Supporting Information

Effect of linker stereochemistry on the activity of indolinobenzodiazepine containing antibody-drug conjugates (ADCs)

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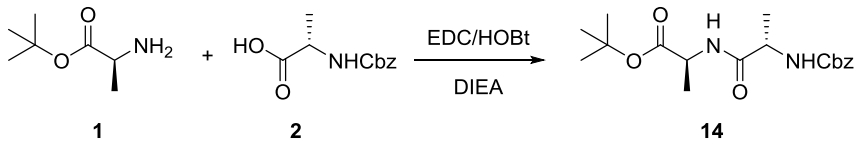
Table of Contents

General Methods:

Unless otherwise stated, all percents, ratios, parts, *etc*. are by weight. Starting materials and reagents purchased from commercial suppliers were used without further purification. Concentration refers to the removal of solvent under reduced pressure using a rotary evaporator. Silica gel column chromatography was performed using a medium pressure automated liquid chromatography on a Teledyne ISCO Combiflash®, Rf200i. Nuclear Magnetic Resonance (NMR) spectra were acquired on a Bruker 400 MHz instrument. Data are reported as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, br s = broad singlet, m = multiplet), and coupling constants (Hz). Mass spectra were acquired on a Bruker Daltonics Esquire 3000 instrument. LCMS were acquired on an Agilent 1260 Infinity LC with an Agilent 6120 single quadrupole MS using electrospray ionization (column: Agilent Poroshell 120 C18, 3.0 x 50 mm, 2.7 μm, 8 min method: flow rate 0.75 mL/min, solvent A: water with 0.1% formic acid, solvent B: MeCN, 5% to 98% of MeCN over 7 min and 98% MeCN for 1 min; 15 min method: column: Agilent Poroshell 120 C18, 3.0 x 100 mm, 2.7 μm, flow rate 0.5 mL/min, solvent A: water with 0.1% formic acid, solvent B: MeCN, 25 to 80% of MeCN over 12 min, 80 to 95% MeCN over 0.5 min and 95% MeCN for 2.5 min). UPLC were acquired on a Waters, Acquity system with a single quadrupole MS Zspray^{TM} (column: Acquity BEH C18, 2.1 x 50 mm, 1.7 μm, 2.5 min method: flow rate 0.8 mL/min, solvent A: water with 0.1% formic acid, solvent B: MeCN, 5 to 95% of MeCN over 2.0 min and 95% MeCN for 0.5 min). Reverse phase semi prep HPLC was performed on a Varian ProStar instrument (column: Kromasil 100-10 C18, 250 x 21.2 mm, 100 Å, 10 μm particle size, Supelco # K08670621, S/N E78646, flow rate: 20 mL/min).

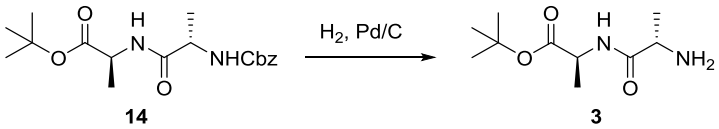
Experimental Procedures:

Synthesis of L-ala-L-ala NHS ester 10



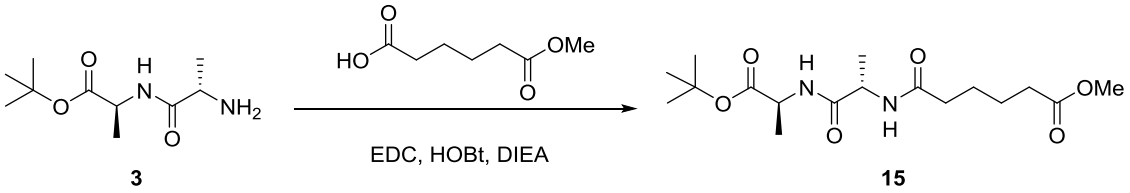
1 **2** **14**

Compound **1** (4.48 g, 24.64 mmol) and compound **2** (5.0 g, 22.40 mmol) were dissolved in DMF (44.8 mL). EDC (4.72 g, 24.64 mmol) and HOBt (3.43 g, 22.40 mmol) were added to the reaction mixture at rt, follow by DIEA (9.75 mL, 56.0 mmol). The reaction stirred under argon at rt overnight. The reaction mixture was diluted with DCM and was washed with sat'd NH_4Cl, sat'd $NaHCO_3$, water, and brine. The organic layer was dried over Na_2SO_4, filtered and concentrated. The crude product was purified via silica gel chromatography (hexanes/EtOAc) to yield compound **14** (6.7 g, 85% yield) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38-7.31 (m, 5H), 6.53-6.42 (m, 1H), 5.42-5.33 (m, 1H), 5.14 (s, 2H), 4.48-4.41 (m, 1H), 4.32-4.20 (m, 1H), 1.49 (s, 9H), 11.42 (d, *J* = 6.8 Hz, 3H), 1.38 (d, *J* = 7.2 Hz, 3H).



14 **3**

Compound **14** (6.7 g, 19.12 mmol) was dissolved in MeOH (60.7 mL) and H_2O (3.03 mL). The solution was purged with argon for 5 min. Pd/C (wet, 10%) (1.017 g, 0.956 mmol) was added slowly while continuing the argon purge for an additional 5 min. The reaction mixture was placed under vacuum and then released to an atmosphere of hydrogen. This was repeated 3 times. The reaction mixture was hydrogenated at 30 psi for 3 h rt. The solution was filtered through Celite, rinsed with MeOH and was concentrated. The crude product was coevaporated with MeOH and ACN (2x) and was placed on the high vacuum to give the amine **3** (4.02 g, 97% yield) which was used directly in the next step without purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.78-7.63 (m, 1H), 4.49-4.42 (m, 1H), 3.55-3.50 (m, 1H), 1.73 (s, 2H), 1.48 (s, 9H), 1.39 (d, *J* = 7.2 Hz, 3H), 1.36 (d, *J* = 6.8 Hz, 3H).

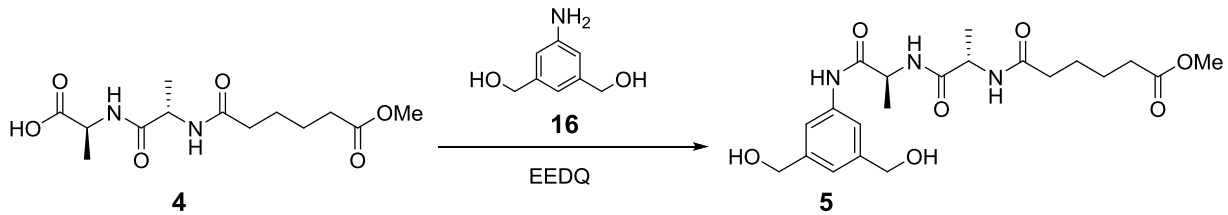


3 **15**

Compound **3** (4.02 g, 18.59 mmol) and 6-methoxy-6-oxo-hexanoic acid (3.03 mL, 20.45 mmol) were dissolved in DMF (62.0 mL). EDC (3.92 g, 20.45 mmol) and HOBt (2.85 g, 18.59 mmol) were added to the reaction mixture at rt, followed by DIEA (6.49 mL, 37.2 mmol) and was stirred. The reaction mixture was diluted with DCM/MeOH (150 mL, 5:1) and was washed with sat'd NH4Cl, sat'd $NaHCO_3$, brine, dried over Na_2SO_4, filtered and concentrated. The compound was coevaporated with ACN (5x), then pumped on the high vacuum at 35 °C to give methyl ester **15** (6.66 g, 100% yield), which was taken onto next step without purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.75 (d, *J* = 6.8 Hz, 1H), 6.44 (d, *J* = 6.8 Hz, 1H), 4.52-4.44 (m, 1H), 4.43-4.36 (m, 1H), 3.65 (s, 3H), 2.35-2.29 (m, 2H), 2.25-2.18 (m, 2H), 1.71-1.60 (m, 4H), 1.45 (s, 9H), 1.36 (t, *J* = 6 Hz, 12.8 Hz, 6H).

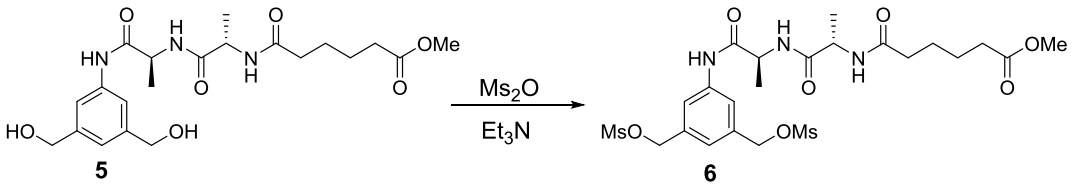
15 **4**

Compound **15** (5.91 g, 16.5 mmol) was stirred in TFA (28.6 mL, 372 mmol) and deionized water (1.5 mL) at rt for 3 h. The crude solution was diluted and coevaporated with ACN (3x) and placed on high vacuum to give compound **4** as a sticky solid (5.88 g, 100% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.21 (d, *J* = 6.8 Hz, 1H), 6.81 (d, *J* = 7.6 Hz, 1H), 4.69-4.60 (m, 1H), 4.59-4.51 (m, 1H), 3.69 (s, 3H), 2.40-2.33 (m, 2H), 2.31-2.24 (m, 2H), 1.72-1.63 (m, 4H), 1.51-1.45 (m, 3H), 1.42-1.37 (m, 3H).

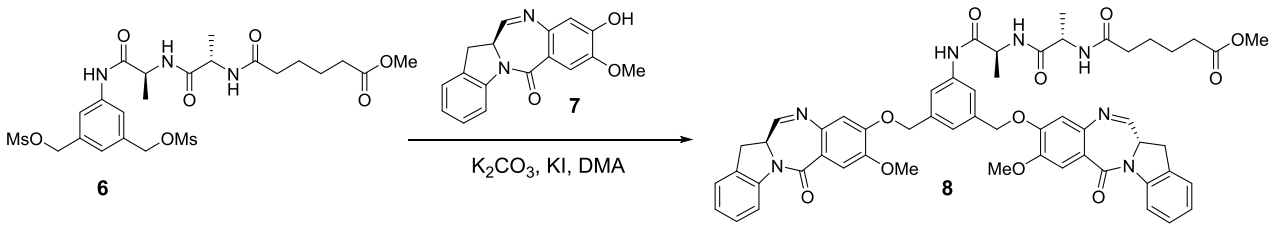


4 **5**

Compound **4** (5.6 g, 18.52 mmol) was dissolved in DCM (118 mL) and MeOH (58.8 mL). EEDQ (8.72 g, 35.3 mmol) was added and the reaction stirred for 1 h, after which compound **16** (2.70 g, 17.64 mmol) was added and the reaction stirred overnight at rt. The reaction mixture was concentrated and EtOAc was added to the residue. The resulting slurry was filtered and the solid was washed with EtOAc and dried over N_2 to give compound **5** (2.79 g, 36% yield). LCMS = 2.887 min (8 min method). ^{1}H NMR (400 MHz, DMSO-*d6*): δ 9.82 (s, 1H), 8.05, (d, *J* = 9.2 Hz, 1H), 8.01 (d, *J* = 7.2 Hz, 1H), 7.46 (s, 2H), 6.95 (3, 1H), 5.21-5.12 (m, 2H), 4.47-4.42 (m, 4H), 4.40-4.33 (, m, 1H), 4.33-4.24 (m, 1H), 3.58 (s, 3H), 2.33-2.26 (m, 2H), 2.16-2.09 (m, 2H), 1.54-1.46 (m, 4H), 1.30 (d, *J* = 7.2 Hz, 3H), 1.22 (d, *J* = 4.4 Hz (3H). LRMS (ESI$^+$): Calc'd for C21H31N3O7 (M + H) 438.21, found 438.30.

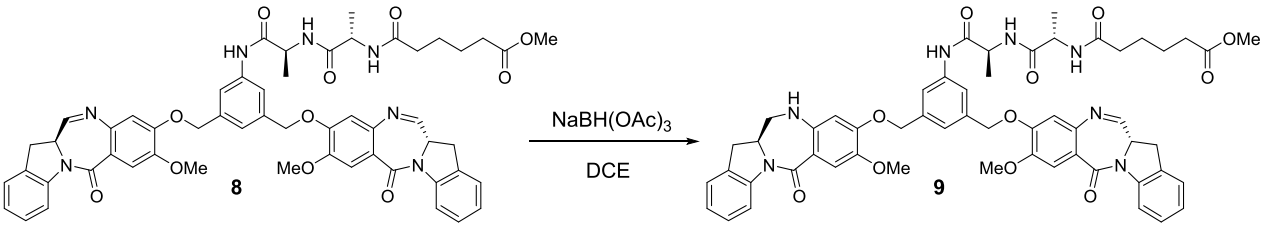


A solution of compound **5** (1.69 g, 3.86 mmol) in DCM (30 mL) and DMF (20 mL) was stirred under argon and cooled to -10 °C in a cooling bath of acetone and ice. Et_3N (2.69 mL, 19.31 mmol) and methanesulfonic anhydride (1.734 g, 9.66 mmol) were sequentially added. The reaction was stirred at -10 °C for 1.5 h. The reaction was quenched with ice water and extracted with cold EtOAc/MeOH (20:1, 2x). The extracts were combined, washed with ice water, brine, dried over Na_2SO_4, filtered and concentrated. The crude product was dried under high vacuum for 2 h to give compound **6** (1.953 g, 3.29 mmol, 85 % yield). LCMS = 4.869 min (15 min method). LRMS (ESI$^+$): Calc'd for C23H35N3O11S2 (M - H) 592.17, found 592.10.



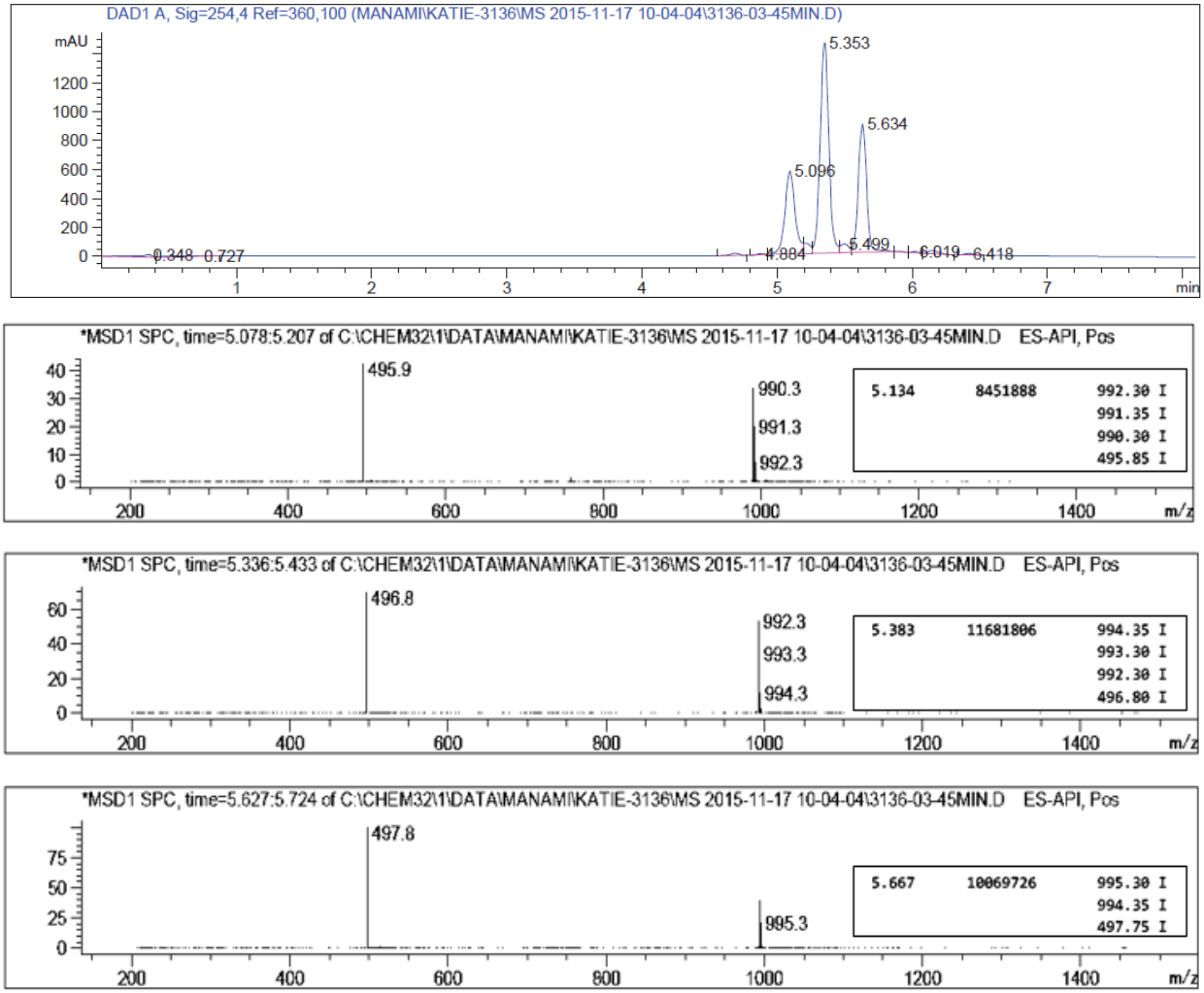
Compound **6** (2.110 g, 7.17 mmol) was added to a stirring solution of compound **7** (1.935 g, 3.26 mmol) and DMA (32.6 mL). K_2CO_3 (1.126 g, 8.15 mmol) and KI (0.541 g, 3.26 mmol) were sequentially added and the reaction was stirred at rt under Ar for 15 h. Water was added to the

reaction mixture. The resulting slurry was stirred for 20 min and was filtered. The collected solids were dissolved in DCM, transferred to a separatory funnel, washed with water, brine, dried over Na_2SO_4, filtered and concentrated. The desired product was purified by silica gel flash chromatography (DCM/MeOH) to give compound **8** (1.84 g, 1.700 mmol, 52% yield). HRMS (ESI^+): calc'd for C55H55N7O11 (M + H) 990.4032, found 990.4015.

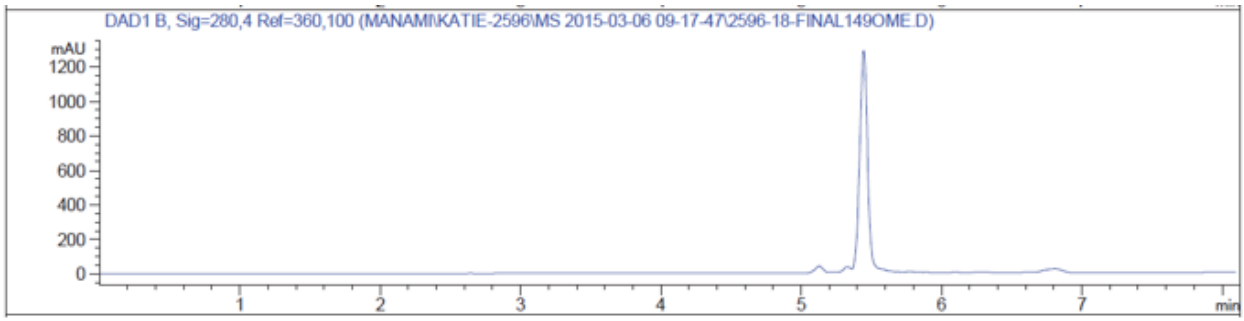


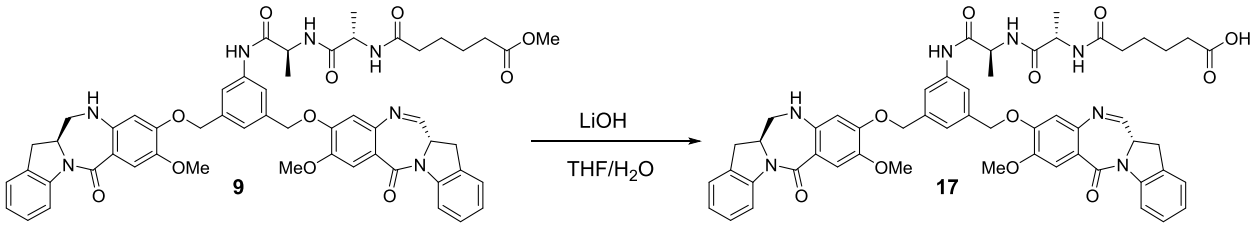
Compound **8** (0.95 g, 0.960 mmol) was dissolved in in DCE (9.6 mL). $NaBH(OAc)_3$ (0.193 g, 0.912 mmol) was added to the reaction mixture and was stirred rt for 1 h. The reaction mixture was diluted with DCM and was quenched with sat'd NH_4Cl. The organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated. The crude product was purified by reverse phase semi-prep HPLC (C18 column, ACN/H_2O). The fractions containing the desired product were frozen and lyophilized to obtain the desired monoimine product **9** as a white solid (196 mg, 20% yield). ^1H NMR (400 MHz, DMSO-d_6) δ 10.06 (s, 1H), 8.21 (d, J=8.4 Hz, 1.3H), 8.12-8.09 (m, 1.6), 8.03-7.97 (m, 2H), 7.72 (m , 2H), 7.42 (s, 0.5H), (m, 9.5H), 6.42 (s, 1H), 6.33 (m, 1H), 5.13 (dd, J= 62.4, 18.8 Hz, 1H), 5.06 (m, 3H), 4.71 (m, 0.2H), 4.54 (m, 0.5H), 4.38 (m, 2H), 4.28 (m, 1H), 4.21 (m, 0.2H), 4.12 (m, 0.2H), 3.86 (d, 1.5H), 3.74 (s, 1.5H), 3.71 (s, 3H), 3.59 (s, 2H) 3.55 (s, 3H), 3.44 (m, 1.4H), 3.27 (m, 1H), 3.09 (m, 0.6H), 2.89 (dd, J= 17.2 Hz, 4.4Hz, 1H), 2.27 (m, 2H), 2.12 (m, 2H), 1.48 (m, 4H), 1.31 (d, J= 6.8 Hz, 3H), 1.20 (d, J= 7.2 Hz, 3H). HRMS (ESI^+): calc'd for C55H57N7O11 (M + H) 992.4189, found 992.4172.

HPLC trace of crude reaction mixture of 8, 9, and direduced IGN

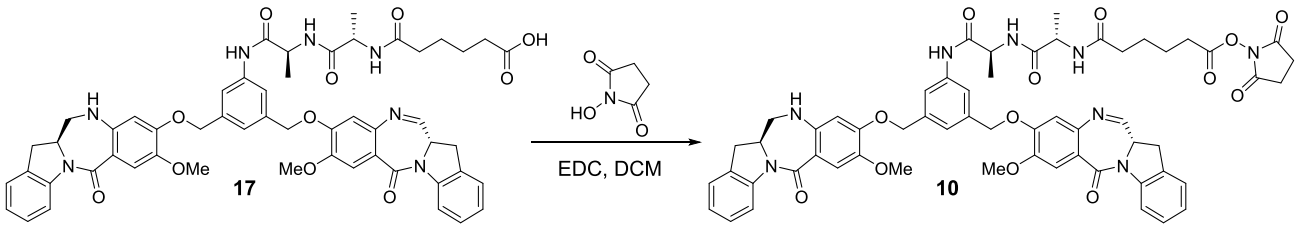


HPLC trace of purified 9



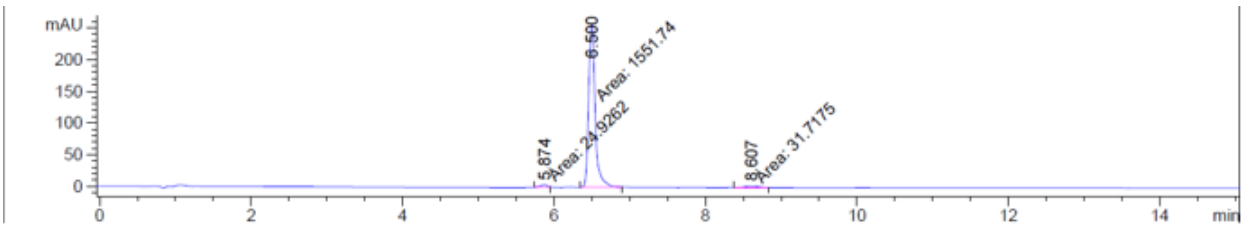


Compound **9** (0.150 g, 0.151 mmol) was co-evaporated with DCM (3x) and was then dissolved in THF (5.7 mL) and H_2O (1.9 mL). LiOH (0.036 g, 1.512 mmol) was added to the reaction mixture and was stirred at rt for 1 h. The reaction mixture was diluted with 30% MeOH/DCM and H_2O, and then acidified slowly with 0.5 M (aq) HCl to pH~3. The aqueous layer was 30% MeOH/DCM (3 x 40 mL). The combined organic layers were washed with water, dried over Na_2SO_4, filtered through Celite, and concentrated to give compound **17** as a yellow solid (152 mg). LCMS = 6.387 min (15 min method). LRMS (ESI^+): Calc'd for C54H55N7O11 (M + H) 977.40, found 978.60.

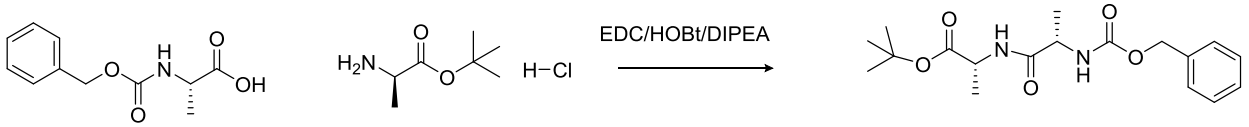


Compound **17** (148 mg, 0.151 mmol) was suspended in DCM (7.60 mL). N-hydroxy succinimide (53.2 mg, 0.453 mmol) and EDC (145 mg, 0.755 mmol) were added at rt and was stirred for 90 min. The reaction mixture was diluted with DCM and H_2O. The organic layer was washed with H_2O, dried over Na_2SO_4, filtered and concentrated. The crude product was purified by reverse phase semi-prep HPLC (C18 column, ACN/H_2O). Fractions containing the desired product were frozen and lyophilized to obtain compound **10** (42 mg, 26% yield). HRMS (ESI^+): calc'd for C58H58N8O13 (M + H) 1075.4196, found 1075.4176.

HPLC trace of purified 10

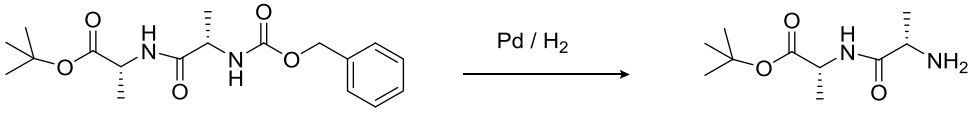


Synthesis of D-ala-L-ala NHS ester 10b



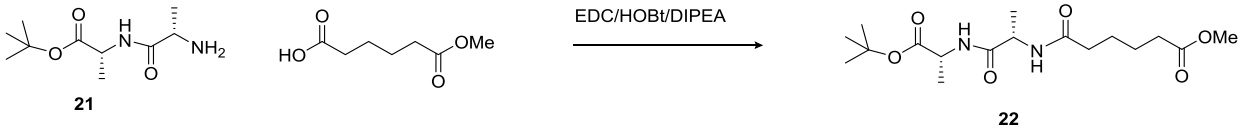
18 19 20

(S)-2-(((benzyloxy)carbonyl)amino)propanoic acid (5 g, 22.40 mmol) and (R)-tert-butyl 2-aminopropanoate hydrochloride (4.48 g, 24.64 mmol) were dissolved in anhydrous *N*,*N*-Dimethylformamide (44.8 ml). 3-(3-Dimethylaminopropyl)-1-ethyl-carbodiimide hydrochloride (4.72 g, 24.64 mmol), 1-Hydroxybenzotriazole hydrate (3.43 g, 22.40 mmol), and then diisopropylethylamine (9.75 ml, 56.0 mmol) were added. The reaction stirred under argon, at room temperature, overnight. The reaction mixture was diluted with dichloromethane and then washed with saturated ammonium chloride, saturated sodium bicarbonate, water, and brine. The organic was dried over sodium sulfate and concentrated. The crude oil was purified via silica gel chromatography (Hexanes/Ethyl Acetate) to yield compound **20** (5.6g, y=71%) ^{1}H NMR (400 Hz, $CDCl_3$): δ 7.39-7.34 (m, 5H), 6.54 (s,1H) 5.28 (s, 1H), 5.15 (s, 2H), 4.47-4.43 (m, 1H), 4.48 (s, 1H), 1.49 (s, 9H), 1.42-1.37 (m, 6H).



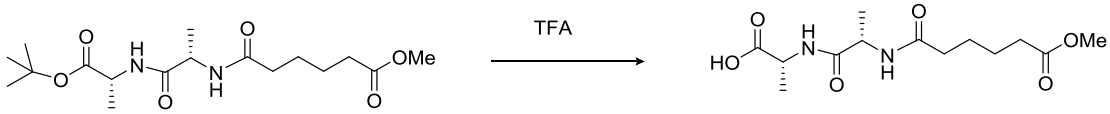
20 21

Compound **20** (R)-tert-butyl2-((S)-2-(((benzyloxy)carbonyl)amino)propanamido) propanoate (5.6g, 15.98 mmol) was dissolved in methanol (50.7 ml) and water (2.54 ml). The solution was purged with argon for five minutes. Palladium on carbon (wet, 10%) (0.850 g, 0.799 mmol) was added slowly. The reaction was stirred overnight under an atmosphere of hydrogen. The solution was filtered through celite, rinsed with methanol and concentrated. It was azeotroped with methanol and acetonitrile and the resulting oil was placed directly on the high vacuum to give compound **21** (3.57g, y=103%) which was used directly in the next step. ^{1}H NMR (400 Hz, $CDCl_3$): δ 7.67 (s,1H), 4.49-4.42 (m,1H), 3.51 (q, J=8Hz), 1.48 (s,1H), 1.4 (d, J=7.2Hz, 3H), 1.36 (d,J=6.8Hz, 3H)



21 22

Compound **21** (3.57 g, 16.51 mmol) and monomethyladipate (Aldrich) (2.69 ml, 18.16 mmol) were dissolved in anhydrous *N*,*N*-Dimethylformamide (55.0 ml). 3-(3-Dimethylaminopropyl)-1-ethyl-carbodiimide hydrochloride (3.48 g, 18.16 mmol) and 1-Hydroxybenzotriazole hydrate ((2.53 g, 16.51 mmol) were added, followed by diispopropylethylamine (5.77 ml, 33.0 mmol). The mixture was stirred overnight at room temperature. The reaction was diluted with dichloromethane/methanol (80 mL, 5:1) and washed with saturated ammonium chloride, saturated sodium bicarbonate, and brine. It was dried over sodium sulfate, filtered and stripped. The compound was azotroped with acetonitrile (5x), then pumped on the high vacuum at 35 °C to give compound **22** (5.91g, y=100%). The crude material was taken onto next step without purification. ^{1}H NMR (400 Hz, $CDCl_3$): δ 6.67 (d, J=6.8Hz, 1H), 6.22 (d, J=7.2Hz , 1H) 4.56-4.49 (m, 1H), 4.46-4.38 (m, 1H), 3.68 (s, 3H), 2.37-2.33 (m, 2H), 2,27-2.24 (m, 2H), 1.70 -1.68 (m, 4H), 1.47 (s, 9H), 1.40 (s, 3H), 1.38 (s, 3H)

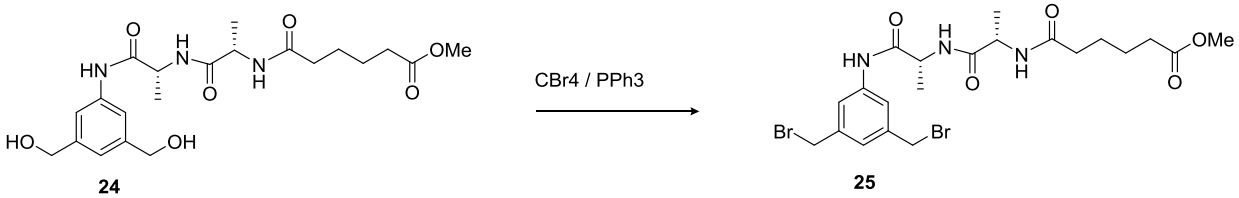


22 23

Compound **22** (5.91 g, 16.5mmol) was stirred in trifluoroacetic acid (25.4 ml, 330 mmol) and deionized water (1.3ml) at room temperature for three hours. It was rotovaped with acetonitrile and placed on high vacuum until the solvents were removed to give crude compound **23** (4.99g y=100%). ^{1}H NMR (400 Hz, $CDCl_3$): δ 7.44 (d, J=7.2Hz, 1H), 6.97 (d, J=8.0 Hz, 1H), 4.81-4.73 (m, 1H), 4.59-4.51 (m, 1H), 3.69 (s, 3H), 2.39-2.32 (m, 2H), 2.31-2.23 (m, 2H), 1.70-1.61 (m, 4H), 1.48(d, J=7.2 Hz, 3H), 1.40 (d, J=7.2 Hz, 3H)

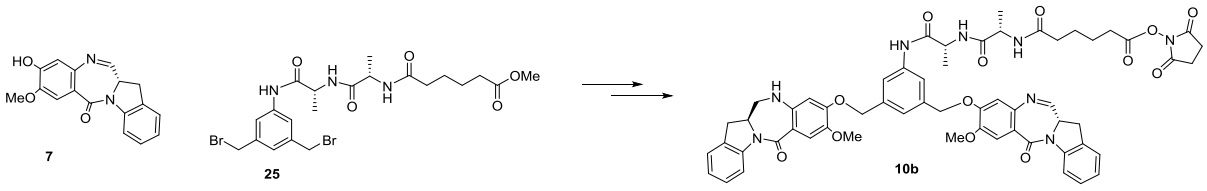
16 23 24

Compound **23** (4.8g, 15.88 mmol) was dissovled in anhydrous dichloromethane (101 ml) and anhydrous methanol (50.4 ml). (5-amino-1,3-phenylene)dimethanol (2.316 g, 15.12 mmol) and N-Ethoxycarbonyl-2-ethoxy-1,2-dihdroquinoline (7.48 g, 30.2 mmol) were added and the reaction was stirred at room temperature, overnight. The solvent was stripped and the crude material purified by silica gel chromatography (dichloromethane/methanol) to give compound **24** (1.65g, y=25%) ^{1}H NMR (400 Hz, DMSO): δ 9.68 (s, 1H), 8.29 (d, J=7.2 Hz, 1H) 8.11 (d, J=6.36 Hz, 1H), 7.52 (s, 2H), 6.97 (s, 1H), 5.15 (s, 2H), 4.45 (s, 4H), 4.39-4.32 (m, 1H), 4,28-4.21 (m, 1H), 3.57 (s, 3H), 2.30-2.27 (m, 2H), 2.17-2.13 (m, 2H), 1.54-1.45 (m, 4H) 1.30 (d, J=7.2Hz. 3H), 1.20 (d, J=7.2 Hz, 3H). LRMS (ESI$^+$): Calc'd for C21H31N3O7 (M + Na) 460.21, found 460.2.



24 **25**

Compound **24** (0.486 g, 1.111 mmol) and carbon tetrabromide (1.105 g, 3.33 mmol) were dissolved in anhydrous *N*,*N*-Dimethylformamide (11.11 ml). Triphenylphosphine (0.874 g, 3.33 mmol) was added and the reaction stirred under argon for four hours. It was diluted with dichloromethane/methanol (10:1) and washed with water and brine. It was dried over sodium sulfate, filtered, and concentrated to remove all sovents. The crude material was purified by silica gel chromatography (dichloromethane/methanol) to give compound **25** (250 mgs, y=40%).^{1}H NMR (400 Hz, DMSO): δ 9.82 (s, 1H), 8.38 (d, J=7.2Hz, 1H), 8.17 (d, J=6.0Hz, 1H), 7.76 (s, 2H), 7.22 (s, 1H), 4.66 (s, 4H), 4.38-4.31 (m, 1H), 4.25-4.19 (m, 1H), 3.56 (s, 3H), 2.30-2.27 (m, 2H), 2.18-2.15 (m, 2H), 1.53-1.51 (m, 4H), 1.32 (d, J=7.2Hz, 3H), 1.21 (d, J=6.8Hz, 3H).

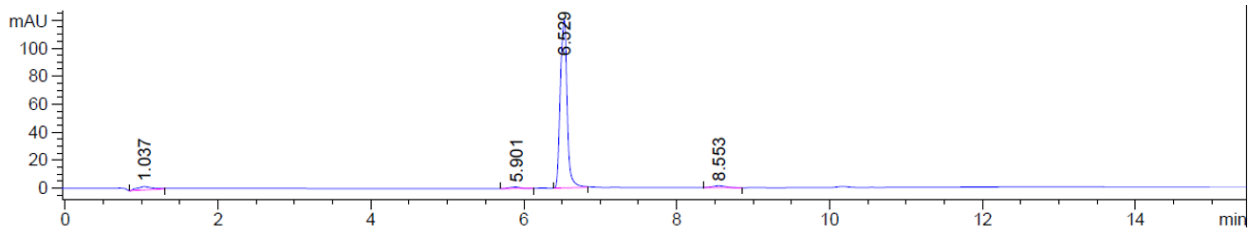


7 **25** **10b**

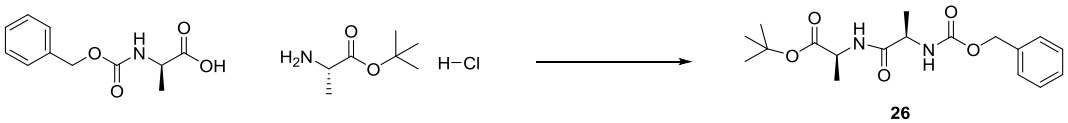
Compound **10b** was prepared from compounds **7** and **25** similarly as compound **10a** from compounds **6** and **7**. The crude material of the last step was purified via RPHPLC (C18 column,

Acetonitrile/Water) to give compound **10** (6.5mg, 97% pure, y=30%). LCMS =6.53 min (15 min method). LRMS (ESI): Calc'd for C58H58N8O13 (M + 1) 1075.41, found 1075.7.

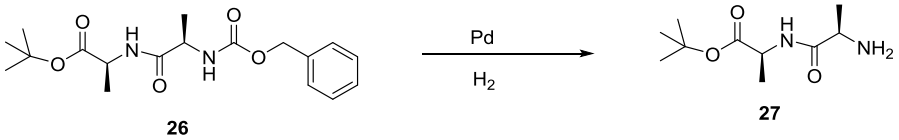
HPLC trace of purified 10b



Synthesis of L-ala-D-ala NHS ester 10c

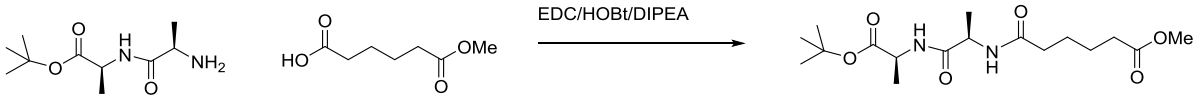


(R)-2-(((benzyloxy)carbonyl)amino)propanoic acid (5 g, 22.40 mmol) and (S)-tert-butyl 2-aminopropanoate hydrochloride (4.48 g, 24.64 mmol) were dissolved in anhydrous *N*,*N*-Dimethylformamide (44.8 ml). 3-(3-Dimethylaminopropyl)-1-ethyl-carbodiimide hydrochloride (4.72 g, 24.64 mmol), 1-Hydroxybenzotriazole hydrate (3.43 g, 22.40 mmol), and then diisopropylethylamine (9.75 ml, 56.0 mmol) were added. The reaction stirred under argon, at room temperature, overnight. The reaction mixture was diluted with dichloromethane and washed with saturated ammonium chloride, saturated sodium bicarbonate, water, and brine. The organic was dried over sodium sulfate and concentrated. The crude oil was purified via silica gel chromatography (Hexanes/Ethyl Acetate) to yield compound **26** (6.01g, y=77%) ^{1}H NMR (400 Hz, DMSO): δ 8.11 (d, J=7.2Hz, 1H), 7.40-7.28 (m, 6H), 5.03 (s, 2H), 4.14-4.04 (m, 2H), 1.39 (s, 9H), 1.24 (d, J=7.2Hz, 3H), 1.20 (d, J=7.2Hz, 3H).



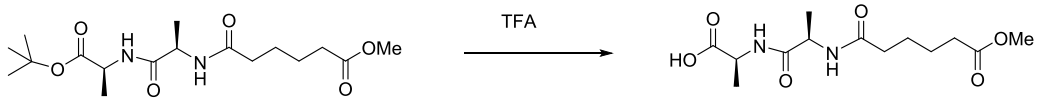
Compound **26** (6.0g, 17.16 mmol) was dissolved in methanol (54.4 ml) and water (2.72 ml). The solution was purged with argon for five minutes. Palladium on carbon (wet, 10%)

(0.913 g, 0.858 mmol) was added slowly. The reaction was stirred overnight under an atmosphere of hydrogen. The solution was filtered through celite, rinsed with methanol and concentrated. It was azeotroped with methanol and acetonitrile and the resulting oil was placed directly on the high vacuum to give compound **27** (3.67g, y=99%) which was used directly in the next step. ^{1}H NMR (400 Hz, DMSO): δ 8.06-7.98 (m, 1H), 4.16-4.09 (m, 1H), 3.38-3.30 (m, 2H), 3.29-3.24 (m, 1H), 1.40 (s, 9H), 1.27 (d, J=15.2Hz, 3H), 1.12 (d, J=6.8Hz, 3H).



27 **28**

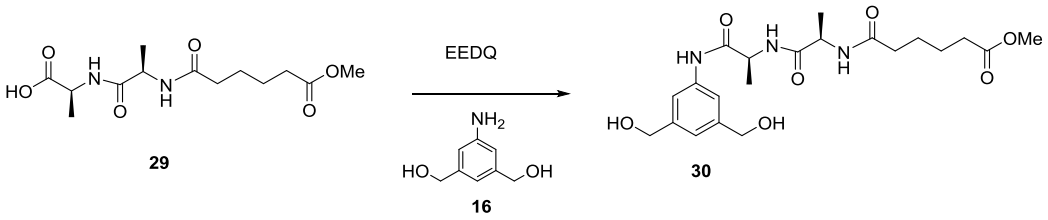
Compound **27** (3.67 g, 16.97 mmol) and monomethyladipate (Aldrich) (2.77 ml, 18.67 mmol) were dissolved in anhydrous *N*,*N*-Dimethylformamide (56.6 ml). 3-(3-Dimethylamino propyl)-1-ethyl-carbodiimide hydrochloride (3.58 g, 18.67 mmol) and 1-Hydroxybenzotriazole hydrate ((2.60 g, 16.97 mmol) were added, followed by diispopropylethylamine (5.93ml, 33.9 mmol). The mixture was stirred overnight at room temperature. The reaction was diluted with dichloromethane/methanol (150 mL, 5:1) and washed with saturated ammonium chloride, saturated sodium bicarbonate, and brine. It was dried over sodium sulfate, filtered and stripped. The compound was azeotroped with acetonitrile (5x), then pumped on the high vacuum at 35 °C to give compound **28** (6.08g, y=100%). The crude material was taken onto next step without purification. ^{1}H NMR (400 Hz, DMSO): δ 8.10 (d, J=7.2Hz, 1H), 7.97-7.89 (m, 1H), 4.37-4.30 (m, 1H), 4.12-4.05 (m, 1H), 3.58 (s, 3H), 2.32-2.26 (m, 2H), 2.15-2.08(m, 2H), 1.53-1.44 (m, 4H), 1.39 (s, (H), 1.23 (d, J=7.6Hz, 3H), 1.17 (d, J=7.2Hz, 3H).



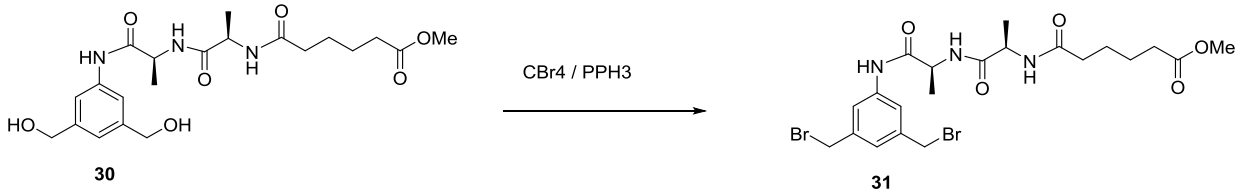
28 **29**

Compound **28** (6.08 g, 16.97 mmol) was stirred in trifluoroacetic acid (26.1 ml, 339 mmol) and deionized water (1.4ml) at room temperature for three hours. It was rotovaped with acetonitrile and placed on high vacuum until the solvents were removed to give crude compound **29** (5.13g y=100%). ^{1}H NMR (400 Hz, DMSO): δ 8.08 (d, J=7.6Hz, 1H), 7.96-7.92 (m, 1H),

4.37-4.29 (m, 1H), 4.23-4.16 (m, 1H), 3.58 (s, 3H), 2.33-2.26 (m, 2H), 2.16-2.08 (m, 2H), 1.55-1.43 (m, 4H), 1.27-1.21 (m, 3H), 1.17 (d, J=6.8Hz, 3H).

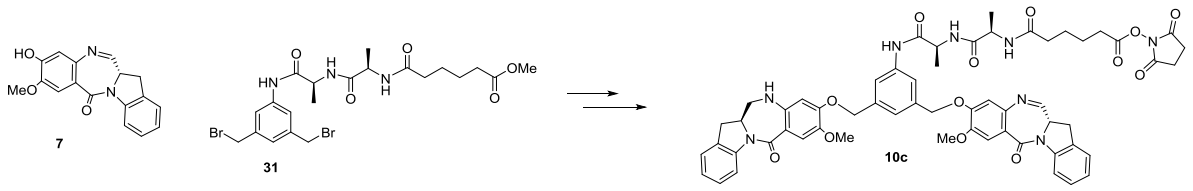


Compound **29** (5.13g, 16.97 mmol) was dissovled in anhydrous dichloromethane (108 ml) and anhydrous methanol (53.9 ml). (5-amino-1,3-phenylene)dimethanol (2.476 g, 16.16 mmol) and N-Ethoxycarbonyl-2-ethoxy-1,2-dihdroquinoline (7.99g, 32.3 mmol) were added and the reaction was stirred at room temperature, overnight. The solvent was stripped and the crude material purified by silica gel chromatography (dichloromethane/methanol) to give compound **30** (1.07g, y=15%) ^{1}H NMR (400 Hz, DMSO): δ 9.68 (s, 1H), 8.28, (d, J=7.6Hz, 1H), 8.11 (d, J=6.4Hz, 1H), 7.52 (s, 2H), 6.97 (s, 1H), 5.20-5.12 (m, 2H), 4.50-4.41 (m, 4H), 4.40-4.31 (m, 1H), 4.28-4.20 (m, 1H), 3.57 (s, 3H), 2.33-2.24 (m, 2H), 2.20-2.09 (m, 2H), 1.57-1.44 (m, 4H), 1.30 (d, J=7.2Hz, 3H), 1.20 (d, J=6.8Hz, 3H). LRMS (ESI): Calc'd for C21H31N3O7 (M + Na) 460.21, found 460.2.



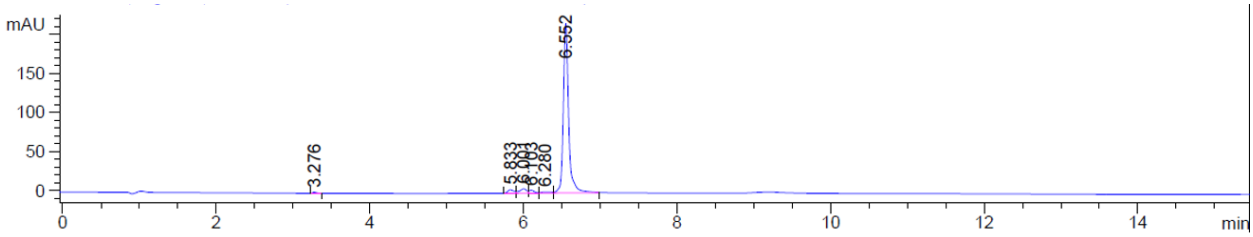
Compound **30** (1.07 g, 2.446 mmol) and carbon tetrabromide (2.433 g, 7.34 mmol) were dissolved in anhydrous *N*,*N*-Dimethylformamide (24.46 ml). Triphenylphosphine (1.924 g, 7.34mmol) was added and the reaction stirred under argon for four hours. It was diluted with dichloromethane/methanol (10:1) and washed with water and brine. It was dried over sodium sulfate, filtered, and concentrated to remove all sovents. The crude material was purified by silica gel chromatography (dichloromethane/methanol) to give compound **31** (411 mgs, y=30%).^{1}H NMR (400 Hz, DMSO): δ 9.82 (s, 1H), 8.38 (d, J=7.6Hz, 1H), 8.17 (d, J=6.4Hz, 1H), 7.77 (s, 1H), 7.76 (s, 1H), 7.22 (s, 1H), 4.66 (s, 4H), 4.38-4.31 (m, 1H)4,26-4.17 (m, 1H),

3.56 (s, 3H), 2.33-2.25 (m, 2H), 2.20-2.11 (m-2H), 1.58-1.45 (m, 4H), 1.32 (d, J=7.2HZ, 3H), 1.21 (d, J=7.2HZ, 3H).

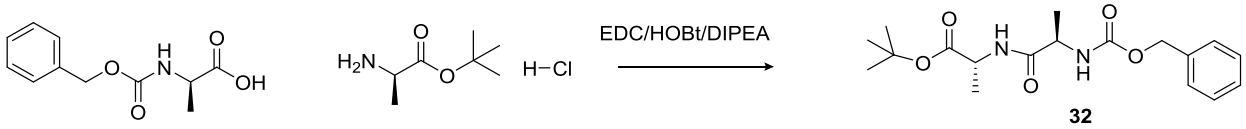


Compound **10c** was prepared from compounds **31** and **7** similarly as compound **10a** from compounds **6** and **7**. The crude material of the last step was purified via RPHPLC (C18 column, Acetonitrile/Water) to give compound **10c** (16.9 mg, 97% pure, y=31%). LCMS =5.4 min (8 min method). LRMS (ESI^+): Calc'd for C58H58N8O13 (M + 1) 1075.41, found 1075.90.

HPLC trace of purified 10c

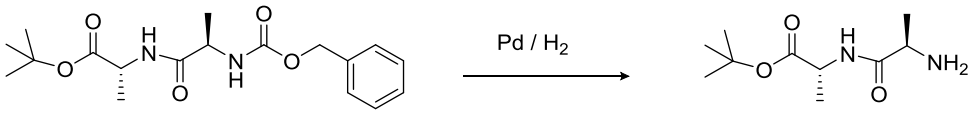


<u>Synthesis of D-ala-D-ala NHS ester 10d</u>



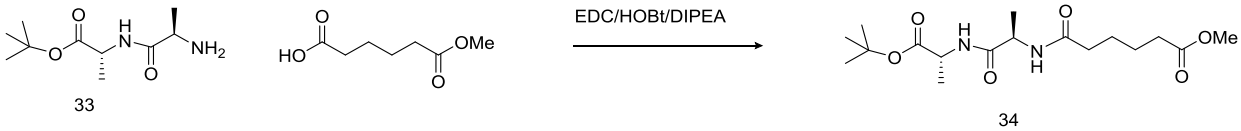
(R)-2-(((benzyloxy)carbonyl)amino)propanoic acid (7.6 g, 34.00 mmol) and (R)-tert-butyl 2-aminopropanoate hydrochloride (6.80 g, 37.50 mmol) were dissolved in anhydrous *N*,*N*-Dimethylformamide (68 ml). 3-(3-Dimethylaminopropyl)-1-ethyl-carbodiimide hydrochloride (7.18 g, 37.5 mmol), 1-Hydroxybenzotriazole hydrate (5.21 g, 34.00 mmol), and then diisopropylethylamine (14.83 ml, 85.0 mmol) were added. The reaction was stirred under argon, at room temperature, overnight. The reaction mixture was diluted with dichloromethane and then washed with saturated ammonium chloride, saturated sodium bicarbonate, water, and brine. The

organic was dried over sodium sulfate and concentrated. The crude oil was purified via silica gel chromatography (Hexanes/Ethyl Acetate) to yield compound **32** as a solid with some residual DMF, assumed 100% yield for next reaction. ^{1}H NMR (400 Hz, DMSO): δ 8.14 (d, J=6.8 Hz, 1H), 7.41-7.32 (m, 6H), 5.01 (s, 2H), 4.12-4.06 (m, 2H), 1.38 (s, 9H), 1.26-1.21 (m, 6H).



32 33

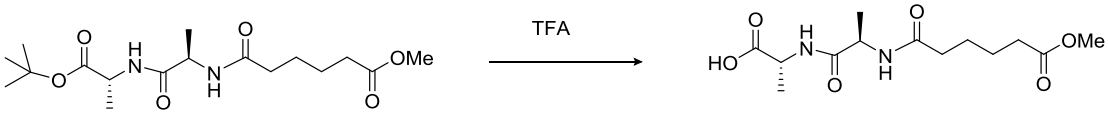
Compound **32** (R)-tert-butyl2-((R)-2-(((benzyloxy)carbonyl)amino)propanamido) propanoate (11.93g, 34.00 mmol) was dissolved in methanol (112 ml) and water (5.6 ml). The solution was purged with argon for five minutes. Palladium on carbon (wet, 10%) (1.87 g, 1.76 mmol) was added slowly. The reaction was stirred overnight under an atmosphere of hydrogen. The solution was filtered through celite, rinsed with methanol and concentrated. It was azeotroped with methanol and acetonitrile and the resulting oil was placed directly on the high vacuum to give compound **33** (7.08g, y=96%) which was used directly in the next step. ^{1}H NMR (400 Hz, DMSO): δ 8.05 (d, J=6.8Hz, 1H), 4.16-4.09 (m, 1H), 3.25 (q, J=6.8Hz, 1H), 1.81 (bs, 2H), 1.39 (s, 9H), 1.25 (d, J=7.2Hz, 3H), 1.12 (d, J=6.8Hz, 3H).



33 34

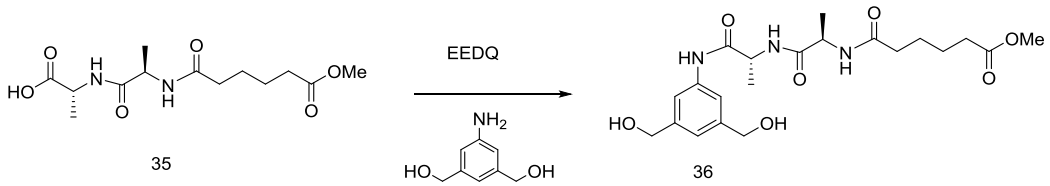
Compound **33** (7.08 g, 32.7 mmol) and monomethyladipate (5.34 ml, 36.0 mmol) were dissolved in anhydrous *N*,*N*-Dimethylformamide (109 ml). 3-(3-Dimethylaminopropyl)-1-ethyl-carbodiimide hydrochloride (6.9 g, 36.0 mmol) and 1-Hydroxybenzotriazole hydrate (5.01 g, 32.7 mmol) were added, followed by diispopropylethylamine (11.43 ml, 65.5 mmol). The mixture was stirred overnight at room temperature. The reaction was diluted with dichloromethane/methanol (300 mL, 5:1) and washed with saturated ammonium chloride, saturated sodium bicarbonate, and brine. It was dried over sodium sulfate, filtered and stripped. The compound was azeotroped with acetonitrile (5x), then pumped on the high vacuum at 35 °C to give compound **34,** assuming 100% yield. The crude material was taken onto the next step without purification. ^{1}H NMR (400 Hz, DMSO): δ 8.14 (d, J=76.8Hz, 1H), 7.96-7.93 (m, 1H),

4.34-4.27 (m, 1H), 4.13-4.05 (m, 1H), 3.58 (s, 3H), 2.31-2.28 (m, 2H), 2.12-2.08(m, 2H), 1.53-1.49 (m, 4H), 1.38 (s, 1H), 1.24 (d, J=7.2Hz, 3H), 1.19 (d, J=7.0Hz, 3H).



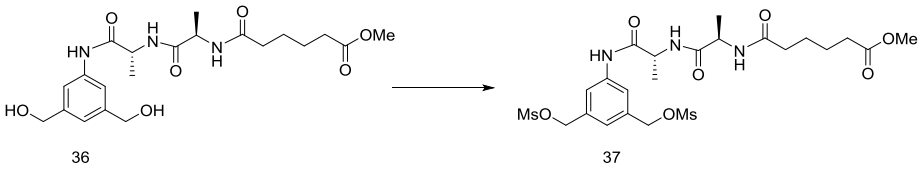
34 35

Compound **34**(11.72 g, 32.7 mmol) was stirred in trifluoroacetic acid (50.4 ml, 654 mmol) and deionized water (2.7ml) at room temperature for three hours. It was rotovaped with acetonitrile and then acetonitrile/water was added, material was frozen and lyophilized to give crude compound **35**. A third of the material was coevaporated with ACN and then DCM, and placed on high vacuum to remove all residual solvents and used in the next reaction without further purification. ^{1}H NMR (400 Hz, DMSO): δ 8.10 (d, J=7.2Hz, 1H), 7.94 (d, J=8Hz, 1H), 4.34-4.27 (m, 1H), 4.23-4.15 (m, 1H), 3.58 (s, 3H), 2.31-2.28 (m, 2H), 2.12-2.09 (m, 2H), 1.53-1.48 (m, 4H), 1.27 (d, J=7.2Hz, 3H), 1.18 (d, J=6.8Hz, 3H).



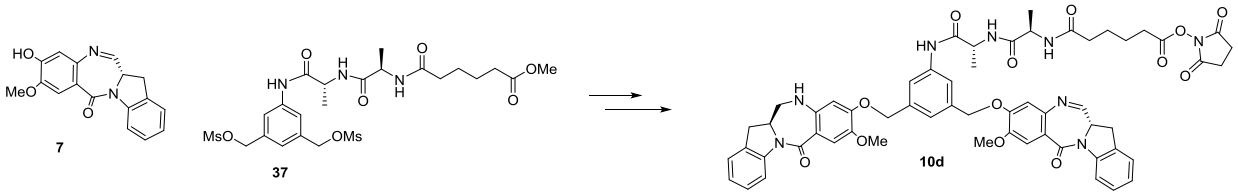
35 36

Compound **35** (5.75g, 19.02 mmol) was dissolved in anhydrous dichloromethane (121 ml) and anhydrous methanol (60ml). (5-amino-1,3-phenylene)dimethanol (2.77 g, 18.11 mmol) and N-Ethoxycarbonyl-2-ethoxy-1,2-dihdroquinoline (8.96g, 36.2 mmol) were added and the reaction was stirred at room temperature, overnight. The solvent was stripped and some of the crude material was purified by two silica gel chromatography columns (dichloromethane/methanol then ethyl acetate/methanol) to give compound **36** (760 mg) ^{1}H NMR (400 Hz, DMSO): δ 9.82 (s, 1H), 8.05-8.00 (m, 2H) J=7.6Hz, 1H), 7.46 (s, 2H), 6.95 (s, 1H), 5.16 (bs, 2H), 4.45 (s, 4H), 4.41-4.34 (m, 1H), 4.32-4.25 (m, 1H), 3.57 (s, 3H), 2.32-2.28 (m, 2H), 2.13-2.11 (m, 2H), 1.51-1.47 (m, 4H), 1.30 (d, J=7.2Hz, 3H), 1.20 (d, J=6.8Hz, 3H). LRMS (ESI^{+}): Calc'd for C21H31N3O7 (M + H) 438.21, found 438.2.



36 37

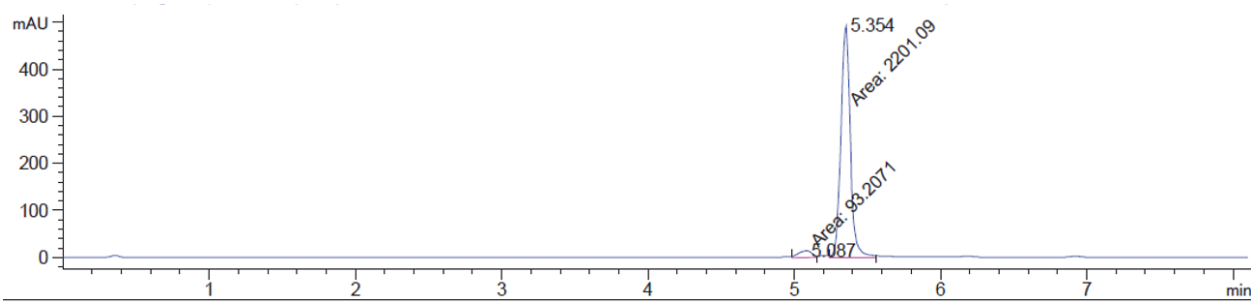
Compound **36** (760mg, 1.737 mmol) was suspended in anhydrous dichloromethane (11.58 ml). Anhydrous DMF was added slowly until most of the solids dissolved (~9 ml). The reaction was cooled to -10°C in an acetone/ice bath and then triethylamine (1.211 ml, 8.69 mmol) and methanesulfonic anhydride (780 mg, 4.34 mmol) were added. The reaction stirred for 1 hour and 30 minutes to give complete conversion of the SM. It was then quenched with ice water and extracted with cold 5% methanol in ethyl acetate twice. The organics were washed with ice water, dried over anhydrous sodium sulfate, filtered and concentrated to give compound **37** (898mgs, y=87%) as a white solid which was used in the next step without further purification. LRMS (ESI$^+$): Calc'd for C23H35N3O11S2 (M - H) 592.17, found 592.2.



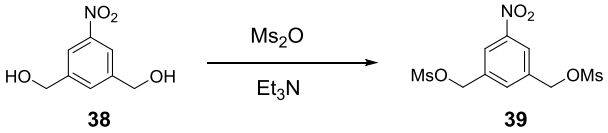
7 37 10d

Compound **10d** was prepared from compounds **37** and **7** similarly as compound **10a** from compounds **6** and **7**. The crude material of the last step was purified via RPHPLC (C18 column, Acetonitrile/Water) to give compound **10d** (63mg, y=45%). LRMS (ESI$^+$): Calc'd for C58H58N8O13 (M + 1) 1075.41, found 1075.30. LCMS = 5.3 min (8 min method).

HPLC trace of purified 10d

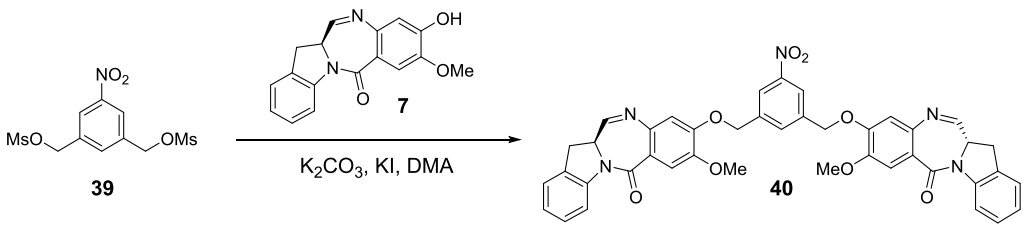


Synthesis of catabolite 13

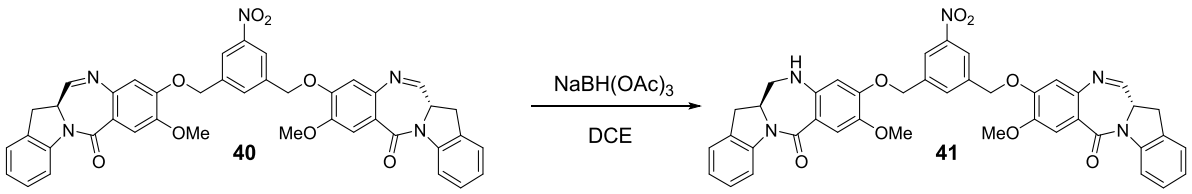


38 39

Compound **38** (1.34 g, 7.02 mmol) was dissolved in DMF (15.61 mL) and DCM (31.2 mL). The reaction mixture was cooled to -10 ºC in an acetone/ice bath. Et_3N (4.89 mL, 35.1 mmol) was added to the reaction mixture, followed by Ms_2O (3.15 g, 17.56 mmol). The reaction was stirred at -10 ºC for 2 h. The reaction mixture was quenched with ice water. The aqueous layer was extracted with EtOAc (2x). The organic layer was washed with H_2O (2x), dried over Na_2SO_4, filtered and concentrated. The crude product was dried under high vacuum for 2 h to give compound **39** as a white solid (2.32 g, 6.84 mmol, 97 % yield). LCMS = 4.481 min (8 min method). LRMS (ESI^+): Calc'd for C10H13NO8S23 (M + Na) 362.01, found 362.00.

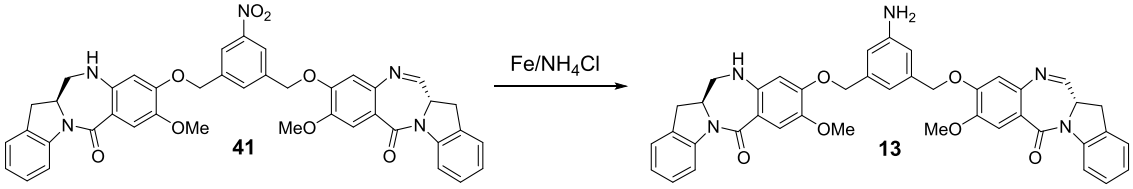


Compound **39** (2.32 g, 6.84 mmol) and compound **7** (4.23 g, 14.36 mmol) were dissolved in DMA (51 mL). K_2CO_3 (1.985 g, 14.36 mmol) and KI (0.568 g, 3.42 mmol) were added to the reaction mixture and was stirred at rt overnight. MeOH (~3 mL), sat'd NH_4Cl (~50 mL) and H_2O (~150 mL) were added to the reaction mixture and the resulting slurry was stirred for 5 min. The slurry was filtered and the solid was washed with H_2O. The solid was redissolved in DCM/MeOH (10:1) and was washed with H_2O, dried over Na_2SO_4, filtered and concentrated to give compound **40** (5.98 g, 6.10 mmol, 89% yield, 75% purity), which was taken onto the next step without purification. LCMS = 7.297 min (12 min method). LRMS (ESI^+): Calc'd for C42H33N5O8 (M + H) 736.23, found 736.5.

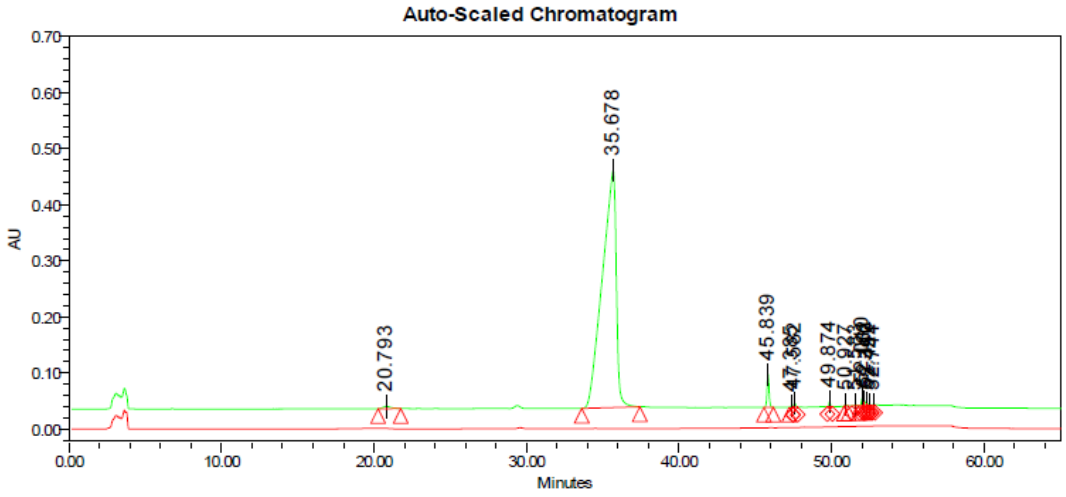


Compound **40** (779 mg, 1.059 mmol) was dissolved in DCE (0.749 mL). $NaBH(OAc)_3$ (225 mg, 1.059 mmol) was added to the reaction mixture and was stirred at rt under Ar for 1 h. The reaction mixture was quenched with MeOH and was stirred for 10 min. The solution was diluted with DCM, washed with sat'd NH_4Cl, brine, dried Na_2SO_4, filtered and concentrated.

The crude product was purified by reverse phase semi-prep HPLC (C18 column, ACN/H_2O). The fractions containing the desired product were frozen and lyophilized to obtain **41** as a white solid (86 mg, 0.117 mmol, 11% yield). LCMS = 6.118 min (8 min method). LRMS (ESI^+): Calc'd for C42H35N5O8 (M + H) 738.25, found 738.70.



Compound **41** (63.5 mg, 0.086 mmol) was dissolved in THF (1.5 mL), MeOH (0.500 mL) and H_2O (0.200 mL). NH_4Cl (46.0 mg, 0.861 mmol) was added to the reaction mixture, followed by iron powder (24.03 mg, 0.430 mmol). The reaction was heated at 50 °C overnight. The reaction mixture was cooled to rt and was diluted with DCM/MeOH (10:1) and was filtered through Celite. The filter cake was washed with DCM and was concentrated. The crude product was purified by reverse phase semi-prep HPLC (C18 column, ACN/H_2O). The fractions containing the desired product were frozen and lyophilized to obtain compound **7** as a white solid (39 mg, 0.052 mmol, 60% yield). HRMS (ESI^+): calc'd for C42H37N5O6 (M + H) 708.2817, found 708.2811.


Auto-Scaled Chromatogram
AU
Minutes
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.00
10.00
20.00
30.00
40.00
50.00
60.00
20.793
35.678
45.839
49.874

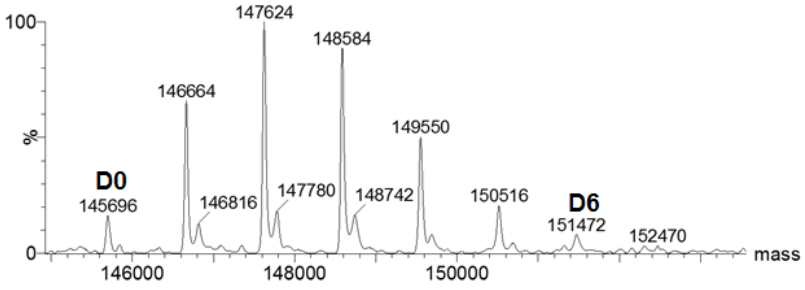
Preparation of anti-FRα ADCs 11a-d

A representative example is shown for anti-FRα **11a**. The remaining ADCs (anti-FRα **11b-d**) were prepared using identical reaction conditions.

A reaction containing 2.0 mg/mL anti-FRα antibody and 3.9 molar equivalents of NHS ester **10a** (pretreated with 5-fold excess of sodium bisulfite in 95:5 DMA:50 mM succinate pH 5.5 for 4 hours at 25 ºC) in 15 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) pH 8.5 buffer and 15% v/v DMA (*N*,*N*-Dimethylacetamide) co-solvent was incubated for 4 hours at 25 ºC. Post-reaction, the conjugate was purified and buffer exchanged into 10 mM succinate, 50 mM sodium chloride, 8.5% w/v sucrose, 0.01% Tween-20, 50 µM sodium bisulfite pH 6.2 formulation buffer using NAP desalting columns (Illustra Sephadex G-25 DNA Grade, GE Healthcare). Dialysis was performed in the same buffer for 4 hours at room temperature and then overnight at 4 ºC utilizing Slide-a-Lyzer dialysis cassettes (ThermoScientific 30,000 MWCO).

The purified conjugate was found to have a final protein concentration of 1.8 mg/mL and an average of 2.7 IGN molecules linked per antibody (by UV-Vis using molar extinction coefficients $\varepsilon_{330\,nm}$= 15,280 $cm^{-1}M^{-1}$ and $\varepsilon_{280\,nm}$= 30,115 $cm^{-1}M^{-1}$ for the IGN effector, and $\varepsilon_{280\,nm}$= 201,400 $cm^{-1}M^{-1}$ for anti-FRα antibody); 98.3% monomer (by size exclusion chromatography); and <1.1% unconjugated IGN effector (by acetone precipitation, reverse-phase HPLC analysis).

MS for deglycosylated anti-FRα **11a** Conjugate

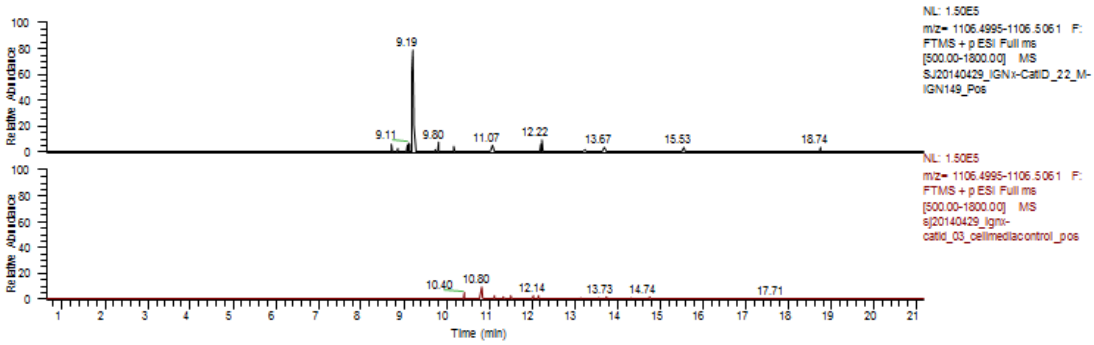


Catabolite enrichment by affinity capture with protein A resin

KB cells expressing FRα were cultured in 5 x T150 tissue culture plates. A saturating amount of anti-FRα **11a** was incubated with these KB cells for 24 hours at 37 °C in a humidified incubator buffered with 5% CO_2. After 24 hours, the media with egressed catabolites was harvested and pooled for assay. Saturating amount of anti-IGN antibody was bound to a slurry of protein A resins by overnight incubation at 4 °C. 1 mL of pre-bound protein A-Anti-IGN antibody complex was incubated with 25 mL of media on an end-to-end rotator for several hours. The resins were centrifuged gently at 1000 rpm, and the supernatant was decanted. The protein-A-anti-IGN antibody resins bound to IGN catabolites were washed with PBS. The catabolites were released into organic phase by acetone extraction. The catabolites were vacuum-dried overnight until the organic solution was completely evaporated. The catabolites were reconstituted with 20% acetonitrile in water, and analyzed by LC/MS.

Results:

anti-FRα **11b** extracted ion chromatogram (XIC): catabolite **12** top, blank on bottom



anti-FRα **11a** extracted ion chromatogram (XIC): catabolite **13** top, blank on bottom