 6.82~6.79(m, 1H), 6.77(s, 2H), 4.03(s, 2H). ^{13}CNMR (100 MHz, DMSO-d_6): δ 157.83, 153.11, 144.56, 141.78, 138.20, 130.34, 129.28, 128.78, 126.75, 125.31, 123.53, 122.54, 118.03, 116. 68, 89.09, 87.71, 38.83. ESI-HRMS: m/z $[M+H]^+$ calcd for 302.1293, found 302.1293.

The preparation of 3-phenyl - 1,1- diethoxyacetone (**3**)[20, 23]:

Ethyl diethoxyacetate (500 mg, 2.84 mmol) was dissolved in fresh anhydrous THF, and the solution was cooled to -78 °C under argon atmosphere. Then benzylmagnesium chloride (642 mg, 4.26 mmol) solution was added via syringe over 15 min, and the reaction was allowed to stir for 3 h. The reaction was quenched by addition of ammonium chloride aqueous solution and then allowed to warm to room temperature. The reagent was evaporated under vacuum and redissolved in ethyl acetate. After extraction by ethyl acetate, the organic layer was washed with saturated sodium chloride aqueous solution. The mixture was concentrated under reduced pressure and then was subjected to chromatography on silica gel (PE/EtOAc 20:1) to give a colorless oil (380mg). Yield: 60%. ^{1}HNMR (400 MHz, DMSO-d_6): δ7.31~7.16 (m, 6H), 4.80(s, 1H) 3.87 (s, 2 H), 3.66~3.53 (m, 4H). ESI-MS: m/z $[M+NH_4]^+$ calcd for 240.16, found 240.5.

The preparation of 2-benzyl-8-benzyl-6-(phenylethynyl)imidazo[1,2-a]pyrazin-3(7*H*)-one (**A1**)[18, 20]:

The mixture of f 2-amino-3-benzyl-5- phenylethynyl -pyrazine (**4-1**, 80 mg, 0.28 mmol) and 3-phenyl - 1,1- diethoxyacetone (**3-1**, 124 mg, 0.5 6mmol) was dissolved in ethanol (3 mL) under argon atmosphere and allowed to stir at room temperature for 10 min. The con.HCl (0.2 mL) in ethanol (1 mL) was then added to the mixture via syringe over 10 min. The reaction was heated to refluxed at 80 °C for 8 h and then allowed to cool to room temperature. The crude was concentrated under vacuum and further purified by chromatography on silica gel (CH_2Cl_2/MeOH 50:1) to give a light brown solid (30 mg). Yield: 26%. Analytical RP HPLC (Phenomenex, C8, 250 x 4.6 mm column): 60% acetonitrile with 0.1% trifluoroacetic acid, 1.0 mL/min at 380 nm, Rt: 7.448 min, 97%. ^{1}HNMR (400 MHz, CD_3OD): δ8.47(s, 1H), 7.58~7.24(m, 15H), 4.59(s, 2H), 4.34(s, 2H). ^{13}CNMR (100 MHz, CD_3OD): δ147.24, 137.12, 134.94, 131.58, 129.75, 128.94, 128.60, 128.45, 128.42, 127.28, 126.60, 120.68, 117.41, 94.22, 80.17, 35.89, 31.07. ESI-HRMS: m/z $[M+H]^+$ calcd for 416.1763, found 416.1757.

2-Benzyl-8-benzyl-6- (4-fluorophenylethynyl)imidazo[1,2-a]pyrazin-3(7*H*)-one (**A2**): yellow solid; yield 35%. Analytical RP HPLC (Phenomenex, C8, 250 x 4.6 mm column): 60% acetonitrile with 0.1% trifluoroacetic acid, 1.0 mL/min at 380 nm, Rt: 7.842 min, 96%. ^{1}HNMR (400 MHz, CD_3OD): δ8.07(s, 1H), 7.64~7.60(m, 2H), 7.38~7.36(m, 2H), 7.29~7.21(m, 7H), 7.19~7.15(m, 3H), 4.42(s, 2H), 4.22(s, 2H). ^{13}CNMR (100 MHz, CD_3OD): δ164.74, 162.25, 147.35, 140.52, 137.03, 134.98, 134.07, 134.03, 133.98, 128.85, 128.38, 128.34, 127.23, 127.10, 126.57, 117.39, 115.84, 115.61, 92.98, 80.05, 35.85, 30.94. ESI-HRMS: m/z $[M+H]^+$ calcd for 434.1669, found 434.1663.

2-Benzyl-8-benzyl-6- (3-fluorophenylethynyl)imidazo[1,2-a]pyrazin-3(7*H*)-one (**A3**): yellow solid; yield 30%. Analytical RP HPLC (Phenomenex, C8, 250 x 4.6 mm column): 60% acetonitrile with 0.1% trifluoroacetic acid, 1.0 mL/min at 370 nm, Rt: 6.173 min, 97%. ^{1}HNMR (400 MHz, CD_3OD): δ8.58(s, 1H), 7.46~7.20(m, 14H), 4.59(s, 2H), 4.34(s, 2H). ^{13}CNMR (100 MHz, CD_3OD): δ163.64, 161.19, 147.39, 137.00, 134.98, 130.58, 130.49, 128.56, 128.40, 128.34, 127.75, 127.22, 126.60, 118.19, 117.95, 117.72, 117.04, 116.82, 92.29, 81.25, 35.98, 30.83. ESI-HRMS: m/z $[M+H]^+$ calcd for 434.1669, found 434.1664.

2-Benzyl-8-benzyl-6- (2-fluorophenylethynyl)imidazo[1,2-a]pyrazin-3(7*H*)-one (**A4**): yellow solid; yield 14%. Analytical RP HPLC (Phenomenex, C8, 250 x 4.6 mm column): 65% acetonitrile with 0.1% trifluoroacetic acid, 1.0 mL/min at 380 nm, Rt: 5.788 min, 97%. ^{1}HNMR (400 MHz, CD_3OD): δ8.57(s, 1H), 7.69~7.65(td, J=7.36Hz, 1.6Hz, 1H), 7.52~7.43(m, 3H), 7.34~7.22(m, 10H), 4.59(s, 2H), 4.34(s, 2H). ^{13}CNMR (100 MHz, CD_3OD): δ164.06, 161.55, 147.41, 140.59, 136.99, 134.98, 133.59, 128.80, 128.40, 128.31, 127.21, 126.60, 124.42, 124.39, 117.71, 115.55, 15.34, 109.38, 109.22, 87.15, 85.15, 35.96, 30.82. ESI-HRMS: m/z $[M+H]^+$ calcd for 434.1669, found 434.1662.

2-Benzyl-8-benzyl-6-(3-methylphenylethynyl)imidazo[1,2-a]pyrazin-3(7*H*)-one (**A5**): yellow solid; yield 40%. Analytical RP HPLC

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(Phenomenex, C8, 250 x 4.6 mm column): 65% acetonitrile with 0.1% trifluoroacetic acid, 1.0 mL/min at 380 nm, Rt: 6.910 min, 95%. ^{1}HNMR (400 MHz, CD_3OD): δ7.70(s, 1H), 7.40~7.18(m, 14H), 4.34(s, 2H), 4.18(s, 2H), 2.36(s, 3H). ^{13}CNMR (100 MHz, CD_3OD): δ147.24, 140.63, 138.54, 138.47, 137.10, 134.99, 134.95, 134.02, 131.94, 130.95, 130.58, 128.86, 128.79, 128.72, 128.56, 128.37, 128.22, 128.12, 127.24, 126.56, 120.95, 120.57, 117.28, 94.50, 79.72, 35.73, 31.06, 20.1. ESI-HRMS: m/z $[M+H]^+$ calcd for 430.1919, found 430.1914.

2-Benzyl-8-benzyl-6-(3-hydroxyphenylethynyl)imidazo[1,2-a]pyrazin-3(7*H*)-one (**A6**): yellow solid; yield 15%. Analytical RP HPLC (Phenomenex, C8, 250 x 4.6 mm column): 60% acetonitrile with 0.1% trifluoroacetic acid, 1.0 mL/min at 370 nm, Rt: 4.946 min, 99%. ^{1}HNMR (400 MHz, CD_3OD): δ7.69(s, 1H), 7.41~7.18(m, 14H), 4.33(s, 2H), 4.18(s, 2H). ^{13}CNMR (100 MHz, CD_3OD): δ157.37, 147.15, 137.09, 134.96, 129.59, 128.80, 128.57, 128.36, 127.25, 126.56, 122.87, 121.52, 117.94, 117.28, 94.44, 79.38, 35.66, 31.11. ESI-HRMS: m/z $[M+H]^+$ calcd for 432.1712, found 432.1701.

The preparation of 2-amino-3-benzyl- 5-((trimethylsilyl)ethynyl)-pyrazine (**4**)[24, 25]:

The suspension of $PdCl_2(PPh_3)_2$ (27 mg, 0.038 mmol), CuI (29 mg, 0.151 mmol) and PPh_3 (20 mg, 0.076 mmol) in 2 mL toluene was added to the solution of 3-benzyl-5-bromo-2-amino-pyrazine (200 mg, 0.76 mmol) in 3 mL toluene under argon atmosphere. Then trimethylsilylacetylene (223 mg, 0.27 mmol) and triethylamine (0.8 mL) were sequentially added. The mixture was stirred at 40 °C for 9 h. Then the mixture was filtered, and the filtrate was collected and evaporated under vacuum. The collection was extracted by ethyl acetate and washed with saturated sodium chloride solution. After dried over anhydrous sodium sulfate and concentrated under reduced pressure, the crude product was further purified by chromatography on silica gel (PE/EtOAc 10:1) to give a light yellow solid (129 mg). Yield: 60%.^{1}HNMR (400 MHz, DMSO-d_6): δ 7.98 (s, 1 H), 7.29~7.20 (m, 5H), 6.79 (s, 2 H), 3.99 (s, 2H). ^{13}CNMR (100 MHz, DMSO-d_6): δ 152.42, 143.94, 140.85, 137.30, 128.36, 127.92, 125.88, 124.25, 103.09, 92.93, 37.91, 0.50. ESI-HRMS: m/z $[M+H]^+$ calcd for 282.1426, found 282.1418.

The preparation of 2-amino-3-benzyl- 5-ethynyl-pyrazine (**5**):

Potassium carbonate (40 mg, 0.284 mmol) was added to the solution of 2-amino-3-benzyl- 5-((trimethylsilyl)ethynyl)-pyrazine (100 mg, 0.356 mmol) in methanol and the mixture was stirred at room temperature for 2h. Then the reagent was evaporated under vacuum, and the crude was redissolved in ethyl acetate. The organic layer was washed with saturated sodium chloride solution after extraction with ethyl acetate. After dried over anhydrous sodium sulfate and concentrated under reduced pressure, the crude product was further purified by chromatography on silica gel (PE/EtOAc 20:3) to give a light yellow solid (50 mg). Yield: 67%. ^{1}HNMR (400 MHz, DMSO-d_6): δ 7.98 (s, 1 H), 7.30~7.20 (m, 5H), 6.76 (s, 2 H), 4.15 (s, 2H), 3.99(s, 2H). ^{13}CNMR (100 MHz, DMSO-d_6): δ 153.38, 144.56, 141.72, 138.13, 129.30, 128.75, 126.73, 124.75, 82.36, 80.32, 38.77. ESI-HRMS: m/z [M+H +] calcd for 210.1031, found 210.1019.

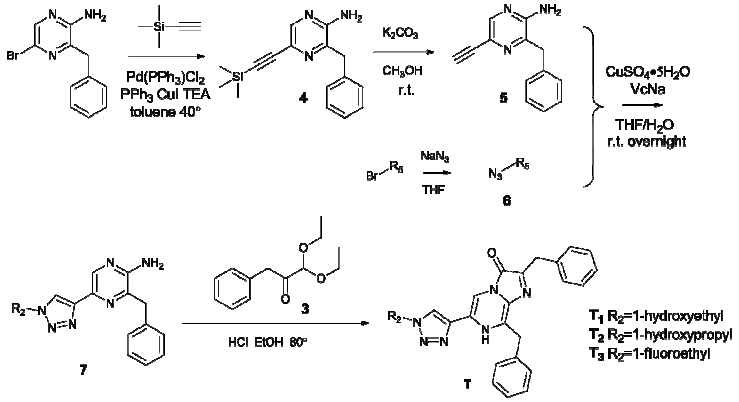
The preparation of 2-azidoethanol (**6-1**)[26]:

2-Bromoethanol (1.76 g, 14.08 mmol) was dissolved in water (3 mL) and then sodium azide (1.83 g, 28.17 mmol) was added to this mixture with stirring. The mixture was stirred at room temperature for 20 h. The mixture was extracted with diethyl ether, and the organic layer was washed with saturated sodium chloride solution. After dried over anhydrous sodium sulfate and concentrated under reduced pressure, the crude product was applied to the next reaction without further purification.

3-Azido-1-propanol (**6-2**) and 1-azido-2-fluoroethane (**6-3**) were prepared with similar method.

The preparation of 2-amino-3-benzyl- 5- [(1-hydroxyethyl)-1, 2, 3-triazol-4]-pyrazine (**7-1**)[22]:

Copper sulfate pentahydrate (2 mg, 7.15 μmol) and sodium ascorbate (4 mg, 1.45 μmol) in water (1.5 mL) were sequentially added to the mixture of 2-amino-3-benzyl-5-ethynyl-pyrazine (30 mg, 0.143 mmol) and 2-azidoethanol (16 mg, 0.186 mmol) in THF (1.5 mL) under argon. Then triethylamine (2 μL) was added and the mixture was stirred at room temperature for 8 h. Then, the mixture was extracted with ethyl acetate and the organic layer was washed



Scheme 2: the synthesis route of triazole series (T) coelenterazine analogs.

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with saturated sodium chloride solution. After dried over anhydrous sodium sulfate and concentrated under reduced pressure, the crude product was further purified by chromatography on silica gel (PE/EtOAc 20:3) to give a light yellow solid (40 mg). Yield: 95%. ^{1}HNMR (400 MHz, $CDCl_3$): δ 8.70 (s, 1H), 8.10(s, 1H), 7.34~7.28 (m, 2H), 7.26~7.22(m, 3H), 4.55(t, J=4Hz, 2H), 4.49(s, 2H), 4.13(m, 4H). ^{13}CNMR (100 MHz, DMSO-d_6): δ 153.52, 146.18, 140.71, 138.47, 137.01, 133.86, 129.19, 128.73, 126.68, 122.12, 60.34, 52.74, 38.93. ESI-HRMS: m/z [M+ H] $^+$ calcd for 297.1464, found 297.1456.

2-Amino-3-benzyl- 5- [(1-hydroxypropyl)-1, 2, 3- triazol-4]-pyrazine (**7-2**): light yellow solid; yield 83%; ^{1}HNMR (400 MHz, $CDCl_3$): δ 8.76 (s, 1H), 8.04(s, 1H), 7.33~7.28 (m, 2H), 7.26~7.22(m, 3H), 4.59(t, *J*=6Hz, 2H), 4.48(s, 2H), 4.14(s, 2H), 3.70(m, *J*=6Hz, 2H), 2.19(m, 2H). ^{13}CNMR (100 MHz, DMSO-d_6): δ 153.54, 146.29, 140.68, 138.47, 137.08, 133.81, 129.17, 128.73, 126.68, 121.80, 57.96, 47.21, 38.92, 33.37. ESI-HRMS: m/z [M+H]$^+$ calcd for 311.1620, found 311.1603.

2-Amino-3-benzyl- 5- [(1-fluoroethyl)-1, 2, 3- triazol-4]-pyrazine (**7-3**): light yellow solid; yield 79%; ^{1}HNMR (400 MHz, DMSO-d_6): δ 8.45 (s, 1H), 8.39(s, 1H), 7.33~7.19 (m, 5H), 6.46(s, 2H), 4.93(t, *J*=4Hz, 1H), 4.80(m, 2H), 4.73(m, 2H), 4.07(s, 2H). ^{13}CNMR (100 MHz, DMSO-d_6): δ 153.62, 146.61, 140.77, 138.44, 137.10, 133.56, 129.16, 128.64, 126.69, 122.08, 66.21, 50.65, 50.45, 38.92. ESI-HRMS: m/z [M+H]$^+$ calcd for 299.1420, found 299.1406.

The preparation of 2-benzyl-8-benzyl-6- [(1-hydroxyethyl)-1, 2, 3-triazol-4]imidazo[1,2-a]pyrazin-3(7*H*)-one (**T1**)[18, 20, 23]:

The mixture of 2-amino-3-benzyl- 5- [(1-hydroxyethyl)-1, 2, 3-triazol-4]-pyrazine (**7-1**, 60 mg, 0.202 mmol) and 3-phenyl-1,1-diethoxyacetone (**3-1**, 90 mg, 0.404 mmol) was dissolved in ethanol (3 mL) under argon atmosphere and allowed to stir at room temperature for 10 min. The con.HCl (0.2 mL) in ethanol (1 mL) was then added to the mixture via syringe over 10 min. The reaction was heated to refluxed at 80 ^{o}C for 8 h and then allowed to cool to room temperature. The crude was concentrated under vacuum and further purified by chromatography on silica gel (CH_2Cl_2/MeOH 50:1) to give yellow solid (60 mg). Yield: 69%. Analytical RP HPLC (Phenomenex, C8, 250 x 4.6 mm column): 45% acetonitrile with 0.1% trifluoroacetic acid, 1.0 mL/min at 370 nm, Rt: 3.923 min, 98%. ^{1}HNMR (400 MHz, CD_3OD): δ 8.35 (s, 1H), 8.02(s, 1H), 7.24~7.05 (m, 10H), 4.44(t, *J*=6Hz, 2H), 4.32(s, 2H), 4.04(s, 2H), 3.86(t, *J*=6Hz, 2H). ^{13}CNMR (100 MHz, CD_3OD): δ148.40, 143.06, 138.07, 136.52, 135.47, 133.85, 128.86, 128.54, 128.37, 128.17, 126.88, 126.80, 124.56, 109.73, 60.10, 52.93, 37.81, 29.08. ESI-HRMS: m/z [M+ +] calcd for 427.1882, found 427.1876.

2-Benzyl-8-benzyl-6- [(1-hydroxypropyl)-1, 2, 3- triazol-4]imidazo[1,2-a]pyrazin-3(7*H*)-one (**T2**): yellow solid; yield 23%. Analytical RP HPLC (Phenomenex, C8, 250 x 4.6 mm column): 50% acetonitrile with 0.1% trifluoroacetic acid, 1.0 mL/min at 370 nm, Rt: 3.888 min, 100%. ^{1}HNMR (400 MHz, CD_3OD): δ 8.44 (s, 1H), 8.15(s, 1H), 7.35~7.12 (m, 10H), 4.57(t, *J*=8Hz, 2H), 4.44(s, 2H), 4.14(s, 2H), 3.58(t, *J*=8Hz, 2H), 2.13(m, 2H). ^{13}CNMR (100 MHz, CD_3OD): δ148.42, 143.09, 136.51, 135.48, 133.72, 128.84, 128.55, 128.39, 128.16, 126.88, 126.80, 124.19, 109.90, 65.52, 57.92, 37.84, 32.49, 29.07. ESI-HRMS: m/z [M+H]$^+$ calcd for 441.2039, found 441.2034.

2-Benzyl-8-benzyl-6- [(1-fluoroethyl)-1, 2, 3- triazol-4]imidazo[1,2-a]pyrazin-3(7*H*)-one (**T3**): yellow solid; yield 25%. Analytical RP HPLC (Phenomenex, C8, 250 x 4.6 mm column): 60% acetonitrile with 0.1% trifluoroacetic acid, 1.0 mL/min at 370 nm, Rt: 3.035 min, 95%. ^{1}HNMR (400 MHz, CD_3OD): δ 8.47 (s, 1H), 8.26(s, 1H), 7.41~7.20 (m, 10H), 4.92(m, 1H), 4.85(m, 3H), 4.46(s, 2H), 4.21(s, 2H). ^{13}CNMR (100 MHz, CD_3OD): δ148.43, 143.66, 138.03, 136.52, 135.46, 134.00, 128.86, 128.55, 128.38, 128.17, 126.88, 126.81, 124.36, 109.68, 82.24, 80.54, 37.85, 29.03. ESI-HRMS: m/z [M+H]$^+$ calcd for 429.1839, found 429.1833.

Luminescence assay

Millipore water was used to prepare all aqueous solutions. Measurements for *Renilla* luciferase bioluminescent assays were determined in 50mM Tris-HCl buffer, pH 7.42. Measurements for *Gaussia* luciferase bioluminescent assays were determined in 25mM Tris-HCl buffer, pH 7.8, with 600 mM NaCl, 1 mM EDTA, 0.05% BSA. The bioluminescence images were captured by using an IVIS Kinetic (Caliper Life Sciences, USA) equipped with a cooled charge-coupled device (CCD) camera. The bioluminescence spectra were performed with F-1200 fluorescence spectrophotometer in luminescence mode. Luciferase was purchased from RayBiotech. The bioluminescence kinetic parameters K_m, V_m and half decay life were calculated using GraphPad Prism software.

Bioluminescence properties measurement (*Renilla* luciferase)

To determine the bioluminescence properties of the novel derivatives, all compounds were freshly dissolved in 95% EtOH as stock and diluted to appropriate concentrations in Tris-HCl (50 mM, pH 7.42) for each measurement. In all the measurements, the final ethanol concentration in the sample solution was kept constant at 0.5% (v/v) to avoid the effect of ethanol on the BL reaction. The *Renilla* luciferase was dissolved in and diluted to 1 μg/mL with Tris-HCl (50 mM, pH 7.42). To measure bioluminescence intensity, the solution of compound (50 μL) was added to a 96-well black flat bottom microscale plate, and luciferase (50 μL) was added and mixed quickly. The final concentration of luciferase was 0.5 μg/mL. The final compound concentrations were 0.25, 0.5, 1, 2, 5, 10 and 25 μM. Bioluminescence intensities of DeepBlueC and the derivatives were immediately measured with an IVIS Kinetic (Caliper Life Sciences, USA) equipped with a cooled charge-coupled device (CCD) camera. The assays were measured in triplicate. The results were reported as total photon flux within an ROI in photons per second. The Michaelis constant K_m and maximum rate V_{max} were estimated with the Michaelis–Menten kinetics equation using GraphPad Prism software.

For the recording of bioluminescence spectra, an aliquot of *Renilla* luciferase solution (0.5 mL, 1 μg/mL) was mixed with derivative solution (0.5 mL, 25 μM) in a quartz cell and the mixture was immediately measured with a F-1200 fluorescence spectrophotometer in luminescence wavelength mode with the lamp off at a scan rate of 3000 nm/min with response time of 2 s. The wavelengths of maximal bioluminescence intensities (λ_{max}) were corrected by the use of coelenterazine and reported data (485

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nm)[15]. Moreover, the quantitative bioluminescence spectra were determined using the instrument software (FL Solutions ver. 2.1).

For the recording of half decay life, an aliquot of *Renilla* luciferase solution (0.5 mL, 1μg/mL) was mixed with derivative solution (0.5 mL, 25 μM) in a quartz cell and the mixture was immediately measured with a F-1200 fluorescence spectrophotometer in luminescence time scan mode with the lamp off at a scan rate of 3000 nm/min with response time of 2s. The measurement was last 300 s. The half decay life was calculated by using GraphPad Prism software.

Bioluminescence properties measurement (*Gaussia* luciferase)

To determine the bioluminescence properties of the novel derivatives, all compounds were freshly dissolved in 95% EtOH as stock and diluted to appropriate concentrations in Tris-HCl (25 mM, pH 7.8, containing 600 mM NaCl, 1 mM EDTA, 0.05% BSA) for each measurement. In all the measurements, the final ethanol concentration in the sample solution was kept constant at 0.5% (v/v) to avoid the effect of ethanol on the BL reaction. The *Gaussia* luciferase was dissolved in and diluted to 0.5 μg/mL with Tris-HCl (25 mM, pH 7.8, containing 600 mM NaCl, 1 mM EDTA, 0.05% BSA). To measure bioluminescence intensity, the solution of compound (50 μL) was added to a 96-well black flat bottom microscale plate, and luciferase (50 μL) was added and mixed quickly. The final concentration of luciferase was 0.25 μg/mL. The final compound concentrations were 0.25, 0.5, 1, 2, 5, 10 and 25 μM. Bioluminescence intensities of DeepBlueC and the derivatives were immediately measured with POLABstar Omega microplate reader or an IVIS Kinetic (Caliper Life Sciences, USA) equipped with a cooled charge-coupled device (CCD) camera. The assays were measured in triplicate. The Michaelis constant K_m and maximum rate V_{max} were estimated with the Michaelis–Menten kinetics equation using GraphPad Prism software.

For the recording of bioluminescence spectra, an aliquot of *Gaussia* luciferase solution (0.5 mL, 0.5 μg/mL) was mixed with derivative solution (0.5 mL, 25 μM) in a quartz cell and the mixture was immediately measured with a F-1200 fluorescence spectrophotometer in luminescence wavelength mode with the lamp off at a scan rate of 3000 nm/min with response time of 2 s. The wavelengths of maximal bioluminescence intensities (λ_{max}) were corrected by the use of coelenterazine and reported data (488 nm)[15]. Moreover, the quantitative bioluminescence spectra were determined using the instrument software (FL Solutions ver. 2.1).

For the measurement of half decay life, the solution of compound (50 μL) was added to a 96-well black flat bottom microscale plate, and luciferase (50 μL) was added and mixed quickly and was immediately measured with a POLABstar Omega microplate reader with a measurement time of 300 s. The half decay life was calculated by using GraphPad Prism software.

Cell culture

ES-2 cells (human ovarian cancers cell line) expressing *Reilla* luciferase (Rluc) were purchased from Shanghai BioDiagnosis Co., Ltd. The ES-2-Rluc cells were cultured in DMEM high glucose supplemented with 10% fetal bovine serum (FBS) at 37 ^{o}C in a humidified atmosphere in a 5% CO2 incubator.

Cell bioluminescence imaging

Cells were grown in black 96-well plates (4×10^5 cells per well). After a 24-h incubation period, the medium was removed, and cells were treated with 100 μL of various concentrations of compounds (range 0.25 μM to 25 μM). Bioluminescence intensity was measured immediately using an IVIS Kinetic imaging system. Luminescent signal (photons per second) for each well was measured and plotted as average values (experiments conducted in triplicate).

Cell concentration dependence assay

Cell were grown in black 96-well plates (0.125, 0.25, 0.5, 1, 2, 4×10^5 cells per well, respectively). After a 24-h incubation period, the medium was removed, and cells were treated with 100 μL of compounds (20 μM). Bioluminescence intensity was measured immediately using an IVIS Kinetic imaging system. Luminescent signal (photons per second) for each well was measured and plotted as average values (experiments conducted in triplicate).

Chemiluminescent assay

For the recording of chemiluminescence spectra, 2 mL of DMSO with 0.5% (v/v) of NaOH (1 M) was mixed with 200 μL of derivative solution (50 μM) in a quartz cell and the mixture was immediately measured with an F-1200 fluorescence spectrophotometer in luminescence wavelength mode with the lamp off at a scan rate of 3000 nm/min with response time of 2 s. The wavelengths of maximal bioluminescence intensities (λ_{max}) and the quantitative chemiluminescence spectra were determined using the instrument software (FL Solutions ver. 2.1).

Fluorescent assay

To measure fluorescent spectra, the solution of compound (200 μL, 100 μM) was added to a 96-well black flat bottom microscale plate and was immediately measured with Varioskan microplate spectrophotometer (Thermo Electron Corporation, Waltham, MA, USA).

Solubility assay

1mg of compound A4, A6 or DeepBlueC was dissolved in deionized water until the solution was clear. The assays were measured in triplicate.

Reasults and discussion

Bioluminescence properties with *Renilla* luciferase

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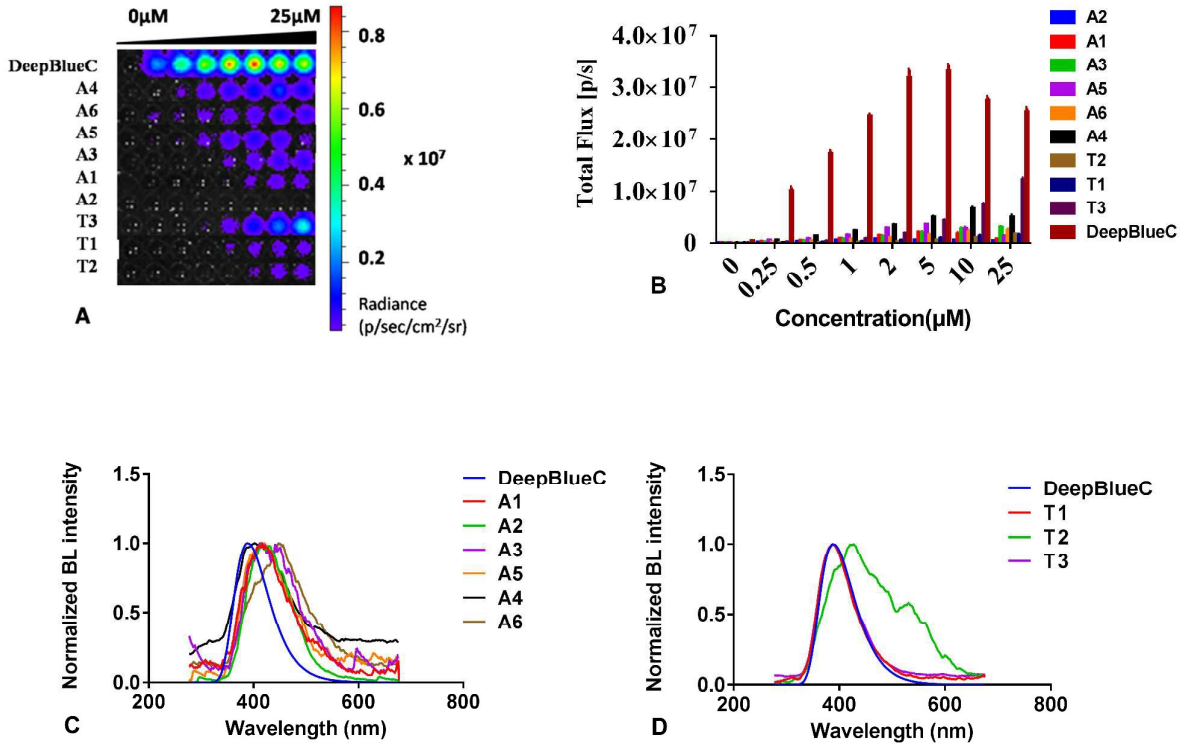

Figure 2. (A)The bioluminescence imaging of A and T series derivatives and DeepBlueC at various concentrations with *Renilla* luciferase. (B)The comparison of bioluminescence intensities of A and T series derivatives and DeepBlueC at various concentrations with *Renilla* luciferase. (C) Corrected bioluminescence spectra of new derivatives and DeepBlueC with *Renilla* luciferase. (D) Corrected bioluminescence spectra of new derivatives and DeepBlueC with *Renilla* luciferase. BL spectra were measured with F-2500 FL Spectrophotometer with response time of 2 s.

All novel compounds were initially evaluated with recombinant *Renilla reniformis* luciferase (Rluc). As shown in Fig. 2 and Table 1, *Renilla* luciferase with them displayed increasing bioluminescence when the rise of concentrations. Moreover, **A4** is the best one of series alkynes, and **T3** is the best one of series triazoles. They emit moderate bioluminescence with *Renilla* luciferase, which is much lower than that of DeepBlueC. However, relative bioluminescence quantum yields (RQY)[17, 27] of all novel compounds seem lower than that of DeepBlueC (Table 1). With increasing of molecular rigidity resulting from the introduction of alkynyl group, series alkynes may have poor activities to interact with luciferase. The 2-fluorophenylethynyl group of compound **A4** may interact with the key active sites of luciferase firmly in contrast with other compounds containing fluoro-group. As for T series, their more flexible of molecular structure may result in humble interaction with active sites of luciferase. However, the results of luminescence spectra disclosed that the maxima of Rluc light emission with analogs except **T1** are significantly red-shifted by 20-60 nm as compared to that with the DeepBlueC (Fig. 2 and Table 1). The maximum emission peak of compound A6 with Rluc is remarkably red-shifted up to 60 nm. Moreover, some compounds such as **A4**, **A6**, **T1** and **T3** with Rluc display the slow kinetic decay of the light signal. Bioluminescence K_m values of novel analogs are in the range of 2-4 μM except **T2** and **T3** (12.97 and 18.43, respectively), which are slightly higher than that of the DeepBlueC. Though the bioluminescence V_{max} values of them are lower than that of DeepBlueC, some of them such as the V_{max} of **T3** approaches that of DeepBlueC.

Table 1. Bioluminescence properties of all compounds with *Renilla* luciferase.

Compounds	Corrected Bioluminescence Emission (nm)	Bioluminescence Half Life[a] (s)	Bioluminescence K_m[b] (μM)	Bioluminescence V_{max}[b] (s/p)	Relative Bioluminescence Quantum Yield (RQY)[c]
DeepBlueC	388	42.23	0.61 ± 0.06	(3.89 ± 0.12) × 10^7	1
A1	416	8.60	2.24 ± 0.15	(2.98 ± 0.09) × 10^6	0.046
A2	416	8.40	2.08 ± 0.44	(1.69 ± 0.21) × 10^6	0.026
A6	448	29.68	3.06 ± 0.41	(2.69 ± 0.11) × 10^6	0.064

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A3	412	13.11	3.56 ± 0.28	(3.53 ± 0.09) × 10^6	0.040
A4	397	141.00	2.68 ± 0.20	(8.14 ± 0.23) × 10^6	0.109
A5	421	20.01	2.10 ± 0.28	(5.25 ± 0.32) × 10^6	0.090
T1	388	80.64	4.50 ± 0.62	(1.82 ± 0.09) × 10^6	0.016
T2	427	16.41	12.97 ± 1.02	(2.44 ± 0.07) × 10^6	0.009
T3	417	232.50	18.43 ± 0.86	(2.08 ± 0.04) × 10^7	0.059

[a] BL half life was calculated using GraphPad Prism software and taken advantage of the values when compounds were 25 μM.
[b] Michaelis constant K_m and maximum rate V_{max} were estimated with the Michaelis–Menten kinetics equation using GraphPad Prism software. The values are shown by means±SD of three independent assays performed in triplicate.
[c] Relative quantum yields of bioluminescence were obtained with an IVIS Kinetic (Caliper Life Sciences, USA) equipped with a cooled charge-coupled device (CCD) camera. The quantum yield (QY) was calculated by dividing the measured total number of photons by the number of substrate molecules in the solution.

Bioluminescence properties with *Gaussia* luciferase

In addition, all novel compounds were evaluated with *Gaussia* luciferase (Table 2). However, all of them including DeepBlueC had poor performance with Gluc. The bioluminescence spectra of T series are similar with of DeepBlueC. All A series compounds with Gluc demonstrate blue-shifted emission by 30-40 nm as compared to that of DeepBlueC. Among them, compound **A4** emits 88% bioluminescence with Gluc as compared to that with DeepBlueC, which is the best performance. All T series compounds have insignificant bioluminescence. Though they have lower V_{max} values, their K_m values are also worse. In a word, our compounds may not be suitable to interact with *Gaussia* luciferase, which leads to underprivileged bioluminescence. The chemiluminescence spectra and fluorescence spectra of all novel compounds were also measured as displayed in Table S1.

Table 2: Bioluminescence properties of novel compounds with *Gaussia* luciferase.

Compounds	Corrected Bioluminescence Emission (nm)	Bioluminescence Half decay life[a] (s)	Bioluminescence K_m [b](μM)	Bioluminescence V_{max} [b](s/p)	Relative Bioluminescence Quantum Yield (RQY)[c]
DeepBlueC	493	27.07	22.97 ± 2.93	(1.24±0.09) ×10^4	1
T2	490	--	--	--	0.078
T1	497	--	--	--	0.028
T3	493	--	--	--	0.059
A1	456	--	1.35±0.12	(1.25±0.03) ×10^3	0.487
A2	451	--	3.24±0.35	(7.96±0.28)×10^2	0.225
A5	452	--	1.26±0.093	(1.50±0.03) ×10^3	0.576
A3	455	--	1.70±0.081	(1.42±0.02) ×10^3	0.548
A4	456	--	0.85±0.064	(2.16±0.04) ×10^3	0.885
A6	458	--	0.54±0.049	(8.93±0.20) ×10^2	0.386

[a] BL half decay life was calculated using GraphPad Prism software and taken advantage of the values when compounds were 25 μM.
[b] Michaelis constant K_m and maximum rate V_{max} were estimated with the Michaelis–Menten kinetics equation using GraphPad Prism software. The values are shown by means±SD of three independent assays performed in triplicate.
[c] Relative quantum yields of bioluminescence were obtained with an IVIS Kinetic (Caliper Life Sciences, USA) equipped with a cooled charge-coupled device (CCD) camera. The quantum yield (QY) was calculated by dividing the measured total number of photons by the number of substrate molecules in the solution.

Bioluminescence *in cellulo*

Subsequently, all compounds were investigated with ES-2 cells expressing *Renilla* luciferase as shown in Fig. 3. It is exciting that our novel compounds behave improved properties in cellular level. Light emission of compounds **A4** and **A6** displays 4- and 6-fold higher emission as compared to that of DeepBlueC, respectively. The performance of compound **A2** is similar to that of DeepBlueC. What's more, bioluminescence emitted by **A4** and **A6** can last longer in contrast with that of DeepBlueC (Fig. 3C). Moreover, bioluminescence emitted by compounds **A4** and **A6** could increase with the rise in cell concentration (Fig. 3D). However, all T series compounds are inferior to DeepBlueC (Fig. S2). Moreover, the mass solubility of **A4**, **A6** and DeepBlueC were determined, and the results were display in Table S2. It is evident that both **A4** and **A6** were easier to dissolve in water at the room temperature as compared to DeepBlueC. The results indicated that compounds **A4** and **A6** with superior water solubility are easier to diffuse into the cells so as to appear improved bioluminescence. Furthermore, the introduction of the electron-rich group, such as a hydroxyl group of compound **A6**, can result in an appropriate lipid-water partition coefficient, which promotes the bioluminescence induced by **A6** in cellular level. Hence, compounds **A4** and **A6** are more potential CTZ-type bioluminescence substrates to be applied at the cellular level compared with DeepBlueC.

Conclusions

We have successfully designed and synthesized two series of CTZ-type derivatives based on the structure of DeepBlueC and then investigated their bioluminescence properties with *Renilla* luciferase and *Gaussia* luciferase *in vitro*. Most of the compounds display moderate bioluminescence in the presence of *Renilla* luciferase and *Gaussia* luciferase. However, they are inferior to DeepBlueC. All compounds of series A behave better in combination with ES-2 cells expressing Rluc. It is of great significance that the compounds **A4**

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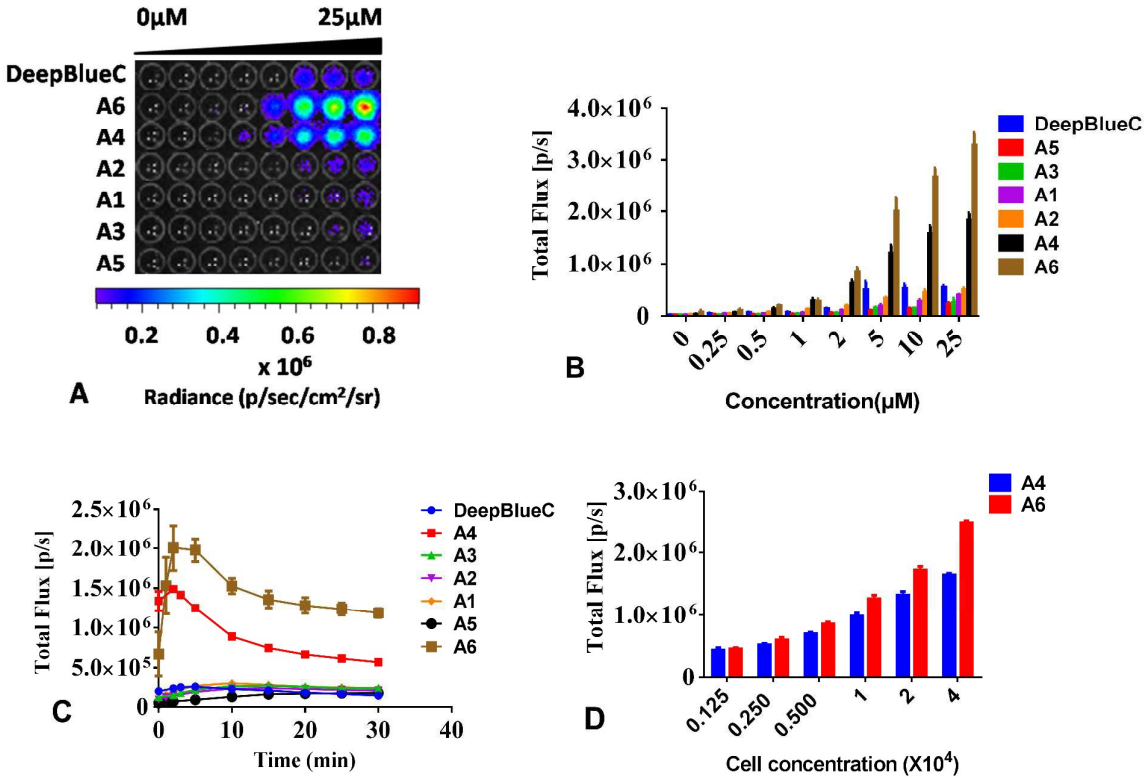

Figure 3: (A) The bioluminescence imaging of A and T series derivatives and DeepBlueC in ES-2 cells expressing *Renilla* luciferase (Rluc) at various concentrations. (B) The comparison of bioluminescence intensities of A series derivatives and DeepBlueC in ES-2 cells expressing *Renilla* luciferase (Rluc) at various concentrations. (C) The time-dependent BL of new derivatives and DeepBlueC in ES-2 cells expressing *Renilla* luciferase (Rluc). (D) The comparison of bioluminescence intensities of new derivatives A4 and A6 in ES-2 cells expressing *Renilla* luciferase (Rluc) at various cell concentrations ($\times 10^4$).

and **A6** that have brighter and longer emission with Rluc in the cellular level are superior to DeepBlueC. The results indicate that introduction of the alkynyl bond could lead to the enhancement of molecular rigidity, which could affect the entrance into the pocket and interaction with active sites of luciferase. The 2-fluorophenylethynyl moiety of compound **A4** may interact with some of the key active sites of luciferase firmly in contrast with other compounds containing fluoro group, which makes it better than others. Moreover, appropriate lipid-water partition coefficient plays an important role in bioluminescence *in cellulo*. The better mass solubility of our novel compounds promotes them to diffuse into cells in order to induce bioluminescence with Rluc. The introduction of electron-rich groups such as a hydroxyl group of compound **A6** can make it have an appropriate lipid-water partition coefficient, which promotes the bioluminescence induced by **A6** *in cellulo*. Both of **A4** and **A6** are suitable and potential bioluminescent substrates that can be applied *in cellulo* with the ability to probe some biological activities. Moreover, these compounds can be further modified to become probes with the use of "caged" strategy [1, 15], thus becoming favorable molecules involved in bioluminescence technique. We consider that our compounds rich the diversity of CTZ-type substrates and the knowledge gained from this work is helpful for the development of novel CTZ-type analogs with superior bioluminescent properties.

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