Core-modified coelenterazine luciferin analogues, synthesis and chemiluminescence properties

Glwadys Gagnot, Vincent Hervin, Eloi P. Coutant, Sophie Goyard, Yves Jacob, Thierry Rose, Fatima Ezzahra Hibti, Alessia Quatela, Yves Louis Janin

Chemistry
A European
Journal

Chemistry Europe
European Chemical Societies Publishing

Accepted Article

Title: Core-modified coelenterazine luciferin analogues, synthesis and chemiluminescence properties

Authors: Glwadys Gagnot, Vincent Hervin, Eloi P Coutant, Sophie Goyard, Yves Jacob, Thierry Rose, Fatima Ezzahra Hibti, Alessia Quatela, and Yves Janin

To be cited as: *Chem. Eur. J.* 10.1002/chem.202004311

Link to VoR: https://doi.org/10.1002/chem.202004311

WILEY-VCH

10.1002/chem.202004311

Core-modified coelenterazine luciferin analogues, synthesis and chemiluminescence properties

Glwadys Gagnot,[ab] Vincent Hervin,[a] Eloi P. Coutant,[a] Sophie Goyard,[c] Yves Jacob,[d] Thierry Rose,[c] Fatima Ezzahra Hibti,[e] Alessia Quatela,[e] Yves L. Janin[a*]

a: Unité de Chimie et Biocatalyse, Institut Pasteur, UMR 3523, CNRS, 28 rue du Dr. Roux, 75724 Paris Cedex 15, France.
b: Université de Paris, 12 rue de l'école de Médecine, 75006 Paris, France.
c: Center for Innovation and Technological Research, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France.
d: Unité de Génétique Moléculaire des Virus à ARN, Institut Pasteur, UMR 3569, CNRS, 28 rue du Dr. Roux, 75724 Paris Cedex 15, France.
e: HORIBA FRANCE SAS, 14 Boulevard Thomas Gobert, Passage Jobin Yvon CS45002, 91120 Palaiseau, France

Abstract

In our work on the design and studies of luciferins related to the blue-hued coelenterazine, we undertook the synthesis of heterocyclic analogues susceptible to produce a photon, possibly at a different wavelength. We describe here the synthesis of *O*-acetylated derivatives of imidazo[1,2-*b*]pyridazin-3(5*H*)-one, imidazo[2,1-*f*][1,2,4]triazin-7(1*H*)-one, imidazo[1,2-*a*]pyridin-3-ol, imidazo[1,2-*a*]quinoxalin-1(5*H*)-one, benzo[*f*]imidazo[1,2-*a*]quinoxalin-3(11*H*)-one, imidazo[1',2':1,6]pyrazino[2,3-*c*]quinolin-3(11*H*)-one and 5,11-dihydro-3*H*-chromeno[4,3-*e*]imidazo[1,2-*a*]pyrazin-3-one thanks to an extensive use of the Buchwald-Hartwig N-arylation. The acidic hydrolysis of these derivatives then gave solutions of the corresponding luciferin analogues which we studied. Not too unexpectedly, even if these were "dressed" with substituents found in actual substrates of the nanoKAZ/NanoLuc luciferase, no bioluminescence was observed with these compounds. However, in a phosphate buffer, all produced a light signal, by chemiluminescence, with extensive variations in their respective intensity and this could be increased by adding a quaternary ammonium salt in the buffer. This aspect was actually instrumental to determine the emission spectra of many of these luciferin analogs.

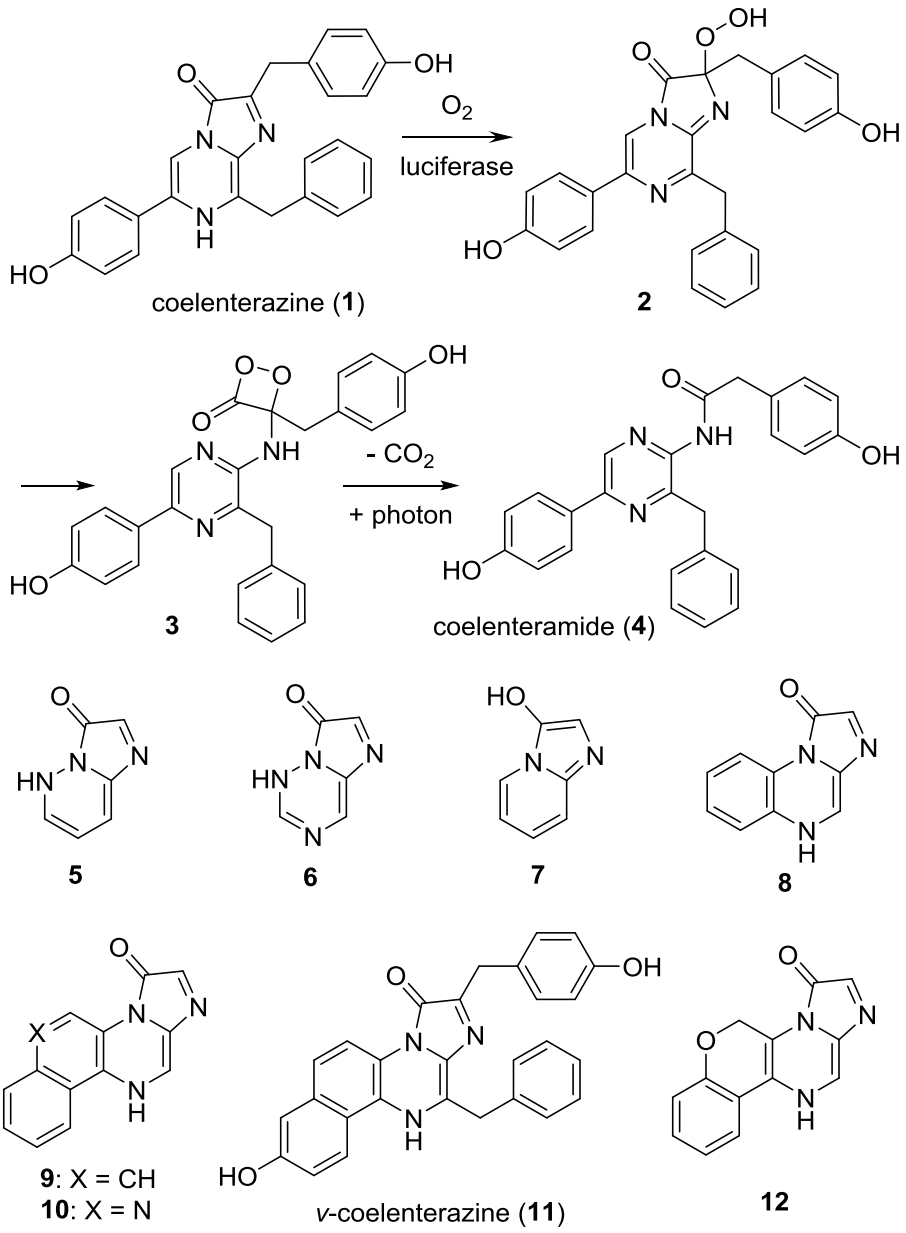
Introduction

Chemiluminescence as well as bioluminescence,[1] its luciferase-catalyzed version, are based on remarkable oxidation reactions of luciferins leading to products in an excited electronic state, which can then relax either by non-radiative processes or by a photon emission.[2] This phenomenon has found numerous applications, especially for detection purposes. In life sciences, a great variety of bioluminescent reporting systems have thus been constructed out of a luciferin and a luciferase. Amongst them, systems based on the marine luciferin coelenterazine (**1**) and the corresponding luciferases or photoproteins from a remarkably diverse number of sea dwelling species[1a, 3] are increasingly used.[4] The mechanism at the source of the photon emission by these systems is depicted in scheme 1. The first step is a reaction with dioxygen leading to the peroxide **2** which, unless stabilized,[5] undergoes a rearrangement into the endoperoxide **3** and then decomposes into carbon dioxide and coelenteramide (**4**). The latter product occurs in an excited electronic state which can relax back to its ground state via the emission of a photon at a "short" wavelength corresponding to a blue color (between 450 and 480 nm), the most visible undersea. Contrary to firefly luciferins, which require an ATP-based activation, this oxidation can also be achieved without a luciferase in the presence of oxygen under a variety of conditions and will lead, via the same mechanism,[6] to a usually far less efficient photon emission phenomenon which is called chemiluminescence. Some current avenue of research are focusing on the design of luciferin/luciferase reporting systems, still based on coelenterazine using enzymes, but emitting a photon at a higher wavelength which is more suitable for *in vivo* studies.[7] This is due to the fact that a red photon will be detectable through the living tissues of a small animal, contrary to a blue one. In the present report, we planned on the use of mutants of luciferases such as nanoKAZ/NanoLuc, which is already accepting a rather large array of imidazo[1,2-*b*]pyridazin-3(5*H*)-ones as substrates,[7i, 8] and designed analogues featuring an altered heterocyclic core which we also "dressed" with the phenyl/bisbenzyls residues found in actual substrates of the nanoKAZ/NanoLuc luciferase. We thus sought accesses to unprecedented derivatives of imidazo[1,2-*b*]pyridazin-3(5*H*)-one **5**, imidazo[2,1-*f*][1,2,4]triazin-7(1*H*)-one **6** or imidazo[1,2-*a*]pyridin-3-ol **7** which could theoretically undergo an electronic cascade leading to the occurrence of hydroperoxide intermediates as depicted for coelenterazine (**1**). The synthesis of a "deaza" coelenterazine analog featuring this last heterocyclic core has actually been reported

10.1002/chem.202004311

previously.[9] We also prepared some imidazo[1,2-*a*]quinoxalin-1(5*H*)-ones **8** which have been the subject of an untranslated patent for their chemiluminescence properties although no mention of the wavelength of the emitted photon appears to be mentioned.[10] A single derivative featuring this ring system has also been claimed in a more recent patent.[11] The synthesis of analogues featuring a benzo[*f*]imidazo[1,2-*a*]quinoxalin-3(11*H*)-one skeleton **9** or imidazo[1',2':1,6]pyrazino[2,3-*c*]quinolin-3(11*H*)-one **10** were also explored as a similar heterocyclic system is found in the structure of *v*-coelenterazine (**11**), a luciferin analogue which is a substrate of the soft coral *Renilla reniformis* luciferase[12] and some of its mutants,[7b] as well as the photoprotein found in the *Aequorea* jellyfish.[13] Finally, we replaced the additional pyridine ring of compound **11** by a pyran ring and designed an access to an unprecedented 5,11-dihydro-3*H*-chromeno[4,3-*e*]imidazo[1,2-*a*]pyrazin-3-one **12** derivative.

10.1002/chem.202004311

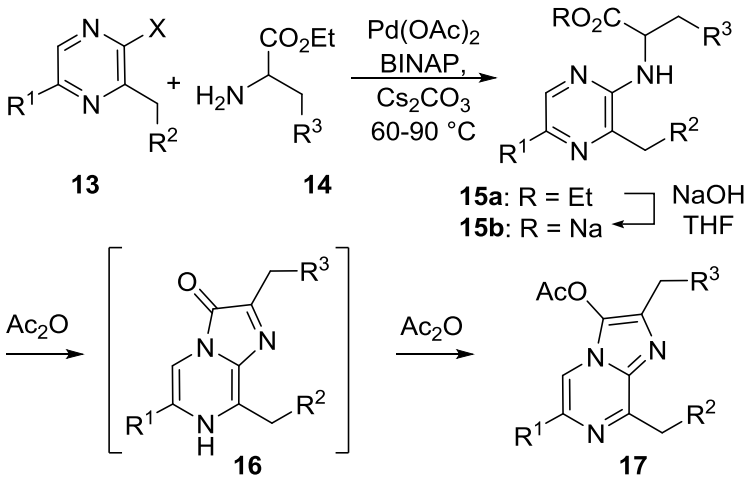


Scheme 1. Luciferase-based oxidative decarboxylation of coelenterazine (**1**) and potential light-producing ring systems investigated in this report

Syntheses

We have previously reported improvements of the nanoKAZ/NanoLuc-based reporting system using an original synthetic pathway leading to enhanced luciferin analogues.[8c, 14] As depicted in scheme 2, these compounds were obtained from chloropyrazines **13** and amino esters **14** via a key N-arylation step to give the stable esters **15a**. Upon treatment with sodium hydroxide under an inert atmosphere, these gave the corresponding sodium salts **15b** and the addition of an excess

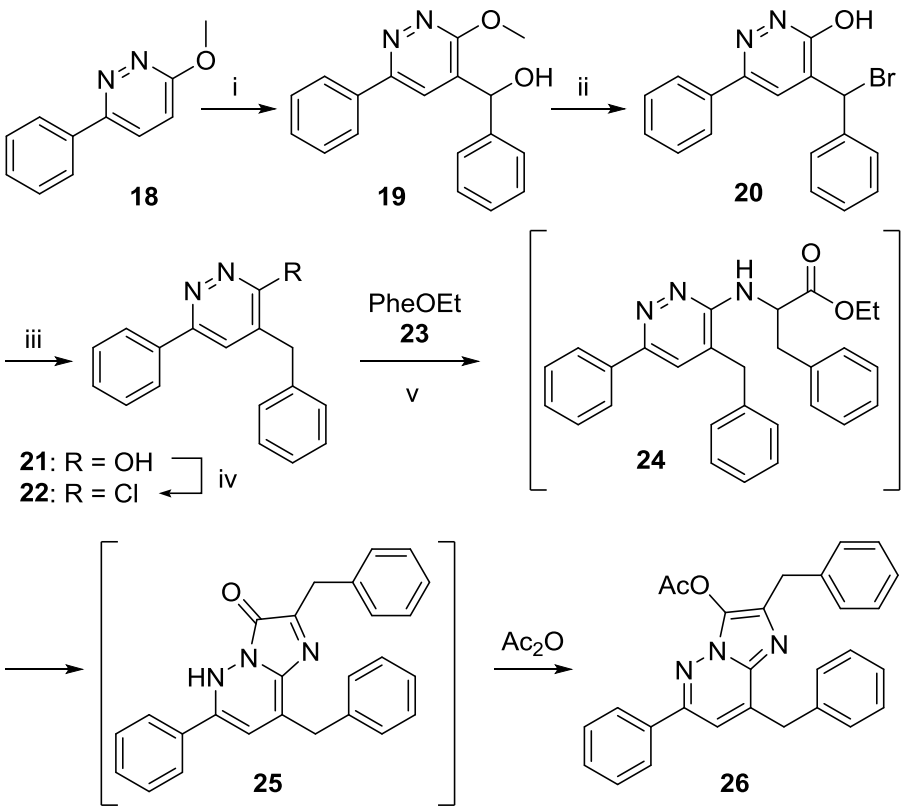
of acetic anhydride led, via the imidazo[1,2-*a*]pyrazin-3(7*H*)-ones **16**, to the far more stable *O*-acetylated luciferins **17**. Then, when required, the proluciferins **17** can be hydrolyzed back into the luciferins **16** under mild conditions to give, after further dilution in appropriate buffer, ready-to-use solution for bioluminescence-based studies.



Scheme 2. Previously reported[8c, 14b] synthesis of *O*-acetyl imidazo[1,2-*a*]pyrazin-3(7*H*)-one luciferins.

*Preparation of an imidazo[1,2-*b*]pyridazin-3(5*H*)-one derivative*. As depicted in scheme 3, the *O*-acetylated compound **26** featuring an imidazo[1,2-*b*]pyridazine ring system was synthesized in 5 steps from phenylpyridazine **18**, readily available from 3-chloro-6-methoxypyridazine and phenylboronic acid using a Suzuki-Miyaura coupling reaction.[15] From this compound, a regioselective deprotonation using lithium diisopropylamide (LDA) followed by the addition of benzaldehyde led to alcohol **19**. The reduction of the hydroxyl function to produce compound **21** turned out to be fraught with difficulties as an over-hydrogenation was often observed. An acceptable yield was eventually found via a palladium-catalyzed hydrogenation of the bromine-bearing 3-hydroxypyridazine **20** readily prepared by treatment of alcohol **19** with hydrogen bromide in acetic acid. From the reduced compound **21**, the 3-chloropyridazine **22** was then obtained upon its treatment with hot phenylphosphonic dichloride. Then, we tried to prepare the *N*-arylated derivative of phenylalanine ethyl ester (**23**) using our previously published method.[8c, 14b] Unexpectedly, under these conditions the corresponding ester **24** directly underwent a cyclization into the corresponding imidazo[1,2-*b*]pyridazin-3(*5H*)-one **25**. However, due its

instability under basic conditions, this intermediate immediately decomposed upon exposure to dioxygen. Accordingly, an excess of acetic anhydride was added to the reaction mixture at the end of this chemical step leading to the far more stable *O*-acetylated imidazo[1,2-*b*]pyridazine derivative **26** which was isolated in a 58% yield from 3-chloropyridazine **22**.



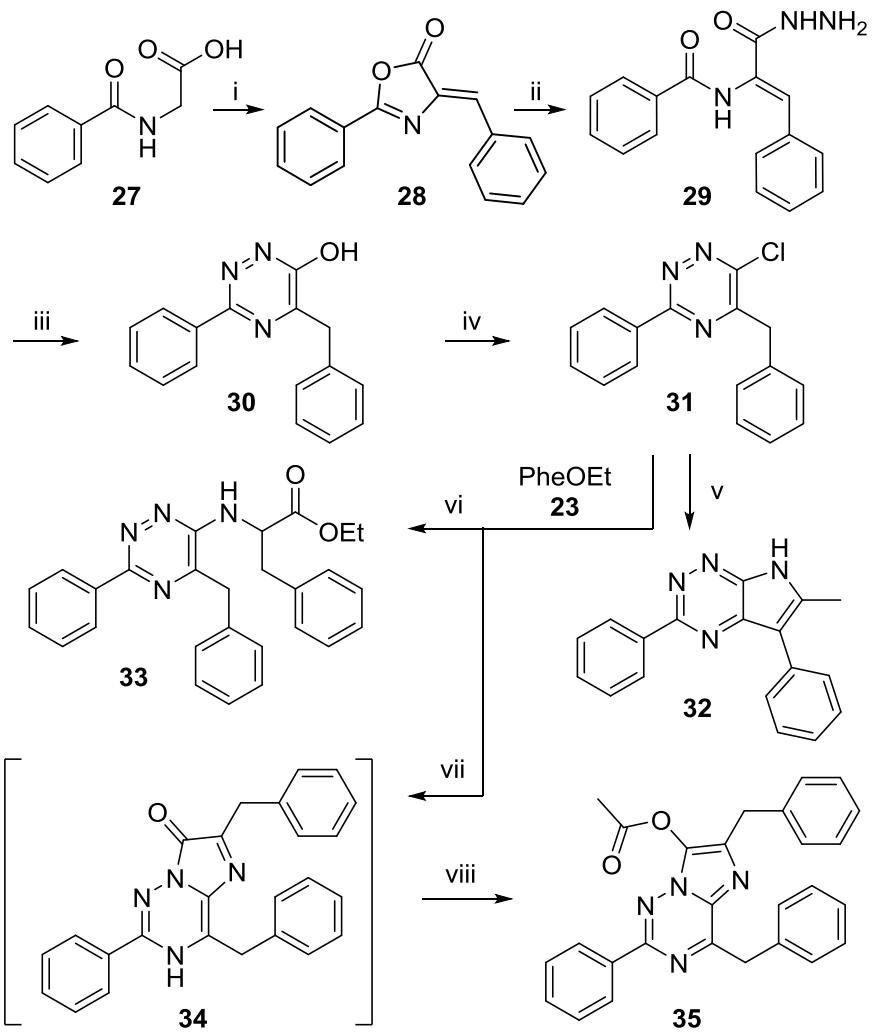
Scheme 3. i: 1) LDA, THF, - 78 °C, 2) PhCHO. ii: HBr/AcOH, 90 °C. iii: H_2, Pd/C, *i*PrOH, 20 °C. iv: $PhPOCl_2$, 100 °C. v: $Pd(OAc)_2$, BINAP, Cs_2CO_3, toluene, 90 °C.

*Preparation of an imidazo[2,1-*f*][1,2,4]triazin-7(1*H*)-one derivative*. As depicted in scheme 4, to reach an imidazo[2,1-*f*][1,2,4]triazine analogue the corresponding 6-hydroxytriazine **30** was made in 3 steps from hippuric acid (**27**) using a previously described method.[16] Unexpectedly, transformation of the 6-hydroxytriazine **30** into the corresponding 6-chlorotriazine **31** turned out to be difficult as low yield and/or decomposition were observed when trying various chlorination agents: thionyl chloride, phosphorus oxychloride, or phenylphosphonic dichloride. The use of mild conditions (phosphorus oxychloride diluted in acetonitrile in the presence of solid potassium carbonate at reflux) led to a rather modest 19-22% yield of the target compound **31**. The ensuing

10.1002/chem.202004311

N-arylation of phenyl alanine ethyl ester (**23**) also turned out to be problematic as an extensive decomposition was observed. Out of the many side products occurring, a minute amount (4%) of the pyrrolotriazine **32**, resulting from a condensation of acetonitrile, was isolated. Switching to toluene at 90 °C actually led to the isolation of the expected amino ester **33** but in a very poor 11% yield. Moreover, the target compound **33** turned out to be unstable in open air under various conditions, such as when left in solution in $CDCl_3$, as seen by LC/MS analysis (*m/z* = 453 and 455). An alternative was eventually designed as we found that the slow uncatalyzed N-arylation of phenylalanine ethyl ester (**23**) by compound **31** was also possible. Indeed, upon heating at 120 °C these compounds in N-methylpyrrolidine (NMP) using triethylamine as a base for 14 days led directly to a solution containing the unstable luciferin **34**. Its *in situ* treatment with an excess of acetic anhydride and additional triethylamine allowed the isolation of the target proluciferin **35** in an 8% overall yield from compound **31**.

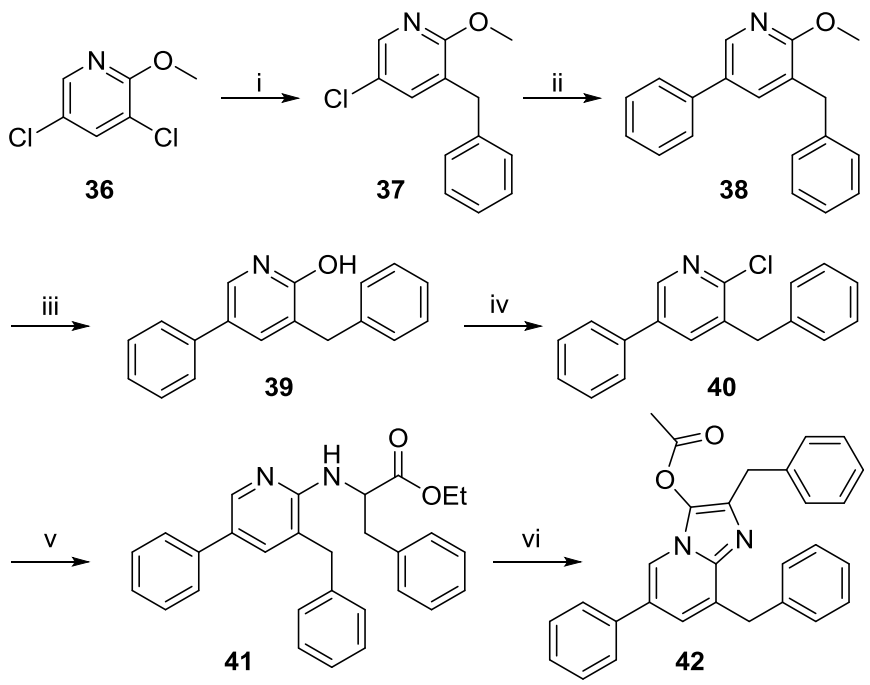
10.1002/chem.202004311



Scheme 4. i: PhCHO, AcONa, Ac_2O, 65 - 90 °C. ii: NH_2NH_2, EtOH, 20 °C. iii: NaOH 1.5N, reflux. iv: $POCl_3$, K_2CO_3, MeCN, reflux. v: $Pd(OAc)_2$, BINAP, Cs_2CO_3, MeCN, 60 °C. vi: $Pd(OAc)_2$, BINAP, Cs_2CO_3, toluene, 90 °C. vii: NEt_3, NMP, 120 °C, 14 days. viii: Ac_2O, NEt_3, 20 °C.

*Preparation of an imidazo[1,2-*a*]pyridin-3-ol derivative.* The original preparation of compound **42** depicted in scheme 5 started with the readily available 2-methoxypyridine derivative **36**.[17] It turned out that a regiospecific Negishi reaction was possible even when using 1.5 equivalents of benzylzincbromide, and the C-3 benzylated derivative **37** was isolated in a 47% yield. The structure of this compound was established by long distance NMR correlation. From this intermediate, a Suzuki-Miyaura coupling provided the phenyl in position 5 and a cleavage of its methoxy group led to the 2-hydroxypiridine derivative **39**. Chlorination of this compound turned

out to require harsher conditions than expected and even heating the reaction at 150 °C for 22 hours gave an overall 56% of the 2-chloropyridine **40** from the C-3 benzylated derivative **37**. The ensuing N-arylation step with phenylalanine ethyl ester (**23**) gave 60% of compound **41**. From this ester, a hydrolysis using sodium hydroxide followed by a cyclisation in the presence of an excess of acetic anhydride led to 32% of the target *O*-acetylated imidazo[1,2-*a*]pyridine **42**.

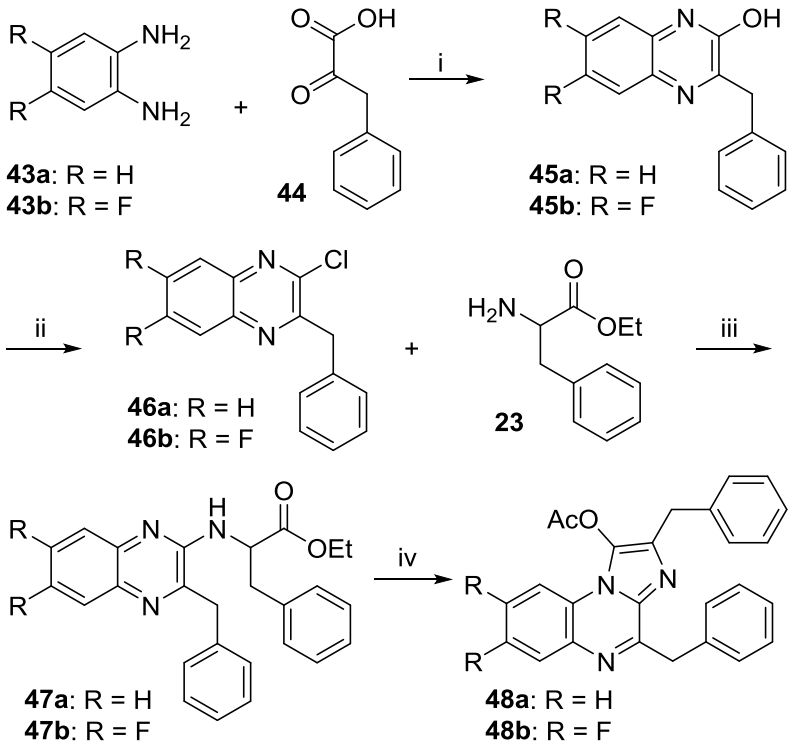


Scheme 5. i: $PhCH_2ZnBr$, $Pd(OAc)_2$, XPhos, THF, 60 °C. ii: $PhB(OH)_2$, $Pd(OAc)_2$, SPhos, dioxane/H_2O 5:1, 90 °C. iii: 33% HBr/AcOH, 100 °C. iv: $PhPOCl_2$, 150 °C. vi: PheOEt (**23**), $Pd(OAc)_2$, BINAP, Cs_2CO_3, toluene, 90 °C. vii: 1) NaOH, THF, 20 °C, 2) Ac_2O, 20 °C.

*Preparation of an imidazo[1,2-*a*]quinoxalin-1(5*H*)-one derivative*. As depicted in scheme 6, an original preparation of luciferin analogues featuring an imidazoquinoxaline ring system was developed. For this, we used the quinoxalinol **45a** made from the condensation between *o*-phenylenediamine (**43a**) and phenylpyruvic acid (**44**) in the presence of hydrochloric acid and 2-mercaptoethanol[18] which led to 79% of **45a**. Of interest is the fact that a control experiment without 2-mercaptoethanol still provided 62% of compound **45a**. From this quinoxalinol, the corresponding 2-chloroquinoxaline **46a** was then obtained in a 90% yield and this was followed by its N-arylation with phenylalanine ethyl ester (**23**) to give 71% of compound **47a**. The ensuing ester hydrolysis using sodium hydroxide followed by a cyclisation using an excess of acetic

10.1002/chem.202004311

anhydride finally gave 69% of the target *O*-acetylated imidazo[1,2-*a*]quinoxaline **48a**. Although a more extensive purification procedure was required to obtain 46% of compound **46b** from the difluorinated *o*-phenylenediamine **43b**, the same synthetic pathway led to the analogue **48b** in a 12% overall yield. Of note was the far slower N-arylation of phenylalanine ethyl ester (**23**) with the fluorine-bearing 2-chloroquinoxaline **46b** as only an 11% yield of the N-arylester **47b** was obtained in 12 hours of heating whereas a 38% yield was achieved when heating for 27 hours.



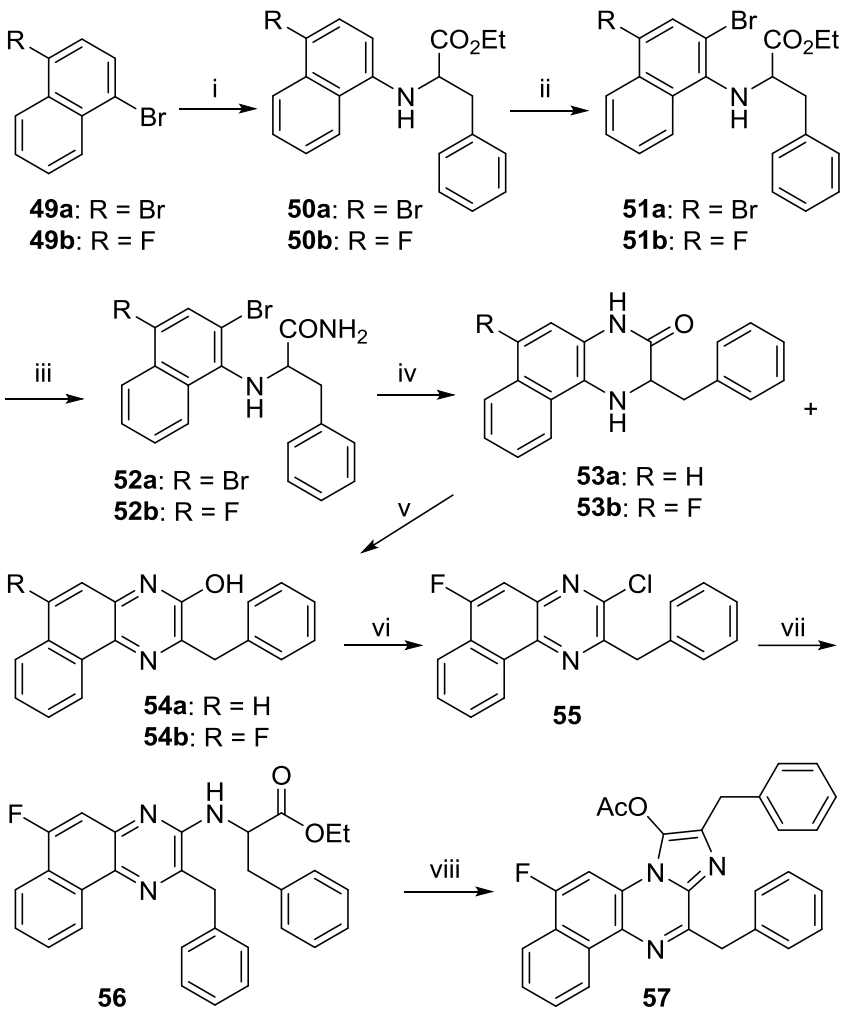
Scheme 6. i: EtOH, $HSCH_2CH_2OH$, 2N $H_3O^+Cl^-$, reflux. ii: $PhPOCl_2$, 100 °C. iii: $Pd(OAc)_2$, BINAP, Cs_2CO_3, toluene, 90 °C. iv: 1) NaOH, THF, 20 °C, 2) Ac_2O, 20 °C.

Preparation of benzo[f]imidazo[1,2-a]quinoxalin-3(11H)-one derivatives. As depicted in scheme 7, an original[13, 19] synthesis of such analogues was designed via the use of three distinct Buchwald-Hartwig N-arylation reactions. The first one allowed the N-arylation of phenylalanine ethyl ester (**23**) by the 2-bromonaphthalene derivatives **49a** and **49b**. Rather long reaction time were required: 22 hours at 105 °C and 64 hours at 100 °C, to secure respectively 67 and 54% yield of compounds **50a** and **50b**. Bromination of the resulting products led selectively to the bis-brominated derivative **51a** and the 2-bromo-4-fluoro derivative **51b**. This was followed by a

10.1002/chem.202004311

quantitative amidification of these esters, using methanolic ammonia in a pressure vessel at 100 °C, which gave the amides **52a** and **52b**. From these, an intramolecular Buchwald-Hartwig reaction was envisaged using Xantphos as the palladium ligand.[20] However, from the bis-brominated derivative **52a**, trials pointed out an extensive decomposition and very little occurrence of compounds **53a** or **54a**, probably due to an un-selective palladium insertion in the carbon-bromine bounds. On the other hand, from the 2-bromo-4-fluoro amide **52b**, the same reaction led to a chromatographic fraction containing mixture of compound **53b**, resulting from the planned cyclization, and compound **54b** resulting from the aromatization of some **53b**. This aromatization was then completed using peracetic acid as the oxidant and compound **54b** was isolated in 54% yield from **52b**. The ensuing chlorination with hot phenylphosphonic dichloride gave 67% of compound **55** which was subjected to the last Buchwald-Hartwig reaction to yield 71% of the *N*-arylated phenylalanine ethyl esters **56**. The last two steps, hydrolysis of the ester function followed *in situ* by a cyclization with an excess of acetic anhydride, gave 65% of the target *O*-acetylated luciferins analogue **57**.

10.1002/chem.202004311

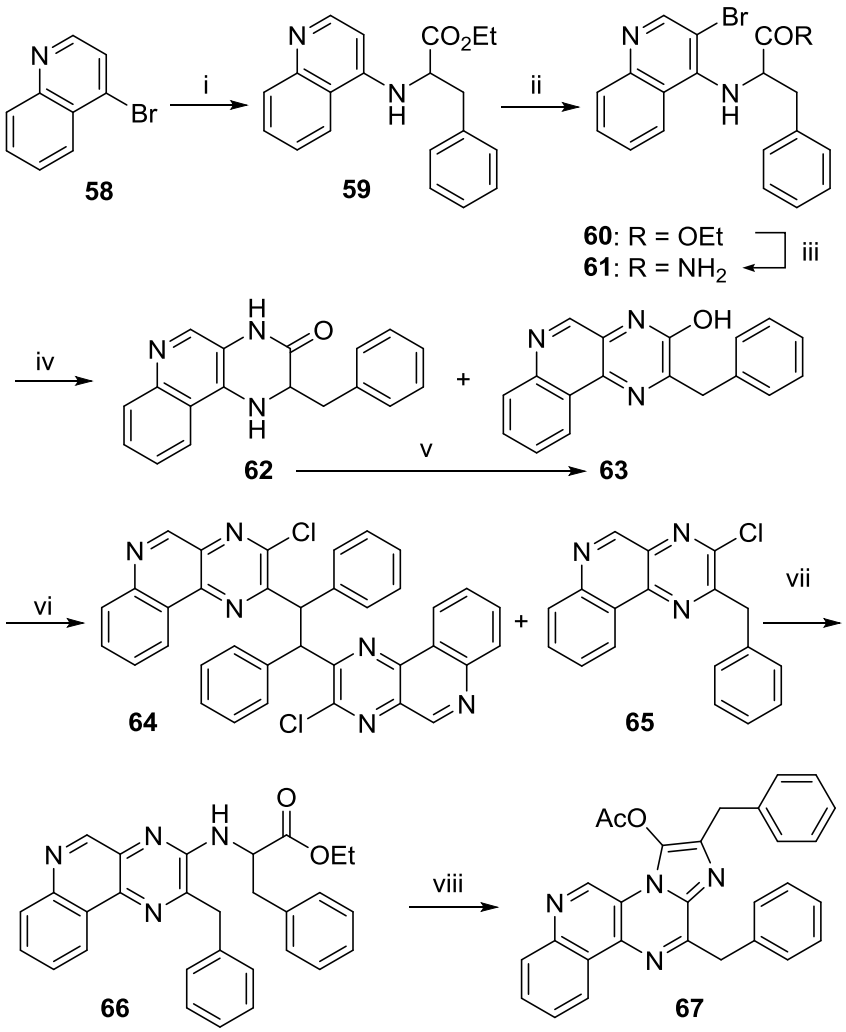


Scheme 7. i: PheOEt (**23**), $Pd(OAc)_2$, BINAP, Cs_2CO_3, toluene, 100 - 105 °C. ii: NBS, CH_2Cl_2, 0-20 °C. iii: 7N NH_3/MeOH, 100 °C. iv: $Pd(OAc)_2$, Xantphos, Cs_2CO_3, THF, 120 °C. v: 35% AcOOH/AcOH, AcOEt, 20 °C. vi: $PhPOCl_2$, 100 °C. vii: PheOEt (**23**), $Pd(OAc)_2$, BINAP, Cs_2CO_3, MeCN, 60 °C. viii: 1) NaOH, THF, 20 °C, 2) Ac_2O, 20 °C.

Preparation of an imidazo[1',2':1,6]pyrazino[2,3-c]quinolin-3(11H)-one derivative. The synthetic pathway described above was used again to prepare the nitrogen-bearing analogue, starting with 4-bromoquinoline (**58**) as depicted in scheme 8. If the first four steps, including the first two Buchwald-Hartwig reactions, proceeded without any difficulties, the aromatization step of compound **62** required sulfur in boiling decaline and gave 67% of the target compound **63**. In the next step, chlorination in hot phenylphosphonic dichloride to obtain compound **65** turned out to be very slow, even at 100 °C. Raising the temperature to 150 °C led to 2.6% of the unexpected

10.1002/chem.202004311

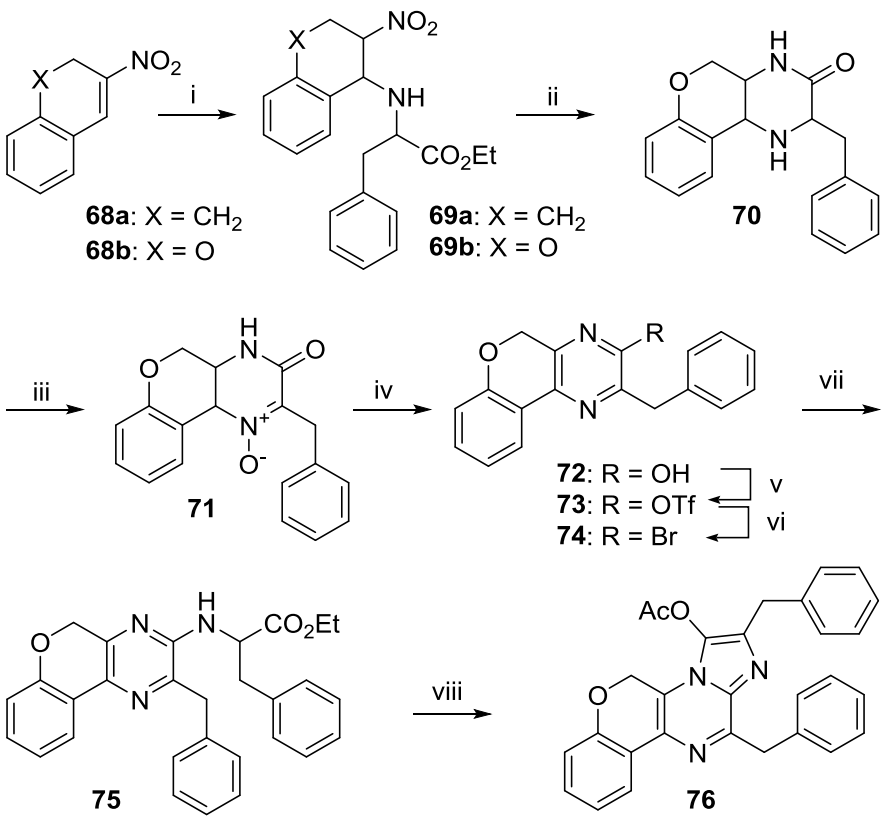
compound **64** resulting from an oxidative dimerization process along with 17% of the target compound **65**. It is only by running the reaction as thoroughly as possible under an argon atmosphere and adding potassium carbonate (presumably to quench any evolving hydrochloric acid) that compound **65** could be obtained in a 38% yield although 19% of the dimer **64** and some unreacted material (less than 8%) were also isolated. This unexpected methylene reactivity, somehow reminiscent of what we previously observed with the chlorotriazine **31** depicted in scheme 4, probably had also some effect on the last Buchwald-Hartwig N-arylation as we could isolate the resulting α-amino ester **66** in only 43% yield. Moreover, a rather high incidence of decomposition was observed in the course of the last synthetic steps and the target *O*-acetylated luciferin analogue **67** was obtained in only a 35% yield.



Scheme 8. i: PheOEt (**23**), $Pd(OAc)_2$, BINAP, Cs_2CO_3, toluene, 95 °C. ii: NBS, CH_2Cl_2, 0-20 °C. iii: 7N NH_3/MeOH, 100 °C. iv: $Pd(OAc)_2$, Xantphos, Cs_2CO_3, THF, 120 °C. v: S_8, decaline, reflux. vi: $PhPOCl_2$, K_2CO_3, 150 °C, argon. vii: PheOEt (**23**), $Pd(OAc)_2$, BINAP, Cs_2CO_3, MeCN, 60 °C. viii: 1) NaOH, THF, 20 °C, 2) Ac_2O, 20 °C.

*Preparation of a 5,11-dihydro-3*H*-chromeno[4,3-*e*]imidazo[1,2-*a*]pyrazin-3-one derivative.* In an attempt to design an alternative pathway to benzo[*f*]imidazo[1,2-*a*]quinoxalin-3(11*H*)-one **14**, we initially contemplated to use our previously reported preparation of chloropyrazines starting with a 1,4-addition of phenyl alanine ethyl ester (**23**) as a free base on nitrostyrene.[8c, 14b] Accordingly, as depicted in scheme 9, we started with the bridged derivative 3-nitro-1,2-dihydronaphthalene (**68a**), readily prepared by nitration of dihydronaphtalene using tetranitromethane.[21] Unexpectedly and despite many trials, no 1,4-addition was observed with this substrate. On the other hand, this 1,4-addition readily took place on 3-nitro-2H-chromene (**68b**)[22] to give the pyran-bearing 1,4-adduct **69b**. We do not have a satisfying explanation for this difference of reactivity although the only example reported of a nitrogen-bearing nucleophilic 1,4-addition on 3-nitro-1,2-dihydronaphthalene (**68a**) involved a far more nucleophilic specie.[23] In any case, from compound **69b**, a reduction of its nitro function using zinc and hydrochloric acid followed by a thermal cyclisation of the resulting amine gave the tetrahydrochromeno[3,4-*b*]pyrazin-3(4*H*)-one **70** as a mixture of diastereoisomers. Many attempts were made to achieve its aromatization, using sulfur in boiling 1,3-dichlorobenzene, iodine as well as other oxidants with a very limited success.[14a] We thus resorted to the preparation of the quite unstable nitrone **71**. From this crude compound, a dehydration reaction using sodium hydroxide[14b] gave the desired 5*H*-chromeno[3,4-*b*]pyrazin-3-ol **72** in 52% yield from compound **70**. The preparation of the corresponding chloro derivative using hot phenylphosphonic dichloride only led to minute amount of this compound. Accordingly, we used a milder alternative[24] via the preparation of the triflate ester **73**, and a displacement reaction with sodium bromide which gave the bromo derivative **74** in 40% yield from the chromenopyrazinol **72**. From compound **74**, the ensuing N-arylation of phenylalanine ethyl ester (**23**) led to 70% of the expected ester **75** and the final steps provided 80% of the target *O*-acetylated luciferin analogue **76**.

10.1002/chem.202004311



Scheme 9. i: PheOEt (**23**; free base), neat, 0 °C. ii: 1) Zn dust, 37% $H_3O^+Cl^-$, dioxane, 0-20 °C, 2) neat, 140 °C. iii: 35% AcOOH/AcOH, AcOEt, 20 °C. iv: NaOH, EtOH, 20 °C. v: Tf_2O, NEt_3, CH_2Cl_2, 20 °C. vii: NaBr, TfOH, DMF, 120 °C. vii: PheOEt (**23**), $Pd(OAc)_2$, BINAP, Cs_2CO_3, toluene, 85 °C. viii: 1) NaOH, THF, 20 °C, 2) Ac_2O, 20 °C.

Hydrolysis of the O-acetylated luciferin analogues and chemiluminescence properties.

The acid hydrolysis of the *O*-acetylated analogues **26**, **35**, **42**, **48a-b**, **57**, **67** and **76**, using a mixture of DMSO, ethanol and hydrochloric acid at 50 °C for two hours gave the luciferins **77-85** depicted in figure 1. Of note is that, in the specific case of compound **35**, which gave compound **79**, this reaction required 4 hours. The LC/MS monitoring of these reactions pointed out the occurrence of clean solutions of the expected compounds **78-85** along with, in the case of compounds **83** and **85**, a slow decomposition taking place over 6 weeks even when the solution was stored at -20 °C. Moreover, hydrolysis of compound **26**, occurred along with the formation of a small proportion of two dimers (*m/z* = 781). A study of this oxidative process pointed out that it took place in the course of the hydrolysis of **26** but further heating of the resulting solution, even in the presence of air or when adding far more hydrochloric acid did not lead to any

10.1002/chem.202004311

additional dimerization of compound **78**. Solution of the compounds **1**, **86** and **87** were also used as references.

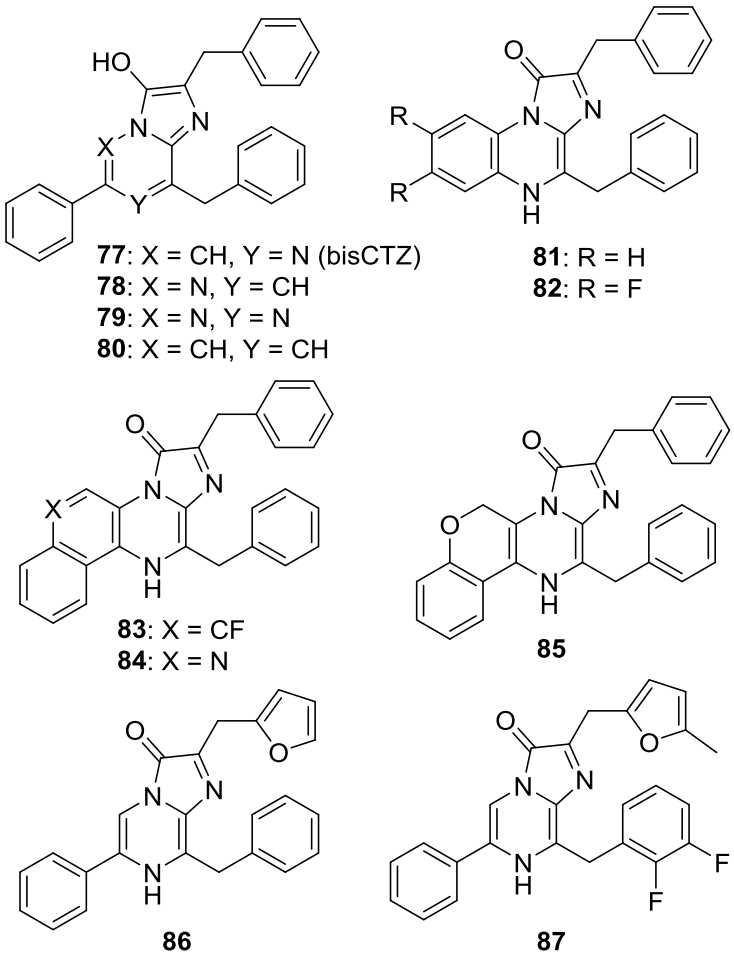


Figure 1. Structures of the luciferin analogues **77**-**87**.

From the resulting solution of compound **78**-**85**, no tangible bioluminescence was observed with compound **78**-**85** when using n nanoKAZ/NanoLuc luciferase under the usual conditions,[14b] contrary to coelenterazine (**1**), bisdeoxycoelenterazine (**77**),[8b] furimazine (**86**)[8a] or the enhanced analogue **87**.[8c] On the other hand, the chemiluminescence properties of these compounds was somewhat more rewarding. In the past, the chemiluminescence properties of coelenterazine (**1**) or some of its analogues have been the focus of many studies.[25] The final concentration of the compounds used in these experiments is always low, varying between 1 µM,[25e] 3 µM,[25i] 10 µM[25a, c, d, f] or in two instances 50 µM.[25g, h] We quickly found out the reason as, at much higher

concentration (i.e.: 1 mM final), we observed remarkable wavelength shift which could be only explained by resonance energy transfers to fluorescent substances arising from the light production process. Interestingly, this requirement for low concentration was not found necessary for the study of the chemiluminescence of firefly luciferins.[26] For our part, as depicted in table 1, for every compound made, their addition to a phosphate buffer (DPBS; pH = 7.4), leading to 5 µM final concentration, led to a chemiluminescence signal as measured by a HIDEX Triathler Gamma Counter. Of note is that the signal intensity varied widely according to the compound studied. Moreover, we confirmed the ammonium-based surfactant enhancement effect reported by Shimomura[1a] since quite remarkable intensity improvement was systematically achieved when adding of 2.5 mMol of cetyltrimethylammonium bromide (CTAB). Concerning the products resulting from this chemiluminescence, LC/MS analysis, using ammonium carbonate as a buffer, pointed out the occurrence of the expected ions typical of the light-producing reaction depicted in scheme 1 (see supplementary info). In the case of coelenterazine (**1**, *m/z* = 424), the ion corresponding to coelenteramide (**4**, *m/z* = 412) and, as reported,[27] the amine (*m/z* = 278) were observed. Concerning the emission spectra of the strong and medium emitters, the use of an Aqualog® (HORIBA Instruments, Inc.), a spectrometer endowed with a sensitive CCD detector (Water-Raman SNR > 20,000:1 - RMS method), gave good results but only upon adding 2.5 mMol of CTAB in the phosphate buffer. Concerning the weak emitters, such as compounds **78**-**81**, we have yet to find a device sensitive enough to determine their emission spectra. Interestingly, even at a 5 µM final concentration a degree of signal quench was observed in one instance (compound **87**) but this was alleviated when measuring the spectra at a 2.5 µM final concentration.

Table 1. Chemiluminescence spectra of the luciferins

Cpd	I_{max} DPBS	I_{max} +2.5M CTAB	λ_{max}
1	4.000	1.100.000	465
77	65.000	2.400.000	462
78	200	28.000[a]	n.s.
79	160	1.400	n.s.
80	250	3.200	n.s.
81	215	602.600[a]	n.s.
82	660	2.400.000	513[b]
83	26.000	19 million[c]	438

84	2.000	596.000	460
85	4.600.000	38 million[c]	458
86	13.000	1.400.000	472
87	4.100	6.100.000	460[d]

a : slow increase over 2 minutes
b: presence of a small peak at 400 nm and a shoulder at 570 nm
c : saturation of the HIDEX Triathler
d : at a 2.5 µM substrate concentration
n.s.: no signal exploitable

Conclusion

In this study, we made an array of compounds which all produced a photon upon their reaction with oxygen via, as confirmed by LC/MS analysis, a mechanism plausibly similar to the one described for imidazo[1,2-*a*]pyrazin-3(7*H*)-one luciferins. Many of these analogues were prepared using our previously reported[8c, 14b] synthetic pathway although unexpected limits were encountered in their preparations. These limitations drove us to use and develop alternative conditions to produce the target compounds, notably using the fact that the N-arylations could sometime take place without a palladium-based catalyst along with a (not too) strong base or the observation that some the resulting esters could undergo a cyclisation into the target luciferin just upon heating. Again, trapping these relatively unstable substances under their O-acetylated form was instrumental in their proper isolation and the success of this work. Concerning their chemiluminescence properties when adding these compounds to a phosphate buffer, a very variable degree of signal intensities was observed. Of interest is the observation that a very strong chemiluminescence is observed for the most rigid analogues such as compound **83** and **85** but unexpectedly not for the "aza" analogue **84**. Aside from a degree of instability (in comparison with other derivatives) which was noted for the O-acetylated precursors of compounds **78**-**80**, it is more difficult to identify a reason for their very low chemiluminescence. Concerning the emission profiles of the compounds endowed with a chemiluminescence of sufficient intensity, the λ_{max} were mainly situated in the blue region (438-472 nm) with the notable exception of the difluorinated derivative **82**, endowed with a green signal at 513 nm. Previous and far more extensive, studies on the chemiluminescence properties of coelenterazine (**1**) have reported on the large impact on the signal wavelength when using a protic or an aprotic organic solvents as well when using a base.[25a] In our case we chose to use only a protic one (water) and a mild base (a phosphate buffer) since we observed little difference between the chemiluminescence (472 nm)

and bioluminescence (455 nm;[8c] with NanoLuc) of the reference compound furimazine (**1**). We thus retained only this set of condition to qualify the emission wavelength of these analogues as a plausibly predictive parameter of their eventual bioluminescence properties (if a luciferase, derived from nanoKAZ/NanoLuc, could be designed to consume them). However, the relatively modest chemiluminescence λ_{max} shifts achieved here do not meet the criteria to initiate a research program aiming at designing a luciferase capable of catalyzing the light-producing combustion of one of these analogues. In any case, the results obtained are providing some additional clues on how to red-shift this type of luciferin and, probably more important, the experimental data presented here should be useful for the design and validation of computer-based models[2] of these light-producing reactions.

Acknowledgement

This work was supported by the Agence Nationale de la Recherche (ANR), grant ANR-11-CRNT-0004, in the context of the investment program 'GLOBAL CARE', an association of the Instituts Carnot 'Pasteur-Maladies Infectieuses', 'Curie-Cancer', 'Voir et Entendre', 'Institut du Cerveau et de la moelle Épinière' and the 'Consortium pour l'Accélération de l'Innovation et de son Transfert dans le domaine du Lymphome' (CALYM). G.G. acknowledges a PhD fellowship from the Université Paris Descartes, Sorbonne Paris Cité and E.P.C. was supported by a fellowship from Global Care. This project also benefited from the Valoexpress funding call of the Institut Pasteur. Prof. Christian Bréchot and Drs. Muriel Delepierre and Daniel Larzul are acknowledged for their interest and support. Finally, we thank Florence Miller from Berthold Technologies for her support and Aline Rifflet from the Unité de Biologie et Génétique at the Institut Pasteur for her great help in LC/MS experiments.

Bibliography

[1] a) O. Shimomura, *Bioluminescence: Chemical Principles And Methods Revised Edition*, World Scientific, Singapore, **2012**, p. 1-468; b) A. C. Love and J. A. Prescher, *Cell Chem. Biol.* **2020**, *27*, 904-920.

[2] M. Vacher, I. Fdez Galván, B. W. Ding, S. Schramm, R. Berraud-Pache, P. Naumov, N. Ferré, Y. J. Liu, I. Navizet, D. Roca-Sanjuán, W. J. Baader and R. Lindh, *Chem. Rev.* **2018**, *118*, 6927-6974.

[3] a) P. J. Herring, *J. Biolumin. Chemilumin.* **1987**, *1*, 147-163; b) S. Inouye, Y. Sahara-Miura, M. Nakamura and T. Hosoya, *Protein Expr. Purif.* **2020**, *171*, 105615; c) M. Tessler, J. P. Gaffney, A. G. Oliveira, A. Guarnaccia, K. C. Dobi, N. A. Gujarati, M. Galbraith, J. D. Mirza, J. S. Sparks, V. A. Pieribone, R. J. Wood and D. F. Gruber, *Sci. Rep.* **2020**, *10*, 17724; d) M. Bessho-Uehara, W. R. Francis and S. H. D. Haddock, *Mar. Biol.* **2020**, *167*, doi.org/10.1007/s00227-00020-03706-w.

[4] a) H. Hoshino, *Expert Opin. Drug Dis.* **2009**, *9*, 374-389; b) A. Roda, M. Guardigli, E. Michelini and M. Mirasoli, *Trends Analyt. Chem.* **2009**, *28*, 307-322; c) T. Ozawa, H. Yoshimura and S. B. Kim, *Anal. Chem.* **2013**, *85*, 590-609; d) E. Michelini, L. Cevenini, M. M. Calabretta and A. Roda, *Anal. Bioanal. Chem.* **2014**, *406*, 5531-5539; e) G. Thouand and R. Marks, *Bioluminescence: Fundamentals and Applications in Biotechnology - Volume 1-3 (Advances in Biochemical Engineering/Biotechnology)*, **2014**, p; f) C. G. England, E. B. Ehlerding and W. Cai, *Bioconjug. Chem.* **2016**, *27*, 1175-1187; g) Z. M. Kaskova, A. S. Tsarkova and I. V. Yampolsky, *Chem. Soc. Rev.* **2016**, *45*, 6048-6077; h) T. Jiang, L. Du and M. Li, *Photochem. Photobiol. Sci.* **2016**, *15*, 466-480; i) L. Mezzanotte, M. van 't Root, H. Karatas, E. A. Goun and C. W. G. M. Löwik, *Trends Biotechnol.* **2017**, *35*, 640-652; j) C. M. Rathbun and J. A. Prescher, *Biochemistry* **2017**, *56*, 5178-5184; k) H. W. Yeh, T. Wu, M. Chen and H. W. Ai, *Biochemistry* **2019**, *58*, 1689-1697; l) H. W. Yeh and H. W. Ai, *Annu. Rev. Anal. Chem.* **2019**, *12*, 129-150; m) V. V. Krasitskaya, E. E. Bashmakova and L. A. Frank, *Int. J. Mol. Sci.* **2020**, 10.3390/ijms21207465 ; n) A. Lopreside, M. M. Calabretta, L. Montali, M. Zangheri, M. Guardigli, M. Mirasoli and E. Michelini, *Luminescence* **2020**, doi.org/10.1002/bio.3948.

[5] J. F. Head, S. Inouye, K. Teranishi and O. Shimomura, *Nature* **2000**, *405*, 372-376.

[6] a) T. Goto, S. Inoue and S. Sugiura, *Tetrahedron Lett.* **1968**, *9*, 3873-3876; b) K. Teranishi and O. Shimomura, *Anal. Biochem.* **1997**, *249*, 37-43.

[7] a) T. Curie, K. L. Rogers, C. Colasante and P. Brûlet, *Mol. Imaging* **2007**, *6*, 30-42; b) A. M. Loening, A. M. Wu and S. S. Gambhir, *Nat. Methods* **2007**, *8*, 641-643; c) G. Giuliani, P. Molinari, G. Ferretti, A. Cappelli, M. Anzini, S. Vomero and T. Costa, *Tetrahedron Lett.* **2012**, *53*, 5114-5118; d) K. Suzuki, T. Kimura, H. Shinoda, G. Bai, M. J. Daniels, Y. Arai, M. Nakano and T. Nagai, *Nat. Commun.* **2016**, *7*, 13718; e) K. A. Rumyantsev, K. K. Turoverov and V. V. Verkhusha, *Sci. Rep.* **2016**, *6*, 36588; f) K. M. Grinstead, L. Rowe, C. M. Ensor, S. Joel, P. Daftarian, E. Dikici, J.-M. Zingg and S. Daunert, *PLoS One* **2016**, *11*, e0158579; g) H. W. Yeh, O. Karmach, A. Ji, D. Carter, M. M. Martins-Green and H. W. Ai, *Nature Methods* **2017**, *14*, 971-974; h) J. Hiblot, Q. Yu, M. D. B. Sabbadini, L. Reymond, L. Xue, A. Schena, O. Sallin, N. Hill, R. Griss and K. Johnsson, *Angew. Chem., Int. Ed.* **2017**, *56*, 14556-14560; i) A. Shakhmin, M. P. Hall, T. Machleidt, J. R. Walker, K. V. Wood and T. A. Kirkland, *Org. Biomol. Chem.* **2017**, *15*, 8559-8567; j) A. Taylor, J. Sharkey, A. Plagge, B. Wilm and P. Murray, *Contrast Media Mol. Imaging* **2018**, 2514796; k) D. C. Alcobia, A. I. Ziegler, A. Kondrashov, E. Comeo, S. Mistry, B. Kellam, A. Chang, J. Woolard, S. J. Hill and E. K. Sloan, *iScience* **2018**, *6*, 280-288; l) R. Nishihara, R. Paulmurugan, T. Nakajima, E. Yamamoto, A. Natarajan, R. Afjei, Y. Hiruta, N. Iwasawa, S. Nishiyama, D. Citterio, M. Sato, S. B. Kim and K. Suzuki, *Theranostics* **2019**, *13*, 2646-2661; m) M. Abe, R. Nishihara, Y. Ikeda, T. Nakajima, M. Sato, N. Iwasawa, S. Nishiyama, R. Paulmurugan, D. Citterio, S. Kim and K. Suzuki, *Chembiochem* **2019**, *20*,

1919-1923; n) H. W. Yeh, Y. Xiong, T. Wu, M. Chen, A. Ji, L. X. and H. W. Ai, *ACS Chem. Biol.* **2019**, *17*, 959-965; o) F. Weihs and H. Dacres, *Trends Analyt. Chem.* **2019**, *116*, 61-73; p) H. Kobayashi, L. P. Picard, A. M. Schönegge and M. Bouvier, *Nat. Protoc.* **2019**, 1084-1107.

[8] a) M. P. Hall, J. Unch, B. F. Binkowski, M. P. Valley, B. L. Butler, M. G. Wood, P. Otto, K. Zimmerman, G. Vidugiris, T. Machleidt, M. B. Robers, H. A. Benink, C. T. Eggers, M. R. Slater, P. L. Meisenheimer, D. H. Klaubert, F. Fan, L. P. Encell and K. V. Wood, *ACS Chem. Biol.* **2012**, *7*, 1848-1857; b) S. Inouye, J. Sato, Y. Sahara-Miura, S. Yoshida, H. Kurakata and T. Hosoya, *Biochem. Biophys. Res. Commun.* **2013**, *437*, 23-28; c) E. P. Coutant, G. Gagnot, V. O. Hervin, R. Baatallah, S. Goyard, Y. Jacob, T. Rose and Y. L. Janin, *Chem. Eur. J.* **2020**, *26*, 948-958.

[9] Y. Lu, R. Hendra, A. Oakley and P. A. Keller, *Tetrahedron Lett.* **2014**, *55*, 6212-6215.

[10] K. Teranishi, (Mie University), JP 2007254352, **2007**

[11] M. Hall, T. Kirkland, T. Machleidt, A. Shakhmin and J. R. Walker, (Promega), US2018030059, **2018**

[12] S. Inouye and O. Shimomura, *Biochem. Biophys. Res. Commun.* **1997**, *233*, 349-353.

[13] O. Shimomura, B. Musicki and Y. Kishi, *Biochem. J.* **1988**, *251*, 405-410.

[14] a) Y. L. Janin, E. P. Coutant, V. Hervin, G. Gagnot, Y. Jacob, S. Goyard and T. Rose, (Institut Pasteur, CNRS), WO 2018197727, **2018**; b) E. P. Coutant, S. Goyard, V. O. Hervin, G. Gagnot, R. Baatallah, T. Rose, Y. Jacob and Y. L. Janin, *Org. Biomol. Chem.* **2019**, *17*, 3709-3713.

[15] I. Parrot, Y. Rival and C. G. Wermuth, *Synthesis* **1999**, 1163-1168.

[16] C. G. Wermuth, J. J. Bourguignon, I. Morin, P. Renard, M. Devissaguet, J. F. de La Faverie and G. Adam, (Adir et compagnie), US 5254548, **1993**

[17] E. Spinner and J. C. B. White, *J. Chem. Soc. B: Phys. Org.* **1966**, 991-992.

[18] a) R. J. Biediger, B. Dupre, L. K. Hamaker, W. W. Holland, J. M. Kassir, W. Li, R. V. Market, N. Nguyen, I. L. Scott, C. Wu and E. R. Decker, (Rockey, Milnamow and Katz, Ltd.), US 20030199692, **2003**; b) H. Arakawa, J. Ishida, M. Yamagushi and M. Nakamura, *Chem. Pharm. Bull.* **1990**, *38*, 3491-3493.

[19] T. Hosoya, R. Iimori, S. Yoshida, Y. Sumida, Y. Sahara-Miura, J. Sato and S. Inouye, *Org. Lett.* **2015**, *17*, 3888-3891.

[20] P. J. Manley and M. T. Bilodeau, *Org. Lett.* **2004**, *6*, 2433-2435.

[21] M. R. Michaelides, Y. Hong, S. DiDomenico Jr, E. K. Bayburt, K. E. Asin, D. R. Britton, C. W. Lin and K. Shiosaki, *J. Med. Chem.* **1997**, *40*, 1585-1599

[22] a) D. Dauzonne and R. Royer, *Synthesis* **1984**, 348-349; b) A. H. Clark, J. D. McCorvy, V. J. Watts and D. E. Nichols, *Bioorg. Med. Chem.* **2011**, *19*, 5420-5431.

[23] K. Imagawa, E. Hata, T. Yamada and T. Mukaiyama, *Chem. Lett.* **1996**, *25*, 291-292.

[24] a) A. R. Katritzky, J. Li, C. Stevens and D. J. Ager, *Org. Prep. Proced. Int.* **1994**, *26*, 439-444; b) S. Murata, K. Kiguchi and T. Sugimoto, *Heterocycles* **1998**, *48*, 1255-1274.

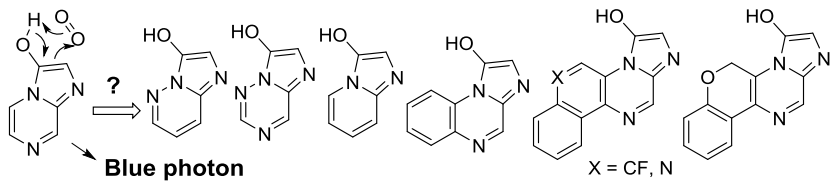
[25] a) K. Teranishi and T. Goto, *Bull. Chem. Soc. Jpn.* **1990**, *63*, 3132-3140; b) T. Hirano, Y. Gomi, T. Takahashi, K. Kitahara, C. F. Qi, I. Mizoguchi, S. Kyushin and M. Ohashi, *Tetrahedron Lett.* **1992**, *39*, 5771-5774; c) K. Teranishi, M. Hisamatsu and T. Yamada, *Luminescence* **1999**, *14*, 297-302; d) H. Kondo, T. Igarashi, S. Maki, H. Niwa, H. Ikeda and T. Hirano, *Tetrahedron Lett.* **2005**, *46*, 7701-7704; e) Y. Takahashi, H. Kondo, S. Maki, H. Niwa, H. Ikeda and T. Hirano, *Tetrahedron Lett.* **2006**, *47*, 6057-6061; f) T. Hirano, Y. Takahashi, H. Kondo, S. Maki, H. Kojima, H. Ikeda and H. Niwa, *Photochem. Photobiol. Sci.* **2008**, *7*, 197-207; g) R. Saito, T. Hirano, S. Maki and H. Niwa, *J. Photochem.*

Photobiol A: Chemistry **2014**, *293*, 12-25; h) M.-L. Yuan, T.-Y. Jiang, L.-P. Du and M.-Y. Li, *Chin. Chem. Lett.* **2016**, *27*, 550-554; i) J. M. Lourenço, J. C. G. Esteves da Silva and L. Pinto da Silva, *J. Luminescence* **2018**, *194*, 139-145.

[26] R. Saito, T. Kuchimaru, S. Higashi, W. L. Shijia, M. Kiyama, S. Iwano, R. Obata, T. Hirano, S. Kizaka-Kondoh and S. A. Maki, *Bull. Chem. Soc. Jpn.* **2019**, *92*, 608-618.

[27] O. Shimomura and F. H. Johnson, *Biochemistry* **1972**, *11*, 1602-1608.

10.1002/chem.202004311



Can extensive alteration of the heterocyclic core of the coelenterazine ring system, a blued-hued luciferin of marine origin, lead to chemiluminescent substances and can this also “red-shift” their emission spectra?

Key words:
Homogeneous catalysis; Synthesis design; Chemiluminescence; Luciferin; Bioluminescence