Chinese Chemical Letters 27 (2016) 550–554

Contents lists available at ScienceDirect

Chinese Chemical Letters

journal homepage: www.elsevier.com/locate/cclet

Original article

Luminescence of coelenterazine derivatives with C-8 extended electronic conjugation

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ARTICLE INFO

Article history:
Received 14 January 2016
Received in revised form 18 February 2016
Accepted 19 February 2016
Available online 3 March 2016

Keywords:
Bioluminescence imaging
Coelenterazine derivatives
Relative quantum yield
Chemiluminescence
Red-shifted

ABSTRACT

Replacement of the methylene group at the C-8 position with an extended electronic conjugation is a new promising method to develop red-shifted coelenterazine derivatives. In this paper, we have described an oxygen-containing coelenterazine derivative with a significant red-shifted (63 nm) bioluminescence signal maximum relative to coelenterazine 400a (DeepBlueC™, **1**). In cell imaging, the sulfur-containing coelenterazine derivative displayed a significantly (1.77 ± 0.09; $P \leq 0.01$) higher luminescence signal compared to coelenterazine 400a and the oxygen-containing coelenterazine derivative exhibited a slightly (0.74 ± 0.08; $P \leq 0.05$) lower luminescence signal. It is beneficial to understand further the underlying mechanisms of bioluminescence.

1. Introduction

Noninvasive bioluminescence imaging (BLI) has emerged as a routine technique in fields such as medical science, biochemistry and molecular biology [1–3]. Wide BLI applications have been discovered including real-time monitoring of gene expression [4], tumor growth [5] and protein–protein interactions [6]. The mechanism depends on an enzymatic oxidation reaction involving an enzyme (luciferase), a substrate (luciferin) and molecular oxygen, resulting in the emission of light.

To avoid absorption and scattering by tissue in small-animal imaging experiments, the wavelength range of light emissions had better be the red to near-infrared (600–900 nm). Firefly luciferase (Fluc, 61 kDa) is the most commonly utilized BLI system due to its favorable emission spectrum (emission spectra in the range of 540–615 nm), stability, and biocompatibility of its bioluminogenic substrate D-luciferin [7]. Unfortunately, however, cofactors such as ATP and Mg^{2+} ion are required which potentially leading to the experimental complexity in bioanalysis. On the other hand, renilla luciferases (Rluc, 36 kDa) in conjunction with coelenterazine (CTZ) or its derivatives as the substrate only require molecular oxygen [8]. Moreover, Rluc has been successfully expressed in mammalian cells without any cytotoxicity. However, the Rluc/CTZ pair produces a blue–green light with are latively short wavelength (450–475 nm). To overcome such a limitation, much effort has been made either by modifications of the RLuc [9] or by synthesis of new red-shifted CTZ analogues [10]. To our knowledge, it seems to be difficult to achieve red-shifted variants of RLuc *via* molecular biology approaches. Hence, we focus on the modifications of CTZ analogues to find a bathochromic and stable substrate of Rluc.

Since the enzymatic recognition mechanism of the Rluc system is not yet fully understood [11], the design of novel valuable CTZ derivatives is challenging, and only few red-shifted substrates have been reported so far. One significant molecule, *v*-coelenterazine owning a more planar and rigid molecular structure with a vinylene bridge at the C-5 position, displays a remarkable red-shifted emission at 512 nm and high quantum yield with RLuc, however at the cost of synthesis and molecular stability [10]. As to other CTZ derivatives modified at the C-2 [12], C-5 [10], C-6 [13] and C-8 [14] positions of the imidazopyrazinone core, few show luminescence properties superior to those of native CTZ, mostly since their structural modifications prevent their enzymatic recognition. In previous work, the luminescence properties of many modified CTZ derivatives are measured in comparison with coelenterazine 400a (DeepBlueC™, **1**). Coelenterazine 400a, a commercially available CTZ derivative, produces a ~400 nm emission peak upon Rluc-mediated oxidation. It is used for bioluminescence resonance energy transfer (BRET) studies because it has minimal interference with the emission of the GFP acceptor, which provides greater signal resolution [6]. Recently, a sulfur-containing CTZ derivative **3** (Fig. 1) was discovered with a 30 nm red-shift compared to coelenterazine 400a in bioluminescence [15]. At the same time,

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http://dx.doi.org/10.1016/j.cclet.2016.02.011

the relative quantum yield (RQY) is 3.6 compared to coelenterazine 400a, measured by mixing substrates and rough cytosolic extract prepared from cells expressing Rluc. However, in cells there are many unwanted substances prone to combine with CTZ derivatives and catalyze the oxidation reaction such as albumin and other intrinsic proteins besides Rluc, thus giving rise to background noise in bioluminescence assays [16].

In this paper, we designed and synthesized a CTZ derivative that contains an oxygen atom in place of the native methylene group at C-8 position (**1**). We hypothesized that this replacement would also produce a red-shifted emission because of similar attributes of the oxygen and sulfuratom. To examine RQY, a new measurement method through an IVIS Kinetic equipped with a cooled charge-coupled-device (CCD) detector had been developed. Besides we employed commercially available purified Rluc enzyme in place of the rough cytosolic extract to decrease enzyme-independent luminescence (autoluminescence) of CTZ derivatives. To further evaluate the luminescence properties of CTZ derivatives, we performed the assay for luminescence activity in the live cell. All results showed that CTZ derivative **2** (Fig. 1) displayed a more significant red-shift (63 nm) in bioluminescence compared to coelenterazine 400a while it had lower quantum yield. In cell imaging photon emission from **3** was significantly higher compared to coelenterazine 400a (1.77 ± 0.09; $P \leq 0.01$), while compound **2** exhibited a 0.74 ± 0.08 slightly lower luminescence signal. Therefore, replacement of the methylene group at the C-8 position with an O or S heteroatom is a new promising method to develop red-shifted CTZ analogs.

2. Experimental

2.1. Materials and apparatus

All reagents for organic synthesis were obtained from commercial suppliers and used without further purification. When necessary, organic solvents were routinely dried and/or distilled prior to use and stored over molecular sieves under argon. Millipore water was used to prepare all aqueous solutions. ^{1}H NMR and ^{13}C NMR were recorded on Bruker AV-300 or AV-600 spectrometers at the College of Chemistry NMR Facility, Shandong University. All chemical shifts are reported in the standard δ notation of parts per million using the peaks of residual proton and carbon signals of the solvent as internal references. Mass spectra were recorded in ESI+ mode (70 eV) in Drug Analysis Center at Shandong University. ESI-HRMS was performed on a Waters SYNAPT G2-Si. The purity of CTZ analogues was confirmed by analytical reverse-phased HPLC (Agilent, 1260 Infinity) on Phenomenex C-18 column (250 × 4.6 mm). Melting points were determined on a Mel-Temp apparatus and were not corrected. Luminescence spectra were recorded using an F-2500 FL Spectrophotometer. The light outputs were determined with an IVIS Kinetic (Caliper Life Sciences, USA) equipped with a cooled CCD camera. All the experiments were carried out at room temperature unless otherwise specified.

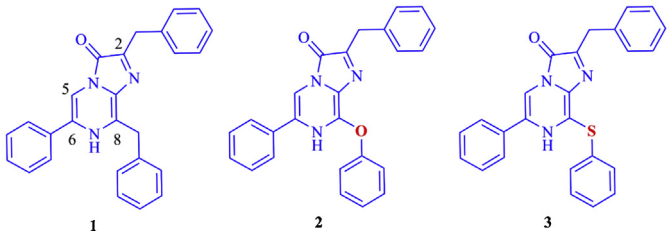

Fig. 1. Structures of the coelenterazine analogs **1–3**.

2.2. Synthesis of CTZ derivatives *1–3*

The synthesis of compound **1–3** was shown in Schemes S1 and S2 in Supporting information. The details for preparation and NMR and HRMS spectra of these compounds were also presented in Supporting information.

2.3. Luminescence spectral analysis

Synthesized CTZ derivatives were dissolved in ethanol at a concentration of 1 mg/mL for use as stock solutions, stored at −20 °C or lower temperature (for long-time storage), and diluted in 50 mmol/L Tris–HCl buffer pH 7.4 (without calcium and magnesium) immediately prior to use. Enzyme assays were conducted using commercially available purified Rluc enzyme (Ray Biotech) at a concentration of 28 nmol/L in 1 mL PBS (pH 7.4) stored at −80 °C.

Luminescence spectra were recorded using an F-2500 FL Spectrophotometer with the excitation lamp turned off and the emission shutter open at a scanning speed of 3000 nm/min. All the spectra were measured at room temperature. The *in vitro* bioluminescence spectra were measured in 50 mmol/L Tris–HCl buffer pH 7.4 containing Rluc protein at a final concentration of 15 nmol/L and initiated by the injection of CTZ derivatives buffer solution at a final concentration of 50 μmol/L. Chemiluminescence spectra were traced by mixing 200 μL of CTZ derivatives buffer solution at a final concentration of 50 μmol/L with 800 μL of dimethylsulfoxide (DMSO). The response time is 2.0 s, and all spectra were not corrected for luminescence decay at spectral scanning. The luminescence spectra are the result of three independent measurements, each one measured in triplicate.

2.4. Relative quantum yield (RQY) and kinetics of in vitro luminescence

The RQY study and kinetic analysis were performed using an IVIS Kinetic (Caliper Life Sciences, USA) which consisted of a cooled charge-coupled device (CCD) camera mounted on a light-tight specimen chamber (dark box), a camera controller, a camera cooling system, and controlled using a computer. The data are represented as pseudocolor images (in photons/s/cm^2/scr) of light intensity (blue—least intense, red—most intense) superimposed over the grayscale reference images. Circular specified regions of interest (ROIs) were drawnon the areas, and the light output were quantified as the total number of photons emitted per second using Living Image software.

To determine the appropriate unsaturated amount of substrate, 10 μL of CTZ derivatives **1–3** between 1 and 100 μmol/L and 90 μL of either DMSO (chemiluminescence) or Rluc enzyme at a final concentration of 15 nmol/L (bioluminescence) were used. The RQY and reaction kinetics was determined by mixing 10 μL of CTZ derivatives (final concentration of 5 μmol/L) with 90 μL of either DMSO or Rluc enzyme (15 nmol/L) onto wells of 96-well black plates to prevent light reflection from well to well. Luminescent signals were measured immediately after mixing and monitored over a period of 25–30 min (luminescence had almost decayed to near-background levels) using the IVIS. Light output was recorded every 5 min with an exposure time of 30 s for chemiluminescence and every 1 min with an exposuretime of 5 s in the first 15 min for bioluminescence. The collected data was analyzed by employing the Prism 5.0 GraphPad software to compute the total light output. As a corresponding blank control, Tris–HCl buffer was added instead of CTZ derivatives solution under the same conditions. All assays were performed in triplicate.