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A pre-biotinylated linker assembly for single-step preparation of novel biosensors

Brent A. Banasik*

Department of Chemistry and Biochemistry, San Diego State University, San Diego, CA 92182-1030, USA

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Abstract: A simple and efficient method for the synthesis of biotin pre-functionalized linkers via orthogonal protecting group elongation strategy is reported for a single-step preparation of new biosensor probes. The target compound *4- (4- (4- (5- ((3S, 4S, 6R)-2-oxohexahydro-1H-thieno[3,4]imidazol-4-yl) pentanamido) butanamido) butanamido) butanoic acid* (**5**) was synthesized in five linear steps with a 40% overall yield.

Keywords: biosensor, linker, biotin, lagunamide. © 2017 ACG Publications. All rights reserved.

1. Introduction

Naturally occurring bioactive molecules are important tools for development of drug-like lead compounds. For example, lagunamide A[1] is a recently discovered potential anti-cancer and anti-malarial therapeutic that has spurred synthetic studies.[2-5] However, the protein binding site remains unknown. Natural products have the distinct advantage of co-evolution with protein targets, usually correlated to extremely high selectivity. Determination of the molecular target(s) of novel bioactive compounds, such as lagunamide A (see Figure 1), would help to determine the mechanism of action and provide a looking glass into the convoluted cellular machinery of inhibition.

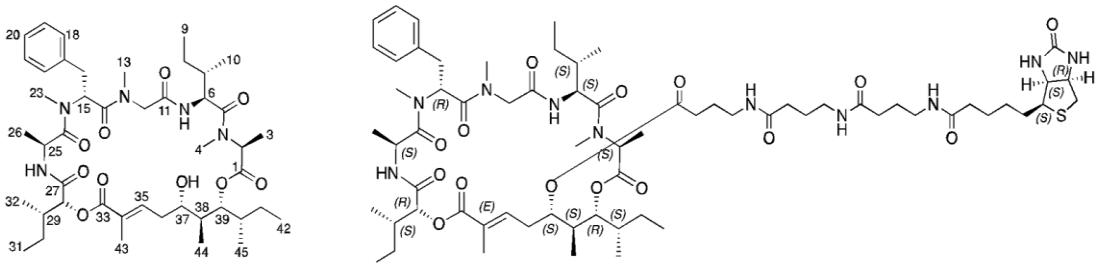

Figure 1. Structure of lagunamide A and proposed biosensor

Biotinylation is a modern technique for determination of protein targets.[6] Biotin has high specific affinity for streptavidin and/or avidin. This tight binding affinity has been exploited for purification (usually affinity chromatography) or detection (usually enzyme reporters such as horseradish peroxidase or fluorescent probes).[7] Convenient methods such as ELISA, electron

* Corresponding author: E-mail: brentbanasik@gmail.com; Tel: (+) 1(509)9395375; Fax: (+) 2063068838

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microscopy and western blots help localize these derivatives. Covalent bonding with biotin tags are unlikely to perturb the function of a molecule and lengthy linkers can probe deep into a number of enzymatic pockets in a proven method for the isolation of natural product receptors (see **Figure 1**).[8]

2. Experimental

IR spectra were recorded on Perkin Elmer FT-IR spectrum RXI. ^{1}H NMR spectra were recorded on a Varian 400, 500, or 600-MHz instrument using $CDCl_3$ or DMSO-d6 with TMS as internal standard (δ = 0 ppm). $CDCl_3$ (δ = 77.00 ppm) or DMSO-d6 (δ = 39.52 ppm) was used as internal references for ^{13}C (100, 126 and 151 MHz) NMR. Preparative HPLC was carried out using Shimadzu SCL-10A/SPD-10A instrument with preparative Varian pursuit 10 C8 50-G 50mm column. Mass spectra were recorded using APCI Expressions instrument. Elemental analysis was conducted by Intertek Pharmaceutical Services (USA) on a Perkin-Elmer 2400 Elemental Analyzer with NIST traceable organic standard. Analytical thin-layer chromatography was performed on Silicycle glass backed 60Å ultra pure silica gel. Flash chromatography was conducted using a Biotage Isolera one instrument with pre-packed silica gel columns (AnaLogix, Sepra Si 50) or self-packed Luknova and Biotage snap columns filled with silica gel (Sorbent Technologies, 60Å, 230-400 mesh). All reactions were conducted under an argon atmosphere and in septum-capped oven-dried glassware unless otherwise specified. All solvents and reagents were purchased from Aldrich, Fisher Scientific, Combi-blocks, TCI America, Chem Impex or Oakwood Scientific.

*2.1. Benzyl 4-((tert-butoxycarbonyl) amino) butanoate (**1**):* To an ice-bath cooled solution of benzyl alcohol (100 µL, 0.9617 mmol) and Boc-GABA-OH (0.391 g, 1.923 mmol) in 10.0 mL freshly distilled DCM was added DCC (0.496 g, 2.404 mmol) and DMAP (0.294 g, 2.404 mmol) in one lot under argon atmosphere and the mixture was stirred for 14 hours. Completion of the reaction was confirmed by TLC and it was quenched with 15 ml sat. NH_4Cl, extracted 4 x 15 mL DCM and the combined organics were dried over $MgSO_4$. The mixture was filtered and concentrated to produce crude solid that was extracted with 10% EtOAc/hexanes (Boc-GABA-OH and other impurities were generally insoluble) and purified via silica gel column chromatography (0-25% EtOAc in hexanes gradient) to afford the title compound, monomer **1** (0.273 g, 97% yield) as white crystals; R_f = 0.5, 25% EtOAc in hexanes; ^{1}H NMR (400 MHz, Chloroform-d) δ 7.37 – 7.23 (m, Ar-H x 5, 5H), 5.09 (s, CH_2Ph, 2H), 4.79 (s, NH, 1H), 3.13 (q, J = 6.6 Hz, NCH_2, 2H), 2.37 (t, J = 7.4 Hz, RCH_2R, 2H), 1.80 (p, J = 7.1 Hz, RCH_2R, 2H), 1.41 (s, OtBu, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.93, 155.92, 135.83, 128.42, 128.09, 128.05, 78.88, 66.13, 39.73, 31.39, 28.30, 25.17; FTIR (neat, cm^{-1}) 3320, 2933, 1731, 1708, 1683, 1540, 1365, 1163, 995, 851, 754, 699, 675. This spectral data is identical to the previously reported literature compound.[17]

*2.2. Benzyl 4- (4- ((tert- butoxycarbonyl) amino) butanamido) butanoate (**2**):* To a solution of carbamate **1** (0.500 g, 1.704 mmol) dissolved in 6.0 mL freshly distilled DCM was added 3 mL TFA (34.04 mmol) and the mixture was stirred for 80 minutes at ambient temperature under argon flow. Consumption of the starting material was confirmed via TLC. The reaction mixture was concentrated under reduced pressure and dried for 2 hrs on HI-VAC to afford a crude (corresponding TFA salt) residue that was re-dissolved in 8 mL CH_2Cl_2 and charged with Boc-GABA-OH (0.693 g, 3.408 mmol), HATU (1.620 g, 4.260 mmol) and DIPEA (1.48 mL, 8.520 mmol) successively under argon at ambient temperature and stirred sealed for 18 h. The reaction mixture was stirred for 3 hours then charged with HOAt (1.5 equiv), resulting in similar yields. The reaction was ultimately quenched with 20 mL cold DI H_2O and extracted with 4 x 15 mL DCM. The combined organics were then washed with 20 mL sat. $NaHCO_3$, 20 mL sat. NH_4Cl, 20 mL brine and dried over Na_2SO4. The resultant combination was filtered and concentrated under reduced pressure then purified via silica gel column chromatography (50-100% EtOAc in hexanes gradient) to afford the title compound, dimer **2** (0.503 g, 78% yield) as a pale yellow solid; R_f = 0.4, 100% EtOAc; ^{1}H NMR (400 MHz, Chloroform-d) δ 7.43 – 7.28 (m, Ar-H x 5, 5H), 6.37 (s, NH, 1H), 5.12 (s, CH_2Ph, 2H), 4.79 (s, NH, 1H), 3.29 (td, J = 6.9, 5.7 Hz, RCH_2R, 2H), 3.14 (q, J = 6.1 Hz, RCH_2R, 2H), 2.43 (t, J = 7.3 Hz, RCH_2R, 2H), 2.18 (t, J = 7.0 Hz, RCH_2R, 2H), 1.86 (p, J = 7.1 Hz, RCH_2R, 2H), 1.81 – 1.71 (m, RCH_2R, 2H), 1.43 (s, OtBu, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.24, 172.82, 156.61, 135.84, 128.59, 128.29, 128.22, 79.47, 66.41,

39.66, 38.97, 33.49, 31.76, 28.39, 26.41, 24.60; FTIR (neat, cm^{-1}) 3422, 2984, 2942, 1709, 1653, 1540, 1366, 1253, 1229, 1167, 847; CHN calc. (%): C (63.47), H (7.99), N (7.40); found: (%): C (65.17), H (7.69), N (7.32).

*2.3. Benzyl 2, 2- dimethyl- 4, 9, 14-trioxo-3-oxa- 5, 10, 15-triazanonadecan-19-oate (**3**):* To a solution of carbamate **2** (0.350 g, 0.925 mmol) dissolved in 8.0 mL freshly distilled DCM was added 3 mL TFA (34.04 mmol) and stirred for 80 minutes at ambient temperature under argon flow. Consumption of starting material was confirmed via TLC. The reaction mixture was concentrated under reduced pressure and dried for 2 hrs on HI-VAC to afford a crude (corresponding TFA salt) residue that was re-dissolved in 10 mL CH_2Cl_2 and charged with Boc-GABA-OH (0.235 g, 1.156 mmol), HATU (0.528 g, 1.388 mmol) and DIPEA (0.645 mL, 3.700 mmol) successively under argon at ambient temperature and stirred for 1 h. The reaction mixture was then charged with HOAt (0.130 g, 0.462 mmol), sealed and stirred 18 h. The reaction was quenched with 20 mL cold DI H_2O and extracted with 4 x 15 mL DCM. The combined organics were then washed with 20 mL sat. $NaHCO_3$, 20 mL sat. NH_4Cl, 20 mL brine and dried over Na_2SO4. The resultant combination was filtered and concentrated under reduced pressure then purified via silica gel column chromatography (solvent mixture was 60:15:15:10 of EtOAc:ACN:MeOH:H_2O isocratic gradient) to afford the title compound, trimer **3** (0.255 g, 66% yield) as pale yellow solid; R_f = 0.6, 100% EtOAc; ^{1}H NMR (500 MHz, Methanol-d4) δ 7.98 (dt, J = 10.3, 5.7 Hz, NH, 1H), 7.33 (dd, J = 21.2, 4.4 Hz, Ar-H x 5, 5H), 5.11 (s, NH x 2, 2H), 4.83 (s, CH_2Ph, 2H), 3.25 – 3.15 (m, RCH_2R x 2, 4H), 3.07 (t, J = 6.9 Hz, RCH_2R, 2H), 2.41 (t, J = 7.4 Hz, RCH_2R, 2H), 2.21 (t, J = 7.5 Hz, RCH_2R x 2, 4H), 1.86 – 1.69 (m, RCH_2R x 3, 6H), 1.43 (s, OtBu, 9H); ^{13}C NMR (101 MHz, CD_3OD) δ 175.37, 175.35, 175.27, 174.36, 158.32, 137.50, 129.45, 129.10, 129.09, 79.79, 67.15, 40.72, 39.74, 39.55, 34.28, 34.26, 32.26, 28.77, 27.21, 26.66, 25.67; FTIR (neat, cm^{-1}) 3327, 2944, 2486, 1740, 1708, 1690, 1652, 1633, 1542, 1526, 1452, 1418, 1365, 1279, 1254, 1224, 1162, 1027, 1003, 852, 736, 693; CHN calc. (%): C (62.18), H (8.05), N (9.06); found: (%): C (61.94), H (7.89), N (9.17).

*2.4. Benzyl 4-(4-(4-(5- ((3aS, 4S, 6aR)-2- oxohexahydro- 1H- thieno [3,4-d]imidazol-4-yl) pentanamido) butanamido) butanamido) butanoate (**4**):* To a solution of carbamate trimer **3** (0.050 g, 0.1079 mmol) dissolved in 5.0 mL freshly distilled DCM was added 0.650 mL TFA and stirred for 90 minutes at ambient temperature under argon flow. Consumption of starting material was confirmed via TLC. The reaction mixture was concentrated under reduced pressure and dried for 2 hrs on HI-VAC to afford a crude (corresponding TFA salt) residue that was re-dissolved in 1.75 mL DMF and charged with D-Biotin (0.027 g, 0.1079 mmol), HOBt (0.020 g, 0.1295 mmol) and EDC-HCl (0.025 g, 0.1295 mmol) under argon atmosphere at ambient temperature. 0.50 mL Et_3N was added dropwise via cannula, and the reaction mixture was sealed and stirred for 18 hrs. To the resultant mixture was added 0.50 mL DCM:DMSO (1:1) that was then acidified to ~pH 1 with concentrated HCl. The mixture was homogenized with the addition of 350 μL DI H_2O. Without further workup, the reaction mixture was injected directly onto prep-HPLC (Varian C-8 30 x 250 mm, 5-95% ACN in H_2O spiked with 0.1% formic acid, 8.5 min retention time) and subsequently lyophilized to afford the title compound **4** (0.060 g, 80% yield) as a white powder; ^{1}H NMR (500 MHz, DMSO-d6) δ 7.81 (t, J = 5.7 Hz, NH, 1H), 7.77 (t, J = 5.4 Hz, NH x 2, 2H), 7.41 – 7.29 (m, Ar-H x 5, 5H), 6.41 (s, NH, 1H), 6.35 (s, NH, 1H), 5.09 (s, CH_2Ph, 2H), 3.09 (m, RCH_2R, 6H), 2.37 (t, J = 7.5 Hz, RCH_2R, 2H), 2.08 – 2.01 (m, RCH_2R, 6H), 1.66 (p, J = 7.1 Hz, RCH_2R, 2H), 1.59 (p, J = 7.2 Hz, RCH_2R, 4H), 1.55 – 1.40 (m, RCH_2R, 2H), 1.31 (dq, J = 15.8, 7.1 Hz, RCH_2R, 2H); ^{13}C NMR (101 MHz, DMSO) δ 172.50, 171.92, 171.72, 171.61, 162.68, 136.22, 128.40, 127.95, 127.88, 65.37, 61.02, 59.18, 55.38, 38.11, 37.73, 35.21, 32.91, 32.87, 30.93, 28.21, 28.01, 25.49, 25.28, 24.56; CHN calc. (%): C (59.06), H (7.35), N (11.88); found: (%): C (58.98), H (7.46), N (11.63).

*2.5. 4- (4- (4- (5- ((3aS, 4S, 6aR)-2-oxohexahydro-1H-thieno [3,4-d]imidazol-4-yl) pentanamido) butanamido) butanamido) butanoic acid (**5**):* To a solution of benzyl ester **4** (0.060 g, 0.102 mmol) dissolved in dry methanol (1 mL, 0.1M) was added 10% Pd/C under inert argon atmosphere. The resulting mixture was evacuated with H_2 (g) and then stirred under H_2 (g) (1 bar) atmosphere for 12 hrs. The mixture was filtered through a celite pad and concentrated under reduced pressure to produce the title compound **5** (95% yield, 85:15 conversion from starting material) ^{1}H NMR (500 MHz,

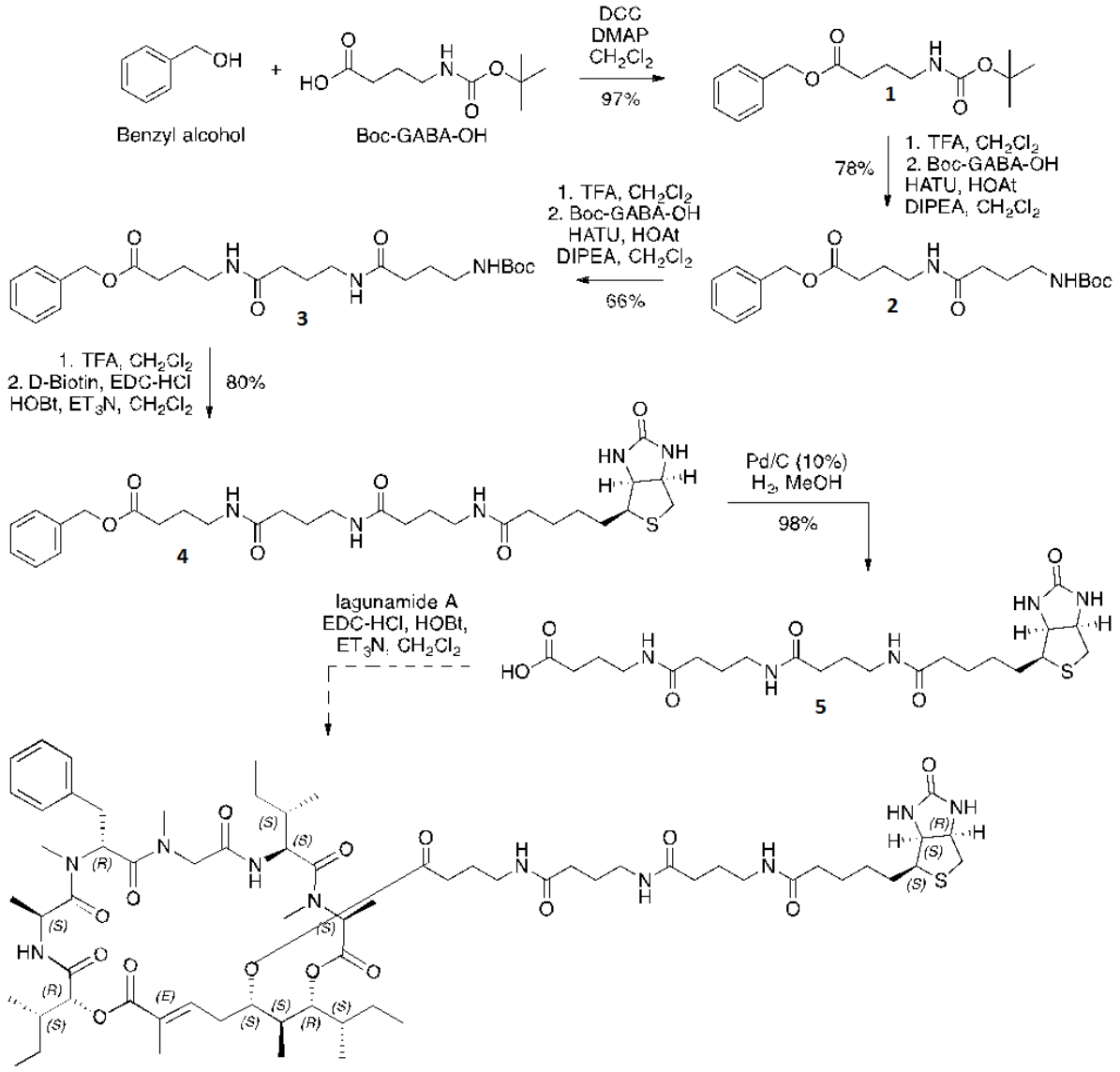
Methanol-d4) δ 4.49 (ddd, J = 7.9, 5.0, 0.9 Hz, 1H), 4.30 (dd, J = 7.9, 4.5 Hz, 1H), 3.19 (td, J = 7.0, 2.1 Hz, 8H), 2.92 (dd, J = 12.7, 4.9 Hz, 1H), 2.70 (d, J = 12.8 Hz, 1H), 2.20 (dtd, J = 11.2, 9.1, 8.2, 2.3 Hz, 9H), 1.78 (dddd, J = 15.2, 8.8, 5.3, 1.7 Hz, 6H), 1.69 – 1.54 (m, 4H), 1.48 – 1.40 (m, 2H); APCI-S *m/z* calcd for $C_{22}H_{36}N_5O_6S$ $[M-H]^-$ 498.6, found 498.6; CHN calc. (%): C (52.89), H (7.46), N (14.02); found: (%): C (53.04), H (7.64), N (14.20).

3. Results and discussion

Sato *et al*.[9] completed an important study to determine that Prohibitin 1 (PHB1) was the binding target protein of aurilide,[10] a potent cytotoxic marine natural product that resulted in mitochondrial-induced apoptosis in cultured human cells.[11] Aurilide is structurally similar and in the same macro-cyclic depsipeptide class of molecule as the more recently discovered lagunamide A. Furthermore, recent biochemical studies by Tripathi *et al*.[12] determined that the cytotoxic effect of lagunamide A might act via mitochondrial-mediated induction of apoptosis as well. Structure-activity relationships (SAR) for aurilide showed that modification of the C35-hydroxy group (comparable to that of the C37-hydroxyl group of lagunamide A) had little to no impact on biological activity.[13] It is proposed that an analogous approach to biotinylation of this hydroxyl moiety would be similarly advantageous. This strategy is applicable to a vast majority of bioactive compounds with similar hydroxyl-containing sights for biosensor derivatization.

The goal of this research was to pre-synthesize an elongated biotin linker that could then be efficiently coupled directly to a bioactive compound. Due to the laborious efforts it takes to isolate and/or synthesize such bioactive molecules, for example complex natural products such as lagunamide A, one would desire the biotin and linker moiety to be a single construct that is subsequently converged in one final step. Conversely, most biosensors are created via chemical derivitization of a bioactive molecule, usually followed by chemical manipulations that finally end in biotinylation. This research succeeded in the construction of a pre-biotinylated linker (**5**) that may be subsequently coupled in a single convergent step. The proposed method is more economical, straightforward, and is a valuable inclusion for the organic chemist's toolbox when compared to other commonplace strategies. Furthermore, a linker of roughly 10-20 covalent bond lengths that positioned the biotin handle well outside of the protein binding pocket is supported by the literature.[14]

In order to synthesize the entirety of the biotin linker prior to conjugation, an orthogonal protecting group strategy for the *C*- and *N*-terminus of the linker was required. Thus, Steglich[15] esterification of commercially available 4-(*tert*-Butoxycarbonylamino)butyric acid (Boc-GABA-OH) with benzyl alcohol mediated by DCC and DMAP in CH_2Cl_2 produced a completely protected monomer unit (**1**) in 97% yield. Repeating units of Boc-GABA-OH made for a step-wise and uniform elongation of the linker via peptide bond fragments with advantageous protecting group strategy (see Scheme 1). When compound **1** was exposed to TFA in CH_2Cl_2, the *N*-Boc protecting group was cleaved while the C-terminus benzyl ester remained protected. Evaporation of this mixture resulted in the crude TFA salt that was successively coupled with a second Boc-GABA-OH unit via HATU/HOAt and excess of DIPEA to produce dimer unit **2** in 78% yield over 2 steps. The identical TFA-mediated *N*-Boc removal and HATU-mediated Boc-GABA-OH coupling protocol was repeated to finalize the trimer fragment (**3**) comprised of the desired chain length in 66% yield over the previous two steps. A number of iterations could be continued to construct any desired chain length. Trimer **3** was *N*-Boc deprotected with TFA and evaporated to produce the crude TFA salt that was subsequently extended by D-Biotin through EDC-HCl mediated coupling to form the biotinylated trimer linker **4** scaffold in 80% yield. Inard *et al*.[16] demonstrated that sulfur containing biotin derivatives were inert towards palladium, and thus palladium-mediated hydrogenation of benzyl ester **4** liberated the free carboxylic acid of the biotinylated linker **5** in 84% yield. Biotinylated linker **5** was equipped for direct esterification with the appropriate bioactive molecule, such as lagunamide A.



Scheme 1. Synthesis of pre-biotinylated linker towards proposed biosensor

4. Conclusion

Since the entire biotin-linker moiety was synthesized prior to combination, valuable steps that preserve the compound of interest are spared in constructing the corresponding biosensor. This strategy is proposed to determine the binding protein for lagunmaide A and will be reported in due course. Conceptually, this combinatory biosensor strategy would be expedient for a number of hydroxyl or amine-comprised small molecule or natural product compounds. In summary, the iterative pathway presented in this article afforded the pre-biotinylated target linker (**5**) in five consecutive steps with a good overall yield of 39.9%.

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