Synthesis of \( N \)-Peptide-6-Amino-\( \delta \)-Luciferin Conjugates with Optimized Fragment Condensation Strategy

Anita K. Kovács\(^{1,2} \) · Péter Hegyes\(^2 \) · Gábor J. Szebeni\(^3 \) · Krisztián Bogár\(^4 \) · László G. Puskás\(^2,3 \) · Gábor K. Tóth\(^1 \)

Accepted: 19 September 2018 / Published online: 22 September 2018
© Springer Nature B.V. 2018

Abstract
The synthesis of peptide-luciferin conjugates has a pivotal role in the development of bioluminescent detection systems that are based on the determination of protease enzyme activity. This work describes the optimized synthesis of an \( N \)-peptide-6-amino-\( \delta \)-luciferin conjugate (Fmoc-Gly-Pro-6-amino-\( \delta \)-luciferin) with a simple fragment condensation method in adequate yields. Fmoc-Gly-Pro-6-amino-\( \delta \)-luciferin was produced from a previously synthesized Fmoc-Gly-Pro-OH and also previously synthesized 6-amino-2-cyanobenzothiazole with an optimized method, to which conjugate cysteine was added in an also improved way. The resulting conjugate was successfully used in a bioluminescent system, in vitro, demonstrating the applicability of the method.

Graphical Abstract

Keywords
Bioluminescence · Protease activity · Aminoluciferin · Conjugate · Fragment condensation

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10989-018-9768-8) contains supplementary material, which is available to authorized users.

\(^{1}\) Department of Medical Chemistry, University of Szeged, Szeged 6720, Dóm tér 8., Hungary

\(^{2}\) Avidin Ltd, Szeged 6726, Alsó Kikötő sor 11/D., Hungary

\(^{3}\) Biological Research Center, Hungarian Academy of Sciences, Szeged 6720, Temesvári krt. 62., Hungary

\(^{4}\) Institute of Pharmaceutical Analysis, University of Szeged, Szeged 6720, Somogyi u. 4., Hungary
Introduction

Bioluminescent detection systems, used for in vivo and in vitro analytical methods, have been in the spotlight of research in the past decades (Jiang et al. 2018; Xu et al. 2016; Sadikot and Timothy 2005; Chollet and Ribault 2012; Sato et al. 2004). The advantage of these systems over fluorescent systems lies in their superior sensitivity and easy handling (De Saint-Hubert et al. 2012; Hu et al. 2012). The most ubiquitous enzyme-substrate system in bioimaging is the American firefly (Photinus pyralis) luciferin-luciferase system (Presiado et al. 2012; Zhang et al. 2012). Substituting this luciferin’s 6-position hydroxyl group with an amino group, the resulting aminoluciferin (aLuc) can form amide bond with a peptide, while retaining the transport and bioluminescent properties of the original substrate, resulting in a good substrate for different important proteases, which can be used for the determination of the enzymatic activity (White et al. 1966).

Earlier this year a general synthesis method for N-peptide-6-amino-d-luciferin conjugates, via a hybrid liquid/solid phase method, was published (Kovács et al. 2018). The applicability of the strategy was demonstrated with the preparation of a known substrate (O’Brian et al. 2005; Hickson et al. 2010), N-Z-Asp-Glu-Val-Asp-6-amino-d-luciferin (N-Z-DEVD-aLuc, Z: benzoyloxy carbonyl) used to measure the activity of caspase-3, and consequently the efficiency of apoptosis-inducing drugs. Our goal was to use the same strategy to produce N-Z-Gly-Pro-6-amino-d-luciferin (N-Z-GP-aLuc), which can be used to measure the activity of other protease enzymes, namely Fibroblast activation protein alpha (FAP) and Prolyl Oligopeptidase (POP/PREP), two prolyl-specific serine proteases, the levels of which are elevated in many cancers and may have roles in promoting angiogenesis and in immunotolerant tumour microenvironment (Christiansen et al. 2013).

Literary Background

Synthesis routes of peptide-6-amino-d-luciferin conjugates have been published five times. The backgrounds were worked out by Geiger and Miska (1991), who developed four methods (phospho-azo and mixed anhydride methods, both with both 6-amino-2-cyanobenzothiazole and carboxyl protected 6-amino-d-luciferin as starting material) and produced 13 different conjugates. The mixed anhydride/6-amino-2-cyanobenzothiazole method was modified in a 2003 Promega patent (O’Brian et al. 2003), and by Gryshuk et al. (2011). O’Brian et al. (2005) followed a different path, when 6-amino-2-cyanobenzothiazole was coupled with a protected peptide with the help of DCC and HOBt. Kovács et al. (2018) synthetized a conjugate with a different, liquid/solid phase synthesis method with better yields than the other methods (unfortunately, in some cases yields and conversion rates were not determined in the literature and the cited patents, also, HPLC, MS and NMR analysis is incomplete or missing (Tables S1–S4)).

Unsuccessful Attempt

Our original plan was to synthesize N-Z-GP-aLuc, applying the above-mentioned 2018 method (Kovács et al. 2018). First the key molecule, 6-amino-2-cyanobenzothiazole (1) was produced (Kovács et al. 2018) which was then coupled to Fmoc-Pro-OH (Fmoc: 9-fluorenylethoxycarbonyl). The resulting protected amino acid-heterocycle conjugate (Fmoc-Pro-6-amino-2-cyanobenzothiazole, 2, Figs. S1, S2) was reacted with d-cysteine. The structure of the resulting Fmoc-Pro-6-amino-d-luciferin was attested with 1H-NMR (Fig. S3) and 13C-NMR (Fig. S4), LC-MS analysis was also carried out (Figs. S5, S6). Then the protected amino acid-aminoluciferin conjugate was attached to p-alkoxybenzyl alcohol resin. However, attaching the protected amino acid-aminoluciferin conjugate to solid support, the loading was only 50%. This meant that we had a significant loss of the conjugate, even though some of it can be regained with different purification methods and reused during further resin attachment. In order to reach higher load, instead of 3 h, the coupling reaction mixture was shaken for 6 h. The determination of load showed that no improvement was achieved in loading and, according to the resulting material’s RP-HPLC profile (Fig. S7) and the mass spectrum (MS) (Fig. S8), 20% of the coupled material was dehydrogenated among the conditions mentioned above. As we were planning to purify the final, completed product, the synthetizing process was not abandoned. The product on the resin was Fmoc-deprotected, which was followed by coupling Z-protected glycine to the proline, and the resulting conjugate was cleaved from the resin (Kovács et al. 2018).

The RP-HPLC profile of the product (Fig. S9) showed a homogeneous material, but, according to the mass spectrum (Fig. S10), it was not the expected N-Z-GP-aLuc, but a fully dehydrogenated conjugate, N-Z-Gly-Pro-6-amino-2-cyanobenzothiazole. This was also attested by its 1H-NMR-spectrum (Fig. S11), on which the α-proton of the 2-thiazoline ring was not detectable; however, an olefin proton was, as proof of dehydrogenation. The driving force behind this dehydrogenation was the aromatization of the 2-thiazoline ring, which led to its transformation into a thiazole ring (Fig. 1). Since this reaction did not occur earlier either, it might have been the result of the longer exposure to the basic conditions.
As dehydroluciferin is a very efficient inhibitor of luciferase (Ciuffreda et al. 2013; Fontes et al. 1997), it is not suitable for the above-mentioned measurement of enzymatic activity. Therefore, our goal was to find a method that is more reliable than the hybrid liquid/solid phase synthesis method, a synthesis route that prevents the side reactions mentioned above. The problem was solved with returning to the fragment-condensation method, which is used to avoid problems occurring during stepwise solid phase synthesis (Nyfeler 1994). However, we had to make modifications to achieve better results than the standard method (O’Brian et al. 2005; Geiger and Miska 1991; O’Brian et al. 2003; Gryshuk et al. 2011).

Results and Discussion

The desired peptide-luciferin conjugate \(N\text{-Fmoc-GP-Luc}\) was reached in a 2-step route:
(a) attachment of the target peptide sequence to 6-amino-2-cyanobenzothiazole → (b) cysteine addition (Fig. 2, Table S5):
The optimized synthesis route of the key molecule and the modifications of the two steps make a significant improvement over the standard method.

---

**Fig. 1** The unsuccessful synthesis route to produce the desired \(N\text{-Z-Gly-Pro-6-amino-\text{-luciferin}}\)

**Fig. 2** The 2-step synthetic route to \(N\text{-Fmoc-GP-Luc}\) (3). Reagents and conditions: a Fmoc-Gly-Pro-OH, TCFH, dry DCM, DIPEA, overnight, rt, yield 68% b \(\text{D-CysHCl\cdotH\text{O}}\), MeOH, \(H\text{\text{2O}}, 5\text{ m/m\% NaHCO}_3\), 2 h, rt, yield 78%


**Synthesis of 6-Amino-2-Cyanobenzothiazole (1)**

We started with the synthesis of the key molecule, 6-amino-2-cyanobenzothiazole (1) with an improved method (Kovács et al. 2018): commercially available 2-chlorobenzothiazole was nitrated with a mixture of cc H2SO4 and KNO3, at 0 °C (Katz 1951). The structure of the resulting 2-chloro-6-nitrobenzothiazole was attested by 1H-NMR spectrum (83% yield corresponding to the isolated pure product). In the next step the nitro group was reduced with ethyl acetate/water/ammonium chloride/iron powder in a Soxlet extractor with a good yield (88%, corresponding to the crude product). The chlorine/nitrile exchange in the 6-amino-2-chlorobenzothiazole was carried out in N,N-dimethylacetamide (DMAA), a polar aprotic non-aqueous solvent, with KCN, at high temperature (110 °C) in 12 h, resulting in relatively high yield (78%, corresponding to the crude product). During procession the remaining KCN was reacted with KH2PO4, keeping pH above 7 to avoid the production of HCN, then first FeSO4 was added, forming K4[Fe(CN)]6, then Fe(III) salt was added, forming insoluble Berlin blue, which can be filtered from the solution. The product was purified with recrystallization (Kovács et al. 2018).

**Synthesis of Fmoc-Gly-Pro-6-Amino-2-Cyanobenzothiazole (2)**

A suitably protected, commercially purchased peptide, Fmoc-Gly-Pro-OH, was coupled with the key molecule, 6-amino-2-cyanobenzothiazole (1). (As during the synthesis only a dipeptide was coupled to the 6-amino-2-cyanobenzothiazole (1), it was reasonable to purchase a ready material, rather than synthesize and purify one. In case of longer peptides, solid phase peptide synthesis can be used.) Due to the deactivated amino group of the 6-amino-2-cyanobenzothiazole (1), the amide bond could not be formed with the usual coupling reagents; therefore, a more powerful coupling agent (Carpino et al. 1996) was necessary. Excellent conversion (97%) of the 6-amino-2-cyanobenzothiazole was obtained with 1.5 equivalents of chloro-N,N,N′,N′-tetramethylformamidinium hexafluorophosphate (TCFH) (Kovács et al. 2018). Obtained yield, corresponding to the crude product: 68%. (Figs. S12, S13).

With this process, we could avoid the extremely long coupling time of the standard mixed anhydride method (O’Brian et al. 2003; Gryshuk et al. 2011) and reached adequate conversion/yield.

**Synthesis of Fmoc-Gly-Pro-6-Amino-γ-Luciferin (3)**

The peptide-heterocycle conjugate (Fmoc-Gly-Pro-6-amino-2-cyanobenzothiazole, 2) was dissolved in methanol (MeOH), then γ-cysteine hydrochloride monohydrate was added. The resulting substance was dissolved in water and the cysteine was released from its salt with NaHCO3. During the reaction (about 25 min) the pH of the solution was kept between 7.3 and 7.4 with the addition of NaHCO3 aqueous solution, the process was continuously monitored with a pH-meter, under argon atmosphere. The Fmoc-protection of the the N-terminal amino-group of the peptide was kept up because it gave higher biological stability to the conjugate. The structure of the resulting conjugate was attested with 1H-NMR (Fig. S14), 13C-NMR (Fig. S15) and LC-MS (Figs. S16, S17).

This method is also an improvement over the standard practice (O’Brian et al. 2005; Geiger and Miska 1991; O’Brian et al. 2003; Gryshuk et al. 2011) as the window between pH 7.3–7.4 a, rules out racemization b, ensures the release of the cysteine from its salt.

**Analysis of Fmoc-Gly-Pro-6-Amino-γ-Luciferin (3) in Enzyme Activity Assays**

The product (3) was tested in bioluminescent-based enzyme activity assays. The substrate specificity of N-Fmoc-GP-aLuc was measured with two human proteases that are involved in cancer, POP/PROP and FAP, and with a bacterial non-specific endoproteinase Pro-C. All three enzymes accepted the substrate and liberated aminoluciferin as a product resulting in increased luminescence signal (Fig. 3). Enzymatic degradation was confirmed with protease inhibitor, which completely abolished bioluminescent signal increase. Our novel substrate, therefore, could be used in different biochemical assays.

**Materials and Methods**

**Materials**

TCFH and d-Cys-HCl·H2O were obtained from AK Scientific Inc. (Union City, CA, USA). Fmoc-Gly-Pro-OH was purchased from Iris Biotech GmbH (Marktredwitz, Germany). Trifluoroacetic acid (TFA) gradient grade came from VWR International (Radnor, PA, USA). POP/PROP and recombinant human FAP alpha were obtained from R&D Systems (Minneapolis, MN, USA). Endoproteinase Pro-C, DTT and Bovine Serum Albumin (BSA) were purchased from Sigma–Aldrich (St. Louis, MO, USA), black plastic microtiter plates were from Tomtec (Budapest, Hungary), Complete protease inhibitor cocktail was from Roche Basel, Switzerland and Luminescence Detection Reagent was from Promega (Madison, WI, USA).

TLC was performed on silica gel plates 60 F254 from Merck (Darmstadt, Germany). pH values were measured with a Hanna HI 8424 pH meter. Analytical reversed-phase
high-performance liquid chromatography (RP-HPLC) was performed on an Agilent 1200 series separations module with diode array and multiple wavelength detector (Waldbronn, Germany), with a Luna C18(2) 100 Å column (10 µm, 250 × 4.6 mm) Phenomenex, (Torrance, CA, USA). The experiments were carried out at room temperature (rt) with a flow rate maintained at 1.2 mL min⁻¹ at 220 nm wavelength (mobile phases solvent A: 0.1% TFA in Milli-Q water and solvent B: 0.1% TFA in acetonitrile (AcN)) using gradient elution. Separation was achieved on a Shimadzu (Kyoto, Japan) semi-preparative system with a Jupiter C18 300 Å column (10 µm, 250 × 21.20 mm), also from Phenomenex (mobile phases solvent A: 0.1% TFA in Milli-Q water and solvent B: 0.1% TFA in AcN) using gradient elution. Mass spectrometry data were collected on Waters (Milford, MA, USA) SQ Detector with atmospheric pressure ionization (API) mass spectrometer in positive ion mode; 1H NMR and 13C NMR spectra were recorded using a Bruker DR X 500 spectrometer at 600 MHz and 150 MHz, in deuterated dimethyl sulfoxide ([D6]DMSO). Chemical shifts were reported on the δ scale and J values were given in Hz.

Methods

Synthesis of Fmoc-Gly-Pro-6-Amino-2-Cyanobenzothiazole (2)

2.03 g (5.145 mmol, 1.5 equiv) anhydrous (anh) Fmoc-Gly-Pro-OH and 1.44 g (5.145 mmol, 1.5 equiv) anh TCFH were solved in 7 mL anh dichloromethane (DCM). The mixture was stirred for 60 min at room temperature. First 1 mL (6.174 mmol, 1.8 equiv) N,N-diisopropylethylamine (DIPEA), then 0.600 g (3.43 mmol, 1 equiv) anh 6-amino-2-cyanobenzothiazole (1) was added. After stirring the reaction mixture overnight at room temperature (according to HPLC analysis the conversion was 97%), it was washed with water (2 × 7 mL), with saturated NaHCO₃-solution (2 × 7 mL), then with water again (2 × 7 mL), and finally with saturated NaCl-solution (2 × 7 mL). It was dried over sicc Na₂SO₄, finally concentrated in vacuo. The resulting crude material was a pale yellow powder, its weight was 1.28 g, yield corresponding to the crude product: 68%. m/z [M + H]+ calcd for C₃₀H₂₅N₅O₄S 551.62, found 552.0 (Fig. S12). RP-HPLC (for the purified compound): 50–100% B in 25 min + 3 min up to 100% B + 100% B in 5 min, tᵣ₁ = 8.973 min: Fmoc-Gly-Pro-OH, tᵣ₂ = 17.868 min: Fmoc-Gly-Pro-6-amino-2-cyanobenzothiazole (Fig. S13).

Synthesis of Fmoc-Gly-Pro-6-Amino-α-Luciferin (3)

5.512 g (0.010 mol, 1 equiv.) Fmoc-Gly-Pro-6-amino-2-cyanobenzothiazole (2) was dissolved in 25 mL MeOH. 2.634 g (0.015 mol, 1.5 equiv.) d-cysteine•HCl•H₂O, solved in 19 mL distilled water, was added to the solution at room temperature, under argon atmosphere, then the mixture was stirred continuously under pH control (starting pH: 2.27).

After 20 min’ stirring at room temperature, 30 mL, 5% (m/m) NaHCO₃ was added dropwise over a period of 1 h to the mixture in order to release cysteine from its salt, while checking pH continuously. Reaching pH 2.6, a fine, yellow

Fig. 3 Luminescence is proportional to protease activity and N-Fmoc-GP-aLuc concentration. Different amount of a POP/PREP, b FAP and c Endoproteinase Pro-C proteases were titrated and assayed with 1–100 µmol N-Fmoc-GP-aLuc for 2 h. (d–f) The effect of protease inhibition was tested using the Complete protease inhibitor cocktail, in each reaction with 10 µmol N-Fmoc-GP-aLuc and 32 pmol/min/reaction protease activity. Each point represents the average of 3 wells ± SD. Values are blank-subtracted (blank = no protease). *p < 0.05; **p < 0.01; ***p < 0.001
solid material, Fmoc-Gly-Pro-6-amino-α-luciferin free carboxylic acid, started to precipitate. At pH 6.3, this material started to dissolve, and at pH 7.40, it dissolved completely. Here the Fmoc-Gly-Pro-6-amino-α-luciferin formed Na-salt, which dissolved under these conditions.

After another 20 min stirring at room temperature, the organic solvent was evaporated. From the remaining aqueous solution, a pale yellow solid material, Fmoc-Gly-Pro-6-amino-α-luciferin Na-salt precipitated partly. This aqueous mixture was extracted with 3 × 15 mL ethyl acetate, in order to get rid of possible impurities. The combined organic layers were extracted with saturated NaCl solution. Having dropped the resulting solution on a mixture of ice and cc HCl, a fine yellow precipitate, Fmoc-Gly-Pro-6-amino-α-luciferin free carboxylic acid, formed. It was allowed to settle for 10 min, filtered and washed with 2 × 5 mL water, then air-dried to constant weight, which was 5.115 g (7.80 mmol), yield corresponding to the crude product: 78%. 1H NMR (500 MHz, [D6]DMSO) δ 10.39 (s, 1H), 8.59 (t, J = 15.85 Hz, 1H), 8.09 (d, J = 8.98 Hz, 1H), 7.88 (d, J = 7.43 Hz, 2H), 7.71 (d, J = 7.48 Hz, 2H), 7.66–7.60 (m, 1H), 7.48 (t, J = 5.65 Hz, 1H), 7.39 (q, J1 = 7.60 Hz, J2 = 15.29 Hz, 2H), 7.30 (q, J1 = 6.78 Hz, J2 = 13.76 Hz, 2H), 5.43 (t, J = 8.98 Hz, 1H), 4.47 (dd, J1 = 2.92 Hz, J2 = 5.21 Hz, 1H), 4.29–4.25 (m, 1H), 4.21 (q, J1 = 6.68 Hz, J2 = 14.87 Hz, 1H), 3.95–3.67 (m, 4H), 3.65–3.48 (m, 4H), 2.20–2.11 (m, 1H), 2.06–1.99 (m, 1H), 1.97–1.88 (m, 2H) (Fig. S14). 13C NMR (125 MHz, [D6]DMSO) δ 171.15, 171.05, 167.43, 164.43, 159.04, 156.55, 148.58, 143.86, 140.70, 138.38, 136.28, 127.61, 127.08, 125.27, 124.20, 120.10, 119.64, 111.52, 78.11, 65.70, 60.47, 46.62, 45.92, 42.72, 34.78, 29.28, 24.52 (Figure S15). m/z [M + H]+ calcd for C33H29N5O6S2 655.74 found 656.0 (Figure S16). RP-HPLC: 70–100% B in 15 min, tR= 12.608 min. TLC: toluene/EtOH 50:30 saturated with water, Rf: 0.44.

Purification of Crude Fmoc-Gly-Pro-6-Amino-α-Luciferin (3)

160 mg crude peptide (45% desired material content, 72 mg) was dissolved in 1 mL N,N-dimethylformamide (DMF), then filtered, using a 0.45µm nylon filter. Gradient elution was used, 40–70% eluent B in 60 min at a 4 mL min⁻¹ flow rate with detection at 220 nm. Pure fractions were collected and lyophilized to give a pale yellow material, the weight of which was 23 mg (0.035 mmol), yield corresponding to the isolated pure product: 32%.

Fmoc-Gly-Pro-aLuc Assay

The following proteases were used in the assay: recombinant human POP/PREP, recombinant human FAP and Endoproteinase Pro-C at equivalent protease activity in tenfold serial dilution starting from 32 fmol/min/reaction to 320 pmol/min/reaction. Assay buffer for POP/PREP and Endoproteinase Pro-C contained 25 mmol tris(hydroxymethyl)aminomethane HCl-salt (Tris·HCl) pH 7.4, 250 mmol NaCl, 2.5 mmol 1,4-dithiothreitol (DTT) and the assay buffer for FAP contained 50 mmol Tris·HCl pH 7.4, 1 M NaCl, 1 mg/mL BSA. The N-Fmoc-GP-aLuc substrate was applied in 1 µmol to 100 µmol in 25 µL final reaction volume in a black plastic microtiter plate. The effect of protease inhibition (Complete protease inhibitor cocktail) was prepared by dissolving one tablet in 2 mL POP/PREP buffer and used in 2.5-fold dilution in each reaction with 10 µmol N-Fmoc-GP-aLuc and 32 pmol/min/reaction protease activity. After 2 h incubation at 37 °C 25 µL Luminescence Detection Reagent was added to each well. Luminescence was recorded within 5 min. The blank wells contained each component except proteases. Presented values are blank-subtracted.

Statistics

Statistical significance was calculated by unpaired t-test (two-tailed, homoscedastic) between untreated and inhibitor containing samples.

Conclusion

We have developed an improved route for the synthesis of N-peptid-6-amino-α-luciferin conjugates. The method is more reliable than the standard practice as the preparation of one of the building blocks and two operations have been improved, which has led to better yields and significantly faster production time. The produced N-Fmoc-GP-aLuc was successfully used to measure FAP and POP/PREP enzyme activity in vitro.

This optimized method provides a practical and scalable way for the preparation of other N-peptide-6-amino-α-luciferin conjugates as well.

Author Contributions Conceptualization: László G. Puskás, Investigation: Anita K. Kovács, Péter Hegyes, Gábor J. Szébeni, NMR analysis: Krisztián Bogár, Writing - original draft, review & editing: Anita K. Kovács, Supervision: Gábor K. Tóth.

Funding This work was partly supported by the following Grants: GINOP-2.3.2-15-2016-00030 and GINOP-2.3.2-15-2016-00001 from the National Research, Development and Innovation Office (NKFI), Hungary, Gábor J. Szébeni was supported by János Bolyai Research Scholarship of the Hungarian Academy of Sciences (BO/00139/17/8).

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.
References

Carpino LA, Triolo SA, Griffin GW, Herman LW, Tarr G, Sole NA, Diekmann E, El-Faham A, Ionescu D, Albericio F (1996) Innovation and Perspectives. In: Solid phase synthesis & combinatorial libraries: peptides, proteins and nucleic acids-small molecule organic chemical diversity. Collected Papers, 4th International Symposium, pp. 41–50, 1996

Chollet R, Ribault S (2012) Use of ATP Bioluminescence for rapid detection and enumeration of contaminants: the milliflex rapid microbiology detection and enumeration system. In Lapota D (ed) Recent advances in oceanic measurements and laboratory applications, InTech, China, pp 99–118, ISBN: 978-953-307-940-0

Christiansen VJ, Jackson KW, Lee KN, Downs TD, McKee PA (2013) Targeting inhibition of fibroblast activation protein-alpha and prolyl oligopeptidase activities on cells common to metastatic tumor microenvironments. Neoplasia 15:348–358

Ciuffreda P, Casati S, Meroni G, Santaniello E (2013) A new synthesis of dehydroluciferin [2-(6’-hydroxy-2’-benzothiazolyl)-thiazole-4-carboxylic acid] from 1,4-benzoquinone. Tetrahedron 69:5893–5897

De Saint-Hubert M, Devos E, Ibrahimi A, Debyser Z, Mortelmans L, Mottaghy FM (2012) Bioluminescence imaging of therapy response does not correlate with FDG-PET response in a mouse model of Burkitt lymphoma. Am J Nucl Med Mol Imaging 2:353–361

Fontes R, Dukhovich A, Sillero A, Sillero MAG (1997) Synthesis of dehydroluciferin by firefly luciferase: effect of dehydroluciferin, coenzyme A and nucleoside triphosphates on the luminescent reaction. Biochem Bioph Res Co 237:445–450

Geiger R, Miska W (1991) US Patent US5035999A

Gryshuk AL, Perkins J, LaTour JV (2011) US Patent US2011022442A1

Hickson J, Ackler S, Klaubert D, Bouska J, Ellis P, Foster K, Oleksijew A, Rodriguez L, Schlessinger S, Wang B, Frost D (2010) Noninvasive molecular imaging of apoptosis in vivo using a modified firefly luciferase substrate, Z-DEVd-aminoluciferin. Cell Death Diff 17:1003–1010

Hu H, Liu JT, Yao LP, Yin JP, Su N, Liu XQ, Cao F, Liang JM, Nie YZ, Wu KC (2012) Real-time bioluminescence and tomographic imaging of gastric cancer in a novel orthotopic mouse model. Oncol Rep 27:1937–1943

Jiang YL, Zhu Y, Moore AB, Miller K, Broome AM (2018) Biotinylated bioluminescent probe for long lasting targeted in vivo imaging of xenografted brain tumors in mice. ACS Chem Neurosci 9:100–106

Katz L (1951) Antituberculous Compunds II. 2-Benzalhydrazinobenzothiazoles. J Am Chem Soc 73:4007–4010

Kovács AK, Hegyes P, Szebeni GJ, Nagy LI, Puskás LG, Tóth GK (2018) Synthesis of N-peptide-6-amino-d-luciferin Conjugates. Front Chem 6:120

Nyfeler R (1994) Peptide synthesis via fragment condensation. Methods Mol Biol 35:303–316

O’Brien MA, Wood KV, Klaubert D, Daily B (2003) US Patent US20030211560A1

O’Brien MA, Daily WJ, Hesselberth PE, Moravec RA, Scurria MA, Klaubert DH, Bulleit RF, Wood KV (2005) Homogeneous, bioluminescent protease assays: caspase-3 as a model. J Biomol Scr 10:137–148

Presiado I, Erez Y, Simkovitch R, Shomer S, Gepshtein R, da Silva LP, da Silva J, Huppert D (2012) Excited-state proton transfer of firefly dehydroluciferin. J Phys Chem A 116:10770–10779

Sadikot RTB, Timothy S (2005) Bioluminescence imaging. Proc Am Thorac Soc 2:337–540

Sato A, Klaunberg B, Tolwani R (2004) In vivo bioluminescence imaging. Comp Med 54:631–634

White EH, Wörther H, Seliger HH, McElroy WD (1966) Amino analogs of firefly luciferin and biological activity thereof 1. J Am Chem Soc 88:10770–10779

Xu TT, Close D, Handagama W, Merr G, Sayer G, Ripp S (2016) The expanding toolbox of in vivo bioluminescent imaging. Front Oncol 6:150

Zhang YM, Pullambhatla M, Latera J, Pomper MG (2012) Influence of bioluminescence imaging dynamics by d-luciferin uptake and efflux mechanisms. Mol Imaging 11:499–506