Tetrahedron Letters 53 (2012) 535–538

Contents lists available at SciVerse ScienceDirect

Tetrahedron Letters

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

Development of a solid-supported biotinylation reagent for efficient biotin labeling of SH groups on small molecules

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ARTICLE INFO

Article history:
Received 31 October 2011
Revised 15 November 2011
Accepted 16 November 2011
Available online 23 November 2011

Keywords:
Solid support
Biotinylation reagent
Npys
Protein modification
SH-specific labeling

ABSTRACT

We report here the design and synthesis of a novel and selective SH-group biotinylating reagent, KSH-1 (**1**), for the biotinylation of small molecules using solid phase chemistry. The results demonstrate that **1** efficiently biotinylated a small molecule, captopril, and afforded the product in high yield and purity.

Introduction

In life sciences research, the biotinylation of bioactive compounds can assist in analyzing the compound's biological properties.[1] This labeling approach is broadly used to label peptides,[1,2] proteins,[3–5] nucleic acids,[6] and antibodies.[7] The affinity and specificity of the binding between avidin and biotin (vitamin H) is extraordinary, and the binding interaction is among the strongest known non-covalent protein–ligand interactions ($K_d = 10^{-15}$ M).[8] This interaction is exploited in a variety of labeling methods, including purification, detection, and immobilization of biomolecules, as well as in viral vector targeting and drug targeting systems.[7] Biotinylation is unlikely to perturb the natural function of a biomolecule because the molecular structure of biotin is small (MW 244).[1] In most cases, biotinylation can be applied rapidly and specifically to particular functional groups of a target molecule using specific reagents.[9] Excess concentrations of the biotinylation reagent are usually used to increase the reaction efficacy. After labeling, the unreacted reagent or by-products produced during the reaction can be removed using purification techniques, usually gel filtration.

The biotinylation of small molecules is more complex, and advanced purification methods, such as reverse-phase HPLC, are required to separate the target compound from the biotin, which can have similar molecular weights. To avoid such laborious purification steps, biotinylation using solid phase chemistry is an attractive method because the unreacted materials or residual product remaining on the solid-support can be easily removed by filtration. The present study describes the design and synthesis of a novel selective SH biotinylation reagent (Fig. 1), for use in biotinylating small molecules using solid phase chemistry techniques. We achieved the efficient biotinylation of an example small molecule, captopril,[10] with excellent product purity.

Results and discussion

Design of the new SH-selective solid-supported biotinylation reagent

As shown in Figure 1, the newly designed solid-supported biotinylation reagent comprised three parts, namely, a solid support, an active disulfide, and a biotin unit. The following three

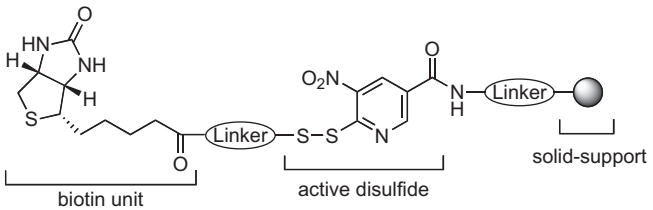

Figure 1. Design of the SH-selective solid-supported biotinylation reagent.

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doi:10.1016/j.tetlet.2011.11.089

principles guided this design. (1) A hydrophilic resin with a polyethyleneglycol (PEG) linker (amino-PEG resin) was chosen as a solid support to realize the biotinylation reaction in either aqueous or organic solution. (2) An Npys[11] (3-nitro-2-pyridinesulfenyl)-type disulfide structure was adopted as a key functional group to realize SH-selective labeling, because Npys was not only useful as a protective group for the SH during the peptide synthesis, but also acted as an attractive active disulfide to enable the specific formation of a disulfide bond with free SH groups under aqueous conditions. (3) The biotin unit was conjugated to a linker that distanced the biotin from the modification site in the target molecule, which reacted with the Npys moiety to form an active disulfide bond. In the present Letter, an aminoethyl chain was used as an example linker. Note that the length of the linker can be altered for a particular purpose, as needed.

Synthesis of KSH-1

KSH-1 (**1**) was synthesized in 10 steps from the commercially available 6-hydroxynicotinic acid[12] **2**, as shown in Scheme 1. Namely, 5-nitro-6-hydroxynicotinic acid **3** was prepared by the nitration of **2** with fuming nitric acid, although the yield was not excellent due to the high water-solubility of the product. The acid **3** was treated with phosphorus pentachloride in phosphorus oxychloride under reflux conditions. After the removal of excess amounts of the phosphorus compound, MeOH was added to give methyl 5-nitro-6-chloronicotinate **4** in moderate yields. This ester was refluxed with benzylmercaptan in the presence of Et_3N, followed by hydrolysis of the ester moiety with LiOH to give 6-benzylsulfanyl-5-nitronicotinic acid **6** in good yield. The acid **6** was then coupled to the linker, H-β-Ala-O^{t}Bu·HCl, in the presence of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride n-hydrate (DMT-MM)[13,14] to obtain the corresponding amide **7**. The conversion of amide **7** to the pyridinesulfenylchloride derivative **8** was achieved using a method reported by Ueki et al., involving chlorination with SO_2Cl_2.[15] The resultant crude chlorosulfenyl derivative was directly coupled with *N*-Boc-aminoethyl *p*-methoxybenzyl sulfide (Boc-NH-C_2H_4-S-PMB) to give the active disulfide **9** in moderate yield. The chemical structure of **9**, which was re-crystallized from chloroform, was confirmed by X-ray crystallography, as shown in Figure 2.[16] In the final steps, the Boc group and *t*-Bu ester of **9** were removed by TFA and subsequently coupled with biotinyl-OSu to give the biotin conjugate **10** in good yield. This product was then attached to the amino-PEG-resin (0.42 mmol/g)[17,18] using the DIPCI-HOBt method[19] to afford the desired solid-supported biotin derivative **1**. Small amounts of the unreacted amino groups on the solid support were capped by acetylation with Ac_2O in the presence of pyridine.

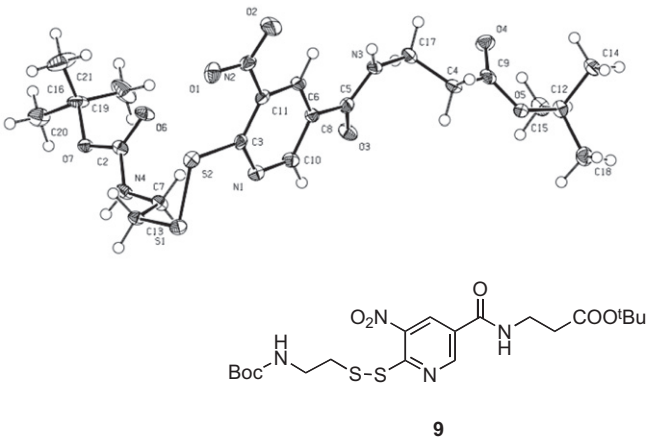
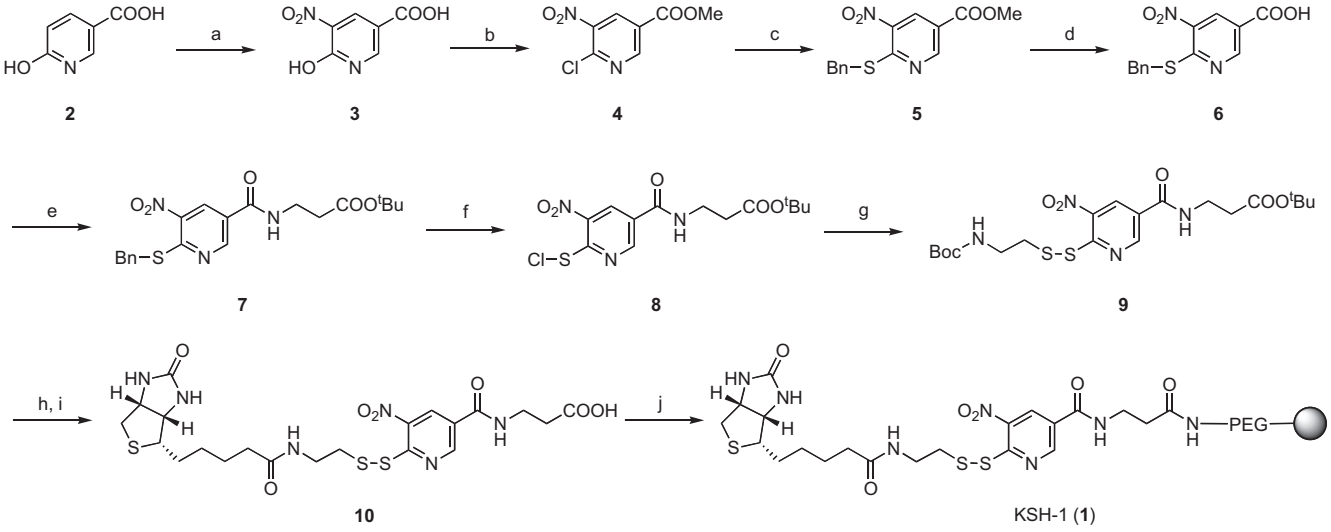


Figure 2. X-ray analysis of intermediate **9**.

Activity of KSH-1 (1) as an SH-selective biotinylation reagent

The ability of KSH-1 (**1**) as a biotinylation reagent was tested using captopril (**11**),[20] an angiotensin converting enzyme (ACE) inhibitor, as a model of SH-containing small molecule (Fig. 3). To determine the reaction conditions for biotinylation with **1**, a solution of **11** in DMF/H_2O (1:1, corresponding to a concentration of 0.011 mol/L for **1**) was mixed with different amounts of **1** (1–4 equiv)[21] in a filtration tube at room temperature. Captopril (**11**) labeling in the reaction mixture was analyzed by HPLC. As shown in Figure 4, the HPLC content of **11** decreased according to the amount of **1**, and the reactant was completely consumed in the presence of 4 equiv of **1**. Therefore, this amount was used to evaluate the isolated yield of biotin labeled captopril (**12**) in a subsequent study. Namely, **11** and **1** (4 equiv)[21] were reacted within



Scheme 1. Reagents and conditions: (a) HNO_3, fuming (1.52), 50 °C, 18 h, 29%; (b) PCl_5, $POCl_3$, 100 °C, 3 h then methanol, 0 °C, 1 h, 57%; (c) benzylmercaptan, Et_3N, MeOH, reflux, 5 h, 92%; (d) LiOH·H_2O, MeOH/H_2O, 0 °C to rt 15 h, quant.; (e) H-β-Ala-O^{t}Bu·HCl, Et_3N, DMT-MM, CH_2Cl_2, rt, 18 h, 85%; (f) SO_2Cl_2, 1,2-dichloroethane, rt, 3 h; (g) Boc–NH–C_2H_4–S–PMB, CH_2Cl_2, −30 °C, 12 h, 83% (two steps); (h) TFA, rt, 30 min; (i) Biotinyl-OSu, Et_3N, DMF, rt, 17 h, 87% (two steps); (j) DIPCI, HOBt·H_2O, amino-PEG-resin (0.42 mmol/g), DMF, rt, 16 h, then Ac_2O, pyridine, DMF, rt, 0.5 h.

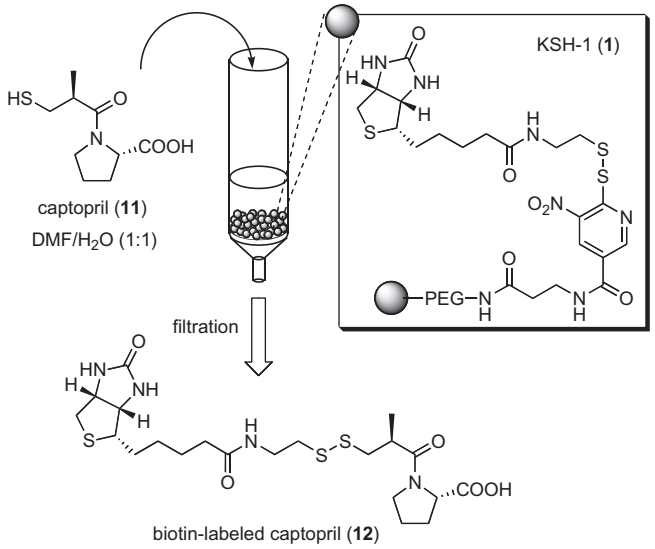


Figure 3. Biotinylation of captopril (**11**) using KSH-1 (**1**).

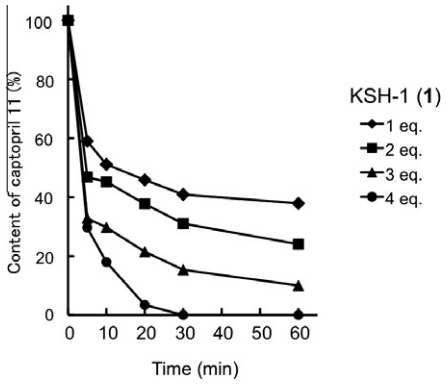


Figure 4. Time course of captopril biotinylation in the presence of **1** (1–4 equiv). An aliquot of the reaction mixture (5 μL) at each time point during the reaction was injected into an HPLC, and the captopril **11** content was calculated according to the corresponding peak areas and positions.

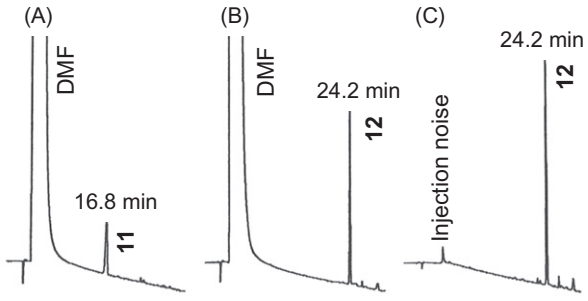


Figure 5. HPLC charts of captopril biotinylation with **1**. Charts of (A) captopril in DMF/H_2O (1:1); (B) the reaction mixture after 30 min; and (C) the final compound after removal of the solvent, resolved in 15% aqueous MeOH. HPLC conditions: YMC-pack ODS-AM (4.6 × 150 mm) with a linear gradient of 0.1% TFA-MeCN (100:0–60:40 over 30 min) at a flow rate of 0.9 mL min^{-1}, detection at 230 nm. A peak with a retention time of 24.2 min in (B and C) corresponded to the biotin-labeled captopril **12**.

DMF/H_2O (1:1, 7 mL, corresponding to a concentration of 0.021 mol/L of **1**) for 30 min. As shown in Figure 5B, the peak for captopril (**11**, rt = 16.8 min, Fig. 5A) completely disappeared, and the biotin-labeled captopril **12** appeared as a new major peak at a retention time of 24.2 min. The reaction mixture was filtered to remove the solid support, and the solvent was removed under reduced pressure. The resultant product was analyzed using HPLC (Fig. 5C). This result demonstrated that the desired product **12** was obtained in a 96% yield and the purity was 87%.

In summary, we report here the design and synthesis of a novel and selective SH biotinylation reagent, KSH-1 (**1**), for the purpose of biotinylating small molecules using solid phase chemistry. This reagent expeditiously biotinylated the small molecule captopril, yielding the product in good purity. This solid phase biotinylation strategy would be applicable to peptides and proteins as well. Furthermore, other tag-based molecular labeling approaches that rely on the preparation of a key Npys-type active disulfide intermediate using solid support approaches are in progress in our laboratory and will be reported in the near future.

General procedure

3-[(6-Chlorosulfanyl-5-nitropyridine-3-carbonyl)amino]propionic acid *tert*-butyl ester (8)

To a mixture of **7** (664 mg, 1.59 mmol) and pyridine (20 μL) in 1,2-dichloroethane (5 mL) was added SO_2Cl_2 (283 μL, 3.50 mmol). After stirring for 3 h, excess amount of reagent and solvent were removed in vacuo. Then, co-evaporated with hexane repeatedly three times, to obtain a yellow color precipitate **8**. This compound was used for the next step without any further purification.

3-{[6-(2-*tert*-Butoxycarbonylaminoethyldisulfanyl)-5-nitro-pyridine-3-carbonyl]amino}propionic acid *tert*-butyl ester (9)

Boc-NH-C_2H_4-S-PMB (synthesized from Boc-NH-C_2H_4-SH and *p*-methoxybenzylchloride, 473 mg, 1.59 mmol) was dissolved in dry CH_2Cl_2 (10 mL) under an Ar atmosphere, followed by cooling to −30 °C in an acetone bath. To this solution was added **8** in dry CH_2Cl_2 (52 mL) dropwise over 30 min. The solution was stirred for 12 h under the same conditions then warmed to room temperature. The solution was sequentially washed with 10% aqueous citric acid, water, and brine. The organic layer was dried over anhydrous Na_2SO_4, followed by concentration in vacuo. The resultant residue was purified by column chromatography over silica gel using hexane/AcOEt (3:1), and elution gave **9** as a yellow oil (660 mg, 83% (2 steps)), which was recrystallized in hexane/$CHCl_3$ as a yellow needle; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.46 (s, 9H), 1.47 (s, 9H), 2.59 (t, J = 5.9 Hz, 2H), 3.01 (t, J = 5.7 Hz, 2H), 3.40 (br m, 2H), 3.73 (dt, J = 5.8, 12 Hz, 2H), 5.65 (br s, 1H), 8.87 (br s, 1H), 9.23 (br s, 1H); HRMS (ES+): m/z 503.1628 ($M+H^+$) (calcd for $C_{20}H_{31}N_4O_7S_2$: 503.1634).

3-[(5-Nitro-6-{2-[5-(2-oxo-hexahydrothieno[3,4-*d*]imidazol-6-yl)pentanoylamino]ethyldisulfanyl}pyridine-3-carbonyl)amino]propionic acid (10)

Compound **9** (516 mg, 1.03 mmol) was dissolved in TFA (10 mL) and stirred for 30 min at room temperature. The solution was concentrated and co-evaporated with hexane three times. A yellow precipitate was obtained. The precipitate was dissolved in dry DMF (25 mL), to which was added Et_3N (660 μL, 5.14 mmol). The solution color changed from yellow to brown, and the biotin-succinimidyl ester (292 mg, 0.856 mmol) was added. After stirring for 17 h, the solvent was removed in vacuo, and AcOEt (20 mL) was added. The obtained yellow precipitate was filtered and washed with AcOEt (10 mL) three times, followed by washing with Et_2O (10 mL) three times. The obtained compound was dried under reduced pressure to give **10** as a yellow powder (425 mg, 87%). ^{1}H NMR spectrum: (400 MHz, CD_3OD) δ 1.28–1.85 (m, 6H), 2.22 (t, J = 7.2 Hz, 2H), 2.58–2.77 (m, 4H), 2.80–3.10 (m, 2H), 3.15–3.27

(m, 1H), 3.40–3.59 (m, 2H), 3.67 (t, J = 6.8 Hz, 2H), 4.26–4.35 (m, 1H), 4.46–4.54 (m, 1H), 8.96 (d, J = 2.0 Hz, 1H), 9.23 (d, J = 2.0 Hz, 2H); HRMS (ES+): m/z 573.1292 ($M+H^+$) (calcd for $C_{21}H_{29}N_6O_7S_3$: 573.1260).

KSH-1 (1)

To a solution of **10** (250 mg, 0.436 mmol) in DMF (4 mL) was added, sequentially, DIPCI (67.5 μL, 0.436 mmol), H_2N-PEG-resin (346 mg, 0.145 mmol), and HOBt·H_2O (73.4 mg, 0.480 mmol). After stirring for 16 h, the solution was filtered and washed with DMF five times. The resin was processed using the Kaiser test, and the reaction was assumed to have reached completion. To cap the small quantities of unreacted $-NH_2$ groups on the resin, Ac_2O (275 μL, 2.91 mmol) and pyridine (236 μL, 2.91 mmol) were added. After 30 min, the solution was filtered. The resin was then sequentially washed with MeOH (2 mL, six times) and Et_2O (2 mL, four times). The resin was dried in vacuo to give KSH-1 (**1**) (410 mg, 0.145 mmol, the total molar quantity of KSH-1 was preserved during the process).

General procedure used for the biotinylation of captopril

KSH-1 (**1**) (410 mg, 0.145 mmol, 4 equiv from captopril) was poured into a filtration tube. A solution of captopril (**11**) (7.89 mg, 36.3 μmol) in DMF/water = 1/1 (7 mL) was added and the solution was stirred for 30 min. The reaction mixture was analyzed by reverse-phase HPLC. After the disappearance of the captopril peak, solutions were separated from the resin by filtration. The resin was washed away using DMF, and the collected filtrate was concentrated in vacuo. The resultant residue was co-evaporated with water and ethanol after drying under reduced pressure to give the biotin-labeled captopril **12** (18.0 mg, 96%) as a colorless solid; (**12** was dissolved in 15% aqueous MeOH and analyzed by reverse-phase HPLC. The purity of **12** was 87%.). ^{1}H NMR (400 MHz, CD_3OD) δ 1.21 (d, J = 6.8 Hz, 2H), 1.28–1.81 (m, 6H), 1.98–2.14 (m, 3H), 2.16–2.41 (m, 3H), 2.66–2.76 (m, 2H), 2.76–2.89 (m, 1H), 2.89–3.06 (m, 2H), 3.08–3.25 (m, 3H), 3.38–3.54 (m, 2H), 3.60–3.80 (m, 2H), 4.26–4.35 (m, 1H), 4.37–4.46 (m, 1H), 4.46–4.54 (m, 1H); HRMS (ES+): m/z 519.1745 ($M+H^+$) (calcd for $C_{21}H_{35}N_4O_5S_3$: 519.1770).

Acknowledgments

This research was supported by the Adaptable and Seamless Technology Transfer Program (A-STEP) through target-driven R&D, Exploratory Research (AS231Z04408F) from the Japan Science and Technology Agency, and by a Grant-in-Aid for Scientific Research (B) (No. 20390036) from MEXT (Ministry of Education, Culture, Sports, Science and Technology) of Japan.

Supplementary data

Supplementary data (the preparation and chemical data for compounds **3**–**7**, including ^{1}H NMR and HRMS) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2011.11.089.

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16. *Crystal structure data for* **9**: Crystallographic data (excluding the structure factors) for the structure of **9** have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 846957. Copies of the data may be obtained, free of charge, upon application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [Fax: (internet) +44 1223-336003 or e-mail: deposit@ccdc.cam.ac.uk].
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18. The resin used was purchased from Watanabe Chemical Industries (polyethyleneglycol spacer on a polystyrene bead (110 mm, 0.42 mmol/g)—NH_2-PEG-resin TG HL-NH_2—cat. # A00303).
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20. Captopril was purchased from Wako Pure Chemical Industries and purified before use. The reagent was purified by preparative HPLC (with a linear gradient of 0–40% CH_3CN in 0.1% aqueous TFA over 40 min). The collected fractions were lyophilized, following recrystallization from hexane/AcOEt to give a colorless solid.
21. The amount of biotin on the resin was calculated based on the original NH_2–loading content.