



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.

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Cell-penetrating peptides (CPPs) are a heterogeneous class of peptides¹ 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 assay⁷ 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.

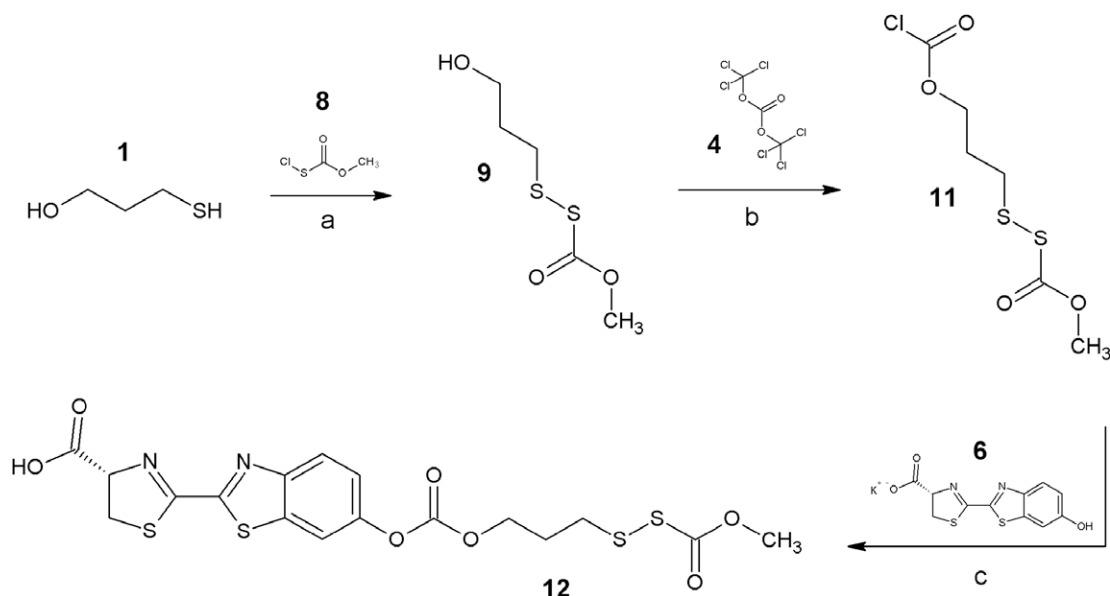
The synthetic route described to the luciferin conjugate with activated disulfide is a concise solution for attachment of luciferin to a transporter. However, in our hands, preparation of the conjugate was hampered by synthetic difficulties. The main issues were (1) time-consuming purification of **3**. (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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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.

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- 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 methoxycarbonylsulfonyl chloride **8** (1.398 g, 11.05 mmol) in ice-cold CH₂Cl₂ (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 (NH₄)₂CO₃ solution (1 g in 20 ml of H₂O), the CH₂Cl₂ phase was dried over MgSO₄ 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) CH₂Cl₂, 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) CH₂Cl₂. The reaction mixture was stirred for 5 h at –10 °C (ice/NaCl bath), and the CH₂Cl₂ 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 H₂O, 0.0745 mmol) was added dropwise to crude **5** according to Jones' method⁷ 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 Na₂CO₃ solution (1 g in 20 ml of H₂O) to remove the side-product **10** instead of using flash-chromatographic purification.

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- 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 CH₂Cl₂, was added dropwise over 10 min to methoxycarbonylsulfonyl chloride **8** (32.7 mg, 0.259 mmol), dissolved in 500 µl of ice-cold CH₂Cl₂. 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) CH₂Cl₂, was added to a CH₂Cl₂ 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) CH₂Cl₂. The reaction mixture was stirred for 5 h at –10 °C (ice/NaCl bath), and the CH₂Cl₂ 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 H₂O, 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 CH₂Cl₂ (3x15 ml). The CH₂Cl₂ phase was dried over MgSO₄ 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 (CDCl₃, 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, OCH₂), 3.90 (s, 3H, CH₃), 3.80 (m, 2H, SCH₂), 2.94 (t, 2H, J = 7.0 Hz, SSCH₂), 2.16 (q, 2H, J = 6.5 Hz, CH₂) ppm. ¹³C NMR (CDCl₃, 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 (OCH₂), 55.5 (CH₃), 35.4 (SCH₂ and SSCH₂), 27.9 (CH₂) ppm.
- 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.