



New red-shifted coelenterazine analogues with an extended electronic conjugation

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ABSTRACT

A new promising approach to the development of red-shifted coelenterazine analogues was described. In order to alter the photochemical properties of native coelenterazine, we have designed and synthesized analogues bearing a new electron-rich structure. The spectroscopic results obtained, in the presence of the target enzyme (Renilla Luciferase), show a bathochromic emission shift of the entire class of new derivatives. Among them, the 2-benzyl-8-(4-chlorophenylthio)-6-(4-hydroxyphenyl)imidazo[1,2-a]pyrazin-3(7H)-one (**8**) shows an emission at 510 nm and uncommon slow kinetic decay.

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The use of bioluminescent enzymes as gene reporters in cell culture and small-animal imaging^{1,2} has become a useful technological tool for life science research and drug discovery. Most of the bioluminescence systems investigated require a luciferase (enzyme), a luciferin (substrate), and molecular oxygen to produce visible light.³ Enzymatic degradation of luciferin components produces intermediates bearing an electron in an excited state. More in detail, the luminescence reaction is initiated by the binding of O₂ at the 2-position of the coelenterazine molecule, giving a peroxide. The peroxide then forms a four-membered ring 'dioxetanone',⁴ that promptly decomposes producing CO₂ and the amide anion of coelenteramide in its excited state. The excited state of the amide anion of coelenteramide emits light when its energy level falls to the ground state, resulting in the emission of blue light (λ_{max} 450–470 nm).⁵ Clearly, the energy of the emitted photons depends on the difference between the two energy states. For imaging experiments in intact animals, photon emissions in the red to near-infrared wavelengths (600–900 nm) are required, since the tissue absorption of photons is reduced in this region of the spectrum.⁶ For this reason the firefly (beetle) luciferase that uses D-luciferin **1** (Fig. 1) as substrate (emission spectra in the range of 540–615 nm) is the preferred bioluminescent system in small-animal imaging applications. Unfortunately, the Mg²⁺- and ATP-

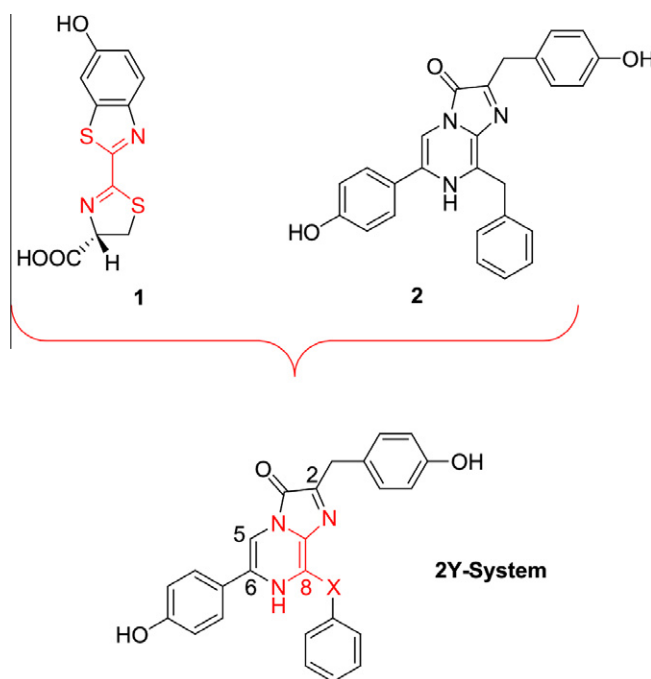


Figure 1. General formula of the derivatives, where X is a not specified heteroatom.

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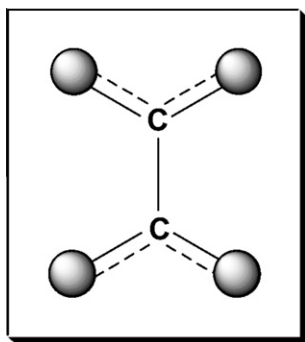


Figure 2. The spheres represent generic heteroatoms, in the 2Y-System in which three of them are nitrogen atoms: N-1, N-4, and N-7 of the original imidazo[1,2-*a*]pyrazin-3(7*H*)-one nucleus.

dependence of the reaction and the poor thermal stability of some of these enzymes limit their versatility.

On the other hand, the reaction of imidazopyrazinone-type luciferases that use coelenterazine **2** as the substrate [e.g. *Renilla* (RLuc), *Oplophorus*, and *Gaussia* luciferases], only require is molecular oxygen. Thus, they do not suffer from such problems, but the emission range of these enzymes is in the blue region (450–475 nm) of the visible spectrum. To overcome such a limitation, the development of red-shifted and stable variants of RLuc obtained via random mutagenesis⁷ used in combination with red-shifted analogues of coelenterazine,⁸ has proven to be a promising approach.⁹ While it seems to be difficult to achieve additional red-shifting by modifications of the enzyme at the current state of knowledge, the synthesis of new coelenterazine analogues with better bathochromic emission shifts may offer some room for improvement. In fact, despite the large number of coelenterazine analogues generated during the past forty years of research, only few red-shifted substrates have been reported thus far. One of such molecules, is *v*-coelenterazine, which displays a 35 nm bathochromic shift and high quantum yield with RLuc, but not *Oplophorus* luciferase.^{8,9} Additional coelenterazine analogues with a conjugated group at the C-8 position of the imidazopyrazinone ring displayed large bathochromic shifts (50–110 nm) of chemiluminescence in polar aprotic solvent, but their bioluminescence in the RLuc reaction was negligibly low.¹⁰

On confronting **1** and **2**, we note that in the *D*-luciferin molecule N and S heteroatoms exist in a particular arrangement (see Fig. 2). We suspect that this electron-rich configuration may play a fundamental role in the formation of the multiple oxyluciferin excited states that result in a broad range of photon-emission colors.¹¹ Thus, we hypothesize that building a similar heteroatomic configuration in the imidazopyrazinone scaffold (which we name 2Y-System, for heteroatoms stand symmetrically at the four tips of a scaffold resembling an upside-down linked double Y) might enhance the stability of lower-energy emitting species of coelenterazine.

As a first step in this direction, we replaced with an S heteroatom the methylene group bonded to the C-8 of the imidazo[1,2-*a*]pyrazin-3(7*H*)-one nucleus of **2**, thus generating a series of hybrid compounds between the *D*-luciferin **1** and coelenterazine **2** (Fig. 1). By means of this simple structural modification, we assumed to modulate the photoemission of the analogues, maintaining the enzymatic binding properties of the native substrate. A number of derivatives (**3–6**, Fig. 3) were made using *bis*-deoxycoelenterazine **3** as the reference, because this compound represents a simpler coelenterazine analogue, which generates a lower-efficiency blue-shifted luminescence (~400 nm) with RLuc. Together with heteroatom interchange, S-phenyl halogen substitution was

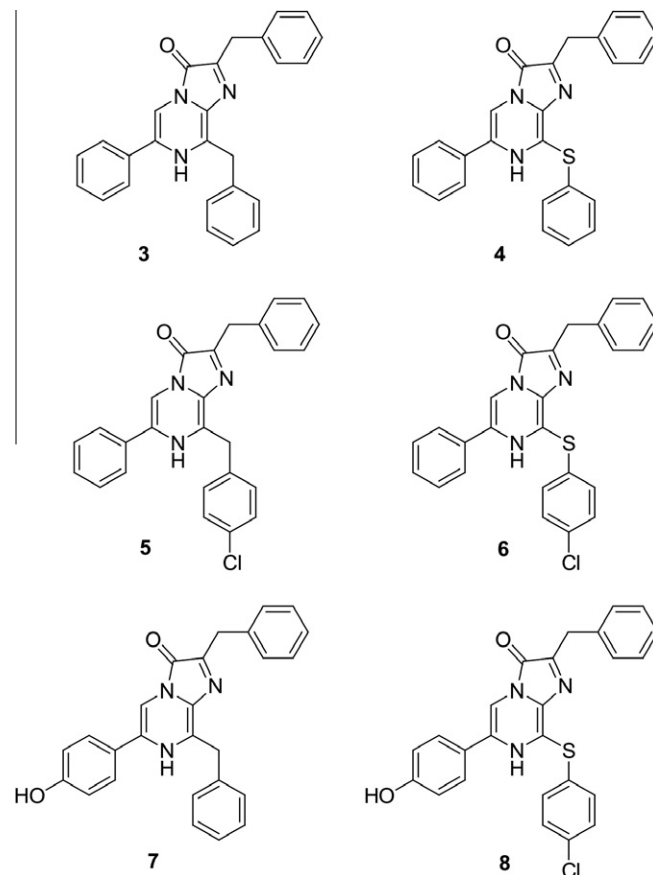


Figure 3. Structures of the newly-synthesized coelenterazine analogues compared to those of reference compounds **3** and **7**.

also explored, in the attempt to further modulate the electron density of the 2Y-System. Similar substitutions were also made on *h*-coelenterazine (**7–8**, Fig. 3), which has RLuc bioluminescent properties indistinguishable from the natural substrate.

The synthesis of the analogues **4,6,8** with sulfur heteroatom was performed from commercially available 2-aminopyrazine (**9**) by means of the procedure reported in Scheme 1 based on the Suzuki coupling¹². Key pyrazine intermediates **11a–c**¹³ were obtained by reaction of bromoderivatives **10a,b**¹⁴ with the appropriate thiol in dry DMF or acetonitrile in the presence of sodium hydride as the base.¹⁵ Demethylation of compound **11c** was performed with pyridine hydrochloride (Py·HCl)^{12d} at 190 °C to obtain the corresponding hydroxyl derivative **11d**.¹³

The final step of the convergent synthesis of the coelenterazine analogues **4,6,8**¹⁶ was achieved by condensation of intermediates **11a,b,d**, with diethoxy derivative¹⁷ in ethanol in the presence of concentrated HCl.¹⁰

The introduction of the sulfur heteroatom in place of the methylene bridge in **3** leading to compound **4** produces a significant (40 nm) bathochromic shift of the chemiluminescence spectrum measured in neutral DMSO (Table 1). The *p*-chlorophenyl substituent did not change the chemiluminescence spectrum in the absence of heteroatom (compound **5**¹⁸) nor did alter the red-shifting effect of sulfur replacement (**6**). A similar red-shift (47 nm) of chemiluminescent emission was observed in the *h*-coelenterazine derivative (**8**, Table 1). This overall bathochromic shifting effect of C-8 heteroatom replacement was also maintained in the bioluminescent reaction with RLuc. The bioluminescence spectra of **4** displayed a 30 nm red-shift compared to **3** (Fig. 4). Likewise, both the minor shoulder (λ_{max} ~387 nm) and the main peak (λ_{max} 471 nm) in the bioluminescent emission of **7** (believed to represent the neutral and the

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