Tetrahedron Letters 50 (2009) 4731–4733

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

An improved synthesis of releasable luciferin–CPP conjugates

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ARTICLE INFO

Article history:
Received 9 March 2009
Revised 23 May 2009
Accepted 5 June 2009
Available online 11 June 2009

Keywords:
Luciferin
Luciferin-linker
Conjugate
Cell-penetrating peptide
Releasable luciferin

ABSTRACT

We have improved the synthesis of a previously published luciferin-linker, used in an assay enabling rapid real-time quantification of luciferin–CPP conjugate uptake and cytosolic cargo release. We also present the synthesis of a new luciferin-linker with the same conjugation ability. Both luciferin-linkers are now available via an efficient one-pot procedure.

Cell-penetrating peptides (CPPs) are a heterogeneous class of peptides[1] which have been used extensively as carriers in biology and medicine.[2] Numerous studies conclusively demonstrate the potential of CPPs, both in vitro and in vivo, for intracellular delivery of various cargoes, that otherwise have difficulties crossing the plasma membrane, such as small molecules, proteins and oligonucleotides.[3] Today, as the number of reported CPPs is growing, reliable assays are urgently needed for evaluating the comparative internalization efficacy and further characterization of CPPs as cellular delivery agents.

Frequently used assays in the CPP field are, for example, dye-labelled CPPs for uptake and distribution experiments,[4] and biological read-out strategy, where peptide nucleic acid (PNA)–CPPs conjugates are used for splice correction.[5] In the former assay, CPPs are bound to the cellular membrane or trapped in intracellular vesicles giving rise to false-positive results.[6] These methods allow neither reliable evaluation of transporters in real time, nor give a direct measure of internalization efficacy.

A recently published assay relying on luminescence from the luciferin/luciferase reaction enables a real-time quantification of a cytosolic luciferin–CPP conjugate in cells and in animal models.[7,8] In this assay, free luciferin is released immediately upon cytosolic entry of the luciferin–CPP conjugate due to the high concentration of cytosolic glutathione. The presence of cytosolic luciferase enzymes results in a light-emitting reaction when free luciferin is converted into oxyluciferin. Due to the luminescent origin of the emitted light, this assay has the advantage of giving a strong signal and low background.

The beauty of the releasable luciferin assay[7] inspired us to investigate its application for the real-time quantification of uptake for a number of CPPs. The synthetic protocol described by Jones et al.[7] (Scheme 1, steps a–c) involves synthesis of activated disulfide **5** by reacting hydroxyl-thiol **1** with three equivalents of 2,2′-dithiodipyridine **2** (2′-aldrithiol). After flash-chromatographic purification, disulfide **3** (97%) was converted into the chloroformate **5** by reacting with triphosgene **4** at room temperature. The organic solvent was removed in vacuo and **5** was used without further purification in the reaction with the potassium salt of luciferin **6**, yielding the carbonate **7**, which after purification (58%) was used for conjugation to an octaarginine transporter.

The synthetic route described to the luciferin conjugate with activated disulfide is a concise solution for attachment of luciferin to a transporter. However, in our hands, preparation of the conjugate was hampered by synthetic difficulties. The main issues were (1) time-consuming purification of **3**. (2) Reaction with triphosgene at room temperature was very problematic and resulted in a very low yield of **7**. (3) Long reaction time for conjugation to a transporter.

We thought that a one-pot method avoiding isolation of intermediates would improve the utility of this assay. To this end, and supported by earlier reported investigations,[9,10] we made improvements to the reported synthesis. In addition, we present a synthetic route to a new luciferin-linker.

2′-Aldrithiol (**2**) is a well-known reagent used for the preparation of unsymmetrical disulfides.[11] Normally, an excess of **2** is reacted with the thiol of interest in good yields (above 60-70%).[11]

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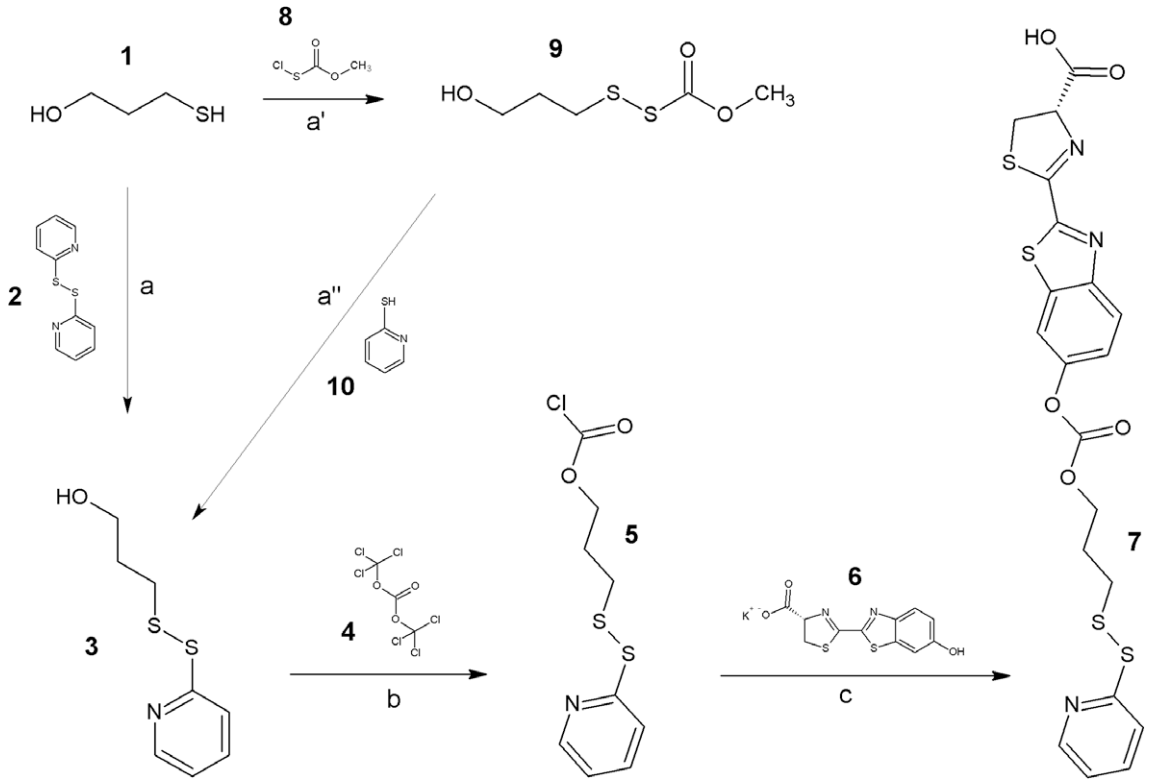
doi:10.1016/j.tetlet.2009.06.038



Scheme 1. Synthetic route to luciferin-linker **7**.

However, the excess of **2** and the formation of mercaptopyridine **10** as a by-product makes laborious chromatographic purification unavoidable. In order to achieve clean conversion into **3** we treated **1** with an equimolar amount of methoxycarbonylsulfenyl chloride **8** and thereafter with an equimolar amount of 2-mercaptopyridine **10** (Scheme 1, steps a′–a″), relying on the reactivity of the *S*-sulfenylcarbomethoxy functionality in compound **9**.[9,10,12] Gratifyingly, we obtained product **3** in nearly quantitative yield and of sufficient purity to use without isolation (Fig. 1, Supplementary data).

In this very efficient route to **3**, the starting materials were completely consumed and the only side-products formed were carbonyl sulfide gas and methanol.[13] This reaction is driven by the release of gaseous carbonyl sulfide and the reaction course is easily monitored by the disappearance of the yellow colour of **10**.

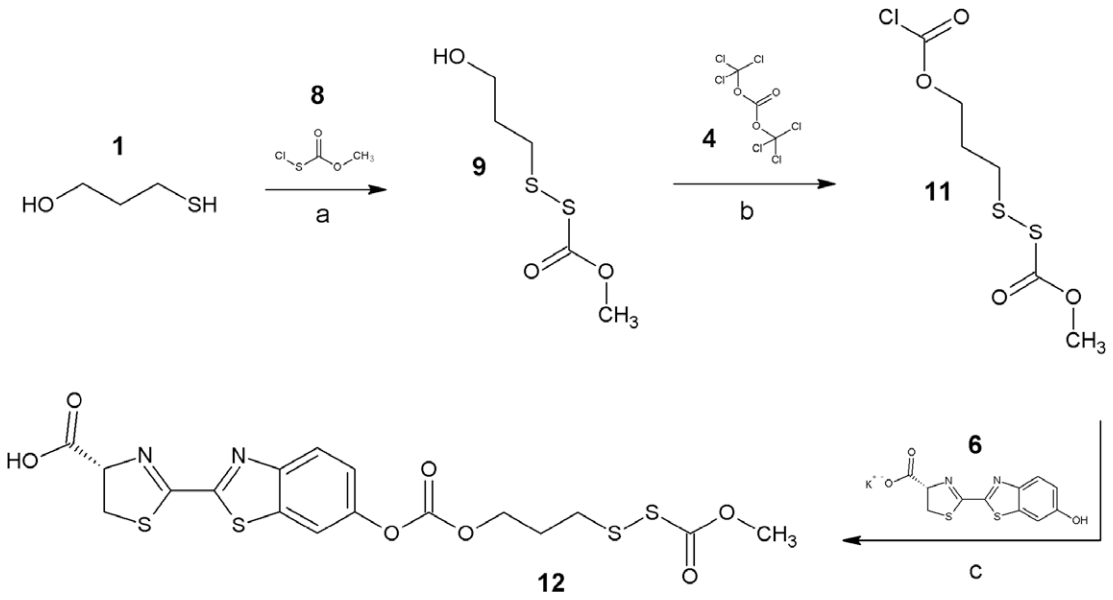
In the next step (Scheme 1, step b), we experienced great difficulties in obtaining chloroformate derivative **5** using triphosgene at room temperature. The main product was a symmetrical dialkylated carbonate side-product, which is in agreement with the fact that it is often essential to keep phosgene/triphosgene reactions at low temperature to minimize the formation of symmetrical dialkyl carbonate side-products.[14] In our hands, decreasing the temperature from room temperature to −10 °C was a crucial factor in the synthesis of **5**. Another important factor that had to be considered was the ratio of the alcohol and triphosgene reactants. We found that a 1:1 ratio of 3-(2-pyridinyldithio)-1-propanol (**3**) and triphosgene (**4**) resulted in a very clean synthesis of 3-(2-pyridinyldithio)-1-propanyl chloroformate (**5**) while a 3:1 ratio gave a 1:1 mixture of product **5** and a symmetrical dialkyl carbonate side-product (Fig. 2, Supplementary data).

Chloroformate **5** was used without purification in accordance with Jones' method.[7] It was treated with luciferin potassium salt **6** in water to form the luciferin-linker **7** (Scheme 1, step c). The luciferin-linker **7** was purified by RP-HPLC and conjugated to three different cysteine-containing CPPs (Table 1, Supplementary data) according to Jones.[7] Also, employing conditions routinely used in our laboratory for similar conjugation reactions, we were able to demonstrate that all the reactions were complete within 30 min (Fig. 3, Supplementary data). This short reaction time allows this procedure to be carried out without an inert gas atmosphere,[15] which further simplifies this step. We also found that a 1:1 molar ratio of luciferin-linker to transporter CPP was as effective as the 1:2 ratio employed by Jones.[7]

In an attempt to further advance our study, we synthesized the novel activated carbonate derivative **12** (Scheme 2).[16] Here, *S*-sulfenylcarbomethoxy-functionalized thiopropanol **9** was directly converted into the chloroformate **11**, which was then reacted with salt **6** to give the desired product **12** in high purity. Both reaction steps a and c go to completion, however, our analysis showed that conversion of **9** into **11** (step b) was not complete (ca. 60% yield). We believe that increasing the reaction time or raising the temperature to −5 °C could improve the yield. Crude product **12** was obtained in 48% yield. The high purity of crude **12** synthesized in one pot (Fig. 4, Supplementary data) is in sharp contrast with that of crude **7**, which is also prepared in one pot (Fig. 5, Supplementary data), despite several attempts to improve the purity. This high purity potentially allows direct conjugation of crude **12** to the carrier.

Pure **12** was equally effective as pure **7** in the conjugation reaction with the three CPPs, giving clean and complete conversion into luciferin-transporters in 30 min (Fig. 6, Supplementary data).[17]

In summary, we have presented an improved and practical protocol for the synthesis of luciferin-linker **7**. We have also described a simple procedure allowing the one-pot preparation of a novel luciferin-linker **12**. Our synthetic route to the activated luciferin carbonate is very effective in terms of time and purity, allowing the total synthesis of the desired luciferin-transporter in two to three days.



Scheme 2. Synthetic route to luciferin-linker **12**.

Acknowledgements

The work presented in this article was supported by the Swedish Research Council (VR-NT); by the Center for Biomembrane Research, Stockholm; by Knut and Alice Wallenberg's Foundation and by the Swedish Governmental Agency for Innovation Systems (VINNOVA-SAMBIO 2006). We thank Dr. Heiner Eckert for valuable advice regarding the triphosgene reactions.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2009.06.038.

References and notes

1. Langel, Ü. *Handbook of Cell-Penetrating Peptides*, 2nd ed.; CRC Press: Boca Raton, 2006.
2. Stewart, K. M.; Horton, K. L.; Kelley, S. O. *Org. Biomol. Chem.* **2008**, *6*, 2242.
3. Mäe, M.; Langel, Ü. *Curr. Opin. Pharmacol.* **2006**, *6*, 509.
4. Holm, T.; Johansson, H.; Lundberg, P.; Pooga, M.; Lindgren, M.; Langel, Ü. *Nat. Prot.* **2006**, *1*, 1001.
5. El Andaloussi, S.; G0075terstam, P.; Langel, Ü. *Nat. Prot.* **2007**, *2*, 2043.
6. Richard, J. P.; Melikov, K.; Vives, E.; Ramos, C.; Verbeure, B.; Gait, M. J.; Chernomordik, L. V.; Lebleu, B. *J. Biol. Chem.* **2003**, *278*, 585.
7. Jones, L. R.; Goun, E. A.; Shinde, R.; Rothbard, J. B.; Contag, C. H.; Wender, P. A. *J. Am. Chem. Soc.* **2006**, *128*, 6526.
8. Wender, P. A.; Goun, E. A.; Jones, L. R.; Pillow, T. H.; Rothbard, J. B.; Shinde, R.; Contag, C. H. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 10340.
9. Kaneko, T.; Willner, D.; Monkovic, I.; Knipe, J. O.; Braslawsky, G. R.; Greenfield, R. S.; Vyas, D. M. *Bioconjug. Chem.* **1991**, *2*, 133.
10. Vlahov, I. R.; Santhapuram, H. K.; Kleindl, P. J.; Howard, S. J.; Stanford, K. M.; Leamon, C. P. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5093.
11. Witt, D.; Klajn, R.; Barski, P.; Grzybowski, B. A. *Curr. Org. Chem.* **2004**, *8*, 1763.
12. The peptides were synthesized in a stepwise manner on an automated peptide synthesizer (Applied Biosystems model 433A, USA) by *tert*-butoxycarbonyl (*t*-Boc) chemistry and purified on a semi-preparative reverse-phase (RP)-HPLC column. Their purity was checked by RP-HPLC and the correct molecular weight was verified by MALDI-TOF mass spectrometry. *Synthesis of luciferin-linker* **7** (Scheme 1): Steps a′–c: 3-mercapto-1-propanol **1** (1.018 g, 11.05 mmol) was added dropwise to methoxycarbonylsulfenyl chloride **8** (1.398 g, 11.05 mmol) in ice-cold CH_2Cl_2 (10 ml), and stirred for 30 min on ice, followed by the addition of 2-mercaptopyridine **10** (1.228 g, 11.05 mmol). The reaction mixture was stirred for 1 h on ice. After washing with $(NH_4)_2CO_3$ solution (1 g in 20 ml of H_2O), the CH_2Cl_2 phase was dried over $MgSO_4$ and evaporated to give crude 3-(2-pyridinyldithio)-1-propanol **3** as a colourless oil. The molecular weight of **3** was verified by MALDI-TOF: calcd monoisotopic mass for $[M+H^+]$ is 202.04, found $[M+H^+]$ 202.02. Pyridine (5.89 mg, 0.0745 mmol) in 300 μl of cold (−10 °C) CH_2Cl_2, was added to crude compound **3** (15.0 mg, 0.0745 mmol) which was then added dropwise over 20 min to triphosgene **4** (22.1 mg, 0.0745 mmol) and dissolved in 650 μl of cold (−10 °C) CH_2Cl_2. The reaction mixture was stirred for 5 h at −10 °C (ice/NaCl bath), and the CH_2Cl_2 was evaporated to give crude 3-(2-pyridinyldithio)-1-propanyl chloroformate **5**. A solution containing luciferin potassium salt **6** (23.7 mg, 0.0745 mmol) dissolved in 2.37 ml of ice-cold water and 149 μl of ice-cold sodium hydroxide solution (0.500 M in H_2O, 0.0745 mmol) was added dropwise to crude **5** according to Jones' method[7] to give luciferin-linker **7** after 4 h reaction on ice. Compound **7** was obtained as a white powder after RP-HPLC purification. The molecular weight of **7** was verified by MALDI-TOF: calcd monoisotopic mass for $[M+H^+]$ is 508.01, found $[M+H^+]$ 507.99.Scheme 1, steps a–c: These reaction steps were carried out according to Ref. 7 with one modification; product **3** (step a) was washed repeatedly with Na_2CO_3 solution (1 g in 20 ml of H_2O) to remove the side-product **10** instead of using flash-chromatographic purification.
13. Brois, S. J.; Pilot, J. F.; Barnum, H. W. *J. Am. Chem. Soc.* **1970**, *92*, 7629.
14. Matzner, M.; Kurkjy, R. P.; Cotter, R. J. *Chem. Rev.* **1964**, *64*, 645.
15. Tam, J. P.; Wu, C. R.; Liu, W.; Zhang, J. W. *J. Am. Chem. Soc.* **1991**, *113*, 6657.
16. *Synthesis of luciferin-linker* ***12*** (Scheme 2): steps a–c: 3-mercapto-1-propanol **1** (21.7 mg, 0.235 mmol), dissolved in 160 μl of ice-cold CH_2Cl_2, was added dropwise over 10 min to methoxycarbonylsulfenyl chloride **8** (32.7 mg, 0.259 mmol), dissolved in 500 μl of ice-cold CH_2Cl_2. The reaction mixture was stirred for 30 min on ice to give crude 3-[(methoxycarbonyl)disulfanyl]-1-propanol **9**. Pyridine (18.6 mg, 0.235 mmol) in 340 μl of cold (−10 °C) CH_2Cl_2, was added to a CH_2Cl_2 solution of crude compound **9** which was then added dropwise over 10 min to triphosgene **4** (69.7 mg, 0.235 mmol), dissolved in 2 ml of cold (−10 °C) CH_2Cl_2. The reaction mixture was stirred for 5 h at −10 °C (ice/NaCl bath), and the CH_2Cl_2 was evaporated to give crude 3-[(methoxycarbonyl)disulfanyl]-1-propanyl chloroformate **11**. Luciferin potassium salt **6** (74.8 mg, 0.235 mmol), dissolved in ice-cold water (3 ml), was added to 470 μl of ice-cold sodium hydroxide solution (0.500 M in H_2O, 0.235 mmol). The luciferin/NaOH solution was added dropwise over 10 min to crude compound **11** and the reaction mixture was stirred for 4 h on ice. The reaction was quenched with 15 ml of 1% TFA and extracted with CH_2Cl_2 (3x15 ml). The CH_2Cl_2 phase was dried over $MgSO_4$ and evaporated to yield luciferin-linker **12**, which after RP-HPLC purification was obtained as a white powder. The molecular weight of **12** was verified by MALDI-TOF: calcd monoisotopic mass for $[M+H^+]$ is 488.99, found $[M+H^+]$ 488.96. ^1H NMR ($CDCl_3$, 500 MHz, 25 °C): δ 8.12 (d, 1H, J = 8.4 Hz, aromatic), 7.79 (s, 1H, aromatic), 7.35 (d, 1H, J = 8.4 Hz, aromatic), 5.43 (m, 1H, NCH), 4.43 (t, 2H, J = 6.1 Hz, OCH_2), 3.90 (s, 3H, CH_3), 3.80 (m, 2H, SCH_2), 2.94 (t, 2H, J = 7.0 Hz, $SSCH_2$), 2.16 (q, 2H, J = 6.5 Hz, CH_2) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz, 50 °C): δ 171.8, 170.1, 167.6, 160.6, 153.3, 151.2, 150.2, 136.9, 125.5 (aromatic CH), 121.1 (aromatic CH), 114.2 (aromatic CH), 78.3 (NCH), 67.1 (OCH_2), 55.5 (CH_3), 35.4 (SCH_2 and $SSCH_2$), 27.9 (CH_2) ppm.
17. *Conjugation of luciferin-linkers to peptides.* Luciferin-linker **7** and a cysteine-containing peptide (Table 1, Supplementary data) were mixed in a 1:1 or a 2:1 ratio at a final peptide concentration of 0.88 mM in either DMF or DMF/acetic acid buffer (pH 5, 50 mM) at room temperature, under nitrogen. Samples taken after 30 min, 2 h, 4 h and 24 h were analyzed by RP-HPLC, the major components collected and their molecular weights verified by MALDI-TOF: calcd monoisotopic mass for luciferin-TP10 $[M+H^+]$ is 2680.42, found $[M+H^+]$ 2680.42; calcd monoisotopic mass for luciferin-pVEC $[M+H^+]$ is 2707.42, found $[M+H^+]$ 2707.42; calcd monoisotopic mass for luciferin-M918 $[M+H^+]$ is 3133.62, found $[M+H^+]$ 3133.61. Luciferin-linker **12** and the peptides were mixed in a 1:1 ratio under the same conditions as above.