Near-Infrared Bioluminescence

A Dual-Color Far-Red to Near-Infrared Firefly Luciferin Analogue Designed for Multiparametric Bioluminescence Imaging**

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Abstract: *Red-shifted bioluminescent emitters allow improved in vivo tissue penetration and signal quantification, and have led to the development of beetle luciferin analogues that elicit red-shifted bioluminescence with firefly luciferase (Fluc). However, unlike natural luciferin, none have been shown to emit different colors with different luciferases. We have synthesized and tested the first dual-color, far-red to near-infrared (nIR) emitting analogue of beetle luciferin, which, akin to natural luciferin, exhibits pH dependent fluorescence spectra and emits bioluminescence of different colors with different engineered Fluc enzymes. Our analogue produces different far-red to nIR emission maxima up to $\lambda_{max} = 706$ nm with different Fluc mutants. This emission is the most red-shifted bioluminescence reported without using a resonance energy transfer acceptor. This improvement should allow tissues to be more effectively probed using multiparametric deep-tissue bioluminescence imaging.*

Bioluminescence imaging (BLI) has revolutionized molecular genetic imaging in biomedical research as a cheap and easy means to longitudinally image the genetic behavior of life and disease processes in whole mammals.[1–4] As they produce the brightest form of bioluminescence,[5] genes from coleopterans are commonly used to localize, track, and quantify cells and molecular or functional events in vivo.[6–8] In a well-studied reaction,[9] beetle luciferin (**1**, Figure 1a) is adenylated by firefly luciferase (Fluc) and this reacts with molecular oxygen to produce an excited state species, oxyluciferin* (**2**), which decays to release a photon with a high quantum yield ($\lambda_{max} = 558$ nm).[5] However, absorption of visible light by hemoglobin (Hb) and melanin restricts image resolution and signal penetration at this wavelength. Between $\lambda = 600–800$ nm, the absorption of light by Hb decreases by a factor of approximately 50,

resulting in less attenuation of red light. This wavelength range is within what is termed the "bio-optical window" and there has been much focus on engineering red-shifted Fluc enzymes that have maximum emission wavelengths in this range,[10–15] but these have peaked at wavelengths less than $\lambda = 645$ nm.

The most red-shifted luciferin analogues to date[16] are based upon amino derivatives (Figure 1b), for example cyclic aminoluciferin (**3a**: $\lambda_{max} = 599$ nm; **3b**: $\lambda_{max} = 607$ nm),[17] seleno-D-aminoluciferin (**4**: $\lambda_{max} = 600$ nm),[18] and a rationally designed 4-(dimethylamino)phenyl derivative conjugated to a thiazoline group (**5**: $\lambda_{max} = 675$ nm).[19] In particular cyclic aminoluciferin derivative **3a** has been shown to give improved bioluminescence imaging compared to luciferin (LH_2; **1**) at dilute concentrations where the intracellular concentration of the luciferin or analogue is limiting.[20] Near-infrared emission has been detected with an aminoluciferin
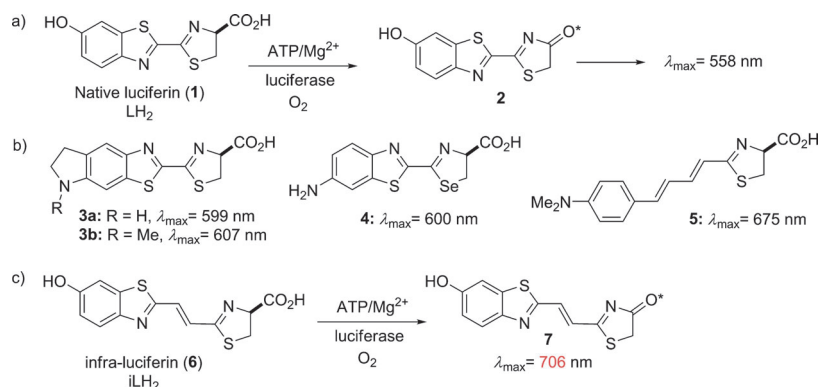
Figure 1. a) Bioluminescence of luciferin (**1**) catalyzed by luciferase. b) Structures of red-shifted bioluminescent amino-luciferin analogues. c) New luciferin analogue iLH₂ **6** that exhibits near-infrared bioluminescence with mutant Fluc. ATP = adenosine triphosphate.

Cy5 conjugate, but this is due to bioluminescence resonance energy transfer (BRET),[21] meaning that the conjugate cannot be used for multiparametric imaging.

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To date, despite a red-shift in emission, no analogues have been reported with the other desirable properties of LH$_2$ **1**, such as a high quantum yield and the ability to produce more than one color with different Fluc mutants. Considering this, and the likely mechanisms of color tuning in Fluc bioluminescence, we describe the design, synthesis, and in vitro and in vivo testing of the first far-red to nIR multicolor-emitting analogue, which can produce the most red-shifted form of true bioluminescence reported to date. Additionally, our far-red-shifted analogue infra-luciferin (**6**, iLH$_2$; Figure 1 c) produces distinct bioluminescent colors with different enzymes akin to native luciferin, and could be of great benefit to multiparametric deep-tissue and tomographic bioluminescence in vivo imaging.

Despite a number of theories, the exact mechanism regulating color tuning in Fluc bioluminescence has not been solved.[11–13] Current measurements and calculations suggest that color modulation is due to perturbing interactions in the microenvironment surrounding the anionic phenolate of excited-state oxyluciferin (**2**) in the Fluc active site.[22–28] Additionally, π–π overlap between the benzothiazole and thiazolone heterocycles in **2** also appears to be important.[29–31] Maki and co-workers demonstrated the importance of extended π-conjugation in luciferin derivatives which led to the development of **5**.[19,32] In our design we proposed that increasing the conjugation of LH$_2$ **1**, and thus **2**, by addition of an alkene linker between the benzothiazole and thiazoline fragments would lead to a red-shifted luciferin analogue (**6**, Figure 1 c) that would be amenable to color modulation with different Fluc mutants. Extended conjugation should reduce the HOMO–LUMO energy gap in the light-emitting phenolate of **7**, which would lead to red-shifting of the emitted light. Our design, in contrast to other established red-shifted luciferin analogues (Figure 1), retained the 6′-hydroxy group. This design was chosen in an attempt to capitalize on the microenvironment effect of different Fluc mutants to generate different bioluminescence emission wavelengths that are essential for multiparametric imaging. We also believed that the increase in the overall shape of the molecule by only one alkene unit may be tolerated by Fluc mutants to facilitate multiwavelength emission.

The molecule iLH$_2$ **6** was synthesized in 10 steps from commercially available starting materials (Scheme 1). During the synthesis we found that once the thiazoline ring had been formed the molecule was incredibly sensitive to epimerization next to the carboxy group. Both the methyl and ethyl ester of iLH$_2$ **6** could be isolated in enantiopure form, but isolation of the free acid after saponification was found to be extremely difficult with epimerization and formation of the thiazole detected. To maximize light output we decided to test the enantiopure esters in vitro and in vivo as it has been shown that esters of LH$_2$ **1** are active in live cells and living mice,[33,34] as they are saponified by esterases. We also synthesized the Maki analogue **5**,[19] the most red-shifted bioluminescent analogue reported to date, to compare its properties to iLH$_2$ **6**.

The fluorescence spectra of iLH$_2$ **6** compared to LH$_2$ **1** showed that at pH 7, the emission maximum of **6** was λ$_{max}$ =
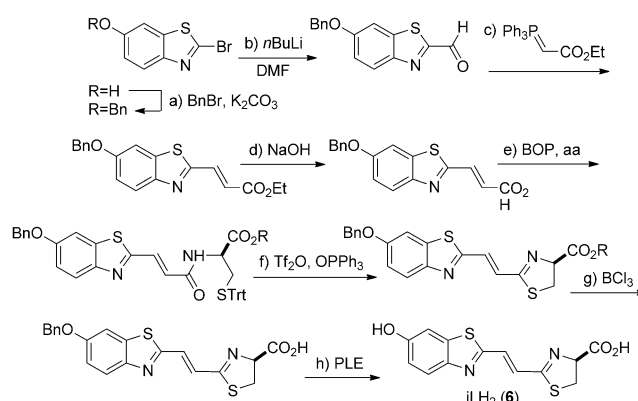
Scheme 1. Synthesis of infra-luciferin **6**. a) BnBr (1.2 equiv), K$_2$CO$_3$ (2.8 equiv), acetone, room temperature, 16 h, 85%; b) *n*BuLi (1.93 M in hexanes, 1.1 equiv), THF, −78°C, 15 min then DMF (4.1 equiv), 1 h, 96%; c) (Carbethoxymethylene)triphenylphosphorane (3 equiv), PhMe, reflux, 3 h, 92%; d) NaOH (1 M), *i*PrOH, 16 h, quantitative yield; e) Et$_3$N (2.4 equiv), DMF, amino acid (aa; 1.2 equiv), 0°C then BOP (1.2 equiv) in CH$_2$Cl$_2$, 2 h (R=Me, 80%, R=Et, 82%); f) Ph$_3$PO (1.3 equiv), Tf$_2$O (2.7 equiv), CH$_2$Cl$_2$, 0°C, 30 min added to benzothiazole in CH$_2$Cl$_2$, 0°C, 10 min, (R=Me, 65%, R=Et, 74%); g) pentamethylbenzene (4.4 equiv), BCl$_3$ (1 M in CH$_2$Cl$_2$, 3 equiv), CH$_2$Cl$_2$, −78°C, (R=Me, 79%, R=Et, 72%); h) PLE, buffer, 37°C, in situ. Bn=benzyl; Tf$_2$O=trifluoromethanesulfonic anhydride; Trt=triphenylmethyl; BOP=(benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; PLE=pig liver esterase.

588 nm, with a red shift of 58 nm compared to **1**. Furthermore, the fluorescence excitation and emission spectra are pH dependent, as measured for **1**. In contrast, the Maki analogue **5** had pH independent fluorescence spectra, producing only one fluorescence excitation color (see Figures S1 a–c in the Supporting Information). This highlights the importance of retaining the 6′-hydroxy group for color modulation.[22–28]

In vitro bioluminescence spectra of iLH$_2$ **6** ethyl ester (saponified with PLE (pig liver esterase) in situ immediately prior to use) with purified wild-type (WT) Fluc, the x5 Fluc mutant (a thermostable Fluc with similar properties to WT but with higher quantum yields),[35,36] and the x5 S284T Fluc mutant (a bright red-shifted point mutant of x5)[37,38] showed marked red-shifted peak maxima of 100 nm magnitude compared to the λ$_{max}$ of each enzyme with **1** (Figure 2, Table 1). This effect is remarkable considering that these mutants were originally engineered for different emission
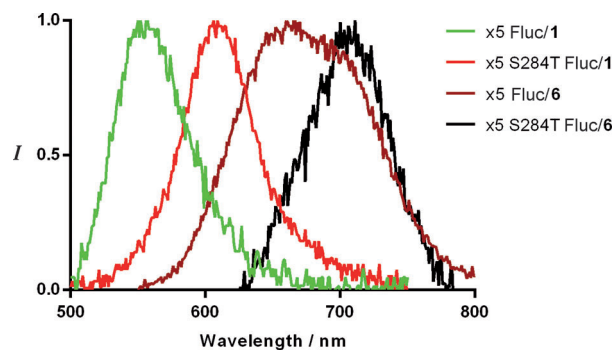
Figure 2. Bioluminescence spectra of native **1** and **6** with x5 and x5 S284T Fluc mutants.

Table 1: Bioluminescence spectral properties of **1**, **5**, and **6** with purified enzymes.

Fluc Mutant	Luciferin Substrate	λ_{max} [nm]	FWHM [nm][a]
WT Fluc	1	558	76
	5	652	76
	6	670	74
X5 Fluc	1	554	62
	5	652	76
	6	646	92
X5 S284T	1	605	56
	5	658	78
	6	706	81

[a] FWHM = bioluminescence full width at half maximum. See Supporting Information for experimental conditions.

colors with LH$_2$ **1**. Our analogue showed bioluminescence with $\lambda_{max} = 706$ nm with Fluc mutant x5 S284T, significantly red-shifted compared to any reported natural or unnatural bioluminescence system. This suggests that Fluc mutants emit different color forms with **1** and **6** in line with the microenvironment effect of the Fluc active sites.[12,22–28] In contrast, bioluminescence of the Maki analogue **5** with different Fluc mutants occurred with almost identical emission wavelength maxima and only very small nonspecific shifts between Fluc mutants (Figure S2a),[19] supporting the theory that the phenolic hydroxy group is crucial for the color-tuning mechanism of luciferin.[22–28] These results suggest that iLH$_2$ **6** would be of use for in vivo multiparametric imaging, whereas the Maki analogue **5** would not be. The other red-shifted luciferin analogues (Figure 1) may also not be suitable because of the substitution of the phenolic hydroxy group for an amine.

The proportion of light output above $\lambda = 600$ nm is a key determinant for transmission efficiency through mammalian tissue.[39] Bioluminescence data were acquired using different band-pass (bp) filters in a Photon Imager and showed that most light from the x5 Fluc mutant with **6** was collected through a $\lambda = 670$–720 nm bp filter and from the x5 S284T mutant through a $\lambda = 700$–750 nm bp filter. The proportion of light emitted over $\lambda = 600$ nm with LH$_2$ **1** was 32% (WT Fluc), 18% (x5 Fluc), and 68% (x5 S284T Fluc), respectively. For iLH$_2$ **6**, this increased dramatically to 95%, 97%, and 100%, respectively (Figure S2b). This should have a significant impact on the amount of transmitted light detected during in vivo imaging (see below).

To compare the in vitro specific activity of the x5 Fluc mutant with the saponified esters (PLE) of LH$_2$ **1**, the Maki analogue **5**, and iLH$_2$ **6**, data was acquired using an IVIS 200 instrument—Caliper Life Sciences, USA (Table 2). With

Table 2: In vitro activity and apparent kinetic parameters of the x5 Fluc mutant with the saponified esters of **1**, **5**, and **6**.[a]
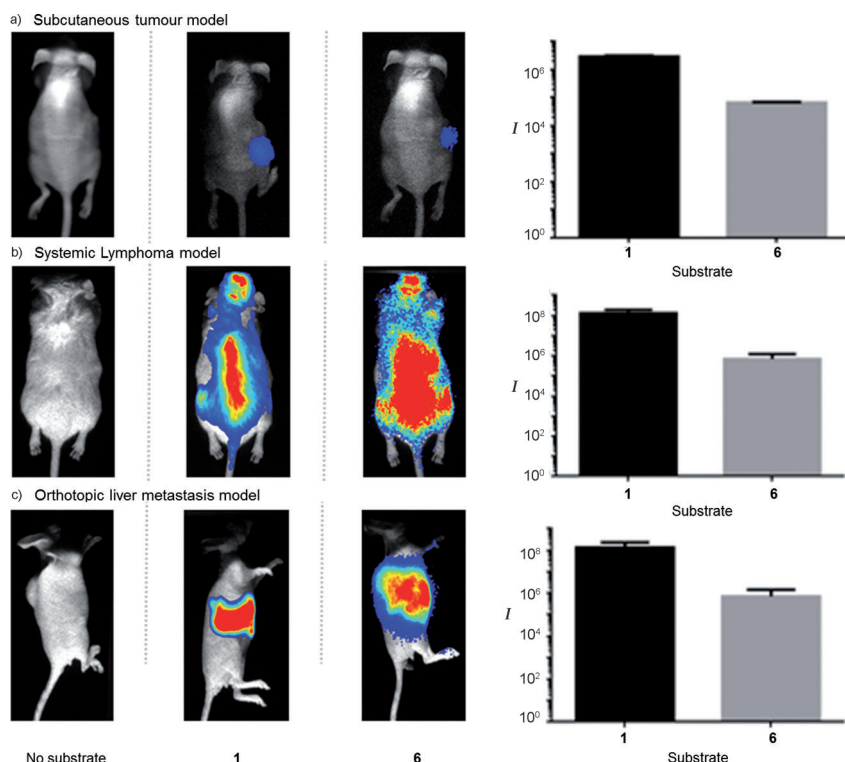
Substrate	Apparent K_m [µM]	Apparent k_{cat} [RLU s$^{-1} \times 10^{15}$]	Apparent k_{cat}/K_m [s^{-1} µM$^{-1} \times 10^{15}$]	Specific Activity/cpm [cm^{-2} mg$^{-1} \times 10^{15}$]
1 Et Ester	2.0	40.0	200	60.9
5 Me Ester	16.7	13.3	8.0	19.7
6 Me Ester	6.0	0.4	0.7	0.6

[a] Data recorded in triplicate. See Supporting Information for experimental conditions. cpm = counts per minute. RLU = relative light units.

purified enzymes, a difference of approximately 100-fold was seen in the specific activity of x5 Fluc from LH$_2$ **1** and iLH$_2$ **6**, whereas **5** displayed a 3-fold lower specific activity than **1**. Apparent kinetic parameters indicate that the affinity (K_m) of x5 Fluc for infra-luciferin (**6**) are more similar to LH$_2$ **1** than **5**, though the turnover (k_{cat}), and therefore the overall catalytic efficiency ($k_{cat} K_m^{-1}$), of **6** was approximately 285-fold lower than **1**, compared to 25-fold lower for **5**. A similar magnitude of a decrease in the k_{cat} value has been reported for commercially available thermostable luciferase mutants compared to WT Fluc with **1**.[36] The full width at half maximum (FWHM) of emission of both x5 Fluc and x5 S284T are markedly wider with infra-luciferin (**6**) than with LH$_2$ **1**, indicating less emitter specificity of the x5 mutant framework (Table 1). It may be possible to engineer Fluc mutants with enhanced activities, kinetic parameters, and/or emitter specificity with infra-luciferin (**6**).[15,35,40,41] Despite **6** having lower activity, the benefit of **6** is that it exhibits near-infrared bioluminescence that is susceptible to color modulation by mutated Fluc enzymes, unlike **5**, and thus shows promise for multiparametric imaging.

Cells transduced with Fluc readily showed bioluminescence activity upon treatment with iLH$_2$ **6**. With this in mind, we established a number of mouse models of cancer with the aim of detecting and imaging these using iLH$_2$ **6** methyl ester and comparing in vivo light yields to native luciferin **1** (Figure 3a–c).[42] A subcutaneous (sc) model was induced by sc injection of 5×10^6 LS174T cells (colon carcinoma cell line) expressing WT Fluc into Nude (MFI NuNu) mice (Figure 3a). After four days post-inoculation, mice were intraperitoneally (ip) injected with LH$_2$ **1** ethyl ester (2 mg) or iLH$_2$ **6** methyl ester and imaged in the Photon Imager. Light emission was readily apparent in mice administered with iLH$_2$ **6** methyl ester. In vivo spectra showed that the maximum emission wavelength for WT Fluc with LH$_2$ **1** is within the $\lambda = 590$–640 nm bp filter, likely as a result of the bathochromic shift of WT Fluc at physiological temperature and also attenuation of the true spectrum by Hb in mouse tissues (Figure S3).[39] However, the in vivo spectrum of WT Fluc with iLH$_2$ **6** displays a λ_{max} in the $\lambda = 700$–750 nm bp filter and its shape appears much less attenuated by mouse tissues. We also found iLH$_2$ **6** methyl ester was active and detectable in a systemic lymphoma mouse model of cancer (Figure 3b). In this case, the same mice were imaged on consecutive days with LH$_2$ **1** ethyl ester or iLH$_2$ **6** methyl ester. One minute background luminescence images were acquired prior to imaging to ensure there was no remaining activity from previous sessions. These images (Figure 3b) show that **6** gives a more even intensity across the whole animal, likely because of the consequence of less attenuation by mouse tissues due to the higher proportion of light output above $\lambda = 600$ nm (Figure S4). Finally, iLH$_2$ **6** was employed to image liver metastases in an orthotopic model in nude mice (Figure 3c, Figure S5), a particularly challenging organ to get meaningful data from because of blood and tissue attenuation. In this case, mice were imaged 15 minutes after ip injection of iLH$_2$ **6** methyl ester (4 mg), or with LH$_2$ **1** potassium salt
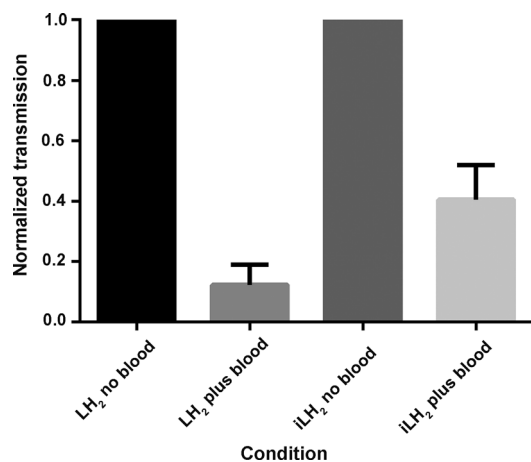
[11] E. H. White, E. Rapaport, H. H. Seliger, T. A. Hopkins, *Bioorg. Chem.* **1971**, *1*, 92–122.

[12] B. R. Branchini, T. L. Southworth, M. H. Murtiashaw, R. A. Magyar, S. A. Gonzalez, M. C. Ruggiero, J. G. Stroh, *Biochemistry* **2004**, *43*, 7255–7262.

[13] N. N. Ugarova, L. Y. Brovko, *Lumin. J. Biol. Chem. Lumin.* **2002**, *17*, 321–330.

[14] K. V. Wood, *J. Biolumin. Chemilumin.* **1990**, *5*, 107–114.

[15] See Ref. [10].

[16] For a recent review, see; S. T. Adams, Jr., S. C. Miller, *Curr. Opin. Chem. Biol.* **2014**, *21*, 112–120.

[17] G. R. Reddy, W. C. Thompson, S. C. Miller, *J. Am. Chem. Soc.* **2010**, *132*, 13586–13587.

[18] N. R. Conley, A. Dragulescu-Andrasi, J. Rao, W. E. Moerner, *Angew. Chem. Int. Ed.* **2012**, *51*, 3350–3353; *Angew. Chem.* **2012**, *124*, 3406–3409.

[19] With our purified WT Fluc we detected a maximum emission wavelength $\lambda_{max} = 652$ nm with the Maki analogue **3** compared to the $\lambda = 675$ nm previously reported: S. Iwano, R. Obata, C. Miura, M. Kiyama, K. Hama, M. Nakurama, Y. Amano, S. Kojima, T. Hirano, S. Maki, H. Niva, *Tetrahedron* **2013**, *69*, 3847–3856. It is not uncommon for bioluminescence wavelength to vary with the strain and purity of luciferase.

[20] M. S. Evans, J. P. Chaurette, S. T. Adams, G. R. Reddy, M. A. Paley, N. Aronin, J. A. Prescher, S. C. Miller, *Nat. Methods* **2014**, *11*, 393–395.

[21] B. R. Branchini, D. M. Ablamsky, J. C. Rosenberg, *Bioconjugate Chem.* **2010**, *21*, 2023–2030.

[22] N. Nakatani, J. Y. Hasegawa, H. Nakatsuji, *J. Am. Chem. Soc.* **2007**, *129*, 8756–8765.

[23] P. Naumov, Y. Ozawa, K. Ohkubo, S. Fukuzumi, *J. Am. Chem. Soc.* **2009**, *131*, 11590–11605.

[24] P. Naumov, M. Kochunnoonny, *J. Am. Chem. Soc.* **2010**, *132*, 11566–11605.

[25] S. Hosseinkhani, *Cell. Mol. Life Sci.* **2011**, *68*, 1167–1182.

[26] I. Navizet, Y.-J. Liu, N. Ferré, D. Roca-Sanjuán, R. Lindh, *ChemPhysChem* **2011**, *12*, 3064–3076.

[27] L. Pinto da Silva, J. C. G. Esteves da Silva, *ChemPhysChem* **2011**, *12*, 951–960.

[28] J. Vieira, L. Pinto da Silva, J. C. G. Esteves da Silva, *J. Photochem. Photobiol. B* **2012**, *117*, 33–39.

[29] D. Cai, M. A. L. Marques, B. F. Milne, F. Nogueira, *J. Phys. Chem. Lett.* **2010**, *1*, 2781–2787.

[30] L. Pinto da Silva, J. C. G. Esteves da Silva, *Photochem. Photobiol. Sci.* **2013**, *12*, 2028–2035.

[31] L. Pinto da Silva, R. Simkovitch, D. Huppert, J. C. G. Esteves da Silva, *ChemPhysChem* **2013**, *14*, 3441–3446.

[32] S. A. Maki, *Ecs Trans.* **2009**, *16*, 1–2.

[33] F. F. Craig, A. C. Simmonds, D. Watmore, F. McCapra, M. R. White, *Biochem. J.* **1991**, *276*, 637–641.

[34] J. Q. Wang, K. E. Pollok, S. Cai, K. M. Stantz, G. D. Hutchins, Q. H. Zhang, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 331–337.

[35] G. H. E. Law, O. A. Gandelman, L. C. Tisi, C. R. Lowe, J. A. H. Murray, *Biochem. J.* **2006**, *397*, 305–312.

[36] A. Jathoul, E. Law, O. Gandelman, M. Pule, L. Tisi, J. Murray in *Biolumin.—Recent Adv. Ocean. Meas. Lab. Appl.* (D. Lapota) (InTech, **2012**), at: http://www.intechopen.com/books/ bioluminescence-recent-advances-in-oceanic-measurements- and-laboratory-applications/development-of-a-ph-tolerant- thermostable-photinus-pyralis-luciferase-for-brighter-in-vivo- imaging.

[37] B. R. Branchini, T. L. Southworth, N. F. Khattak, E. Michelini, A. Roda, *Anal. Biochem.* **2005**, *345*, 140–148.

[38] B. R. Branchini, D. M. Ablamsky, M. H. Murtiashaw, L. Uzasci, H. Fraga, T. L. Southworth, *Anal. Biochem.* **2007**, *361*, 253–262.

[39] H. Zhao, T. C. Doyle, O. Coquoz, F. Kalish, B. W. Rice, C. H. Contag, *J. Biomed. Opt.* **2005**, *10*, 041210.

[40] Y. Nakajima, T. Yamazaki, S. Nishii, T. Noguchi, H. Hoshino, K. Niwa, V. R. Viviani, Y. Ohmiya, *Plos One* **2010**, *5*, e10011.

[41] K. R. Harwood, D. M. Mofford, G. R. Reddy, S. C. Miller, *Chem. Biol.* **2011**, *18*, 1649–1657.

[42] A comparison with in vivo luminescent images of the Maki analogue **5** was attempted. While this may be just a special case, the mouse which was administered the methyl ester of Maki analogue **5** (2 mg) became sick, and on ethical grounds we ceased in vivo studies of this analogue.