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COMMUNICATION

Gram-Scale Synthesis of Luciferins Derived from Coelenterazine and Original Insights in Their Bioluminescence Properties

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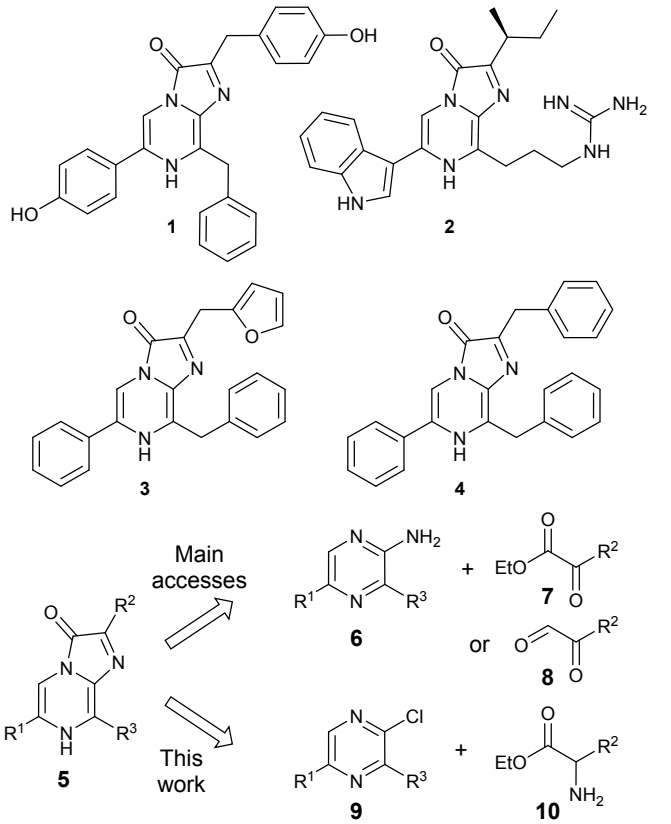
An original gram-scale synthesis of *O*-acetylated forms of coelenterazine, furimazine or hydroxy-bearing analogues of these luciferins is described. The comparisons over two hours of their bioluminescence, using the nanoKAZ/NanoLuc luciferase, is providing remarkable insights usefull for the selection of a substrate adapted for a given application.

Bioluminescence is based on the combination of at least oxygen, a small chemical cofactor and an enzyme. As depicted in scheme 1, extensive research,[1] led to the discovery that many sea-dwelling bioluminescent species[2, 3] are using coelenterazine (CTZ, **1**) or varguline (**2**) and harbor a large variety of luciferases or photoproteins (which are calcium-dependent luciferases) to produce light with these. Across the years, because of the many uses of bioluminescence-based tools in life sciences,[4-10] attempts were made to improve the signal intensity, duration and/or the emission wavelength. This started with the isolation of a wide range of coelenterazine-using luciferases and their combination with analogues of coelenterazine (**1**). It then moved to the analysis of the effect of luciferase mutations on the bioluminescence properties and the best results obtained so far were achieved by the association of mutated luciferases and luciferins analogues.[11-25] It is the combinations of an extensively mutated form of the catalytic subunit of *Oplophorus gracilirostris* luciferase (nanoKAZ/NanoLuc)[20, 21] and furimazine (**3**),[20] or bisdeoxycoelenterazine (*bis*-CTZ, **4**),[21] which appears to be the current state of the art in regard with signal intensity and duration.



Scheme 1. Structures of coelenterazine (**1**), varguline (**2**), furimazine (**3**), and bisdeoxycoelenterazine (**4**) and retrosynthesis of imidazo[1,2-*a*]pyrazine-3(7*H*)-ones

Concerning the chemistry of these luciferins, they are oxygen-sensitive and readily decompose in solution especially in the presence of a base, or upon light exposure.[26] As depicted above, many[27] if not all[28-30] the reported preparations of imidazo[1,2-*a*]pyrazin-3(7*H*)-ones **5** are requiring an aminopyrazine (**6**) prior to the construction of the imidazole ring via a condensation with α-ketoesters (**7**) or α-ketoaldehydes (**8**). Recent improvements have extended this access to original luciferins[24, 26] but to avoid some of its inherent limitations, we focused on an alternative

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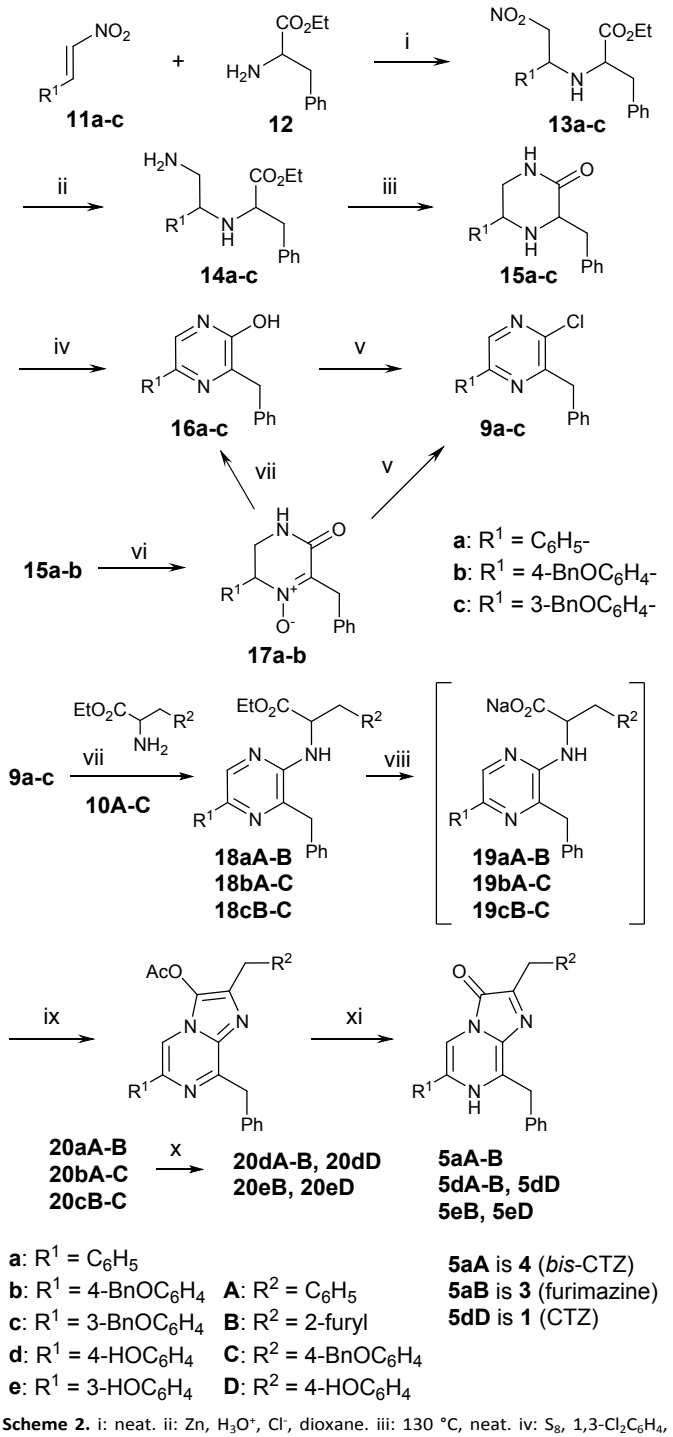
initially explored on a model compound.[31] This path not only avoids the use of the non-trivial intermediates **7-8**, but it also offers, via a key N-arylation of halogenopyrazines (**9**), the recourse to a wide range of the far more available α-amino esters (**10**).[32-34] As depicted in scheme 2, an original preparation of chloropyrazines **9a-c** was achieved starting with a 1,4-addition reaction between β-nitrostyrenes **11a-c** and phenylalanine ethyl ester (**12**).



Scheme 2. i: neat. ii: Zn, H_3O^+, Cl^-, dioxane. iii: 130 °C, neat. iv: S_8, 1,3-$Cl_2C_6H_4$, reflux. v: $PhPOCl_2$, 100 °C. vi: AcOOH, AcOEt. vii: chlorobenzene, reflux or NaOH, EtOH, reflux. . vii: Cs_2CO_3, $Pd(OAc)_2$, BINAP, MeCN, 60 °C. viii: NaOH, THF, 20 °C. ix: Ac_2O, 20 °C. x: H_2, Pd/C, AcOEt, AcOH, EtOH. xi: EtOH, DMSO, H_3O^+, Cl^-, 50 °C.

Indeed, in our cases, it is only when removing the solvent that the 1,4 adducts **13a-c** were formed. The use of zinc and hydrochloric acid in dioxane for their reduction provided an access to the diamines **14a-c** including a compatibility with the benzyloxy groups of **13b-c**. Their cyclization to give the (separable) piperazinone diastereoisomers **15a-c** was then achieved with heat. The previously unreported use of sulfur as an oxidant was initially essential for their aromatization into the corresponding hydroxypyrazines **16a-c**. Later on, we found an alternative via an original dehydration of the *N*-oxide **17a-b** (obtained by the peroxyacetic acid treatment of **15a-b**) using either heat or more preferably sodium hydroxide. Finally, from the hydroxypyrazines **16a-c**, hot phenylphosphonic dichloride[42] was essential to prepare the chloropyrazines **9a-c**, and this reagent could also be used to directly transform, for instance, *N*-oxide **17a** into the chloropyrazine **9a**. This was followed by the key Buchwald-Hartwig palladium-catalyzed N-arylation of the readily available[33, 34] α-amino esters **10A-C** by chloropyrazines **9a-c**. Starting from related precedents,[43-48] it quickly turned out that a mild temperature was required. The best conditions we found, 60 °C in acetonitrile with cesium carbonate for 12 hours using BINAP and palladium(II) acetate, led to the *N*-arylesters **18** in 69-90% yields. For the next step, we observed that the inherent instability of the target luciferins **5aB** limited its purification to a precipitation. To avoid this, we prepared the far more stable *O*-acetylated derivatives **20** in one pot from the *N*-arylesters **18** via the acid salts **19**, generated *in situ*, and an ensuing treatment with an excess of acetic anhydride. These pro-luciferins turned out to be stable enough to withstand a chromatography but, even better, a simple recrystallization provided compounds **20aA-B** in up to grams amount. For the synthesis of the phenol-bearing luciferins, a catalytic hydrogenation of *O*-benzyl-bearing compounds **20bA-bC** and **20cB-cC** provided the corresponding *O*-acetylated luciferins **20dA-dB**, **20dD**, **20eB** and **20eD**. Then, as seen by LC/MS (supporting information, figure S1), a treatment of these O-acetylated luciferins **20** with a mixture of hydrochloric acid, ethanol and DMSO at 50 °C provided concentrated solutions of the pure luciferins **5** which could be used immediately, upon a dilution in the relevant buffer, or stored at low temperature. As depicted in figure 2, and described in the supporting information section, the bioluminescence properties of these luciferins were then studied using a purified recombinant nanoKAZ/NanoLuc luciferase. In comparison with the very low intensity of coelenterazine (**1**), or for that matter "isocoelenterazine" (**5eD**), furimazine (**3**) and bisdeoxycoelenterazine (**4**) were, as previously reported,[20, 21] providing vastly improved bioluminescence signals lasting at least two hours. Interestingly, the two monohydroxy-bearing analogues *h*-coelenterazine (**5dA**) and **5dB** led to at least twice more intense signals but which lasted only minutes (figure 2B). Such initial intensity has actually been reported before for *h*-coelenterazine (**5dA**).[21] On the other hand, the isomeric mono hydroxy-bearing compound **5eB** displayed a far more stable bioluminescence profile pretty much identical with the one observed for furimazine (**3**). With all these luciferins, the light intensity decreased with time: rather quickly for the "flash" and

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much more slowly for the "glow" substrates. For the "flash" substrates, luciferins **5dA** and **5dB**, the decrease fitted with a first order equation. Adding further substrate after losing 90% of the initial light emission intensity did not, at best, produce any changes and could even causes a decrease of the remaining signal intensity. On the other hand, as depicted in figure 2C, adding instead the same amount of enzyme used at the start led to a recovery of the signal. For the others substrates, this enzyme death was less pronounced but took place anyway (see supporting information).

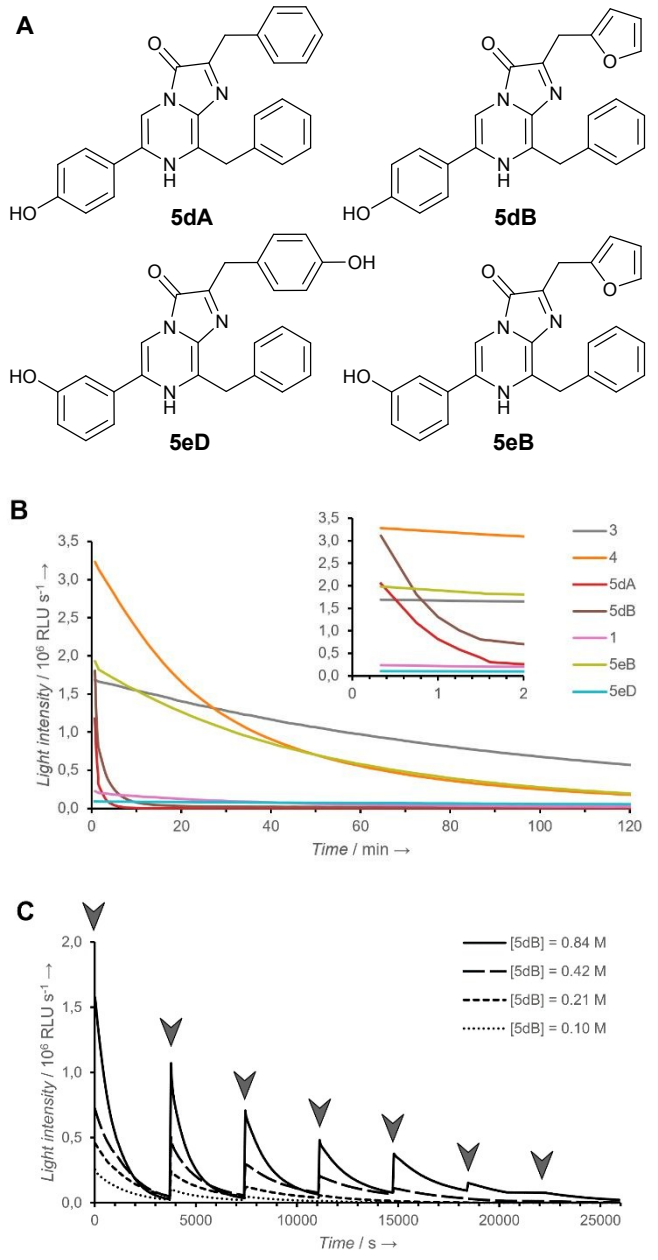

Figure 2. Bioluminescence signals of the luciferins **5** using recombinant nanoKAZ/NanoLuc. (**A**) Luciferin structures; compounds **1**, **3**, and **4** are depicted in figure 1. (**B**) Light intensity in RLU s^{-1} plotted vs. time over two hours, the insert zooms on the first two minutes of the reaction. (**C**) Bioluminescence profiles of compound **5dB** at different concentrations along with the repeated addition (arrows) of luciferase.

Accordingly, the enzyme is irreversibly inactivated by a reaction product in all the cases with a constant (k_{inact}) dependent on the substrate used. Of note is that when all the substrate has been consumed, the area below these curves is providing a molecules consumed per RLU produced ratio (see table 1). Concerning the kinetics of these reactions, they are fitting with a Michaelis-Menten model, if we assume that the number of detected photons per consumed substrate molecule is constant whatever the substrate concentration (see the supporting information for a discussion). The K_M and V_{max} values were computed considering: 1) the luciferins (S) as the limiting substrates, O_2 as saturating substrate 2) an inhibition of the enzyme E by excess of substrate through the binding of a second substrate (ESS) on the Michaelis' complex (ES) with the dissociation constant K_I and 3) a stochastic inactivation of the enzyme (E*) with the kinetic constant k_{inact}. As seen in table 1 and figure 3, coelenterazine (**1**) has a very high k_{cat} but a poor photon emission efficiency. Interestingly, furimazine (**3**) and its isomer **5eB** are the most efficient photon emitter per substrate molecule catalysis with the same k_{cat} but furimazine (**3**) is somehow providing a longer life time of active enzyme (low k_{inact}). All substrates are sensitive to substrate concentration beyond the K_M, but two luciferins, coelenterazine (**1**) and **5eD**, are more affected than others as seen with their low dissociation constants (K_I). Of note is that the strong light intensity produced by the catalyzed oxidation of **5dB** in a very short time (flash) does not mean the production of a high level of light intensity cumulated in two hours (ΣI) and the very long half-life ($t_{1/2}$) with low light intensity (glow) provided by compound **5eD** neither. Among these substrates, furimazine (**3**) is, so far, providing the best compromise. Also quite unexpected is the reason behind the fact that the natural substrate coelenterazine (**1**) leads to a signal two orders of magnitudes less intense than furimazine (**3**). Indeed, it is not because of a lesser catalytic activity but it is mostly due to a pretty much counter intuitive lesser number of photons detected by luciferin consumed.

Table 1. Kinetic parameters for each substrate.

	3	**4**	**5dA**	**5dB**	**1**	**5eB**	**5eD**
$\mathbf{I_{max}}$ (10^6 RLU s^{-1})	1.69	3.28	2.05	3.11	0.23	1.98	0.10
t½ (min)	74.29	19.34	3.22	0.33	20.96	32.27	148
ΣI (10^6 RLU)	84.98	80.85	5.61	11.57	6.29	62.97	6.04
$\mathbf{K'_M}$ (10^{-6} M)	3.40	2.33	3.71	3.45	8.8	4.23	7.02
$\mathbf{k'_{cat}}$ (10^{18} RLU s^{-1})	1.80	3.50	2.32	3.60	0.44	2.33	0.17
Molecules/RLU	1775	4581	12067	4463	101169	1804	70020
$\mathbf{K_I}$ (10^{-6} M)	104.94	115.21	140.55	101.64	22.48	84.02	35.42
$\mathbf{K_M}$ (10^{-6} M)	2.22	3.02	5.90	3.88	6.80	3.30	2.94
$\mathbf{k_{cat}}$ ($mol.s^{-1}.mol_E^{-1}$)	106	534	932	535	1500	140	362
$\mathbf{k_{inact}}$ (10^{-4} s^{-1})	1.5	5.2	425	375	3.8	4.5	3.1

An alternative relative depiction of some of these variables is provided figure 3.

Figure 3 is probably providing a better visual representation of these differences. In comparison with the central equidistant triangle representing three characteristics of furimazine (**3**): maximum intensity (Imax), the sum of the signal (ΣI), and half-life of the signal over two hours ($t_{1/2}$), the values for the other substrates can vary widely. Accordingly, the next stage of our research will be to find out how specific structural features of the luciferins have an influence on these characteristics and more importantly, can such changes lead to even better luciferins.

Conclusions

In conclusion, the results of this work should herald more researches focusing/based on bioluminescent reporting systems using imidazo[1,2-*a*]pyrazine-3(7*H*)-one luciferins. Indeed, we describe here a simple mean to prepare these rather expensive luciferins in such amount that it should provide scientists with many more opportunities to design and use such reporting systems.

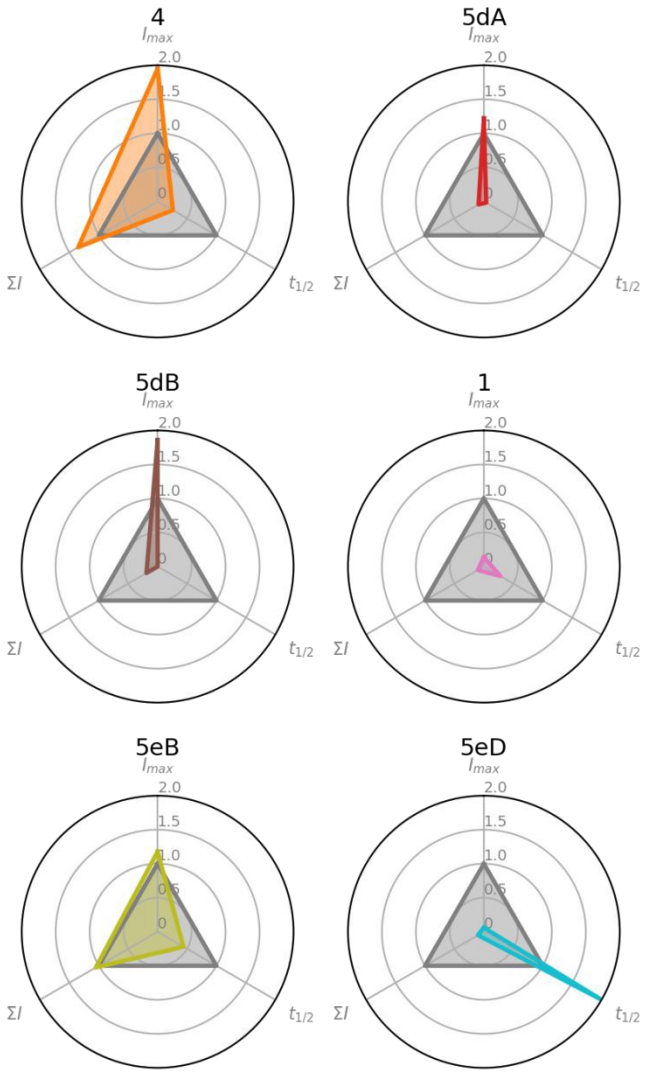

Figure 3. Depiction of the maxima of intensities (I_{max}), integrated signals (ΣI), and half-time durations ($t_{1/2}$) for all the luciferins, relative to furimazine (**3**) represented in light grey.

The kinetic analyses of light emission presented here are providing rather unexpected insights for distinct applications. The glowing property of some substrates is appropriate for high throughput *in vitro* bioassays and long imaging dynamics *in vivo* or *in cellulo*, whereas the flashier profile of other is appropriate for high sensitivity acquisition systems requiring more light in a short time. Moreover, it appears that if the actual catalytic efficiency of nanoKaz/NanoLuc is high, as it is in the 10^2-10^3 $mol_S/s \cdot mol_E$ range, the detection of emitted photons is remarkably modest: only one RLU for more than 1800 decarboxylated molecules at best. Also of note is the fast stochastic inactivation seen for some substrate in contrast with other which may reflect the existence of two distinct inactivation mechanisms which we are also trying to investigate. In any case, in view of all this, we believe that further luciferases mutagenesis and/or design of original luciferin analogues could lead to even more improved bioluminescence profiles.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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