Theses of doctoral (Ph.D.) dissertation

OPTIMIZED SYNTHESIS ROUTES AND BIOLOGICAL APPLICATION OF *N*-PEPTIDE-6-AMINO-D-LUCIFERIN CONJUGATES

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1. INTRODUCTION AND AIMS

In the recent years, numerous *in vivo* and *in vitro* analytical methods, based on fluorescence and bioluminescence, have been developed for various biological objectives, including immunoassays, gene expression assays, bioimaging, investigation of infectious diseases etc. Plate based, high-throughput viability assays addressing the detection of protease activity is in the focus of intensive research. The advantage of bioluminescent systems over fluorescent ones lies in their superior sensitivity and easy handling.

In the bioluminescent methods, diverse sets of luciferases and their substrates, luciferins have been applied in different cellular and animal models, the most ubiquitous enzyme-substrate system is the American firefly (*Photinus pyralis*) luciferin-luciferase system.

Substituting the 6-position hydroxyl group of native luciferin with an amino group, the resulting 6-amino-D-luciferin (2-(6-aminobenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid, hereinafter: aLuc, Figure 1) can form an 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.

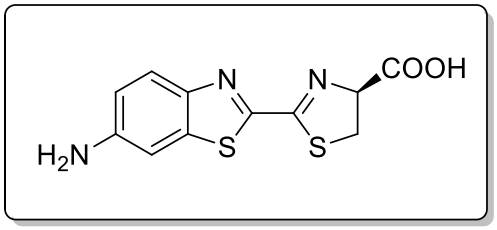


Figure 1 6-amino-D-luciferin

Due to this feature, these conjugates can be used for the determination of the enzymatic activity the following way: the protease enzyme to be measured recognizes the peptide part of the conjugate with the suitable peptide sequence, then cleaves the amide bond between the peptide and the aLuc, thus aLuc is released, which, in the presence of luciferase enzyme, emits light (Figure 2).

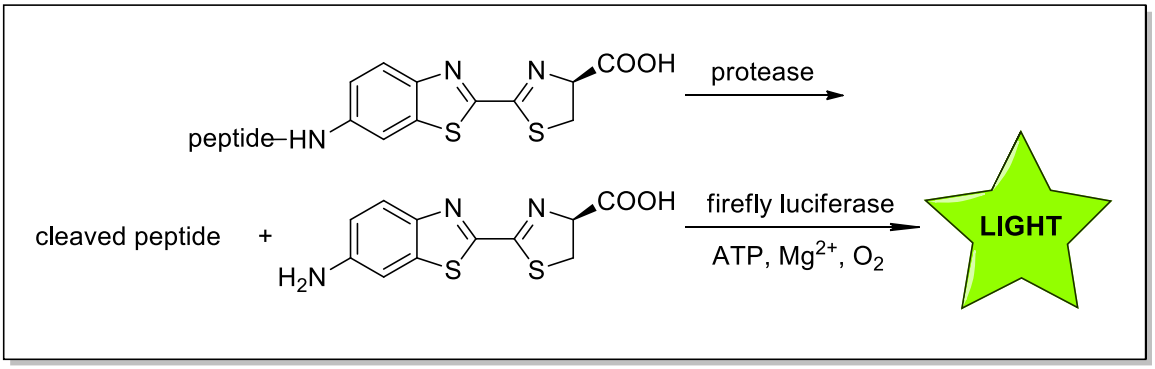


Figure 2 The operation of the bioluminescent system

The activity of the given protease enzyme can be determined from the amount of emitted light, as the emitted light is directly proportional to the activity of the enzyme.

Unfortunately, the synthesis methods of these substrates published so far are complicated; and the very few commercially available conjugates are very expensive. The aim of this dissertation was to introduce novel routes for the scalable and economical synthesis of *N*-peptide-aLuc conjugates.

The preparative work focused on preparing a precursor (6-amino-2-cyanobenzothiazole, **3**), two conjugates (*N*-Z-Asp-Glu-Val-Asp-aLuc, **6**, *N*-Fmoc-Gly-Pro-aLuc, **8**) and an intermediate (*N*-Boc-aLuc, **10**).

2. MATERIALS AND METHODS

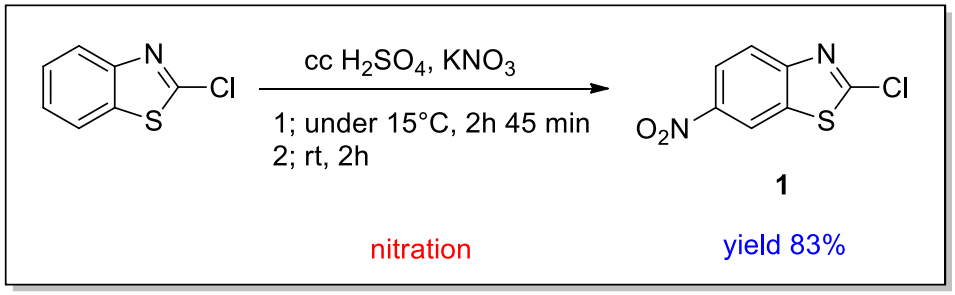
During the synthetic work, most of the reactions were performed on a millimolar scale. Reactions were monitored with RP-HPLC analysis and thin-layer chromatography. Products were purified with RP-HPLC or with simple filtration and recrystallization. The molecular structures of products were determined by one-dimensional NMR technique combined with mass spectrometric measurements. The applicability of substrates *N*-Z-Asp-Glu-Val-Asp-aLuc (**6**) and *N*-Fmoc-Gly-Pro-aLuc (**8**) were tested in biochemical assays with caspase-3, POP/PREP, FAP alpha and endoproteinase Pro-C enzymes. In vivo measurements were also conducted with A549 non-small cell lung carcinoma cells and with SCID mice inoculated with U87-Luc cells.

3. RESULTS AND DISCUSSION

3.1 Synthesis of the precursor

3.1.1 2-chloro-6-nitrobenzothiazole (1) synthesis

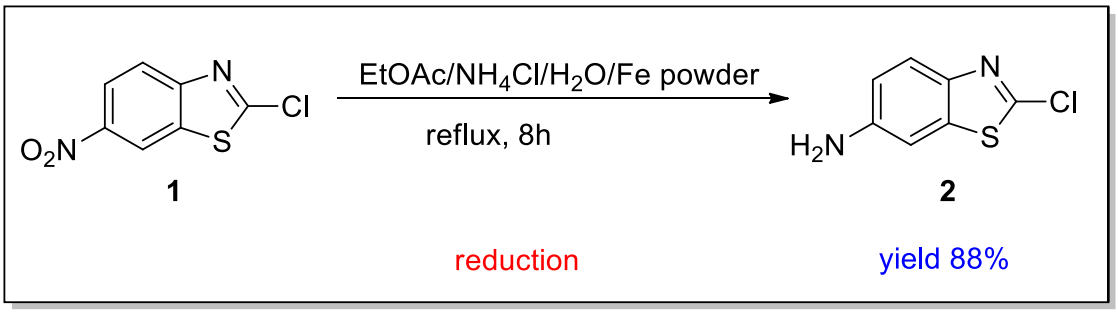
As starting material, cheap, commercially available 2-chlorobenzothiazole was used, which was nitrated with a mixture of KNO_3 and cc H_2SO_4, keeping the temperature first under 15ºC then at room temperature (Scheme 1).



Scheme 1 Synthesis of 2-chloro-6-nitrobenzothiazole (**1**)

3.1.2 6-amino-2-chlorobenzothiazole (2) synthesis

The nitro group of the 2-chloro-6-nitrobenzothiazole (**1**) was reduced with the application of EtOAc/H_2O/NH_4Cl/Fe powder system, with a good yield (88%) (Scheme 2).

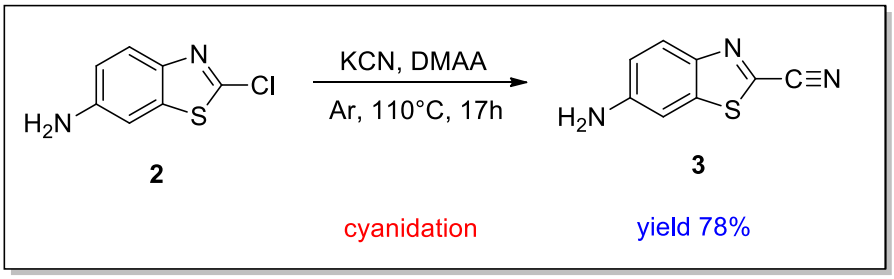


Scheme 2 Synthesis of 6-amino-2-chlorobenzothiazole (**2**)

Using a Soxhlet extractor turned out to be a solvent-sparing, thus environmentally-friendly method and processing the obtained product was also simple: the solution had to be decanted in order to get rid of the Fe powder and then extracted.

3.1.3 6-amino-2-cyanobenzothiazole (3)

The chlorine/nitrile exchange in the 6-amino-2-chlorobenzothiazole (**2**) is the key step in the production of the desired precursor. The satisfactory rate of KCN dissolution requires a polar aprotic non-aqueous solvent (DMAA), high temperature (110 °C) and long reaction time (17h) (Scheme 3).



Scheme 3 Synthesis of 6-amino-2-cyanobenzothiazole (**3**)

3.2 *N*-peptide-aLuc conjugate synthesis

The low nucleophilicity of the amino group makes its protection with a protecting group problematic, therefore, a more reliable method was necessary.

Mixed liquid/solid phase method

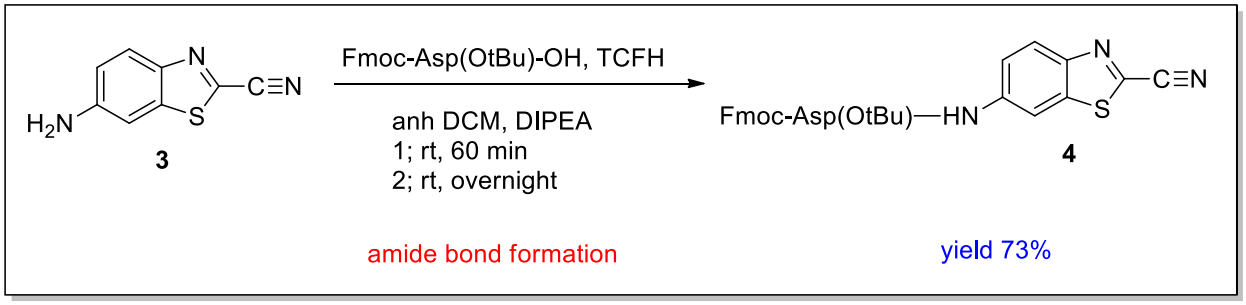
3.2.1 N-peptide aLuc conjugate synthesis with liquid/solid phase Fmoc strategy

The desired aLuc conjugate (*N*-Z-Asp-Glu-Val-Asp-aLuc, **6**) was reached in a 5-step route:

a) attachment of the *C*-terminal amino acid of the target sequence → b) cysteine addition → c) attachment to resin → d) solid-phase peptide synthesis → e) cleavage from resin

3.2.1.1 N-Fmoc-Asp(OtBu)-6-amino-2-cyanobenzothiazole (4) synthesis

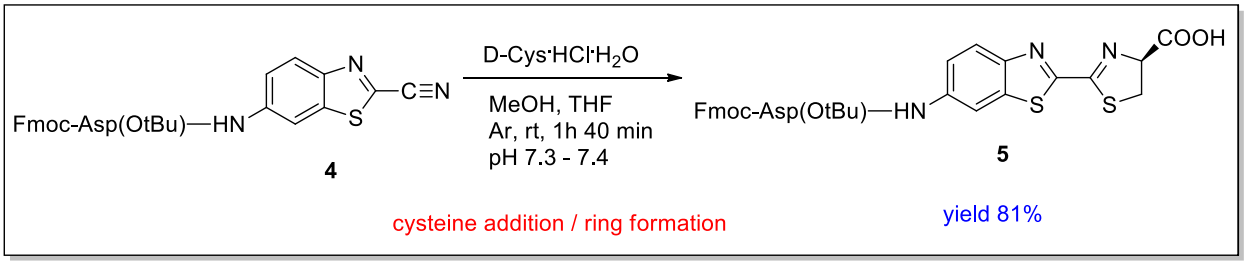
An Fmoc-protected amino acid (Fmoc-Asp(OtBu)-OH) was coupled to the 6-amino-2-cyanobenzothiazole (**3**). As, due to the deactivated amino group, the amide bond could not be formed with a usual coupling reagent like DCC, we used 1.5 equivalents of TCFH, a powerful coupling agent (Scheme 4).



Scheme 4 Synthesis of *N*-Fmoc-Asp(OtBu)-6-amino-2-cyanobenzothiazole (**4**)

*3.2.1.2 N-Fmoc-Asp(OtBu)-aLuc (**5**) synthesis*

During the D-cysteine addition, the amino acid-heterocycle conjugate was dissolved in THF and MeOH, then D-cysteine hydrochloride monohydrate was added. In order to promote the release of the cysteine from its salt, and also to prevent racemization, the pH of the solution was kept between 7.3-7.4 by the addition of $NaHCO_3$ aqueous solution (Scheme 5).



Scheme 5 Synthesis of *N*-Fmoc-Asp(OtBu)-aLuc (**5**)

*3.2.1.3 Attachment of N-Fmoc-Asp(OtBu)-aLuc (**5**) to solid support*

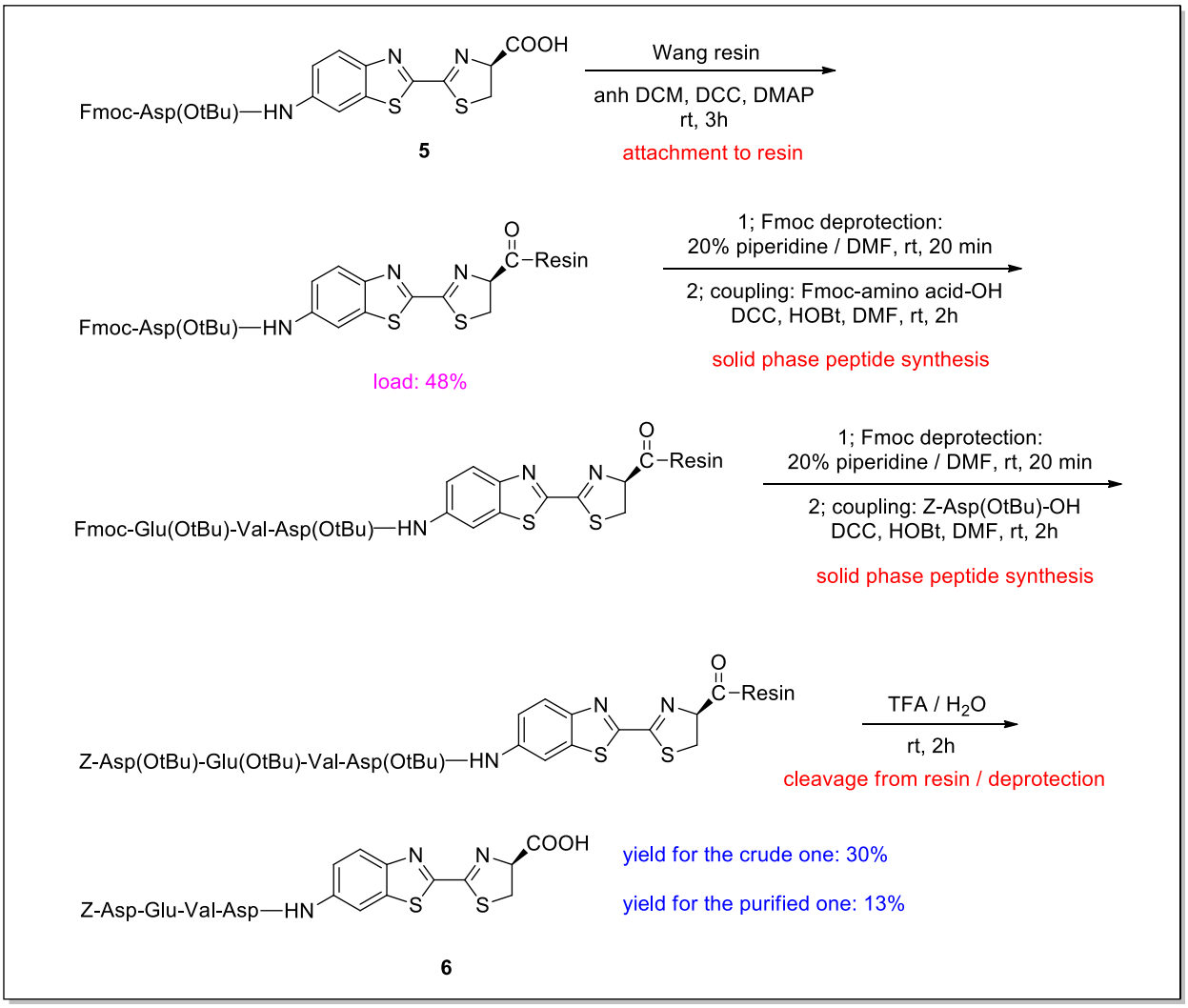
Having carried out tests, to get better loading, the conjugate was attached to Wang resin, not 2-chlorotrityl chloride.

3.2.1.4 Building the peptide chain

The peptide chain was built with solid phase Fmoc strategy, during which the *N*-terminal amino acid was always Z-protected, as this protecting group gives higher biological stability to the peptide (Scheme 6).

3.2.1.5 Cleavage of the peptide from the resin

The obtained *N*-peptide-aLuc conjugate was removed from the resin with the mixture of TFA/water (95:5 v/v) (Scheme 6).



Scheme 6 Solid phase synthesis of *N*-Z-Asp-Glu-Val-Asp-aLuc (**8**)

3.2.1.6 Limitations of the mixed liquid/solid phase method

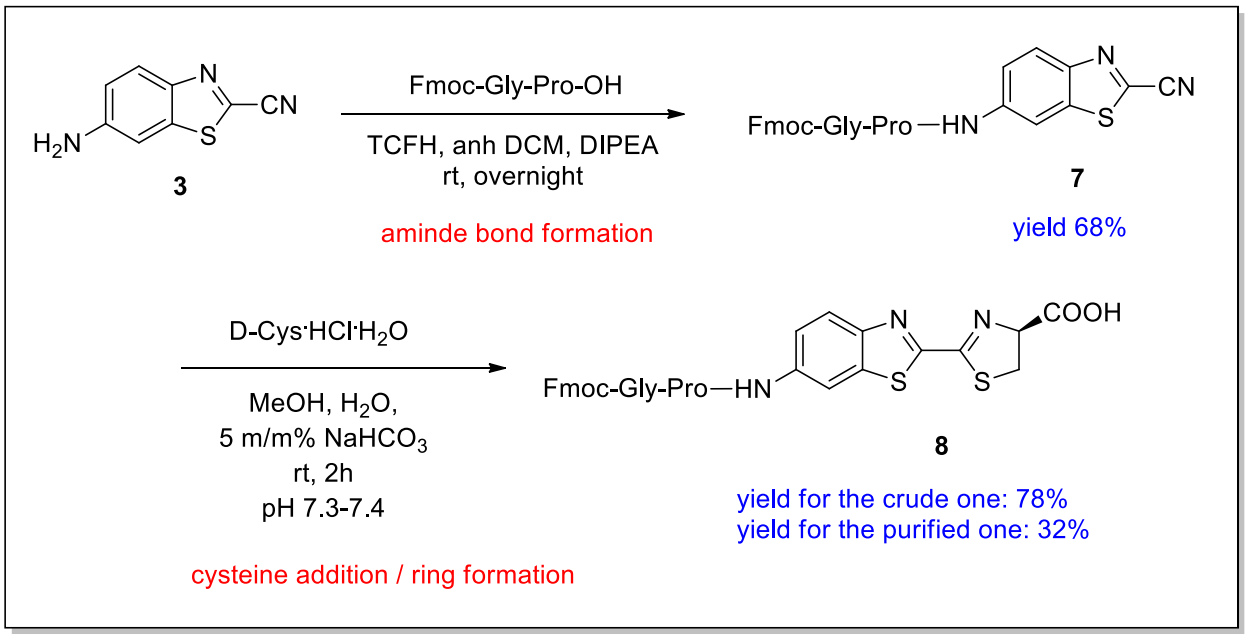
Our method gives better yield than the published methods, however, the high risk of dehydrogenation poses limitations to it.

Fragment condensation method

3.2.2 N-peptide aLuc conjugate synthesis with fragment condensation strategy

Having made modifications to achieve better results than the standard fragment condensation methods, the desired *N*-peptide-luciferin conjugate (*N*-Fmoc-Gly-Pro-aLuc, **8**) was reached in a 2-step route:

a) attachment of the target peptide sequence (*N*-Fmoc-Gly-Pro-OH) to 6-amino-2-cyanobenzothiazole (**3**)→ b) cysteine addition (Scheme 7):



Scheme 7 Synthesis of *N*-Fmoc-Gly-Pro-aLuc (**8**)

3.2.2.1 N-Fmoc-Gly-Pro-6-amino-2-cyanobenzothiazole (7) synthesis

A suitably protected, commercially purchased peptide, *N*-Fmoc-Gly-Pro-OH, was coupled with the key molecule, 6-amino-2-cyanobenzothiazole (**3**). Due to the deactivated amino group of the 6-amino-2-cyanobenzothiazole (**3**), the amide bond could not be formed with the usual coupling reagents; therefore, a more powerful coupling agent was necessary. Excellent conversion (97%) of the 6-amino-2-cyanobenzothiazole (**3**) was obtained with 1.5 equivalents of TCFH.

With this process, we could avoid the extremely long coupling time of the standard fragment condensation method and reached adequate yield (68%).

*3.2.2.2 N-Fmoc-Gly-Pro-aLuc (**8**) synthesis*

During the D-cysteine addition, the amino acid-heterocycle conjugate was dissolved in MeOH, then D-cysteine hydrochloride monohydrate was added. In order to promote the release of the cysteine from its salt, and also to prevent racemization, the pH of the solution was kept between 7.3-7.4 by the addition of $NaHCO_3$ aqueous solution (Scheme 7). 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.

3.2.2.3 Limitations of the fragment condensation method

Although the optimized synthesis route of the key molecule (**3)**, and the modifications of the two steps make a significant improvement over the standard methods, it has certain limitations: a) when attaching longer peptides (ones that contain more than ten amino acids), solubility problems may occur, which may make coupling difficult, b) when not glycine or proline is chosen as the *C*-terminal amino acid, racemization might occur.

Immediate precursor for Boc strategy solid phase method

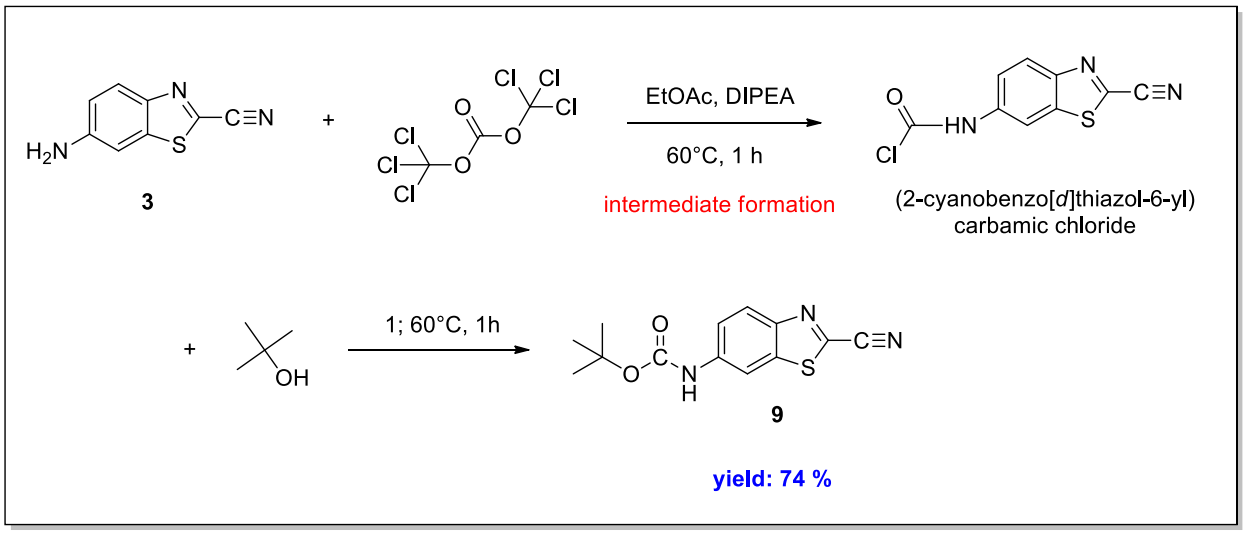
3.2.3 Building block production for the Boc-strategy solid phase synthesis of N-peptide-aLuc conjugates

The cornerstone of Boc-strategy *N*-peptide-aLuc conjugate synthesis is the availability of Boc protected aLuc in large quantities. Our aim was to synthetize a general, all-purpose Boc protected aLuc to which any peptide sequence can be attached. The desired substance was obtained in a 2-step route:

a) *N*-Boc-protection of 6-amino-2-cyanobenzothiazole (**3**) $\rightarrow$ b) cysteine addition

*3.2.3.1 N-Boc-6-amino-2-cyanobenzothiazole (**9**) synthesis*

6-amino-2-cyanobenzothiazole (**3**) was dissolved in EtOAc, then DIPEA and triphosgene was added, producing 2-cyanobenzo[*d*]thiazol-6-yl)carbamic chloride as intermediate (Scheme 8). After the addition of tBuOH, the acidic solution was neutralized with 28% aqueous NaOH solution.

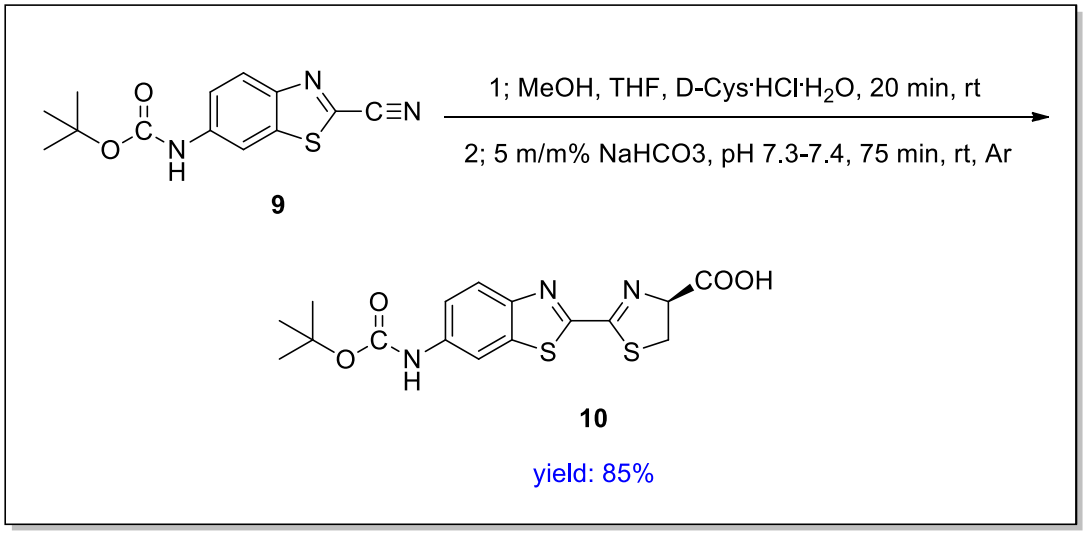


3 **9**

Scheme 8 Synthesis of *N*-Boc-6-amino-2-cyanobenzothiazole (**9**)

*3.2.3.2 N-Boc-aLuc (**10**) synthesis*

N-Boc-6-amino-2-cyanobenzothiazole (**9**) was dissolved in a mixture of MeOH and THF, then aqueous solution of D-cysteine hydrochloride monohydrate was added. In order to promote the release of the cysteine from its salt, and also to prevent racemization, the pH of the solution was kept between 7.3-7.4 by the addition of $NaHCO_3$ aqueous solution (Scheme 9).



9 **10**

Scheme 9 Synthesis of *N*-Boc-aLuc (**10**)

3.2.4 Biological testing

*3.2.4.1 Biochemical and cellular testing of N-Z-Asp-Glu-Val-Asp-aLuc (**6**)*

The biological relevance of our *N*-Z-Asp-Glu-Val-Asp-aLuc (**6**) substrate was confirmed in a biochemical reaction using a serial dilution of recombinant caspase-3. We also verified the applicability of our *N*-Z-Asp-Glu-Val-Asp-aLuc (**6**) substrate to detect cellular apoptotic cell death caused by a drug candidate molecule, a curcumin analogue C150 in A549 non-small cell lung carcinoma cells.

*3.2.4.2 In vivo testing of N-Z-Asp-Glu-Val-Asp-aLuc (**6**)*

We were able to measure apoptosis directly in animals with optical imaging performed *in vivo*, administrating *N*-Z-Asp-Glu-Val-Asp-aLuc (**6**) to SCID mice, previously inoculated with the stably expressing luciferase cell line U87-Luc that had been treated with apoptosis inducing agent, Ac-915. The drug enhanced the bioluminescent signal already at 6 hours, while significantly fewer signals were detected from control mouse having no treatment representing the basal level of apoptosis.

*3.2.4.3 Biochemical testing of N-Fmoc-Gly-Pro-aLuc (**8**)*

The substrate specificity of *N*-Fmoc-Gly-Pro-aLuc (**8**) was measured with two human proteases that are involved in cancer, POP/PREP 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. Enzymatic degradation was confirmed with a protease inhibitor, which completely abolished bioluminescent signal.

3.3. Summary

Efficient routes have been developed for the scalable synthesis of *N*-peptide-aLuc conjugates. Two routes are optimized versions of already published methods, while our third finding, an all-purpose building block, opens up the possibility to synthesize theoretically any kind of peptide-aLuc conjugate.

PUBLICATIONS

Papers related to the thesis

[1] **Anita K. Kovács**, Péter Hegyes, Gábor J. Szebeni, Lajos I. Nagy, László G. Puskás, Gábor K. Tóth:

Synthesis of *N*-peptide-6-amino-D-luciferin conjugates

Front. Chem. **2018**, *6* (120), 1-11., doi: 10.3389/fchem.2018.00120 **IF: 4.155**

[2] **Anita K. Kovács**, Péter Hegyes, Gábor J. Szebeni, Krisztián Bogár, László G. Puskás, Gábor K. Tóth:

Synthesis of *N*-peptide-6-amino-D-luciferin conjugates with optimized fragment condensation strategy

Int J Pept Res Ther **2018**, (ahead of print), doi: 10.1007/s10989-018-9768-8 **IF: 1.132**

Other publications

[3] Anikó Angyal, András Demjén, Edit Wéber, **Anita K. Kovács**, János Wölfling, László G. Puskás, Iván Kanizsai

Lewis acid-catalyzed diastereoselective synthesis of multisubstituted *N*-acylaziridine-2-carboxamides from 2H-azirines via Joullié-Ugi three-component reaction

J. Org. Chem. **2018**, *83* (7), 3570–3581., doi: 10.1021/acs.joc.7b03189 **IF: 4.805**

[4] Gyula Telegdy, **Anita K. Kovács**, Kinga Rákosi, Márta Zarándi, Gábor K. Tóth

Antiamnesic properties of analogs and mimetics of the tripeptide human urocortin 3

Amino Acids **2016**, *48* (9), 2261-2266., doi: 10.1007/s00726-016-2268-2 **IF: 3.173**

Selected scientific lectures

Anita K. Kovács, Péter Hegyes, Gábor J. Szebeni, László G. Puskás, Gábor K. Tóth

Synthesis of peptide-6-amino-D-luciferin conjugates

18th International Symposium on Bioluminescence and Chemiluminescence

Uppsala, Sweden, June 23-28, 2014, oral presentation

Anita K. Kovács, Péter Hegyes, Gábor J. Szebeni, László G. Puskás, Gábor K. Tóth

Boc strategy for the synthesis of peptide-6-amino-D-luciferin conjugates

19th International Symposium on Bioluminescence and Chemiluminescence

Tsukuba, Japan, 29 May – 02 June, 2016, oral presentation

Anita K. Kovács, Péter Hegyes, Gábor J. Szebeni, László G. Puskás, Gábor K. Tóth

Synthesis methods of peptide-6-amino-D-luciferin conjugates for protease activity detection

8th Conference Chemistry towards Biology

Brno, Czech Republic, August 28 – September 01, 2016, oral presentation

Anita K. Kovács, Péter Hegyes, Gábor J. Szebeni, László G. Puskás, Gábor K. Tóth

Comparison of Fmoc-, Boc- and fragment condensation strategies in the synthesis of peptide-6-amino-D-luciferin conjugates

34th European Peptide Symposium

Leipzig, Germany, September 04-09, 2016, poster presentation

Anita K. Kovács, Péter Hegyes, Gábor J. Szebeni, László G. Puskás, Gábor K. Tóth

Comparison of Fmoc-, Boc- and fragment condensation strategies in the synthesis of peptide-6-amino-D-luciferin conjugates

12th Australian Peptide Conference

Noosa Heads, Victoria, Australia, October 15-20, 2016, poster presentation