Synthesis of a pH-Sensitive Nitrilotriacetic Linker to Peptide Transduction Domains to Enable Intracellular Delivery of Histidine Imidazole Ring-Containing Macromolecules

Supporting Information

1. Methods
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Methods

All commercial chemicals were reagent grade and were used without further purification except where otherwise specified. N^{ε}-benzyloxycarbonyl-$_L$-lysine *tert*-butyl ester hydrochloride and Fmoc Aspertic acid were purchased from Bachem (Torrance, CA). All the protected amino acids and coupling agents required for peptide synthesis were purchased from Nova Biochem (San Diego, CA).

Thin-layer chromatography was performed on precoated silica gel plates (Kieselgel $60F_{254}$, Merck) and preaparative silica gel flash chromatography was performed on Combi Flash Rf (Teledyne Isco) using Redi Sep Rf Columns. ^{1}H NMR spectra were recorded on a Varian 300 Mercury spectrometer at 300 MHz in $CDCl_3$. Mass spectra were recorded at UCSD Chemistry and Biochemistry Mass Spectroscopy Facility utilizing either an LCQDECA (Finnigan) ESI with a quadrupole ion trap or an MAT900XL (ThermoFinnigan) FAB double focusing mass spectrometer.

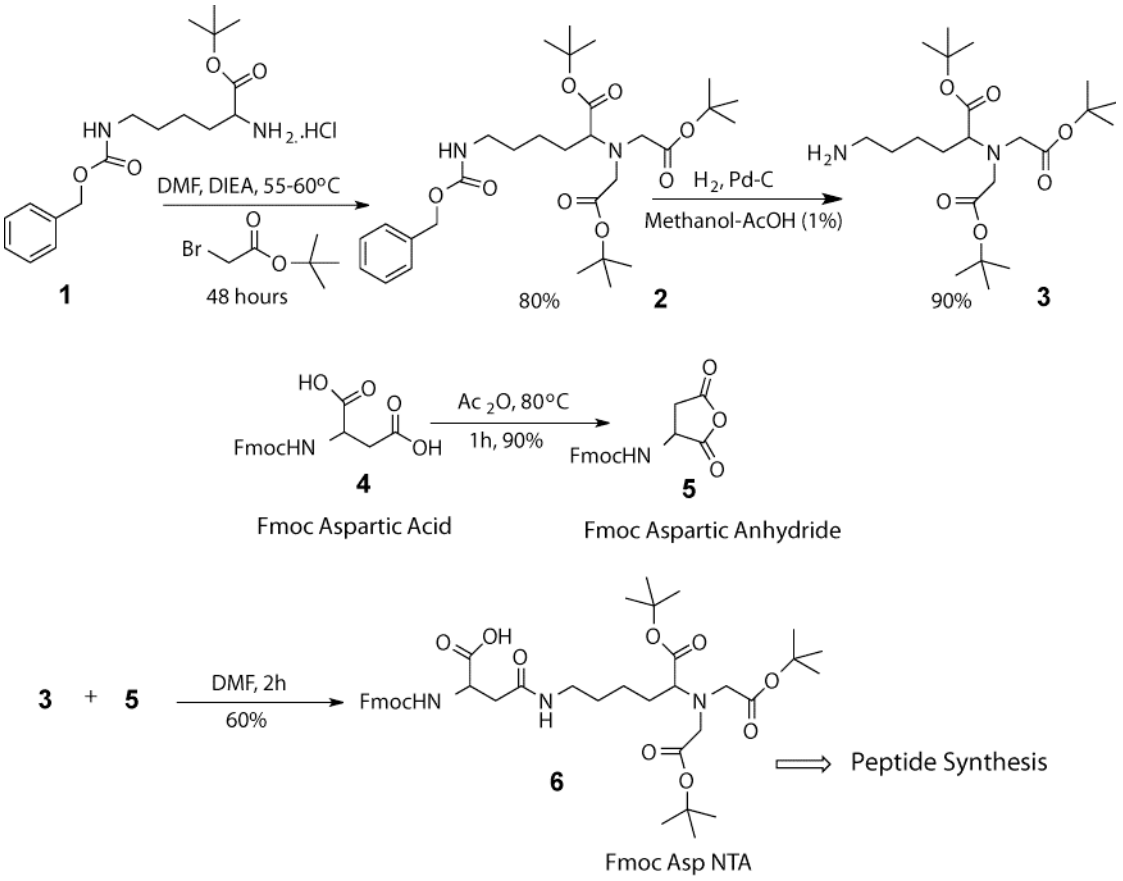
Peptide synthesis was performed in 25μM scale using Fmoc solid phase peptide synthesis protocol on a Symphony Quartet peptide synthesizer (Ranin) and rink-amide MBHA resin was used as the solid support. All peptides were cleaved and deprotected using standard conditions (95% TFA, 1% water, 1% TIS). Crude peptides were precipitated using cold diethylether and purified using prep-scale RP-HPLC with an Agilent Prep C18 30 x 250 mm column. Peptide purity was confirmed by mass spectrometry using α-CHCA matrix (Voyager, Applied Biosystems DE-Pro MALDI-TOF).

H1299 cells were cultured in DMEM with 5% FBS and antibiotics. CHOLSL-eGFP cells[1] were cultured in F12 medium with 10% FBS and antibiotics. Cells were maintained at 37 °C in the presence of 5% CO_2. For experiments, 20-30 thousand cells were plated in 24 well plates and allowed to attach for 18 hours. Cells were washed with PBS or serum-free media prior to protein transductions. NTA-PTD-Cargo complexes were prepared by mixing 1 equivalent of NTA-PTD with 3 equivalents of $NiSO_4$ (Aldrich, St. Louis, MO) followed by addition of 1

equivalent of cargo (6xHis-Rhodamine or 6xHis-Cre Recombinase[2] and incubation at 37 °C for 10 minutes prior to dilution in serum-free media. 6xHis-β-Galactosidase transduction products were performed by adding 1 equivalent of NTA-PTD and 6xHis-β-Galactosidase and 3 equivalents of $NiSO_4$ to media. 6xHis-tagged proteins were made by transforming BL21 Codon Plus *e. coli.* (Stratagene, La Jolla, Ca) and purified using Ni-NTA-agarose, ion exchange (6xHis-Cre), or gel filtration (6xHis-β-Galactosidase). Following transductions, cells were washed 3 times with 0.5 mg/mL Heparin in PBS and trypsinized.[3] For cell association studies, cells were resuspended in media and immediately assayed. β-Galactosidase activity was determined by resuspending the cells in media with a final concentration of 1 μM followed by incubation for 1 hr and cytometry. For LSL-excision experiments, cells were replated following trypsinization and assayed after 24 hours. For flow cytometry, 5-10 thousand live events were collected using an LSRII flow cytometer (BD Biosciences Franklin Lakes, NJ). Autofluorescence was monitored using a dummy channel, and cells were gated and quantified using BD FACS DIVA software. Overlay histograms were constructed using FlowJo (Tree Star, Ashland, OR). Digital images were acquired using an Axiovert 200M microscope equipped with a CCD camera (Carl Zeiss Microimaging, Thornwood, NJ). All images were acquired with the same exposure and thresholded identically.

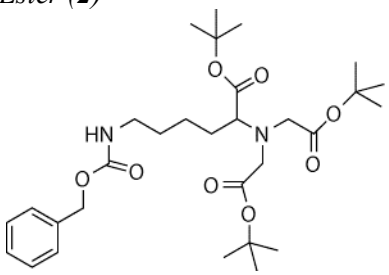
Transgenic ROSA26 loxP-Stop-loxP Luciferase mice[4] (Jackson Labs) were injected subcutaneously with 50 μl of 20 μM 6xHis-Cre or NTA-PTD-6xHis-Cre to activate luciferase gene expression via removal of a loxP-STOP-loxP DNA transcriptional terminator genetic element (Day 0). For Luciferase imaging, D-Luciferin (150 mg/kg) was administrated subcutaneouslly and luciferase expression monitored by live animal imaging (IVIS Spectrum Xenogen) for 10-25 min postluciferin injection, twice daily per mouse each day for 6 days.

Monomer Synthesis



Scheme S1: Synthesis of Fmoc Asp NTA

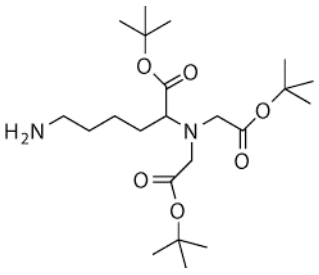
*Synthesis of N^{α}, N^{α}-Bis[(tert-butyloxycarbonyl)methyl]-N^{ε}-benzyloxycarbonyl-$_L$-lysine tert-Butyl Ester (**2**)*



The synthesis was performed adopting the procedure from Lata et al.[5] tert-Butyl bromoacetate (1.59 ml, 10.8 mmol) and DIEA (2.30 ml, 13.5 mmol) were added sequentially to a solution of N^{ε}-benzyloxycarbonyl-L-lysine tert-butyl ester hydrochloride

(**1**) (1.00 g, 2.7 mmol) in DMF (25 ml). The reaction vessel was purged with N_2 and then continuously stirred for 48 hours at 55°C. The volatiles were evaporated in vacuo at 60°C. Hexane:ethylacetate (3:1, 15 ml) solution was added to the partially solidified reaction mixture. The slurry so obtained was filtered over sintered glass funnel and the precipitate was washed three times with the same solvent (3 x 10 ml). The filtrate was concentrated under reduced pressure and the residue was purified by flash silica gel chromatography using hexane/ethylacetate (3:1) as the moving phase. The fractions containing the product was removed which afforded 1.2 g of **2**. Yield 80%. ESI MS for $C_{30}H_{48}N_2O_8$ calculated 564.34 found 565.12 $[M+H]^+$, 587.18 $[M+Na]^+$ ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.46 - 1.32 (m, 27H), 1.54 - 1.47 (m, 2H), 1.60 (s, 2H), 3.20 - 3.07 (m, 2H), 3.25 (t, J = 7.3 Hz, 1H), 3.53 - 3.32 (m, 4H), 5.11 - 4.98 (m, 2H), 5.32 - 5.17 (m, 2H), 7.40 - 7.17 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 23.19, 28.29, 28.40, 29.40, 30.31, 40.98, 54.06, 65.25, 66.57, 80.88, 81.27, 128.12, 128.25, 128.61, 136.99, 156.71, 170.89, 172.58.

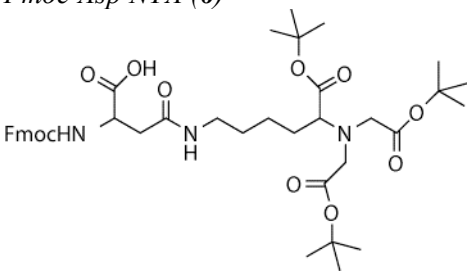
*Synthesis of N^{α}, N^{α}-Bis[(tert-butyloxycarbonyl)methyl]-$_L$-lysine tert-Butyl Ester (**3**)*



N^{α}, N^{α}-Bis[(tert-butyloxycarbonyl)methyl]-N^{ε}-benzyloxycarbonyl-$_L$-lysine tert-Butyl Ester (**2**) (1 g, 1.78 mmol) was dissolved in 30 ml Methanol containing 0.5% acetic acid). The solution was purged with Argon and 20 mg of Pd/C (10% Pd) was added. The reaction mixture was vigorously stirred under H_2 atmosphere (balloon pressure) at room temperature for 6 hours.

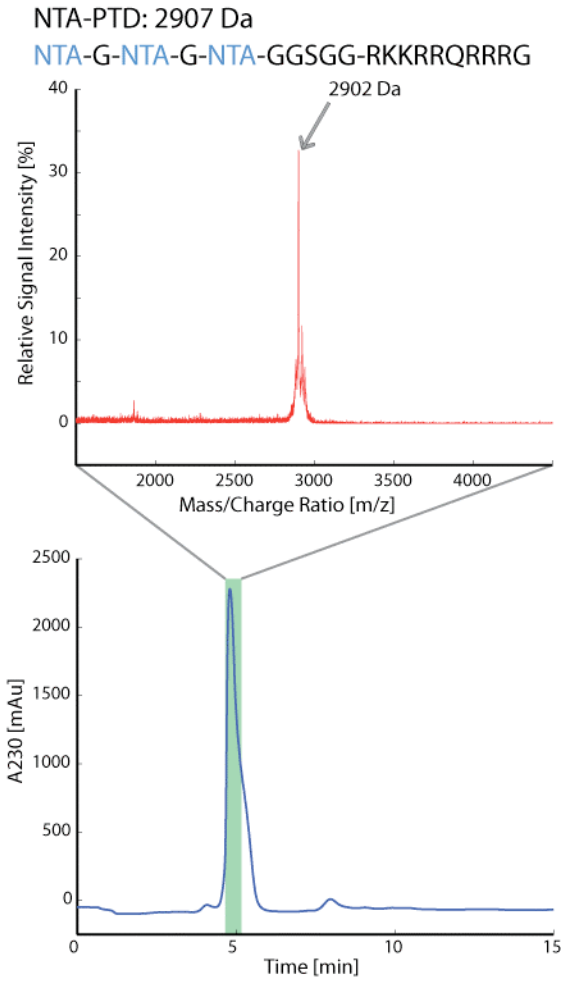
After TLC analysis showed the complete conversion of the starting material, the catalyst was filtered off through celite and the volatiles from the filtrate were removed under reduced pressure afforded 720 mg of **3**. Yield: 95%. ESI MS for $C_{22}H_{42}N_2O_6$ calculated 430.3 found 431.22 $[M+H]^+$ ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.29 - 1.45 (m, 27H), 1.45 - 1.57 (m, 4H), 2.7(m, 2H) 3.20 - 3.07 (m, 2H), 3.25 (t, *J* = 7.3 Hz, 1H), 3.29 - 3.49 (m, 4H)

*Synthesis of N^{ε}-[Fmoc Asp] N^{α}, N^{α}-Bis[(tert-butyloxycarbonyl)methyl]-$_L$-lysine tert-Butyl Ester Fmoc Asp NTA (**6**)*



The synthesis was performed adopting slight modification of the procedure from Morgan *et. Al.*[6] Fmoc aspartic anhydride[7] (**5**) (0.55 g, 1.63 mmol) dissolved in 5 ml anhydrous DMF was added to N^{α},N^{α}-bis[(*tert*-butyloxycarbonyl) methyl]-L-lysine *tert*-butyl ester (**3**) (0.7 g, 1.63 mmol). The solution was stirred for 2 h at room temperature. The solvent was removed under reduced pressure and the residue was dissolved in 100 ml EtOAc. The organic layer was washed with water (2 x 30 ml) and then brine (2 x 30) and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure and purified by flash silica gel chromatography using EtOAc -AcOH (99:1). The fractions containing the product was removed which afforded (**6**) as white foamy solid. Yield 0.8 g, 65% ESI MS for $C_{41}H_{57}N_3O_{11}$ calculated 767.4 found 768.25 $[M+H]^+$, 790.23 $[M+Na]^+$ ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.26 - 1.48 (m, 28H), 1.51 - 1.72 (m, 4H), 2.61 - 2.84 (m, 1H), 3.0 (m, 1H), 3.17 - 3.68 (m, 5H), 4.08-4.62 (m, 4H), 4.7 (m, 2H) 7.20 - 7.49 (m, 4H), 7.60 (d, *J* = 7.0 Hz, 2H), 7.77 (d, *J* = 7.5 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 23.2, 28.3, 28.40, 29.40, 30.3, 47.0, 54.1, 65.2, 66.5, 80.6, 81.2, 121.1, 126.1, 128.25, 128.6, 143.9, 156.2, 169.2, 170.4,170.9, 172.8.

Analytical Data for Synthesized NTA-PTD



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