**Targeting Cell Surface Alpha(v)beta(3,5) Integrins Increases Therapeutic Efficacies of a Legumain Protease-Activated Auristatin Prodrug**

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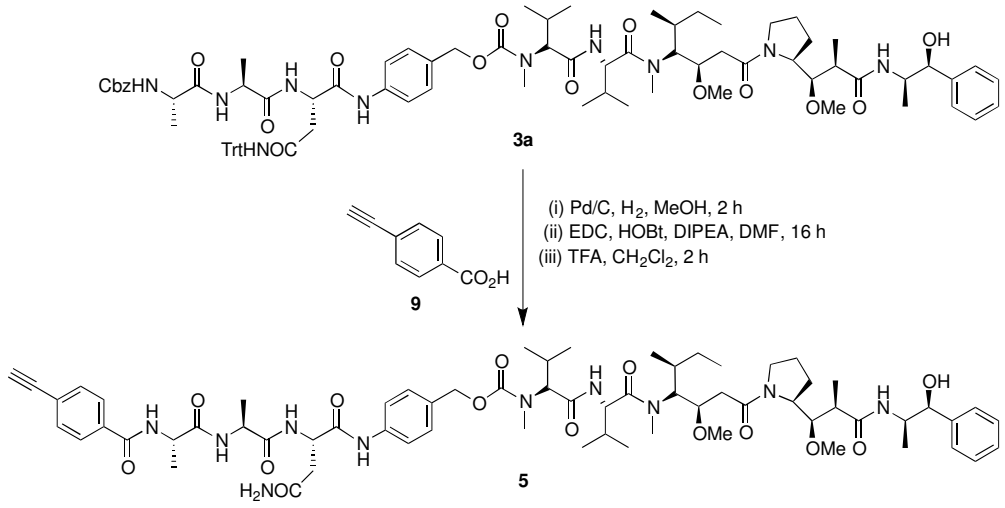
# Supporting Information

## 1. General Methods

All solvents and reagents were used as obtained from commercial sources unless otherwise indicated. All starting materials were also obtained from commercial sources. All reactions were performed under argon unless otherwise noted. In the course of aqueous work-ups, organic layers were washed with water, brine, dried over anhydrous $Na_2SO_4$ and evaporated at 40 °C under reduced pressure ("standard work-up"). $^1H$ and $^{13}C$ NMR spectra were recorded on Bruker and Varian instruments 300, 400, and 500 using deuterated chloroform (99.8%D) or DMSO-d6 (99.8%D) as solvents. $^1H$ Chemical shifts values (*d*) are reported in ppm downfield from tetramethylsilane as standard. Mass spectra were measured in positive mode electrospray ionization (ESI) on Agilent LC/MSD TOF instrument. TLC was performed on silica gel 60 $F_{254}$ glass plates; column chromatography was performed using silica gel (35-75 mesh). All final compounds sent for biological assay were further purified by HPLC. Analytical HPLC was performed using a Shimadzu LC-10AD system, equipped with a Waters 484 tunable absorbance detector set at 254, 280, 310 or 360 nm.

## 2. Synthesis of prodrug 8

### 2.1. Intermediate 5 (Scheme SI-1)




**Scheme SI-1.** Synthesis of the alkyne intermediate **5**.

To a solution of compound **3**[1] (250 mg, 0.17 mmol) in MeOH (3 mL) was added Pd/C (20 mg), and the mixture was hydrogenated under atmospheric pressure using an $H_2$-filled balloon for 1 h.

It was then filtered through a short pad of Celite, and the filter cake was washed with MeOH. The filtrate and the washings were combined and concentrated in *vacuo* to give free amine, which was taken to next reaction without further purification.

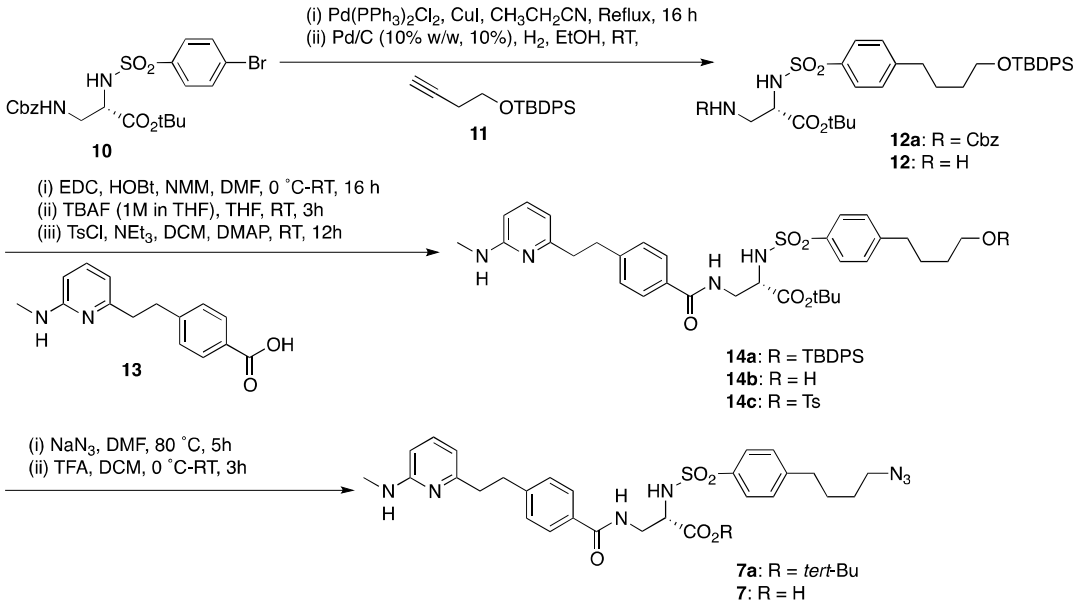
EDC (36 mg, 0.19 mmol), HOBt (26 mg, 0.19 mmol) and DIPEA (0.1 mL, 0.51 mmol) were added sequentially to a solution of 4-ethynylbenzoicacid **9**, (38 mg, 0.25 mmol) and the above-described free amine (227 mg, 0.17 mmol) in DMF (2 mL) at 0 ˚C, and the mixture was stirred at 0 ˚C to room temperature for 12 h. The reaction mixture was worked-up using aqueous $NH_4Cl$ solution and $CH_2Cl_2$. The combined organic extracts were washed with water and dried ($Na_2SO_4$), filtered and concentrated in *vacuo*. Purification of the crude material using column chromatography (silica gel, 6-7% MeOH in $CH_2Cl_2$) afforded the coupled product (162 mg, 65% yield). $R_f$ = 0.45 (silica gel, 10% MeOH in $CHCl_3$). $^1$H NMR ($CDCl_3$, 500 MHz): δ 8.74 (br s, 1H), 8.17 (s, 1H), 7.57-7.54 (m, 3H), 7.36-7.21 (m, 15H), 7.15-7.02 (m, 14H), 6.62 (bs, 1H), 6.54-6.51 (m, 1H), 5.21-5.14 (m, 1H), 5.12-5.00 (m, 2H), 4.81-4.79 (m, 1H), 4.60 (bs, 1H), 4.22-4.00 (m, 6H), 3.41 (s, 3H), 3.30 (s, 3H), 3.21 (s, 1H), 3.14-3.06 (m, 1H), 3.00 (s, 3H), 2.86 (s, 3H), 2.61-2.50 (m, 1H), 2.48-2.35 (m, 3H), 2.10-1.92 (m, 4H), 1.82-1.75 (m, 6H), 1.32-1.23 (m, 12H), 1.11-0.70 (m, 21 H). LCMS (ESI): (m/z) $(M+H)^+$ 1493, $(M+Na)^+$ 1515.

Trifluoroacetic acid (0.2 mL) was added to a solution of the above-described coupled product (162 mg, 0.1 mmol) in $CH_2Cl_2$ (2 mL), and the mixture was stirred at 0 °C for 24 h. The reaction mixture was then concentrated in *vacuo* to give compound **5** (91 mg, 67%)**.** LCMS (ESI): (m/z) $(M+H)^+$ 1252, $(M+Na)^+$ 1257.

**2.2. Intermediate 7** (Scheme SI-2)

Alkyne **11** (3.0 g, 9.75 mmol) in degassed propionitrile (10 mL) was added dropwise over an hour to a refluxing solution of compound **10**[2] (2.0 g, 3.9 mmol), CuI (37 mg, 0.19 mmol), $PdCl_2(PPh_3)_2$ (69 mg, 0.1 mmol) and $Et_3N$ (3 mL) in propionitrile (30 mL), and the mixture was stirred at reflux temperature for 16 h. The mixture was diluted with water (20 mL), extracted with EtOAc (2x40 mL), washed with brine, dried over anhydrous $Na_2SO_4$, and filtered through Celite. The Filtrate was concentrated in *vacuo* and purified by chromatography (EtOAc/Hexane, 1:3) to give the coupled product **12a** as an oil (4.1 g, 87 %). $^1$H NMR ($CDCl_3$, 300 MHz): δ 7.75-7.67 (m, 7H), 7.46-7.29 (m, 12H), 5.64 (d, $J$ = 5.2 Hz, 1H), 5.21 (t, $J$ = 5.3 Hz, 1H), 5.12-5.02

(m, 2H), 3.85 (t, *J* = 7.3 Hz, 2H), 3.57-3.41 (m, 2H), 2.68 (t, *J* = 7.2 Hz, 2H), 1.25 (s, 9H), 1.07 (s, 9H). HRMS (ESI): Calcd for $C_{41}H_{49}N_2O_7SSi$, 741.3167 $(M+H)^+$; Found, *m/z* 741.2987.




**Scheme SI-2.** Synthesis of the azide intermediate **7**.

To a solution of compound **12a** (4.1 g, 5.53 mmol) in EtOH (20 mL) was added Pd/C (100 mg), and the mixture was hydrogenated under atmospheric pressure using an $H_2$-filled balloon for 12 h. It was then filtered through a short pad of Celite, and the filter cake was washed with MeOH. The filtrate and the washings were combined and concentrated in *vacuo* to give free amine **12** (3.3 g, 5.53 mmol), which was used directly for the next reaction without further purification

EDC (1.05 g, 5.52 mmol), HOBt (746 mg, 5.52 mmol) and NMM (2.7 mL, 25.1 mmol) were added sequentially to a solution of acid, **13**[2], (1.29 g, 5.02 mmol) and free amine **12**, (3.0 g, 5.02 mmol) in DMF (5 mL) at 0 ˚C, and the mixture was stirred at 0 ˚C to room temperature for 12 h. The reaction mixture was worked-up using aqueous $NH_4Cl$ solution and $CH_2Cl_2$. The combined organic extracts were washed with water and dried ($Na_2SO_4$), filtered and concentrated in *vacuo*. Purification of the crude material using column chromatography (silica gel, 2-3% MeOH in $CH_2Cl_2$) afforded 1**4a** (3.3 g, 3.89 mmol). $^1$H NMR ($CDCl_3$, 300 MHz): δ 7.91 (d, *J* = 8.3 Hz, 2H), 7.81-7.67 (m, 2H), 7.63-7.56 (m, 2H), 7.40-7.21 (m, 15H), 6.32 (d, *J* = 8.7 Hz, 1H), 6.16 (d, *J* = 7.3 Hz, 1H), 4.48- 4.41 (m, 1H), 3.61 (t, *J* = 7.3 Hz, 2H), 3.10-2.90 (m, 2H), 2.88-2.85 (m, 5H), 2.65-2.58 (m, 2H), 1.77-1.62 (m, 2H), 1.58-1.52 (m, 2H), 1.38-1.31 (m, 3H), 1.27 (s, 9H),

1.07 (s, 9H). HRMS (ESI): Calcd for $C_{48}H_{60}N_4O_6SSiNa$, 871.4411 $(M+Na)^+$; Found, *m/z* 871.3417.

TBAF (1M in THF, 3.9 mL, 3.9 mmol) was added to a solution of compound **14a** (3.3 g, 3.89 mmol) in dry THF (20 mL) at 0 ˚C. The reaction mixture was warmed to room temperature and stirred for 3 h. It was quenched with saturated aqueous $NH_4Cl$ solution (10 mL), extracted with EtOAc (2x50 mL), washed with brine (10 mL), dried ($Na_2SO_4$), and concentrated in *vacuo*. Purification of the crude product by column chromatography (silica gel, 5-6% MeOH in $CHCl_3$ as eluants) afforded pure primary alcohol **14b** (2.2 g, 95%) as colorless oil. $^1H$ NMR ($CDCl_3$, 300 MHz): δ 7.67 (d, *J* = 8.2 Hz, 2H), 7.56-7.51 (m, 3H), 7.37 (t, *J* = 8.3 Hz, 1H), 7.17-7.12 (m, 6H), 6.73 (d, *J* = 8.6 Hz, 1H), 6.31 (d, *J* = 7.2 Hz, 1H), 6.12 (d, *J* = 7.4 Hz, 1H), 4.67- 4.59 (m, 1H), 3.98-3.87 (m, 1H), 3.84-3.70 (m, 1H), 3.56 (t, *J* = 5.4 Hz, 2H), 2.99-2.91 (m, 2H), 2.82-2.79 (m, 2H), 2.60-2.51 (m, 2H), 1.67-1.42 (m, 4H), 1.27 (s, 9H). HRMS (ESI): Calcd for $C_{32}H_{42}N_4O_6SNa$, 633.3117 $(M+Na)^+$; Found, *m/z* 633.2984.

TsCl (1.1 g, 5.4 mmol) and DMAP (44 mg, 0.36 mmol) were added sequentially to a solution of compound **14b** (2.2 g, 3.6 mmol) and $Et_3N$ (1.5 mL, 10.8 mmol) in $CH_2Cl_2$ (20 mL) at 0 ˚C, and the mixture was stirred at room temperature for 12 h. The reaction was worked-up using a saturated $NH_4Cl$ solution and $CH_2Cl_2$. The combined organic extracts were washed with water, brine, dried ($Na_2SO_4$) and concentrated in *vacuo*. Purification of the crude product using column chromatography afforded the tosyl derivative **14c**, which underwent next step without further purification.

$NaN_3$ (343 mg, 5.28 mmol) was added to a solution of compound **14c** (2.7 g, 3.52 mmol) in DMF (30 mL), and the mixture was heated at 70 ˚C for 7 h. The reaction was worked-up using a saturated $NH_4Cl$ solution and EtOAc. The combined organic extracts were washed with brine, dried ($Na_2SO_4$) and concentrated in *vacuo*. Purification of the residue using slica gel column chromatography (90-95% Ethyl acetate in petroleum ether as eluants) afforded **7a** (1.4 g, 65%). $R_f$ = 0.33 (silica gel, 100% EtOAc). $^1H$ NMR ($CDCl_3$, 300 MHz): δ 7.69 (d, *J* = 8.1 Hz, 2H), 7.63-7.59 (m, 3H), 7.31 (t, *J* = 8.1 Hz, 1H), 7.23-7.12 (m, 6H), 6.69 (t, *J* = 8.5 Hz, 1H), 6.31 (d, *J* = 7.1 Hz, 1H), 6.18 (d, *J* = 7.2 Hz, 1H), 4.63- 4.53 (m, 1H), 3.89-3.82 (m, 1H), 3.64-3.52 (m, 1H), 3.24-3.22 (m, 2H), 3.02-2.96 (m, 1H), 2.86-2.78 (m, 5H), 2.60-2.51 (m, 2H), 1.67-1.49 (m, 4H), 1.29 (s, 9H). HRMS (ESI): Calcd for $C_{32}H_{42}N_7O_5S$, 636.2987 $(M+NH)^+$; Found, *m/z*

636.2889.

Trifluoroacetic acid (2 mL) was added to a solution of **7a (**1.4 g, 2.20 mmol) in $CH_2Cl_2$ (10 mL) and the mixture was stirred at 0 °C for 12 h, and then the reaction mixture was concentrated in *vacuo* to give compound **7**. HRMS (ESI): Calcd for $C_{28}H_{33}N_7O_5SH$, 580.2262 $(M+H)^+$; Found, *m/z* 580.2259.

**2.3. Prodrug 8** (See Figure 1 in Paper)

A mixture of alkyne **5** (108 mg, 0.09 mmol), azide **7** (50 mg, 0.09 mmol), Cu (4 mg, 0.065 mmol), $CuSO_4$-solution (1 M, 20 μL, 0.02 mmol) in DMF (2 mL) was heated at 40 ˚C for 24 h. Solvents were removed under reduced pressure, and the residue was then purified using HPLC affording compound **8** (90 mg, 57%). HRMS (ESI): Calcd. for $C_{94}H_{128}N_{17}O_9S$ $(M+H)^+$ 1830.9217; Found, 1830.9117.

**3. HPLC purity analysis of compound 8**

| Compd No. | Solvent system | Wavelength (λmax) | $t_R$ (min) | Purity |
|---|---|---|---|---|
| **8** | A | 254 | 21.3 | 99% |

HPLC Condition: (A) Gradient from 20% acetonitrile in water (0.1% TFA) to 1% water in acetonitrile (0.07% TFA) at 1.0 mL/min.; 35 mins. per cycle.

HLPC column: ODS-A (A-300-CC, 50 x 4.6 mm, 12 nm).

**4. References**


(1) Bajjuri, K. M.; Liu, Y.; Liu, C.; Sinha, S. C. The Legumain Protease-Activated Auristatin Prodrugs Suppress Tumor Growth and Metastasis without Toxicity. *ChemMedChem* **2011**, *6*, 54–9.

(2) Sinha, S. C., Das, S.; Li, L. S.; Lerner, R. A.; Barbas, C. F. III. Preparation of integrin α(v)β(3)-targeting Ab 38C2 constructs. *Nature Protocols* **2007**, *2*, 449–56.