

Tailored linker chemistries for the efficient and selective activation of ADCs with KSPi payloads

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General information

All reactants or reagents of which the preparation has not been described herein were purchased from generally accessible commercial sources and were used without further purification. For all other reactants or reagents for which the preparation likewise is not described herein and which were not commercially available or were obtained from sources which are not generally accessible, a reference is given to the published literature in which their preparation is described. Some of the compounds and intermediates are also described in a patent application or in previous publications of this group.^{S1,S2}

LC-MS analysis of the toxophores was performed using one of the following methods:

Method 1 (LC-MS): Instrument: Waters ACQUITY SQD UPLC system; column: Waters Acquity UPLC HSS T3 1.8 μ 50 x 1 mm; mobile phase A: 1 L of water + 0.25 mL of 99% strength formic acid; mobile phase B: 1 L of acetonitrile + 0.25 mL of 99% strength formic acid; gradient: 0.0 min 90% A \rightarrow 1.2 min 5% A \rightarrow 2.0 min 5% A oven: 50°C; flow rate: 0.40 mL/min; UV detection: 208 – 400 nm.

Method 2 (LC-MS): Instrument: Waters ACQUITY SQD UPLC system; column: Waters Acquity UPLC HSS T3 1.8 μ 50 x 1 mm; mobile phase A: 1 L of water + 0.25 mL of 99% strength formic acid; mobile phase B: 1 L of acetonitrile + 0.25 mL of 99% strength formic acid; gradient: 0.0 min 95% A \rightarrow 6.0 min 5% A \rightarrow 7.5 min 5% A oven: 50°C; flow rate: 0.35 mL/min; UV detection: 210–400 nm.

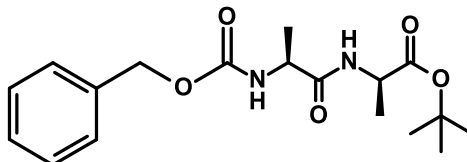
Method 3 (LC-MS): MS instrument type: Thermo Scientific FT-MS; instrument type UHPLC+: Thermo Scientific UltiMate 3000; column: Waters, HSST3, 2.1 x 75 mm, C18 1.8 μ m; mobile phase A: 1 L of water + 0.01% formic acid; mobile phase B: 1 L of acetonitrile + 0.01% formic acid; gradient: 0.0 min 10% B \rightarrow 2.5 min 95% B \rightarrow 3.5 min 95% B; oven: 50°C; flow rate: 0.90 mL/min; UV detection: 210 nm/ Optimum Integration Path 210–300 nm.

Method 4 (UPLC-HRMS): Instrument: Waters TOF; Instrument UPLC: Waters Acquity I-CLASS; column: Waters, HSST3, 2.1 x 50 mm, C18 1.8 μ m; mobile phase A: 1 L water + 0.01% formic acid; mobile phase B: 1 L acetonitrile + 0.01% formic acid; gradient: 0.0 min 2% B \rightarrow 0.5 min 2% B \rightarrow 7.5 min 95% B \rightarrow 10.0 min 95% B; oven: 50°C; flow rate: 1.00 mL/min; UV-detection: 210 nm.

1 Synthesis of peptidic precursors

Intermediate 1

tert-Butyl-*N*-[(benzyloxy)carbonyl]-L-alanyl-D-alaninate

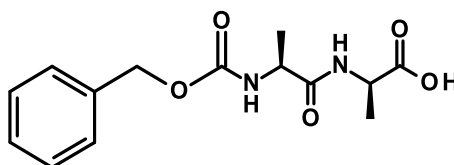


A solution of *N*-[(benzyloxy)carbonyl]-L-alanine (2.00 g, 8.96 mmol) in DMF (100 mL) was supplemented with *tert*-butyl-D-alaninate-hydrogen chloride (1.79 g, 9.86 mmol), *N,N*-diisopropylethylamine (4.2 mL, 24 mmol) and HATU (4.43 g, 11.6 mmol) and the reaction was stirred for 2 hours at room temperature. The mixture was concentrated to dryness under reduced pressure and the residue was purified by preparative HPLC to yield 1.44 g of the product.

LC-MS (Method 1): $R_t = 0.9$ min; MS (ESIpos): $m/z = 351(M+H)^+$.

Intermediate 2

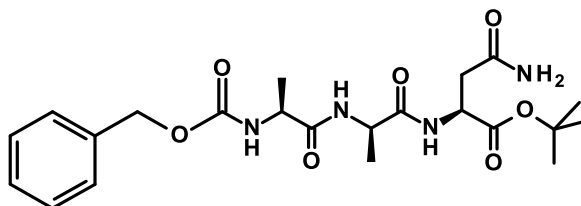
N-[(benzyloxy)carbonyl]-L-alanyl-D-alanine



Tert-butyl-*N*-[(benzyloxy)carbonyl]-L-alanyl-D-alaninate (3.12 g, 8.90 mmol) was diluted in dichloromethane (100 mL). Trifluoroacetic acid (25 mL) was added and the reaction was stirred for 6 hours at room temperature. The mixture was concentrated under reduced pressure and the residue was diluted in acetonitrile/water and lyophilized to yield 1.57 g of the product.

Intermediate 3

tert-Butyl *N*-[(benzyloxy)carbonyl]-L-alanyl-D-alanyl-L-asparaginate



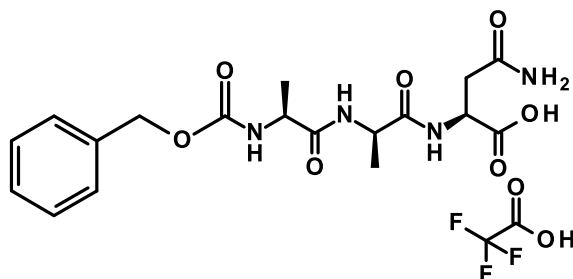
A solution of *N*-[(benzyloxy)carbonyl]-L-alanyl-D-alanine (2.62 g, 8.90 mmol) in DMF (150 mL) was supplemented with *tert*-butyl L-asparaginate (1.84 g, 9.79 mmol), *N,N*-diisopropylethylamine (4.7 mL, 27 mmol) and HATU (4.40 g, 11.6 mmol) and the reaction was

stirred at room temperature for 1 hour. The mixture was concentrated to dryness under reduced pressure and the residue was purified by preparative HPLC to yield 3.8 g of the product.

LC-MS (Method 1): $R_t = 0.73$ min; MS (ESIpos): $m/z = 465$ (M+H)+.

Intermediate 4

N-[(benzyloxy)carbonyl]-L-alanyl-D-alanyl-L-asparagine—trifluoroacetic acid salt

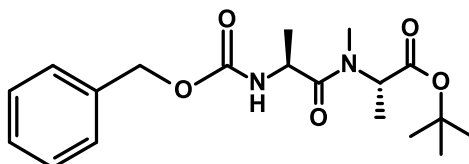


Tert-butyl-*N*-[(benzyloxy)carbonyl]-L-alanyl-D-alanyl-L-asparaginate (600 mg, 1.29 mmol) was diluted in dichloromethane (20 mL). Trifluoroacetic acid (15 mL) was added and the reaction was stirred for 90 minutes at room temperature. The mixture was concentrated to dryness under reduced pressure and the residue was purified by preparative HPLC to yield 238 mg of the product.

LC-MS (Method 1): $R_t = 0.53$ min; MS (ESIpos): $m/z = 409$ (M+H)+.

Intermediate 5

tert-Butyl-*N*-[(benzyloxy)carbonyl]-L-alanyl-*N*-methyl-L-alaninate

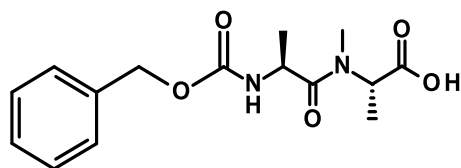


A solution of *N*-[(benzyloxy)carbonyl]-L-alanine (750 mg, 3.36 mmol) in DMF (25 mL) was supplemented with *tert*-butyl *N*-methyl-L-alaninate hydrogen chloric acid salt (723 mg, 3.70 mmol), *N,N*-diisopropylethylamine (1.6 mL, 9.1 mmol) and HATU (1.66 g, 4.37 mmol) and the reaction was stirred at room temperature for 30 minutes. The mixture was concentrated to dryness under reduced pressure and the residue was purified by preparative HPLC to yield 1.14 g of the product.

LC-MS (Method 1): $R_t = 1.01$ min; MS (ESIpos): $m/z = 364$ (M+H)+.

Intermediate 6

N-[(benzyloxy)carbonyl]-L-alanyl-*N*-methyl-L-alanine

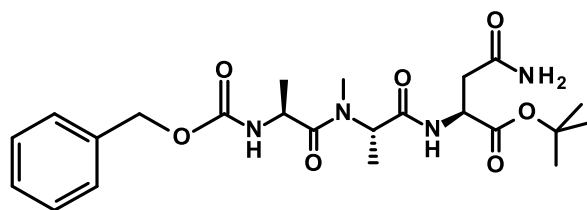


Tert-butyl-*N*-[(benzyloxy)carbonyl]-L-alanyl-*N*-methyl-L-alaninate (1.14 g, 3.11 mmol) was diluted in dichloromethane (20 mL). Trifluoroacetic acid (20 mL) was added and the reaction was stirred for 1 hour at room temperature. The mixture was concentrated under reduced pressure and the residue was diluted in acetonitrile/water and lyophilized to yield 1.16 g of the product.

LC-MS (Method 1): R_t = 0.68 min; MS (ESIpos): m/z = 308 (M+H)+.

Intermediate 7

tert-Butyl *N*-[(benzyloxy)carbonyl]-L-alanyl-*N*-methyl-L-alanyl-L-asparaginate

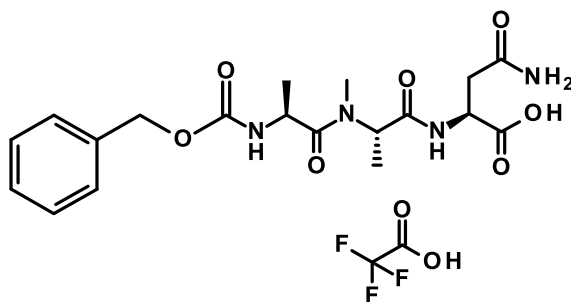


A solution of *N*-[(benzyloxy)carbonyl]-L-alanyl-*N*-methyl-L-alanine (592 mg, 1.92 mmol) in DMF (32 mL) was supplemented with *tert*-butyl L-asparaginate (398 mg, 2.11 mmol), *N,N*-diisopropylethylamine (1.0 mL, 5.8 mmol) and HATU (949 mg, 2.50 mmol), and the reaction was stirred for 1 hour at room temperature. The mixture was concentrated to dryness under reduced pressure and the residue was purified by preparative HPLC to yield 898 mg of the product.

LC-MS (Method 1): R_t = 0.75 min; MS (ESIpos): m/z = 478 (M+H)+.

Intermediate 8

N-[(benzyloxy)carbonyl]-L-alanyl-*N*-methyl-L-alanyl-L-asparagine

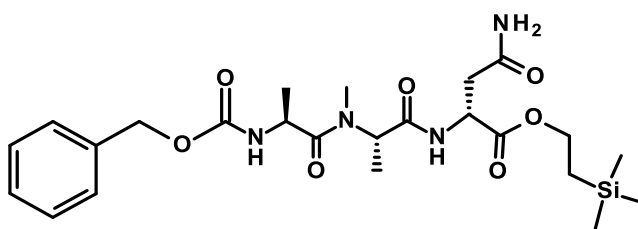


Tert-butyl-*N*-[(benzyloxy)carbonyl]-*L*-alanyl-*N*-methyl-*L*-alanyl-*L*-asparaginate (2.81 g, 5.88 mmol) was diluted in dichloromethane (66 mL). Trifluoroacetic acid (33 mL) was added and the reaction was stirred for 3 hours at room temperature. The mixture was concentrated to dryness under reduced pressure and the residue was purified by preparative HPLC to yield 848 mg of the product.

LC-MS (Method 1): R_t = 0.57 min; MS (ESIpos): m/z = 422 ($M+H$)⁺.

Intermediate 9

2-(trimethylsilyl)ethyl *N*-[(benzyloxy)carbonyl]-*L*-alanyl-*N*-methyl-*L*-alanyl-*D*-asparaginate

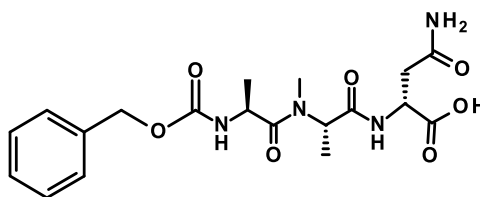


A solution of *N*-[(benzyloxy)carbonyl]-*L*-alanyl-*N*-methyl-*L*-alanine (86.0 mg, 279 μ mol) in DMF (25 mL) was supplemented with acetic acid-2-(trimethylsilyl)ethyl *D*-asparaginate (680 μ L, 330 μ mol), *N,N*-diisopropylethylamine (150 μ L, 840 μ mol) and HATU (127 mg, 335 μ mol), and the reaction was stirred for 1 hour at room temperature. The mixture was concentrated to dryness under reduced pressure and the residue was purified by preparative HPLC to yield 898 mg of the product.

LC-MS (Method 1): R_t = 0.94 min; MS (ESIpos): m/z = 523 ($M+H$)⁺.

Intermediate 10

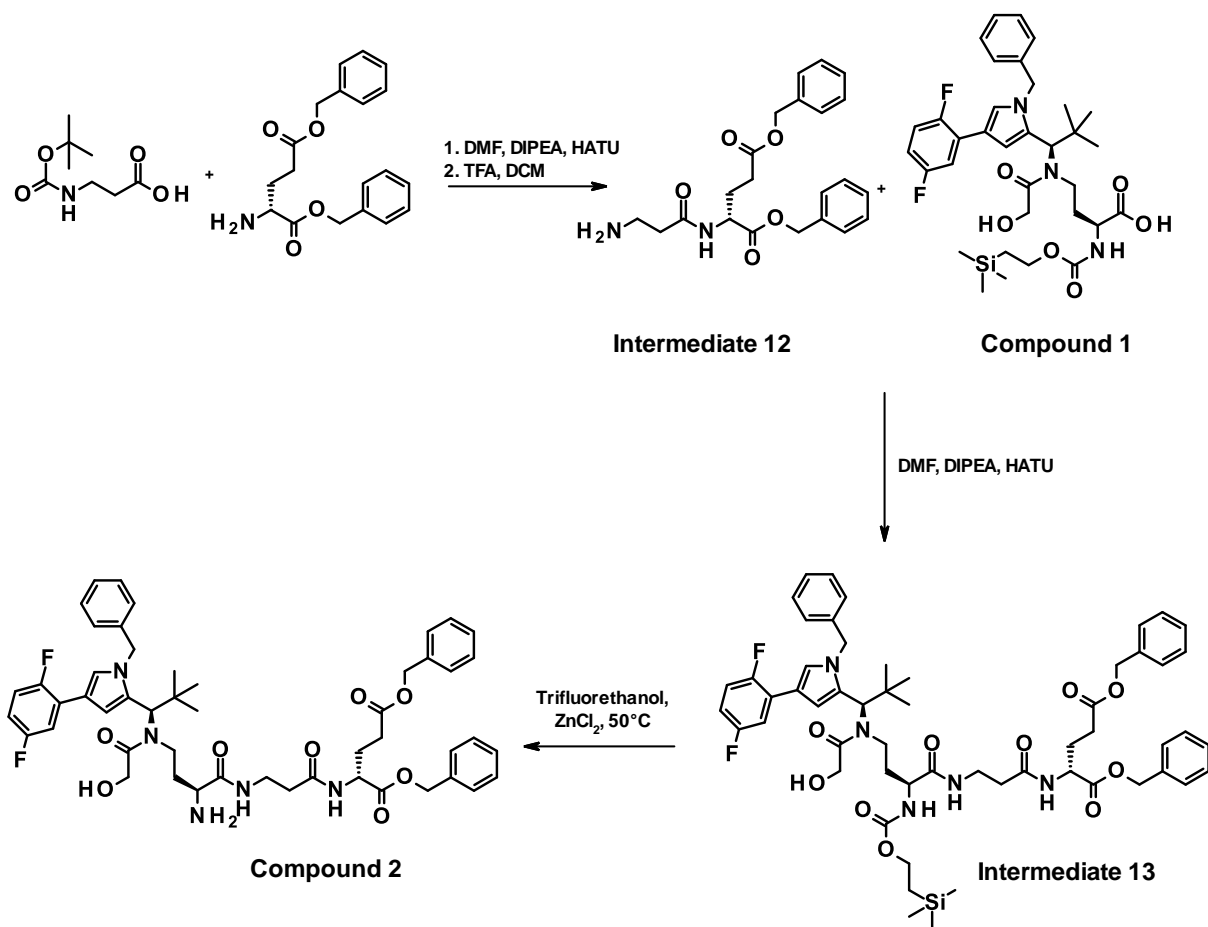
N-[(benzyloxy)carbonyl]-*L*-alanyl-*N*-methyl-*L*-alanyl-*D*-asparagine



2-(trimethylsilyl)ethyl *N*-[(benzyloxy)carbonyl]-*L*-alanyl-*N*-methyl-*L*-alanyl-*D*-asparaginate (45.0 mg, 86.1 μ mol) was dissolved in 5 mL of 2,2,2-trifluoroethanol. Zinc chloride (70 mg, 517 μ mol) was added and the reaction was stirred at 50 °C for 3 hours. The mixture was diluted with water and 151 mg (517 μ mol) ethylene diamine-*N,N,N,N*-tetraacetic acid was added. The mixture was stirred for a few minutes. The reaction was concentrated under reduced pressure and the residue was purified by preparative HPLC to yield 10.5 mg of the product.

LC-MS (Method 1): R_t = 0.58 min; MS (ESIpos): m/z = 423 [$M+H$]⁺

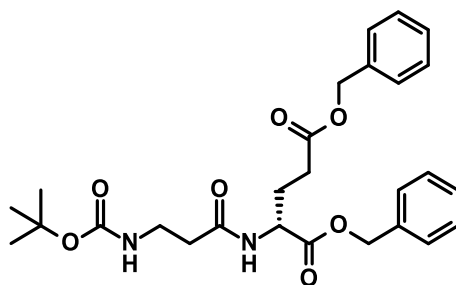
2 Synthesis of Compound 2



Scheme S1: Synthesis of Compound 2

Intermediate 11

Dibenzyl *N*-(*tert*-butoxycarbonyl)-beta-alanyl-D-glutamate

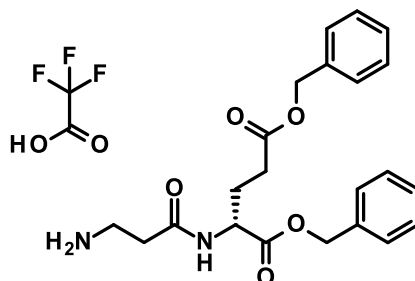


A solution of dibenzyl-D-glutamate (3.78 g, 11.5 mmol) and *N*-(*tert*-butoxycarbonyl)-beta-alanine (2.40 g, 12.7 mmol) in DMF (75 mL) was supplemented with *N,N*-diisopropylethylamine (6.0 mL, 35 mmol) and HATU (5.27 g, 13.9 mmol), and the reaction was stirred for 1 hour at room temperature. The reaction was concentrated to dryness under reduced pressure, and the residue was purified by preparative HPLC to yield 7.00 g of the product.

LC-MS (Method 1): Rt = 1.10 min; MS (ESIpos): m/z = 498 (M+H)+.

Intermediate 12

Dibenzyl beta-alanyl-D-glutamate

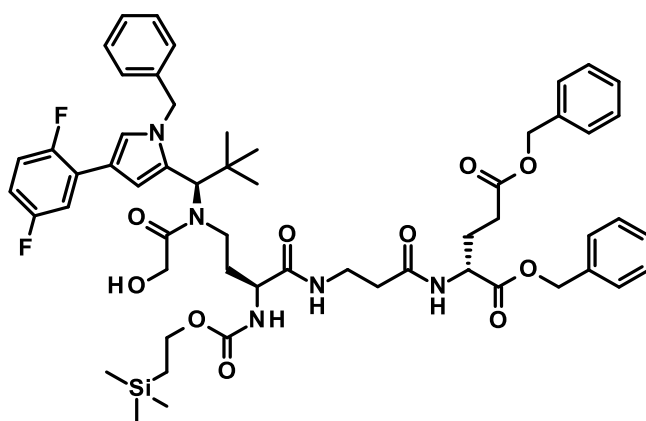


A solution of dibenzyl *N*-(*tert*-butoxycarbonyl)-beta-alanyl-D-glutamate (7.50 g, 14.2 mmol) in dichloromethane (70 mL) was supplemented with trifluoroacetic acid (50 mL), and the reaction was stirred for 1 hour at room temperature. The mixture was then concentrated to dryness under reduced pressure, and the residue was suspended in diethyl ether and pentan and the mixture was filtrated. The residue was dissolved in acetonitrile/water (1:1) and the mixture was lyophilized to yield 6.5 g of the product.

LC-MS (Method 1): Rt = 0.66 min; MS (ESIpos): m/z = 398 (M+H)+.

Intermediate 13

Dibenzyl *N*-[[(2*S*)-4-[[[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-2-[[2-(trimethylsilyl)ethoxy]carbonyl]amino)butanoyl]-β-alanyl-D-glutamate



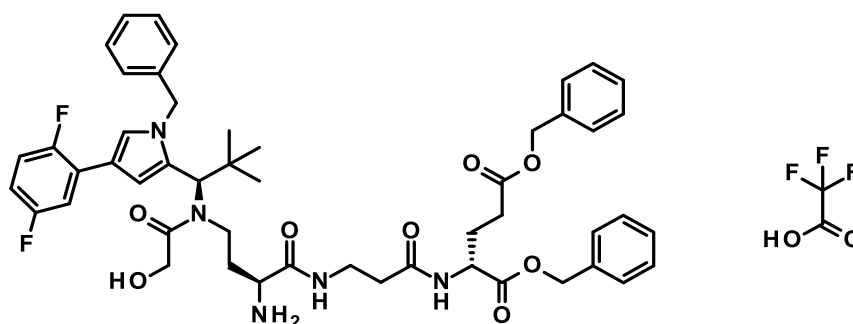
The solution of (2S)-4-[(1R)-1-[1-benzyl-4-(2,5-difluorophenyl)-1H-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-2-([2-(trimethylsilyl)ethoxy]carbonyl)amino)butanoic acid^{S2} (500 mg, 760 µmol) in DMF (75 mL) was supplemented with dibenzyl beta-alanyl-D-glutamate (584 mg, 1.14 mmol), *N,N*-diisopropylethylamine (400 µL, 2.3 mmol) and HATU (434 mg, 1.14 mmol). The reaction was stirred at room temperature for 1 h and then concentrated to

dryness under reduced pressure. The residue was purified by preparative HPLC to yield 552 mg of the product.

LC-MS (Method 1): $R_t = 1.54$ min; MS (ESIpos): $m/z = 1038$ (M+H)+.

Compound 2

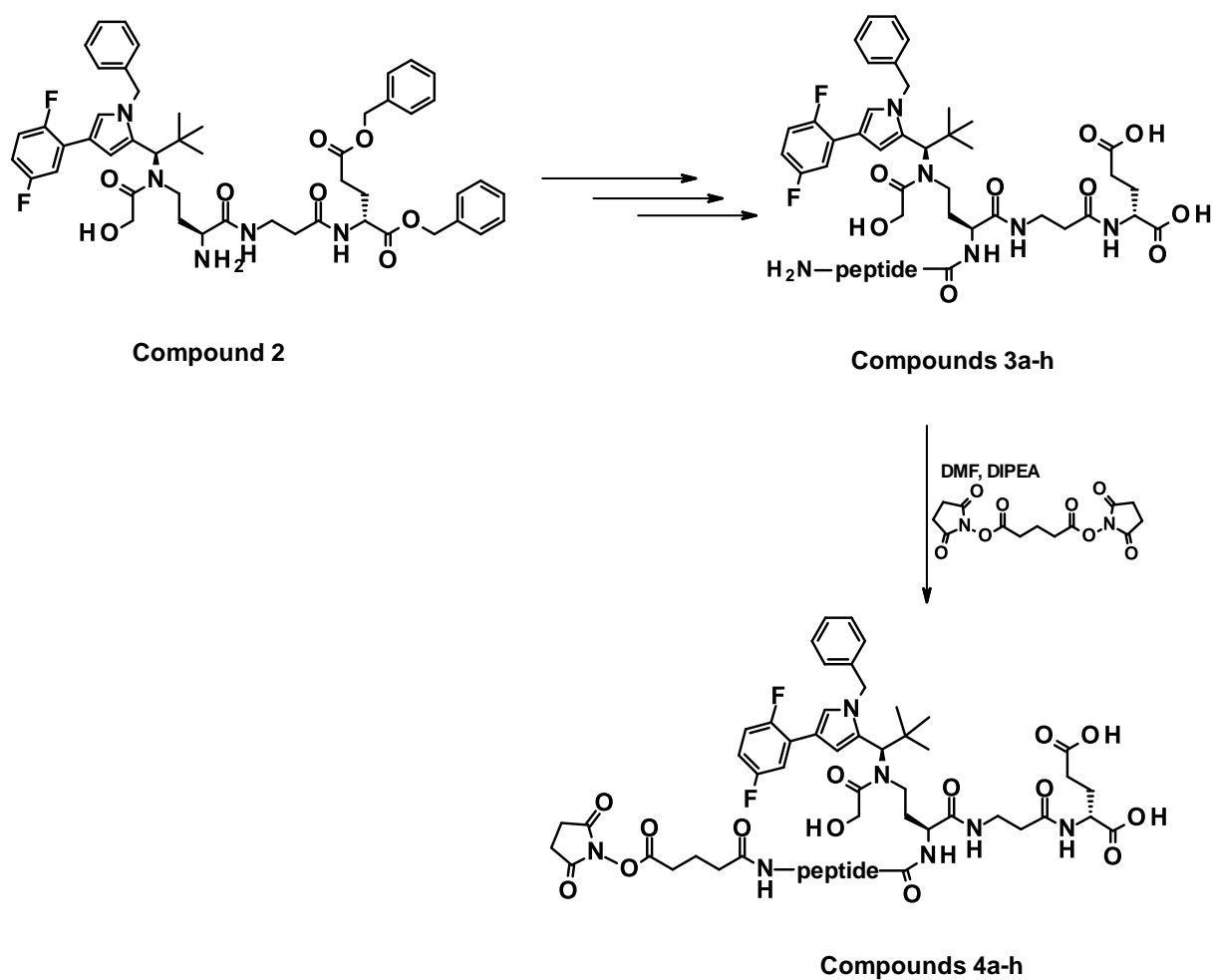
Dibenzyl *N*-{(2*S*)-2-amino-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]butanoyl}-beta-alanyl-D-glutamate trifluor acetic acid salt



Dibenzyl *N*-[(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl} (hydroxyacetyl)amino]-2-([2-(trimethylsilyl)ethoxy]carbonyl)amino]butanoyl]-beta-alanyl-D-glutamate (553 mg, 533 μ mol) was dissolved in 15 mL of 2,2,2-trifluoroethanol. Zinc chloride (871 mg, 6.39 mmol) was added, and the reaction was stirred at 50 °C for 2 hours. The mixture was diluted with water/acetonitrile (1:1), supplemented with 1.87 g (6.39 mmol) of ethylene diamine-*N,N,N,N*-tetraacetic acid and stirred for a few minutes. The reaction was concentrated under reduced pressure and the residue was purified by preparative HPLC. Concentration of the appropriate fractions and lyophilization of the residue from acetonitrile/water yielded 371 mg of the product.

LC-MS (Method 1): $R_t = 1.02$ min; MS (ESIpos): $m/z = 894$ [M+H]⁺

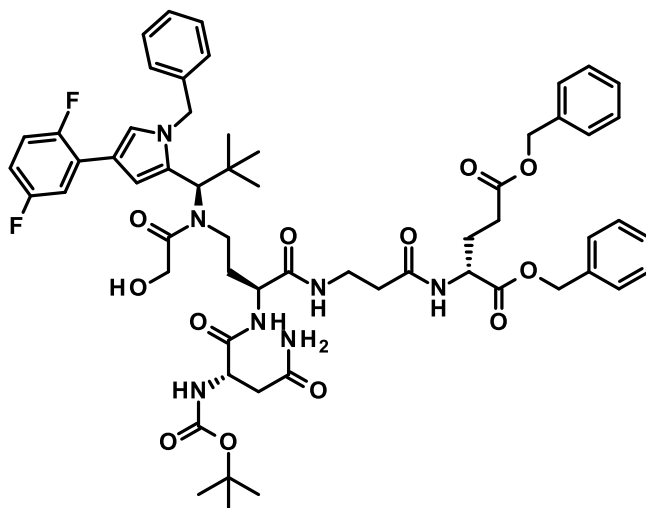
3 Synthesis of ADCs precursors



Scheme S2: Synthesis of Compound 4a-h

Intermediate 14

Dibenzyl *N*-[(2*S*)-4-[[[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-2-[[*N*²-(*tert*-butoxycarbonyl)-*L*-asparaginy]amino]butanoyl]- β -alanyl-D-glutamate

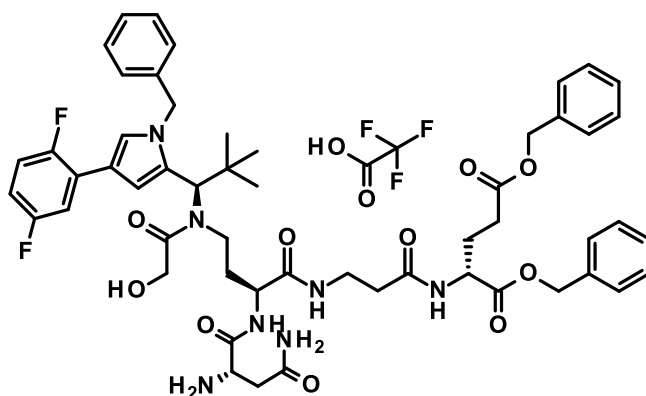


Dibenzyl *N*-{(2*S*)-2-amino-4-[[[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]butanoyl}- β -alanyl-D-glutamate (500 mg, 515 μ mol) and 2,5-dioxopyrrolidin-1-yl *N*²-(*tert*-butoxycarbonyl)-*L*-asparaginate (254 mg, 772 μ mol) were dissolved in DMF (20 mL) and the mixture was supplemented with *N,N*-diisopropylethylamine (270 μ L, 1.5 mmol). The reaction was stirred overnight and the mixture was concentrated to dryness under reduced pressure. The residue was purified by preparative HPLC to yield 516 mg of the product.

LC-MS (Method 1): R_t = 1.35 min; MS (ESIpos): m/z = 1108 $[M+H]^+$

Intermediate 15

Dibenzyl *N*-{(2*S*)-2-(*L*-asparaginylamino)-4-[[[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]butanoyl}- β -alanyl-D-glutamate

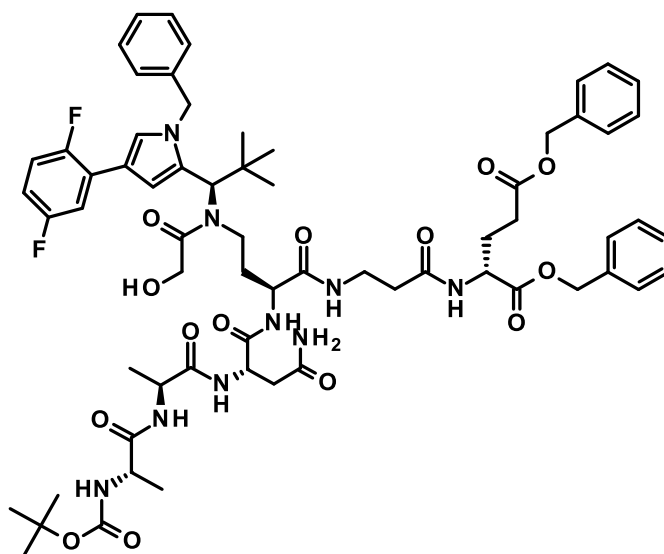


Dibenzyl *N*-[(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]-2-[[*N*²-(*tert*-butoxycarbonyl)-*L*-asparaginy]amino]butanoyl]- β -alanyl-D-glutamate (1.02 g, 917 μ mol) was dissolved in 20 mL of 2,2,2-trifluoroethanol. Zinc chloride (750 mg, 5.50 mmol) of were added, and the reaction was stirred at 50 °C for 30 minutes. The mixture was diluted with 10 mL water/acetonitrile (1:1) and supplemented with 1.61 g (5.50 mmol) of ethylene diamine-*N,N,N,N*-tetraacetic acid. The mixture was stirred for a few minutes. The reaction was concentrated under reduced pressure and the residue was purified by preparative HPLC. Concentration of the appropriate fractions and lyophilization yielded 745 mg of the product.

LC-MS (Method 1): R_t = 1.05 min; MS (ESIpos): m/z = 1008 [M+H]⁺

Intermediate 16

Dibenzyl (2*R*)-2-[[[(6*S*,9*S*,12*S*,15*S*)-12-(2-amino-2-oxoethyl)-15-{2-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]ethyl]-2,2,6,9-tetramethyl-4,7,10,13,16,20-hexaoxo-3-oxa-5,8,11,14,17-pentaazaicosan-20-yl]amino]pentanedioate

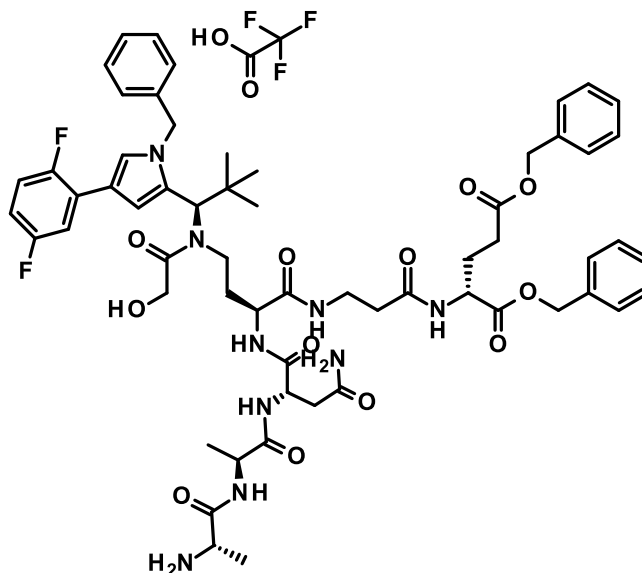


A solution of dibenzyl *N*-{(2*S*)-2-(*L*-asparaginylamino)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]butanoyl}- β -alanyl-D-glutamate (100 mg, 89.1 μ mol) in DMF (10 mL) was supplemented with dibenzyl *N*-(*tert*-butoxycarbonyl)-*L*-alanyl-*L*-alanine (27.8 mg, 107 μ mol), *N,N*-diisopropylethylamine (62 μ L, 360 μ mol) and HATU (40.7 mg, 107 μ mol) and the reaction was stirred for 10 minutes at room temperature. The mixture was concentrated to dryness under reduced pressure and the residue was purified by preparative HPLC to yield 99 mg of the product.

LC-MS (Method 1): R_t = 1.30 min; MS (ESIpos): m/z = 1250 (M+H)⁺.

Intermediate 17

Dibenzyl (2R)-2-[[[(6S,9S,12S,15S)-15-amino-9-(2-amino-2-oxoethyl)-6-{2-[[[(1R)-1-[1-benzyl-4-(2,5-difluorophenyl)-1H-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]ethyl}-12-methyl-5,8,11,14-tetraoxo-4,7,10,13-tetraazahexadecan-1-oyl]amino}pentanedioate

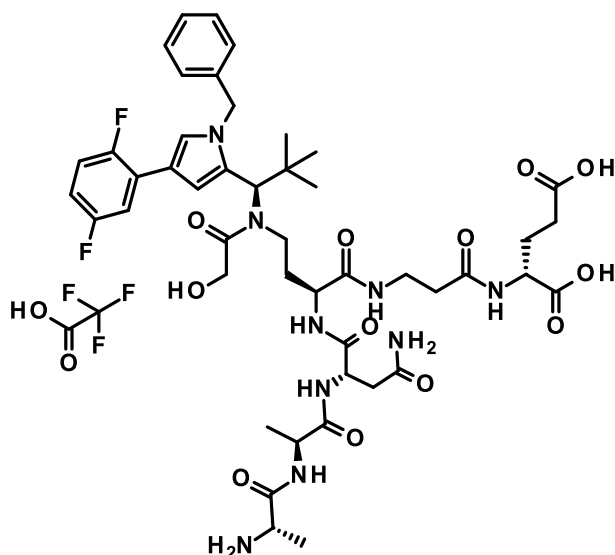


Dibenzyl (2R)-2-[[[(6S,9S,12S,15S)-12-(2-amino-2-oxoethyl)-15-{2-[[[(1R)-1-[1-benzyl-4-(2,5-difluorophenyl)-1H-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]ethyl}-2,2,6,9-tetramethyl-4,7,10,13,16,20-hexaoxo-3-oxa-5,8,11,14,17-pentaazaicosan-20-yl]amino}pentanedioate (99.0 mg, 79.2 μ mol) was dissolved in 10 mL of 2,2,2-trifluoroethanol. Zinc chloride (64.7 mg, 475 μ mol) was added, and the reaction was stirred at 50 °C for 30 minutes. The mixture was diluted with 10 mL water and supplemented with 200 μ L of TFA and 139 mg (475 μ mol) of ethylene diamine-*N,N,N,N*-tetraacetic acid. The mixture was stirred for a few minutes. The reaction was filtrated and the filtrate was concentrated under reduced pressure. The residue was purified by preparative HPLC. The appropriate fractions were concentrated and lyophilized to yield 86 mg of the product.

LC-MS (Method 1): R_t = 1.05 min; MS (ESIpos): m/z = 1150 $[M+H]^+$

Compound 3a

L-alanyl-L-alanyl-*N'*-{[(2S)-4-[[[(1R)-1-[1-benzyl-4-(2,5-difluorophenyl)-1H-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-1-[(3-[[[(1R)-1,3-dicarboxypropyl]amino]-3-oxopropyl]amino]-1-oxobutan-2-yl]-L-aspartamide—trifluoroacetic acid salt

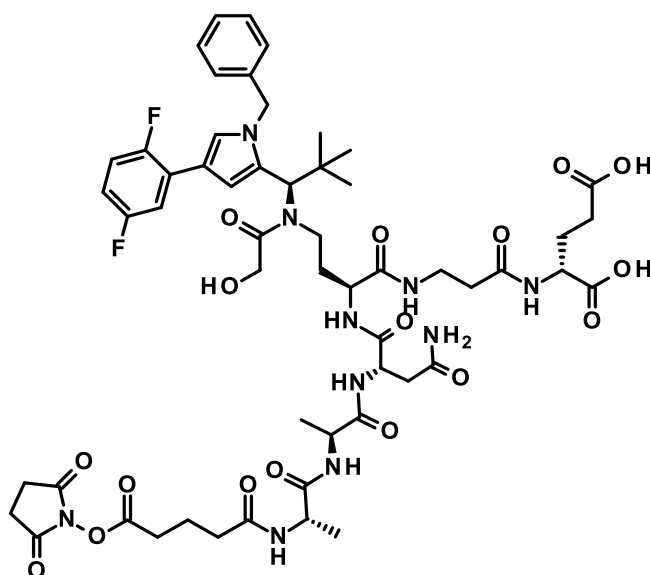


A solution of dibenzyl (2R)-2-[[[(6S,9S,12S,15S)-15-amino-9-(2-amino-2-oxoethyl)-6-{2-[[[(1R)-1-[1-benzyl-4-(2,5-difluorophenyl)-1H-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]ethyl}-12-methyl-5,8,11,14-tetraoxo-4,7,10,13-tetraazahexadecanan-1-oyl]amino]pentanedioate (40.0 mg, 31.6 μ mol) in ethanol (10 mL) was supplemented with palladium on charcoal (10%, 5 mg). The mixture was hydrogenated at ambient pressure for 1 hour. The solids were filtered off and the residue was washed several times with acetonitrile/water (1:1). The filtrate was concentrated under reduced pressure and the remaining residue was lyophilized to yield 34 mg of the title product.

LC-MS (Method 1): R_t = 0.76 min; MS (ESIpos): m/z = 970 $[M+H]^+$

Compound 4a

N-{5-[(2,5-dioxopyrrolidin-1-yl)oxy]-5-oxopentanoyl}-L-alanyl-L-alanyl-*N*¹-{(2S)-4-[[[(1R)-1-[1-benzyl-4-(2,5-difluorophenyl)-1H-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-1-[(3-[[[(1R)-1,3-dicarboxypropyl]amino]-3-oxopropyl]amino]-1-oxobutan-2-yl]-L-aspartamide



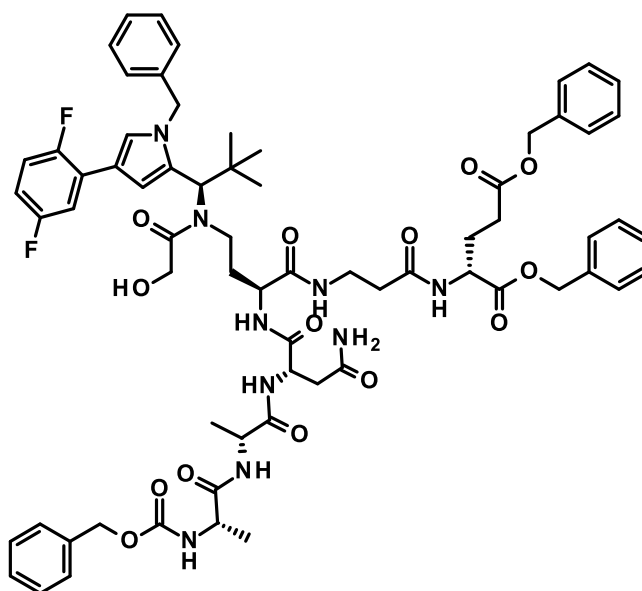
L-alanyl-L-alanyl-*N*¹-{(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]-1-[(3-[(1*R*)-1,3-dicarboxypropyl]amino)-3-oxopropyl]amino]-1-oxobutan-2-yl)-L-aspartamide) (34.0 mg, 31.4 μ mol) and 1,1'-[(1,5-dioxopentane-1,5-diyl)bis(oxy)]di(pyrrolidine-2,5-dione) (12.3 mg, 37.6 μ mol) were dissolved in DMF (3 mL) and *N,N*-diisopropylethylamine (11 μ L, 63 μ mol) was added. The reaction was stirred for 30 minutes. The mixture was concentrated under reduced pressure and the residue was purified by preparative HPLC. Concentration of the appropriate fractions and lyophilization yielded 18 mg of the product.

LC-MS (Method 1): R_t = 0.93 min; MS (ESIpos): m/z = 1203 $[M+Na]^+$

HPLC-HRMS (Method 4): R_t = 0.94 min; MS (ESI_{neg}): m/z : calcd for $C_{55}H_{69}N_{10}O_{17}F_2$ $[M-H]^-$: 1179.4810; found: 1179.4827 $(M-H)^-$.

Intermediate 18

Dibenzyl (2R)-2-[[[(5S,8R,11S,14S)-11-(2-amino-2-oxoethyl)-14-{2-[[[(1R)-1-[1-benzyl-4-(2,5-difluorophenyl)-1H-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]ethyl}-5,8-dimethyl-3,6,9,12,15,19-hexaoxo-1-phenyl-2-oxa-4,7,10,13,16-pentaazanonadecan-19-yl]amino}pentanedioate

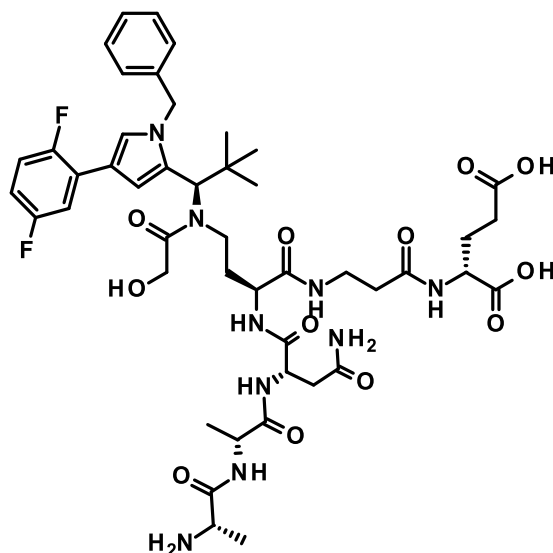


A solution of dibenzyl *N*-{(2*S*)-2-amino-4-[[[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]butanoyl}-β-alanyl-D-glutamate (80.0 mg, 78.4 μmol) in DMF (10 mL) was supplemented with *N*-[(benzyloxy)carbonyl]-L-alanyl-D-alanyl-L-asparagine (51.5 mg, 94.1 μmol), *N,N*-diisopropylethylamine (41 μL, 240 μmol) and HATU (44.7 mg, 118 μmol) and the reaction was stirred for 1 hour at room temperature. The mixture was concentrated to dryness under reduced pressure and the residue was purified by preparative HPLC to yield 73 mg of the product.

LC-MS (Method 1): $R_t = 1.33$ min; MS (ESIpos): $m/z = 1284$ $[M+H]^+$

Compound 3b

L-alanyl-D-alanyl-*N*¹-{(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]-1-[(3-[(1*R*)-1,3-dicarboxypropyl]amino)-3-oxopropyl]amino]-1-oxobutan-2-yl}-L-aspartamide

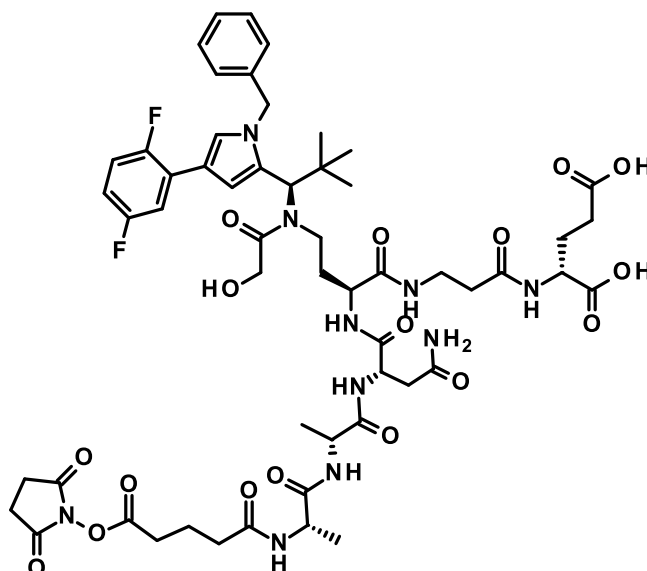


A solution of dibenzyl (2*R*)-2-[(5*S*,8*R*,11*S*,14*S*)-11-(2-amino-2-oxoethyl)-14-{2-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]ethyl)-5,8-dimethyl-3,6,9,12,15,19-hexaoxo-1-phenyl-2-oxa-4,7,10,13,16-pentaazononadecan-19-yl]amino}pentanedioate (73.0 mg, 56.8 μ mol) in ethanol (20 mL) was supplemented with palladium on charcoal (10%, 7 mg). The mixture was hydrogenated at ambient pressure for 1 hour. The solids were filtered off and the residue was washed several times with acetonitrile/water (1:1). The filtrate was concentrated under reduced pressure and the remaining residue was lyophilized to give 34 mg of the title product.

LC-MS (Method 1): R_t = 0.77 min; MS (ESIpos): m/z = 970 $[M+H]^+$

Compound 4b

N-{5-[(2,5-dioxopyrrolidin-1-yl)oxy]-5-oxopentanoyl}-L-alanyl-D-alanyl-*N*¹-{(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]-1-[(3-[(1*R*)-1,3-dicarboxypropyl]amino)-3-oxopropyl]amino]-1-oxobutan-2-yl}-L-aspartamide



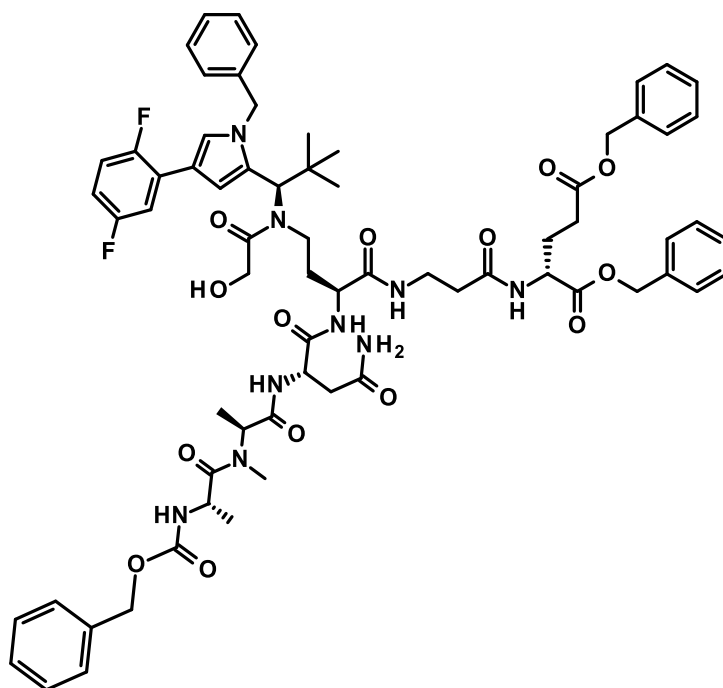
L-alanyl-D-alanyl-*N*¹-{(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]-1-[(3-[(1*R*)-1,3-dicarboxypropyl]amino)-3-oxopropyl]amino]-1-oxobutan-2-yl]-L-aspartamide (12 mg, 12.4 μ mol) and 1,1'-[(1,5-dioxopentane-1,5-diyl)bis(oxy)]di(pyrrolidine-2,5-dione) (10 mg, 30.9 μ mol) were dissolved in DMF (8 mL) and *N,N*-diisopropylethylamine (7.8 μ L, 45 μ mol) was added. The mixture was stirred for 1 hour. The mixture was concentrated under reduced pressure and the residue was purified by preparative HPLC. Concentration of the appropriate fractions and lyophilization yielded 8 mg of the product.

LC-MS (Method 1): R_t = 0.94 min; MS (ESIpos): m/z = 1180 $[M+Na]^+$

HPLC-HRMS (Method 4): R_t = 0.94 min; MS (ESI): m/z : calcd for $C_{55}H_{69}N_{10}O_{17}F_2$ $[M-H]^-$: 1179.4810; found: 1179.4808 $(M-H)^-$.

Intermediate 19

Dibenzyl (2*R*)-2-[(5*S*,8*S*,11*S*,14*S*)-11-(2-amino-2-oxoethyl)-14-{2-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]ethyl}-5,7,8-trimethyl-3,6,9,12,15,19-hexaoxo-1-phenyl-2-oxa-4,7,10,13,16-pentaazanonadecan-19-yl]amino}pentanedioate

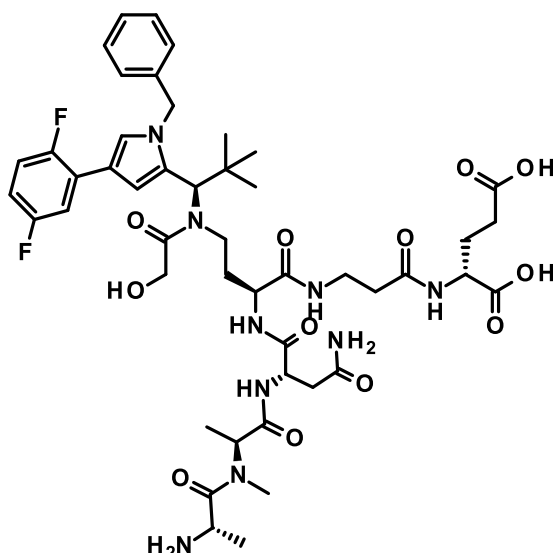


A solution of dibenzyl *N*-{(2*S*)-2-amino-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]butanoyl}-β-alanyl-D-glutamate (548 mg, 544 μmol) in DMF (69 mL) was supplemented with *N*-[(benzyloxy)carbonyl]-L-alanyl-*N*-methyl-L-alanyl-L-asparagine (281 mg, 598 μmol), *N,N*-diisopropylethylamine (280 μL, 1.6 mmol) and HATU (269 mg, 707 μmol) and the reaction was stirred for 1.5 hours at room temperature. The mixture was concentrated to dryness under reduced pressure and the residue was purified by preparative HPLC to yield 591 mg of the product.

LC-MS (Method 1): $R_t = 1.33$ min; MS (ESIpos): $m/z = 1298$ $[M+H]^+$

Compound 3c

L-alanyl-*N*-methyl-L-alanyl-*N'*-{(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]-1-[(3-[(1*R*)-1,3-dicarboxypropyl]amino)-3-oxopropyl]amino]-1-oxobutan-2-yl]-L-aspartamide

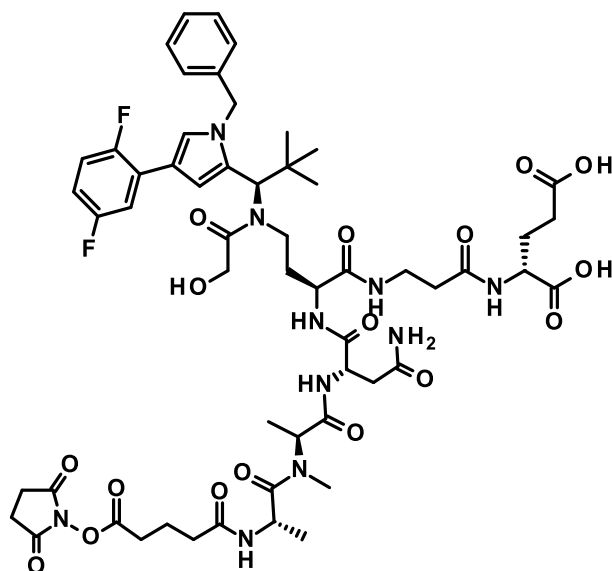


A solution of dibenzyl (2R)-2-[[[(5S,8S,11S,14S)-11-(2-amino-2-oxoethyl)-14-{2-[[[(1R)-1-[1-benzyl-4-(2,5-difluorophenyl)-1H-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]ethyl]-5,7,8-trimethyl-3,6,9,12,15,19-hexaoxo-1-phenyl-2-oxa-4,7,10,13,16-pentaazononadecan-19-yl]amino}pentanedioate (593 mg, 457 μ mol) in DCM/methanol (1.1, 75 mL) was supplemented with palladium on charcoal (10%, 50 mg). The mixture was hydrogenated at ambient pressure for 1 hour. The solids were filtered off and the residue was washed several times with acetonitrile/water (1:1). The filtrate was concentrated under reduced pressure and the remaining residue was lyophilized to give 449 mg of the title product.

LC-MS (Method 1): R_t = 0.80 min; MS (ESIpos): m/z = 984 $[M+H]^+$

Compound 4c

N-{5-[(2,5-dioxopyrrolidin-1-yl)oxy]-5-oxopentanoyl}-L-alanyl-*N*-methyl-L-alanyl-*N*'-{(2S)-4-[[[(1R)-1-[1-benzyl-4-(2,5-difluorophenyl)-1H-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]-1-[(3-[[[(1R)-1,3-dicarboxypropyl]amino]-3-oxopropyl)amino]-1-oxobutan-2-yl]-L-aspartamide



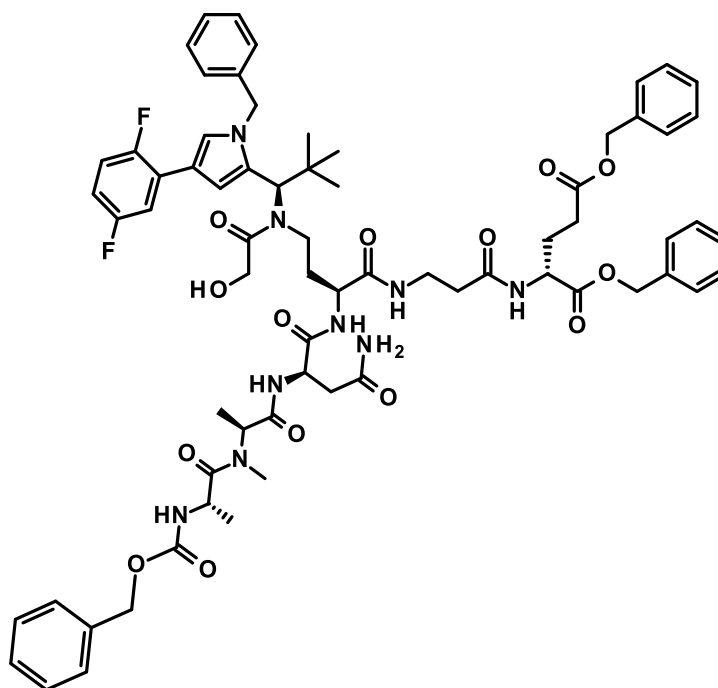
L-alanyl-*N*-methyl-L-alanyl-*N'*-{(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]-1-[(3-[(1*R*)-1,3-dicarboxypropyl]amino)-3-oxopropyl]amino]-1-oxobutan-2-yl}-L-aspartamide (100 mg, 102 μ mol) and 1,1'-[(1,5-dioxopentane-1,5-diyl)bis(oxy)]di(pyrrolidine-2,5-dione) (82.9 mg, 254 μ mol) were dissolved in DMF (10 mL) and *N,N*-diisopropylethylamine (53 μ L, 300 μ mol) was added. The reaction was stirred for 30 minutes. The mixture was concentrated under reduced pressure and the residue was purified by preparative HPLC. Concentration of the appropriate fractions and lyophilization yielded 86 mg of the product.

LC-MS (Method 1): R_t = 0.93 min; MS (ESIpos): m/z = 1195 $[M+Na]^+$

HPLC-HRMS (Method 4): R_t = 0.96 min; MS (ESI): m/z : calcd for $C_{56}H_{71}N_{10}O_{17}F_2$ $[M-H]^-$: 1193.4967; found: 1193.4991 $(M-H)^-$.

Intermediate 20

Dibenzyl (2*R*)-2-[(5*S*,8*S*,11*R*,14*S*)-11-(2-amino-2-oxoethyl)-14-{2-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]ethyl}-5,7,8-trimethyl-3,6,9,12,15,19-hexaoxo-1-phenyl-2-oxa-4,7,10,13,16-pentaazanonadecan-19-yl]amino}pentanedioate

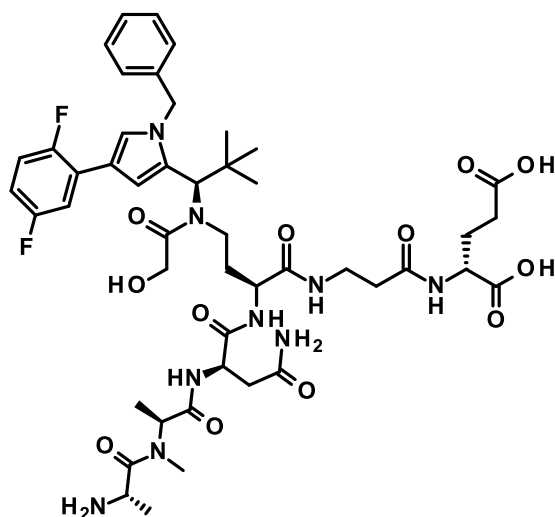


A solution of dibenzyl *N*-{(2*S*)-2-amino-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]butanoyl}-β-alanyl-D-glutamate (18.4 mg, 18.3 μmol) in DMF (3 mL) was supplemented with *N*[(benzyloxy)carbonyl]-L-alanyl-N-methyl-L-alanyl-D-asparagine (8.50 mg, 20.1 μmol), *N,N*-diisopropylethylamine (9.6 μL, 55 μmol) and HATU (9 mg, 23.8 μmol) and the reaction was stirred for 2 hours at room temperature. The mixture was concentrated to dryness under reduced pressure and the residue was purified by preparative HPLC to yield 13 mg of the product.

LC-MS (Method 1): $R_t = 1.33$ min; MS (ESIpos): $m/z = 1299$ $[M+H]^+$

Compound 3d

L-alanyl-*N*-methyl-L-alanyl-*N'*-{(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]-1-[(3-[(1*R*)-1,3-dicarboxypropyl]amino)-3-oxopropyl]amino]-1-oxobutan-2-yl]-D-aspartamide

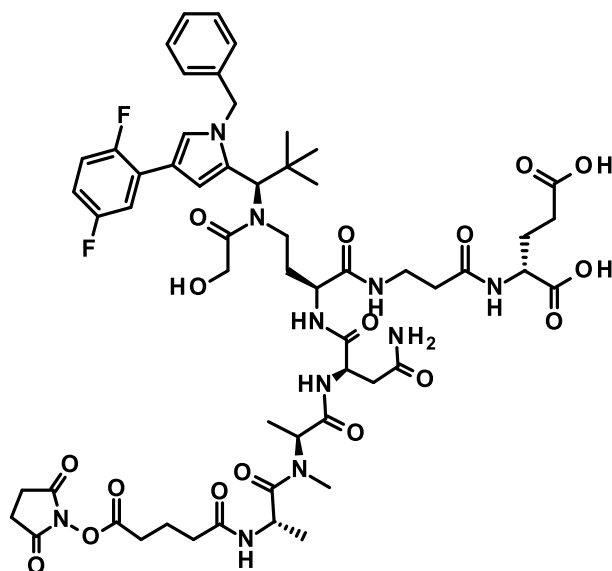


A solution of dibenzyl (2R)-2-[[[(5S,8S,11R,14S)-11-(2-amino-2-oxoethyl)-14-{2-[[[(1R)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]ethyl}-5,7,8-trimethyl-3,6,9,12,15,19-hexaoxo-1-phenyl-2-oxa-4,7,10,13,16-pentaazononadecan-19-yl]amino}pentanedioate (13.0 mg, 10.0 μ mol) in DCM/methanol (1:1, 5 mL) was supplemented with palladium on charcoal (10%, 5 mg). The mixture was hydrogenated at ambient pressure for 1 hour. The solids were filtered off and the residue was washed several times with acetonitrile/water (1:1). The filtrate was concentrated under reduced pressure and the remaining residue was lyophilized to yield 9 mg of the product.

LC-MS (Method 2): R_t = 2.53 min; MS (ESIpos): m/z = 984 $[M+H]^+$

Compound 4d

N-{5-[(2,5-dioxopyrrolidin-1-yl)oxy]-5-oxopentanoyl}-L-alanyl-*N*-methyl-L-alanyl-*N'*-{(2S)-4-[[[(1R)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-1-[(3-[[[(1R)-1,3-dicarboxypropyl]amino]-3-oxopropyl]amino]-1-oxobutan-2-yl]-D-aspartamide

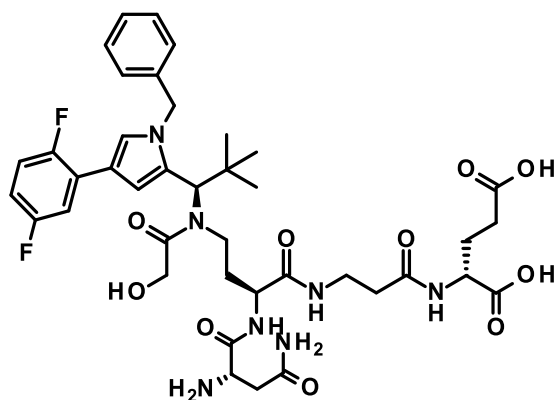


L-alanyl-*N*-methyl-L-alanyl-*N'*-{((2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino)-1-[(3-[(1*R*)-1,3-dicarboxypropyl]amino)-3-oxopropyl]amino]-1-oxobutan-2-yl}-D-aspartamide (9.00 mg, 9.15 μ mol) and 1,1'-[(1,5-dioxopentane-1,5-diyl)bis(oxy)]di(pyrrolidine-2,5-dione) (7.5 mg, 23 μ mol) were dissolved in DMF (4 mL) and *N,N*-diisopropylethylamine (4.8 μ L, 27 μ mol) was added. The reaction was stirred for 1.5 hours at room temperature. The mixture was concentrated under reduced pressure and the residue was purified by preparative HPLC. Concentration of the appropriate fractions and lyophilization yielded 8 mg of the product.

LC-MS (Method 2): R_t = 3.01 min; MS (ESIpos): m/z = 1195 $[M+H]^+$

Compound 3e

N-{((2*S*)-2-(L-asparaginylamino)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]butanoyl}-beta-alanyl-D-glutamic acid



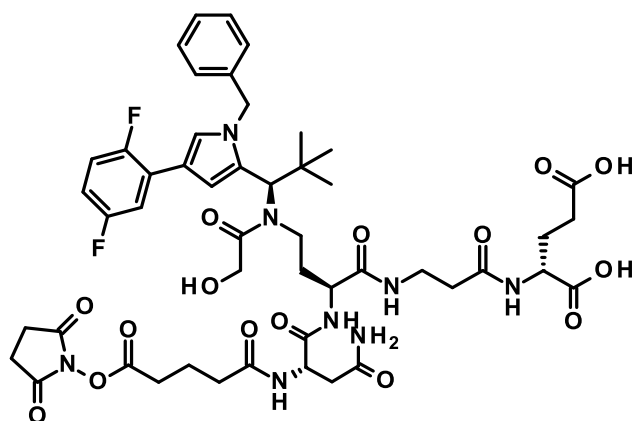
A solution of dibenzyl *N*-{((2*S*)-2-(L-asparaginylamino)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]butanoyl}-beta-alanyl-D-glutamate (50.0 mg, 42.6 μ mol) in DCM/methanol (1:1, 10 mL) was supplemented with

palladium on charcoal (10%, 10 mg). The mixture was hydrogenated at ambient pressure for 1 hour. The solids were filtered off and the residue was washed several times with acetonitrile/water (1:1). The filtrate was concentrated under reduced pressure and the remaining residue was lyophilized to yield 35 mg of the product.

LC-MS (Method 1): $R_t = 0.79$ min; MS (ESIpos): $m/z = 828$ $[M+H]^+$

Compound 4e

N-{(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]-2-[(*N*²-{5-[(2,5-dioxopyrrolidin-1-yl)oxy]-5-oxopentanoyl}-L-asparaginy]amino]butanoyl}-beta-alanyl-D-glutamic acid



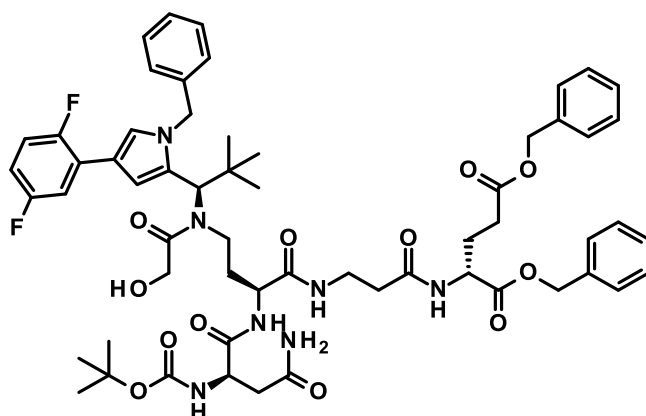
N-{(2*S*)-2-(L-asparaginylamino)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]butanoyl}-beta-alanyl-D-glutamic acid (18.0 mg, 21.3 μ mol) and 1,1'-[(1,5-dioxopentane-1,5-diyl)bis(oxy)]di(pyrrolidine-2,5-dione) (17.3 mg, 53 μ mol) were dissolved in DMF (5 mL) and *N,N*-diisopropylethylamine (15 μ L, 85 μ mol) was added. The reaction was stirred for 1 hour. The mixture was concentrated under reduced pressure and the residue was purified by preparative HPLC. Concentration of the appropriate fractions and lyophilization yielded 14 mg of the product.

LC-MS (Method 1): $R_t = 0.95$ min; MS (ESIpos): $m/z = 1037$ $[M+H]^+$

HPLC-HRMS (Method 4): $R_t = 0.95$ min; MS (ESI): m/z : calcd for $C_{49}H_{59}N_8O_{15}F_2$ $[M-H]^-$: 1037.4068; found: 1037.4054 $(M+H)^+$.

Intermediate 21

Dibenzyl *N*-[(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]-2-[(*N*²-(*tert*-butoxycarbonyl)-D-asparaginy]amino]butanoyl}-beta-alanyl-D-glutamate

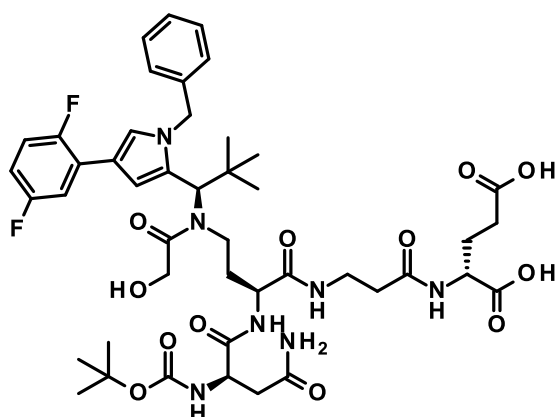


A solution of dibenzyl *N*-[(2*S*)-2-amino-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]butanoyl]-beta-alanyl-D-glutamate (27.0 mg, 28.2 μ mol) and *N,N*-diisopropylethylamine (15 μ L, 85 μ mol) in DMF (5.0 mL) was supplemented with *N*^{*t*}-(*tert*-butoxycarbonyl)-D-asparagine (7.87 mg, 33.9 μ mol) and the reaction was stirred at room temperature for 2 hours. The mixture was concentrated to dryness under reduced pressure and the residue was purified by preparative HPLC to yield 22 mg of the product.

LC-MS (Method 1): R_t = 1.37 min; MS (ESIpos): m/z = 1108 $[M+H]^+$

Intermediate 22

N-[(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-2-[[*N*^{*t*}-(*tert*-butoxycarbonyl)-D-asparaginyl]amino]butanoyl]-beta-alanyl-D-glutamic acid

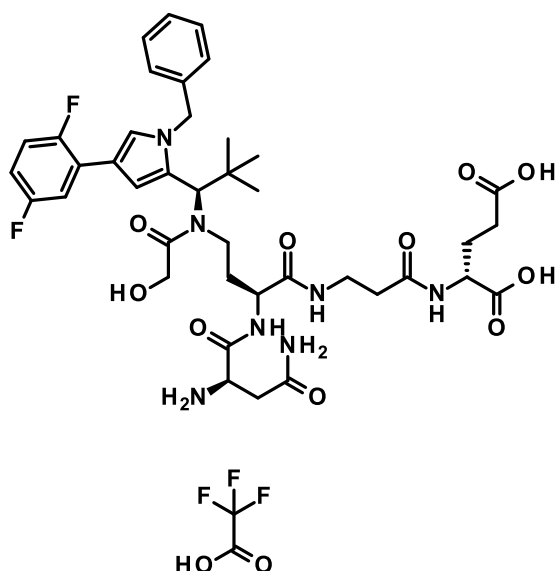


A solution of dibenzyl *N*-[(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-2-[[*N*^{*t*}-(*tert*-butoxycarbonyl)-D-asparaginyl]amino]butanoyl]-beta-alanyl-D-glutamate (22.5 mg, 20.3 μ mol) in DCM/methanol (1.1 mg, 10 mL) was supplemented with palladium on charcoal (10%, 15 mg). The mixture was hydrogenated at ambient pressure for 1.5 hours. The solids were filtered off and the residue was washed several times with acetonitrile/water (1:1). The filtrate was concentrated under reduced pressure and the remaining residue was lyophilized to yield 15 mg of the product.

LC-MS (Method 1): $R_t = 1.05$ min; MS (ESIpos): $m/z = 928$ $[M+H]^+$

Compound 3f

N-{[(2*S*)-2-(D-asparaginylamino)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]butanoyl}-beta-alanyl-D-glutamic acid

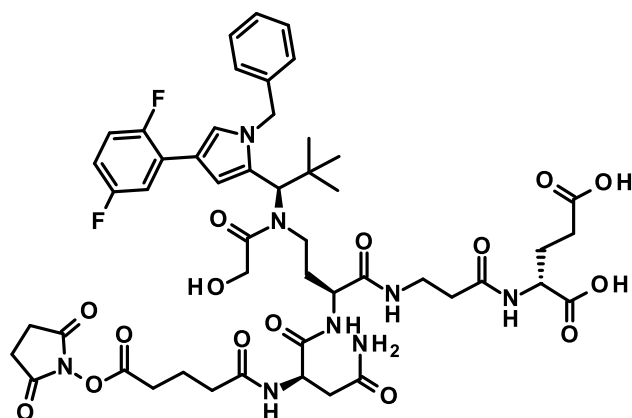


N-[(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-2-[[*N*²-(*tert*-butoxycarbonyl)-D-asparaginy]amino]butanoyl]-beta-alanyl-D-glutamic acid (15.0 mg, 16.2 μ mol) was dissolved in 2,2,2-trifluoroethanol (3mL). Zinc chloride (13 mg, 97 μ mol) was added, and the reaction was stirred at 50 °C for 4 hours. The mixture was diluted with water and 28 mg (97 μ mol) of ethylene diamine-*N,N,N,N*-tetraacetic acid was added. The mixture was stirred for a few minutes. The reaction was concentrated under reduced pressure and the residue was purified by preparative HPLC to yield 6.4 mg of the product.

LC-MS (Method 3): $R_t = 1.44$ min; MS (ESIpos): $m/z = 828$ $[M+H]^+$

Compound 4f

N-{[(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-2-[[*N*²-{5-[(2,5-dioxopyrrolidin-1-yl)oxy]-5-oxopentanoyl}-D-asparaginy]amino]butanoyl}-beta-alanyl-D-glutamic acid

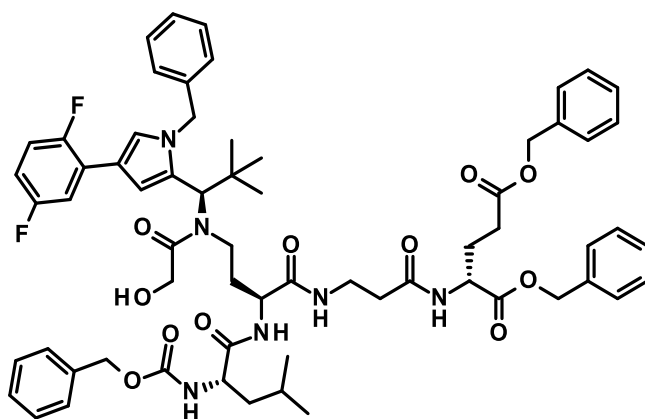


N-((2*S*)-2-(D-asparaginylamino)-4-(((1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl)(hydroxyacetyl)amino]butanoyl)-beta-alanyl-D-glutamic (6.40 mg, 6.79 μ mol) and 1,1'-[(1,5-dioxopentane-1,5-diyl)bis(oxy)]di(pyrrolidine-2,5-dione) (5.5 mg, 17 μ mol) were dissolved in DMF (4 mL) and *N,N*-diisopropylethylamine (4.7 μ L, 27 μ mol) was added. The reaction was stirred over night at room temperature. The mixture was concentrated under reduced pressure and the residue was purified by preparative HPLC. Concentration of the appropriate fractions and lyophilization yielded 1.6 mg of the product.

LC-MS (Method 3): R_t = 1.69 min; MS (ESIpos): m/z = 1037 $[M+H]^+$

Intermediate 23

Dibenzyl *N*-[(2*S*)-4-(((1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl)(hydroxyacetyl)amino]-2-({*N*-[(benzyloxy)carbonyl]-L-leucyl}amino)butanoyl]-beta-alanyl-D-glutamate



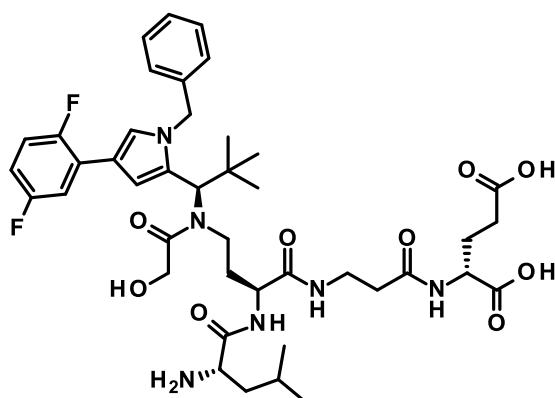
A solution of dibenzyl *N*-[(2*S*)-2-amino-4-(((1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl)(hydroxyacetyl)amino]butanoyl)-beta-alanyl-D-glutamate (75.0 mg, 74.4 μ mol) and *N,N*-diisopropylethylamine (39 μ L, 220 μ mol) in DMF (5.0 mL) was supplemented with 2,5-dioxopyrrolidin-1-yl *N*-[(benzyloxy)carbonyl]-L-leucinate (33.7 mg, 93.0 μ mol) and the reaction was stirred at room temperature for 2 hours. The mixture was concentrated to dryness

under reduced pressure and the residue was purified by preparative HPLC to yield 67 mg of the product.

LC-MS (Method 1): $R_t = 1.51$ min; MS (ESIpos): $m/z = 1141$ $[M+H]^+$

Compound 3g

N-[(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-2-(*L*-leucylamino)butanoyl]-beta-alanyl-D-glutamic acid

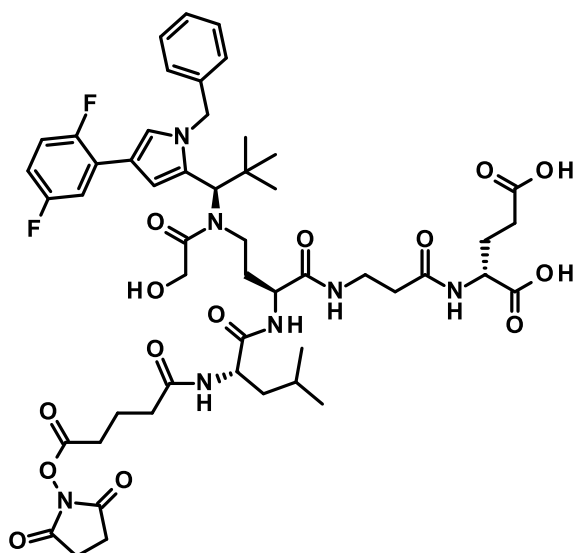


A solution of dibenzyl *N*-[(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-2-[(*N*-(benzyloxy)carbonyl)-*L*-leucyl]amino]butanoyl]-beta-alanyl-D-glutamate (67.0 mg, 58.7 μ mol) in DCM (5 mL) and methanol (5 mL) was supplemented with palladium on charcoal (10%, 15 mg). The mixture was hydrogenated at ambient pressure for 2 hours. The solids were filtered off and the residue was washed several times with acetonitrile/water (1:1). The filtrate was concentrated under reduced pressure and the remaining residue was lyophilized to give 49 mg of the title product.

LC-MS (Method 1): $R_t = 0.84$ min; MS (ESI_{neg}): $m/z = 825$ $[M-H]^-$

Compound 4g

N-[(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-2-[(*N*-{5-[(2,5-dioxopyrrolidin-1-yl)oxy]-5-oxopentanoyl}-*L*-leucyl)amino]butanoyl]-beta-alanyl-D-glutamic acid

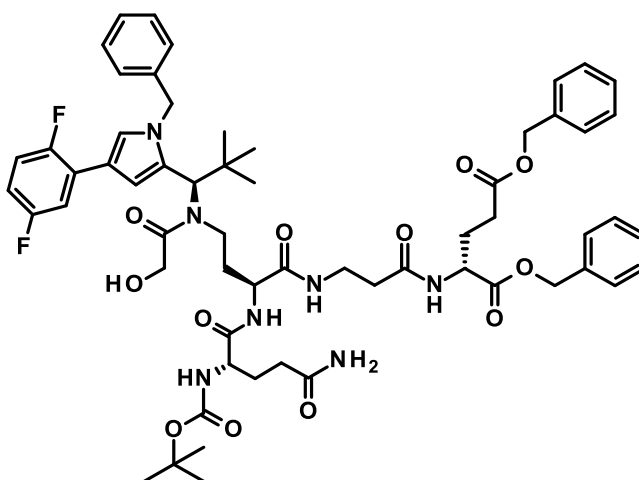


N-[(2*S*)-4-[[[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl) amino]-2-(*L*-leucylamino)butanoyl]-beta-alanyl-D-glutamic acid (35.0 mg, 42.3 μ mol) and 1,1'-[(1,5-dioxopentane-1,5-diyl)bis(oxo)]di(pyrrolidine-2,5-dione) (34.5 mg, 130 μ mol) were dissolved in DMF (4 mL) and *N,N*-diisopropylethylamine (22 μ L, 130 μ mol) was added. The reaction was stirred for 30 minutes at room temperature. The mixture was concentrated under reduced pressure and the residue was purified by preparative HPLC. Concentration of the appropriate fractions and lyophilization yielded 21.7 mg of the product.

LC-MS (Method 1): R_t = 1.02 min; MS (ESIpos): m/z = 1038 $[M+H]^+$

Intermediate 24

Dibenzyl *N*-[(2*S*)-4-[[[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-2-[[*N*²-(*tert*-butoxycarbonyl)-*L*-glutaminy]amino]butanoyl]-beta-alanyl-D-glutamate



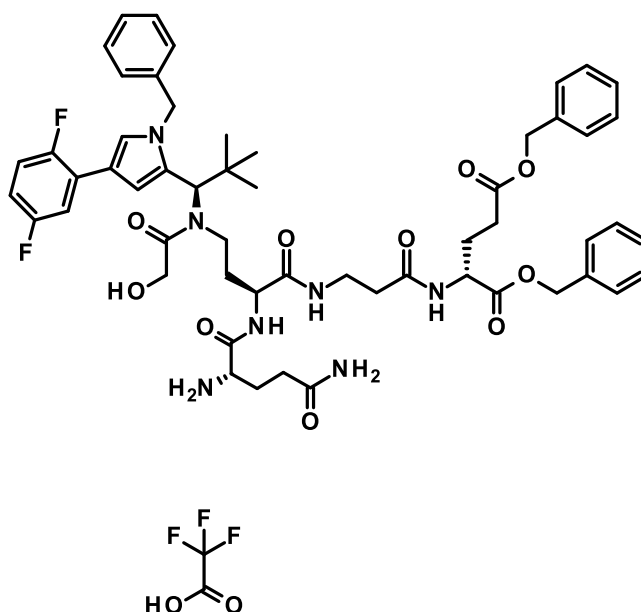
Dibenzyl *N*-{(2*S*)-2-amino-4-[[[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]butanoyl]-beta-alanyl-D-glutamate (30.0 mg, 31.4 μ mol)

and 2,5-dioxopyrrolidin-1-yl *N*²-(*tert*-butoxycarbonyl)-L-glutamate (12.9 mg, 37.6 μmol) were dissolved in DMF (5 mL) and *N,N*-diisopropylethylamine (16 μL, 94 μmol) was added. The reaction was stirred for 2 hours at room temperature. The mixture was concentrated under reduced pressure and the residue was purified by preparative HPLC. Concentration of the appropriate fractions and lyophilization yielded 24.5 mg of the product.

LC-MS (Method 1): *R*_t = 1.37 min; MS (ESIpos): *m/z* = 1123 [M+H]⁺

Intermediate 25

Dibenzyl *N*-[(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-2-(L-glutaminylamino)butanoyl]-beta-alanyl-D-glutamate

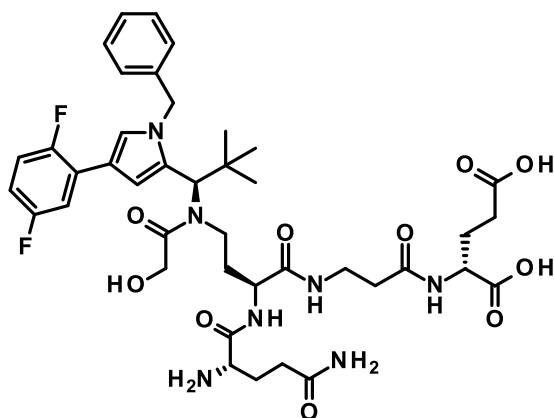


Dibenzyl *N*-[(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-2-[(*N*²-(*tert*-butoxycarbonyl)-L-glutaminyl)amino]butanoyl]-beta-alanyl-D-glutamate (24.5 mg, 20.9 μmol) was dissolved in 2,2,2-trifluoroethanol (6 mL). Zinc chloride (17 mg, 125 μmol) was added, and the reaction was stirred at 50 °C for 1.5 hours. The mixture was diluted with water and 37 mg (125 μmol) of ethylene diamine-*N,N,N,N*-tetraacetic acid was added. The mixture was stirred for a few minutes. The reaction was concentrated under reduced pressure and the residue was purified by preparative HPLC to yield 23 mg of the product.

LC-MS (Method 3): *R*_t = 2.15 min; MS (ESIpos): *m/z* = 1022 [M+H]⁺

Compound 3h

N-[(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-2-(L-glutaminylamino)butanoyl]-beta-alanyl-D-glutamic acid

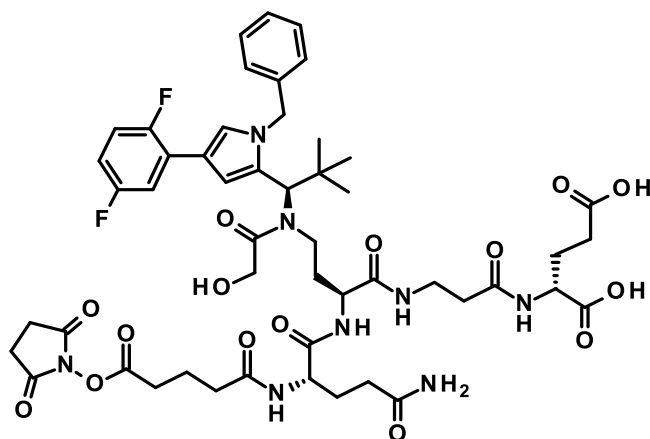


A solution of dibenzyl *N*-[(2*S*)-4-[[[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-2-(*L*-glutaminylamino)butanoyl]-beta-alanyl-D-glutamate (23.0 mg, 20.2 μ mol) in methanol (10 mL) was supplemented with palladium on charcoal (10%, 3 mg). The mixture was hydrogenated at ambient pressure for 1 hour. The solids were filtered off and the residue was washed several times with acetonitrile/water (1:1). The filtrate was concentrated under reduced pressure and the remaining residue was lyophilized to yield 16 mg of the product.

LC-MS (Method 1): R_t = 0.81 min; MS (ESIpos): m/z = 842 $[M+H]^+$

Compound 4h

N-[(2*S*)-4-[[[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-2-[(*N*²-{5-[(2,5-dioxopyrrolidin-1-yl)oxy]-5-oxopentanoyl}-*L*-glutaminyl)amino]butanoyl]-beta-alanyl-D-glutamic acid



N-[(2*S*)-4-[[[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-2-(*L*-glutaminylamino)butanoyl]-beta-alanyl-D-glutamic acid (16.0 mg, 14.3 μ mol) and 1,1'-[(1,5-dioxopentane-1,5-diyl)bis(oxy)]di(pyrrolidine-2,5-dione) (14 mg, 43 μ mol) were dissolved in DMF (4 mL) and *N,N*-diisopropylethylamine (12 μ L, 72 μ mol) was added. The reaction was stirred for 1 hour at room temperature. The mixture was concentrated under

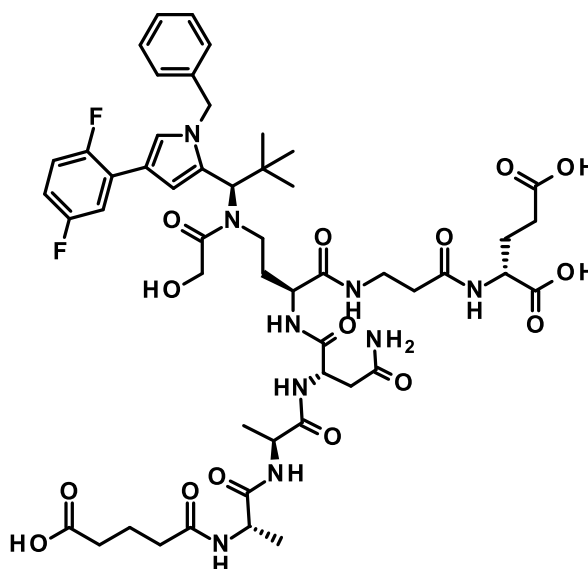
reduced pressure and the residue was purified by preparative HPLC. Concentration of the appropriate fractions and lyophilization yielded 9.4 mg of the product.

LC-MS (Method 1): $R_t = 0.92$ min; MS (ESIpos): $m/z = 1052$ $[M+H]^+$

4 Synthesis of SMOL tool compounds

Compound 5a

N-(4-carboxybutanoyl)-L-alanyl-L-alanyl-*N*'-{(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]-1-[(3-[(1*R*)-1,3-dicarboxypropyl]amino)-3-oxopropyl]amino]-1-oxobutan-2-yl]-L-aspartamide

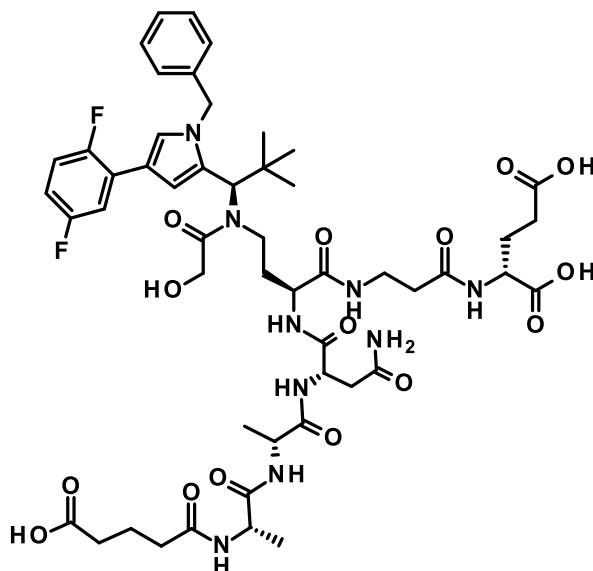


A solution of *N*-{5-[(2,5-dioxopyrrolidin-1-yl)oxy]-5-oxopentanoyl}-L-alanyl-L-alanyl-*N*'-{(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]-1-[(3-[(1*R*)-1,3-dicarboxypropyl]amino)-3-oxopropyl]amino]-1-oxobutan-2-yl]-L-aspartamide (7.00 mg, 5.93 μ mol) in acetonitrile/water (1:1, 2 mL), was supplemented with a saturated solution of sodiumhydrogencarbonate (100 μ L) and the reaction was stirred for 20 hours at room temperature. TFA (15 μ L) was added and the mixture was concentrated under reduced pressure. The residue was purified by preparative HPLC. Concentration of the appropriate fractions and lyophilization yielded 1.9 mg of the product.

LC-MS (Method 1): $R_t = 0.87$ min; MS (ESIpos): $m/z = 1082$ $[M-H]^-$

Compound 5b

N-(4-carboxybutanoyl)-L-alanyl-D-alanyl-*N*¹-{(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]-1-[(3-[(1*R*)-1,3-dicarboxypropyl]amino)-3-oxopropyl]amino]-1-oxobutan-2-yl]-L-aspartamide

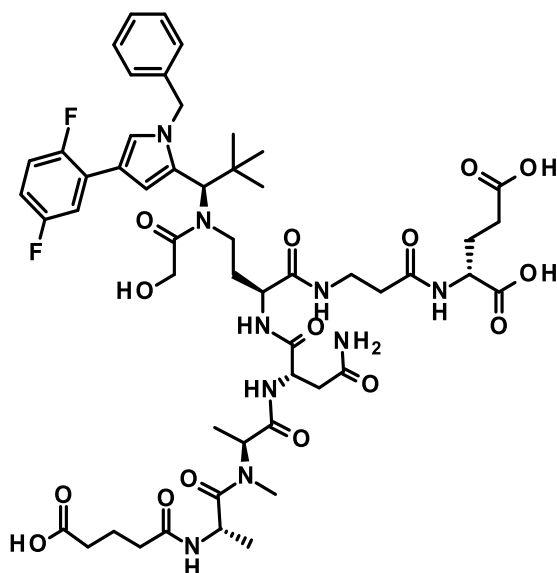


A solution of *N*-{5-[(2,5-dioxopyrrolidin-1-yl)oxy]-5-oxopentanoyl}-L-alanyl-D-alanyl-*N*¹-{(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]-1-[(3-[(1*R*)-1,3-dicarboxypropyl]amino)-3-oxopropyl]amino]-1-oxobutan-2-yl]-L-aspartamide (7.00 mg, 5.93 μ mol) in acetonitrile/water (1:1, 2 mL) was supplemented with a saturated solution of sodiumhydrogencarbonate (100 μ L) and the reaction was stirred for 20 hours at room temperature. TFA (15 μ L) was added and the mixture was concentrated under reduced pressure. The residue was purified by preparative HPLC. Concentration of the appropriate fractions and subsequent lyophilization yielded 2.4 mg of the product.

LC-MS (Method 1): R_t = 0.91 min; MS (ESIpos): m/z = 1082 [M-H]⁺

Compound 5c

N-(4-carboxybutanoyl)-L-alanyl-*N*-methyl-L-alanyl-*N*¹-{(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]-1-[(3-[(1*R*)-1,3-dicarboxypropyl]amino)-3-oxopropyl]amino]-1-oxobutan-2-yl]-L-aspartamide

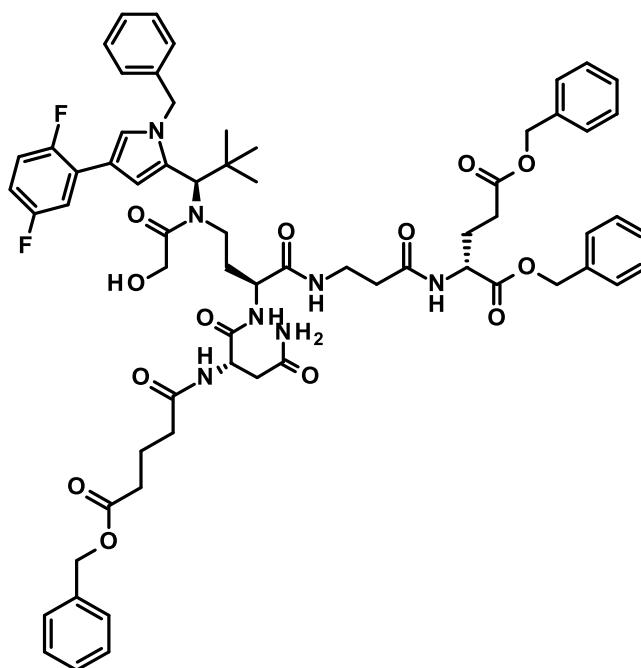


A solution of *N*-{5-[(2,5-dioxopyrrolidin-1-yl)oxy]-5-oxopentanoyl}-L-alanyl-*N*-methyl-L-alanyl-*N*¹-{(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]-1-[(3-[(1*R*)-1,3-dicarboxypropyl]amino)-3-oxopropyl]amino]-1-oxobutan-2-yl}-L-aspartamide (12.0 mg, 10.0 μ mol) in acetonitrile/water (1:1, 4 mL) was supplemented with a saturated solution of sodiumhydrogencarbonate (480 μ L) and the reaction was stirred for 2 hours at room temperature. TFA (50 μ L) was added and the mixture was concentrated under reduced pressure. The residue was purified by preparative HPLC. Concentration of the appropriate fractions and lyophilization yielded 8 mg of the product.

LC-MS (Method 1): R_t = 0.92 min; MS (ESIpos): m/z = 1096 [M-H]⁺

Intermediate 26

Dibenzyl *N*-[(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]-2-({*N*²-[5-(benzyloxy)-5-oxopentanoyl]-L-asparaginy]amino)butanoyl]-beta-alanyl-D-glutamate

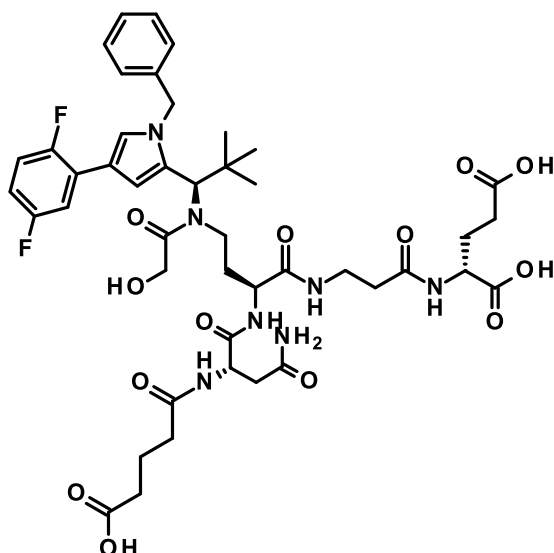


A solution of dibenzyl *N*-{(2*S*)-2-(*L*-asparaginylamino)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]butanoyl}-beta-alanyl-D-glutamate (14.0 mg, 12.5 μ mol) was supplemented with *N,N*-diisopropylethylamine (6.5 μ L, 37 μ mol) and HATU (7.12 mg, 18.7 μ mol) and the reaction was stirred for 2 hours at room temperature. The mixture was concentrated to dryness under reduced pressure and the residue was purified by preparative HPLC to yield 12 mg of the product.

LC-MS (Method 3): R_t = 2.53 min; MS (ESIpos): m/z = 1212 $[M+H]^+$

Compound 5e

N-[(2*S*)-4-[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl}(hydroxyacetyl)amino]-2-[[*N*²-(4-carboxybutanoyl)-*L*-asparaginyl]amino]butanoyl]-beta-alanyl-D-glutamic acid

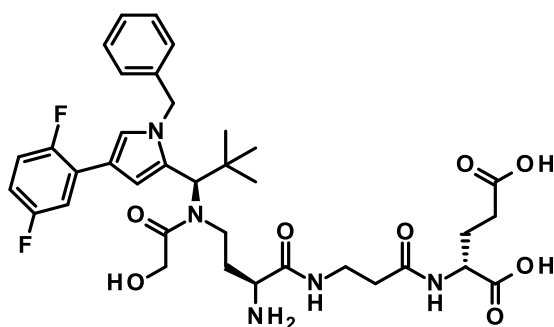


A solution of dibenzyl *N*-[(2*S*)-4-[[[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]-2-({*N*²-[5-(benzyloxy)-5-oxopentanoyl]-L-asparaginy] amino)butanoyl]-beta-alanyl-D-glutamate (12.4 mg, 10.2 μ mol) in DCM/methanol (1.1, 5 mL) was supplemented with palladium on charcoal (10%, 5.8 mg). The mixture was hydrogenated at ambient pressure for 1 hour. The solids were filtered off and the residue was washed several times with acetonitrile/water (1:1). The filtrate was concentrated under reduced pressure and the remaining residue was lyophilized to yield 9.6 mg of the product.

LC-MS (Method 1): R_t = 0.88 min; MS (ESIpos): m/z = 940 $[M-H]^+$

Active metabolite 8

N-[(2*S*)-2-amino-4-[[[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]butanoyl]-beta-alanyl-D-glutamic acid



A solution of dibenzyl *N*-[(2*S*)-2-amino-4-[[[(1*R*)-1-[1-benzyl-4-(2,5-difluorophenyl)-1*H*-pyrrol-2-yl]-2,2-dimethylpropyl](hydroxyacetyl)amino]butanoyl]-beta-alanyl-D-glutamate (Compound 2, 70.0 mg, 66.3 μ mol) in methanol (15 mL) was supplemented with palladium on charcoal (10%, 7 mg). The mixture was hydrogenated at ambient pressure for 1 hour. The solids were filtered off and the residue was washed several times with acetonitrile/water (1:1). The filtrate was

concentrated under reduced pressure and the remaining residue was lyophilized to yield 54 mg of the product.

LC-MS (Method 1): $R_t = 0.80$ min; MS (ESIpos): $m/z = 714$ $[M+H]^+$

5 Synthesis of ADCs

The antibodies used herein are described in WO 2015/096982 A1,^{S1} WO2015/189143 A1,^{S3} and WO2016/207094 A1.^{S4}

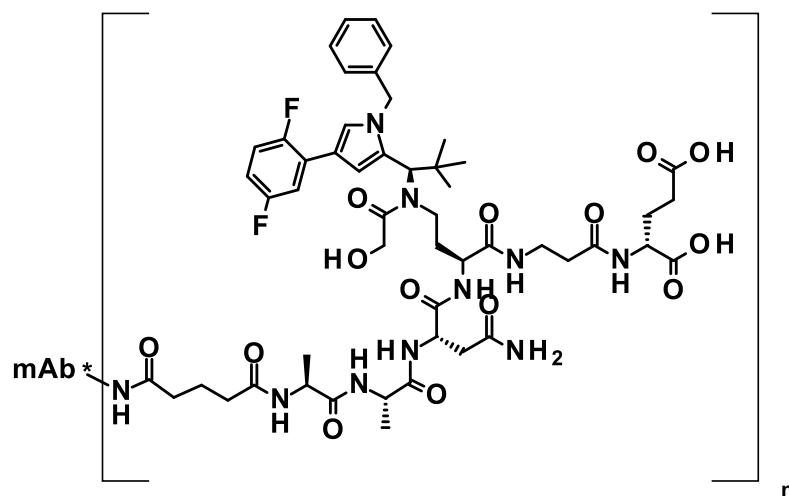
TPP-1015: an anti-HER-2 antibody (IgG1), an in-house produced version of Trastuzumab having the same peptide sequence as Trastuzumab;^{S1}

TPP-7007: an antagonistic anti-TWEAKR antibody (IgG1);^{S4}

TPP-2658: an agonistic anti-TWEAKR-antibody; (IgG1);^{S1,S3}

TPP-5657: an isotype control antibody, (IgG1)^{S4}

ADCs 6a and 7a



Anti-TWEAKR ADC with TPP-7007 (Compound 6a):

A solution of anti-TWEAKR antibody TPP-7007 in PBS buffer (1 mL, 10 mg/mL) was supplemented with Compound 4a (0.38 mg, 0.34 μ mol) dissolved in DMSO (50 μ L) and the mixture was stirred for 1 hour at room temperature. Compound 4a (0.38 mg, 0.34 μ mol) dissolved in DMSO (50 μ L) was added. After stirring for an additional hour at room temperature, the mixture was diluted to 5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The sample was diluted

to a total volume of 14 mL with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 7.81 mg/mL

Drug-to-antibody ratio (DAR): 3.2

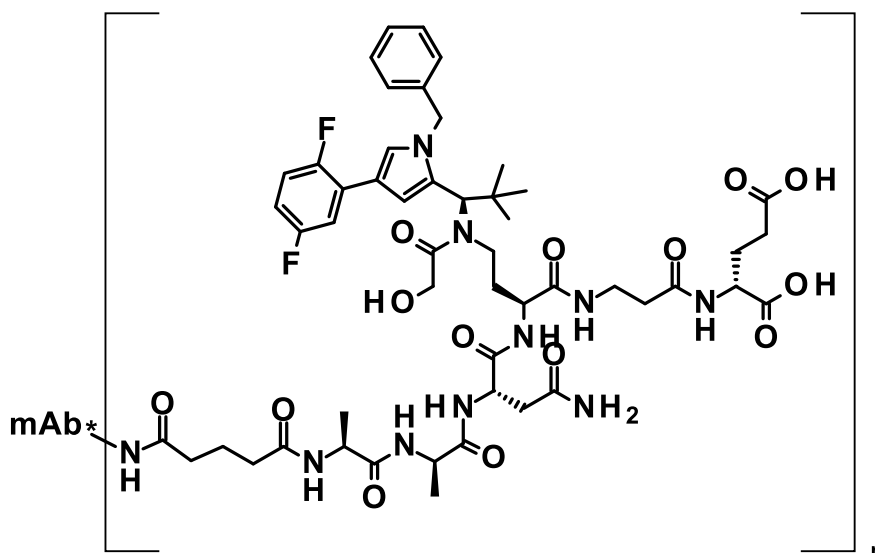
Anti-HER2 ADC with TPP-1015 (Compound 7a):

A solution of trastuzumab TPP-1015 in PBS buffer (0.5 mL, 10 mg/mL) was supplemented with Compound 4a (0.2 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) and the mixture was stirred for 1 hour at room temperature. Compound 4a (0.2 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) was added. After stirring for an additional hour at room temperature the mixture was diluted to 2.5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 2.06 mg/mL

DAR: 6.3

ADCs 6b, 6b*, 7b and isotype 6b



Anti-TWEAKR ADC with TPP-7007 (Compound 6b):

A solution of anti-TWEAKR antibody TPP-7007 in PBS buffer (4 mL, 10 mg/mL) was supplemented with Compound 4b (1.58 mg, 1.34 μ mol) dissolved in DMSO (200 μ L) and the mixture was stirred for 1 hour at room temperature. Compound 4b (1.58 mg, 1.34 μ mol) dissolved in DMSO (200 μ L) was added. After stirring for an additional hour at room temperature

the mixture was diluted to 5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The sample was diluted to a total volume of 28 mL with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 6.92 mg/mL

DAR: 4.6

Anti-TWEAKR ADC with TPP-2658 (Compound 6b*)

A solution of the anti-TWEAKR antibody TPP-2658 in PBS buffer (0.307 mL, 16.3 mg/mL) was supplemented with Compound 4b (0.23 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) and the mixture was stirred for 1 hour at room temperature. Compound 4b (0.23 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) was added. After stirring for an additional hour at room temperature the mixture was diluted to 2.5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 2.21 mg/mL

DAR: 5.7

Anti-HER2 ADC with TPP-1015 (Compound 7b):

A solution of Herceptin antibody TPP-1015 in PBS buffer (0.9 mL, 11 mg/mL) was supplemented with Compound 4b (0.39 mg, 0.33 μ mol) dissolved in DMSO (50 μ L) and the mixture was stirred for 1 hour at room temperature. Compound 4b (0.39 mg, 0.33 μ mol) dissolved in DMSO (50 μ L) was added. After stirring for an additional hour at room temperature the mixture was diluted to 2.5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 1.47 mg/mL

DAR: 3.0

Isoype control ADC with TPP-5657 (Compound isotype 6b)

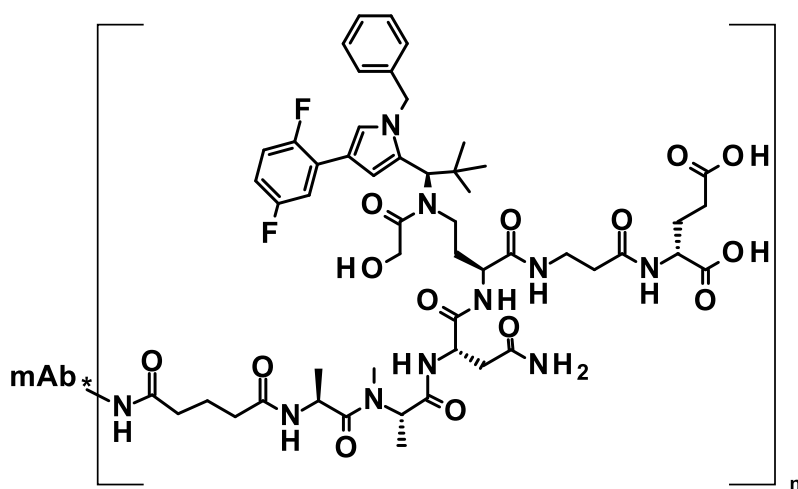
A solution of the isotype antibody TPP-5657 in PBS buffer (4 mL, 10 mg/mL) was supplemented with Compound 4b (1.57 mg, 1.33 μ mol) dissolved in DMSO (200 μ L) and the mixture was stirred for 1 hour at room temperature. Compound 4b (1.57 mg, 1.33 μ mol) dissolved in DMSO (200

μL) was added. After stirring for an additional hour at room temperature the mixture was diluted to 5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 6.70 mg/mL

DAR: 4.9

ADCs 6c, 6c*, 7c and isotype 6c



Anti-TWEAKR ADC (TPP-7007) (Compound 6c):

A solution of anti-TWEAKR antibody TPP-7007 in PBS buffer (4 mL, 10 mg/mL) was supplemented with Compound 4c (1.59 mg, 1.33 μmol) dissolved in DMSO (200 μL) and the mixture was stirred for 1 hour at room temperature. Compound 4c (1.59 mg, 1.33 μmol) dissolved in DMSO (200 μL) was added. After stirring for an additional hour at room temperature the mixture was diluted to 5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The sample was diluted to a total volume of 28 mL with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 6.98 mg/mL

DAR: 4.3

Anti-TWEAKR ADC with TPP-2658 (Compound 6c*)

A solution of the TWEAKR antibody TPP-2658 in PBS buffer (0.5 mL, 10 mg/mL) was supplemented with Compound 4c (0.20 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) and the mixture was stirred for 1 hour at room temperature. Compound 4c (0.20 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) was added. After stirring for an additional hour at room temperature the mixture was diluted to 2.5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 2.07 mg/mL

DAR: 5.6

Anti-HER2 ADC (TPP-1015) (Compound 7c):

A solution of Herceptin antibody TPP-1015 in PBS buffer (0.5 mL, 10 mg/mL) was supplemented with Compound 4c (0.20 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) and the mixture was stirred for 1 hour at room temperature. Compound 4c (0.20 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) was added. After stirring for an additional hour at room temperature the mixture was diluted to 2.5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 2.05 mg/mL

DAR: 5.4

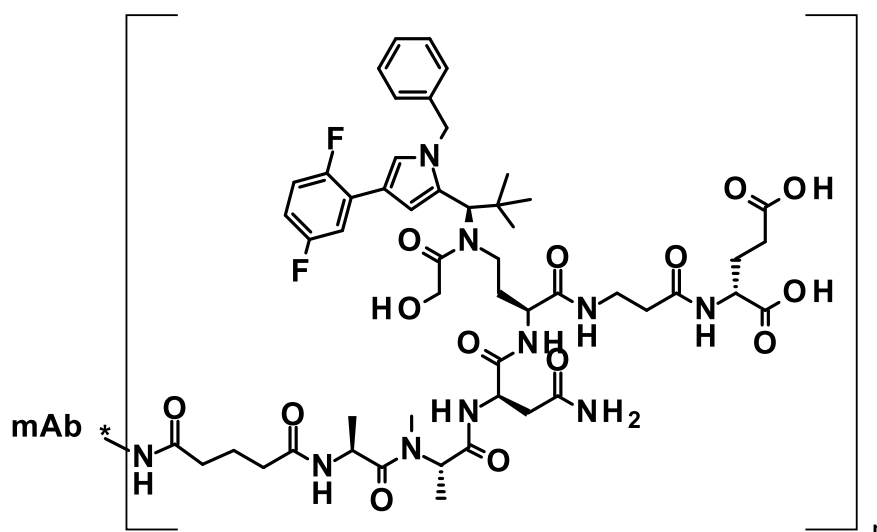
Isotype control ADC with TPP-5657 (Compound isotype 6c)

A solution of the isotype antibody TPP-5657 in PBS buffer (4 mL, 10 mg/mL) was supplemented with Compound 4c (1.59 mg, 2.67 μ mol) dissolved in DMSO (200 μ L) and the mixture was stirred for 1 hour at room temperature. Compound 4c (1.59 mg, 2.67 μ mol) dissolved in DMSO (200 μ L) was added. After stirring for an additional hour at room temperature the mixture was diluted to 5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 6.88 mg/mL

DAR: 5.6

ADC 6d

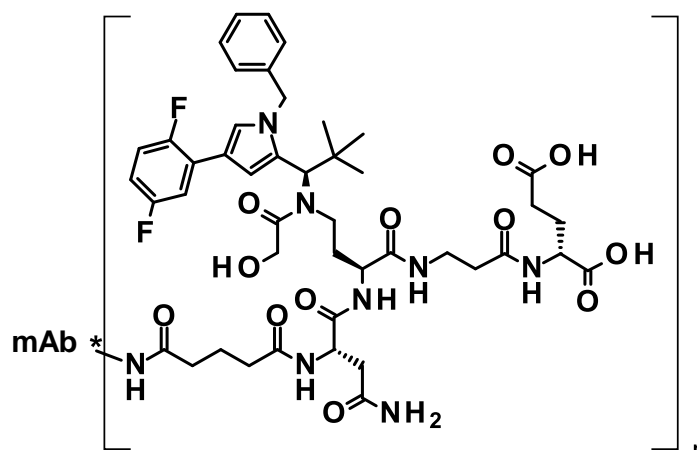


A solution of anti-TWEAKR antibody TPP-7007 in PBS buffer (0.5 mL, 10 mg/mL) was supplemented with Compound 4d (0.2 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) and the mixture was stirred for 1 hour at room temperature. Compound 4d (0.2 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) was added. After stirring for an additional hour at room temperature the mixture was diluted to 5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 2.23 mg/mL

DAR: 4.5

ADCs 6e, 6e*, 7e and isotype 6e



Anti-TWEAKR ADC with TPP-7007 (Compound 6e):

A solution of TWEAKR antibody TPP-7007 in PBS buffer (4 mL, 10 mg/mL) was supplemented with Compound 4e (1.39 mg, 1.33 μ mol) dissolved in DMSO (200 μ L) and the mixture was stirred for 1 hour at room temperature. Compound 4e (1.39 mg, 1.33 μ mol) dissolved in DMSO (200 μ L) was added. After stirring for an additional hour at room temperature the mixture was diluted to 5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 7.47 mg/mL

DAR: 6.6

Anti-TWEAKR ADC with TPP-2658 (Compound 6e*)

A solution of the TWEAKR antibody TPP-2658 antibody in PBS buffer (0.4 mL, 11 mg/mL) was supplemented with Compound 4e (0.17 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) and the mixture was stirred for 1 hour at room temperature. Compound 4e (0.17 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) was added. After stirring for an additional hour at room temperature the mixture was diluted to 2.5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The ADC solution was concentrated by ultracentrifugation, rediluted with PBS buffer (pH = 7.2).

Protein concentration: 1.66 mg/mL

DAR: 7.0

Anti-HER2 ADC with TPP-1015 (Compound 7e):

A solution of the Trastuzumab antibody TPP-1015 in PBS buffer (0.41 mL, 11 mg/mL) was supplemented with Compound 4e (0.17 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) and the mixture was stirred for 1 hour at room temperature. Compound 4e (0.17 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) was added. After stirring for an additional hour at room temperature the mixture was diluted to 2.5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 2.32 mg/mL

DAR: 6.9

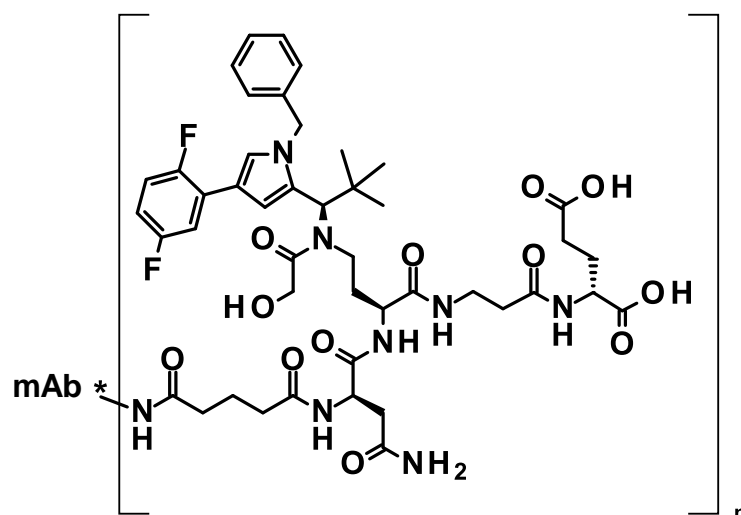
Isoype control ADC with TPP-5657 (Compound isotype 6e)

A solution of the isotype antibody TPP-5657 in PBS buffer (0.4 mL, 10 mg/mL) was supplemented with Compound 4e (1.39 mg, 1.33 μ mol) dissolved in DMSO (200 μ L) and the mixture was stirred for 1 hour at room temperature. Compound 4e (1.39 mg, 1.33 μ mol) dissolved in DMSO (200 μ L) was added. After stirring for an additional hour at room temperature the mixture was diluted to 5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 7.59 mg/mL

DAR: 6.0

ADCs 6f and 7f



Anti-TWEAKR ADC with TPP-7007 (Compound 6f):

A solution of TWEAKR antibody TPP-7007 in PBS buffer (0.5 mL, 8 mg/mL) was supplemented with Compound 4f (0.15 mg, 0.13 μ mol) dissolved in DMSO (50 μ L) and the mixture was stirred for 1 hour at room temperature. Compound 4f (0.15 mg, 0.13 μ mol) dissolved in DMSO (50 μ L) was added. After stirring for an additional hour at room temperature the mixture was diluted to 2.5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 2.56 mg/mL

DAR: 3.4

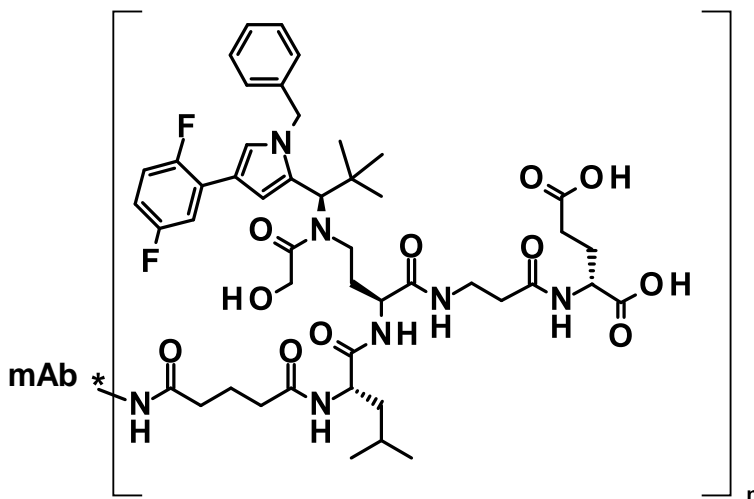
Anti-HER2 ADC with TPP-1015 (Compound 7f):

A solution of Trastuzumab antibody TPP-1015 in PBS buffer (0.5 mL, 8 mg/mL) was supplemented with Compound 4f (0.15 mg, 0.13 μ mol) dissolved in DMSO (50 μ L) and the mixture was stirred for 1 hour at room temperature. Compound 4f (0.15 mg, 0.13 μ mol) dissolved in DMSO (50 μ L) was added. After stirring for an additional hour at room temperature the mixture was diluted to 2.5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 2.54 mg/mL

DAR: 5.4

ADCs 6g and 7g



Anti-TWEAKR ADC with TPP-7007 (Compound 6g):

A solution of TWEAKR antibody TPP-7007 in PBS buffer (0.5 mL, 10 mg/mL) was supplemented with Compound 4g (0.17 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) and the mixture was stirred for 1 hour at room temperature. Compound 4g (0.17 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) was added. After stirring for an additional hour at room temperature the mixture was diluted to 2.5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 1.89 mg/mL

DAR: 4.5

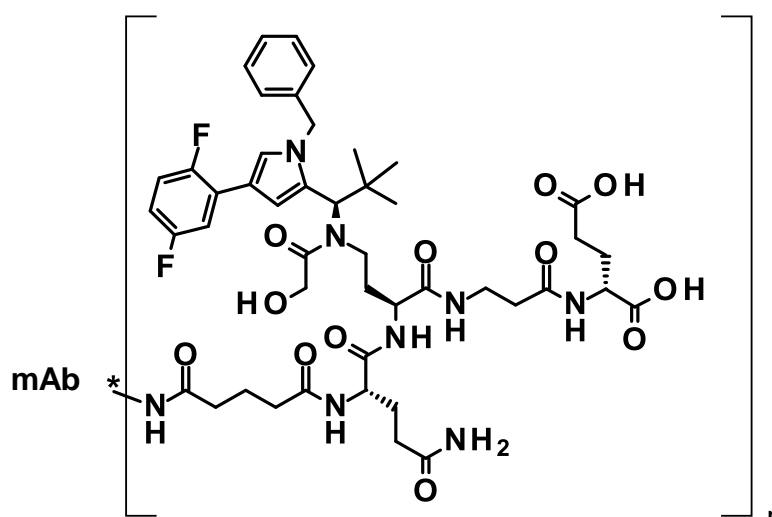
Anti-HER2 ADC with TPP-1015 (Compound 7g):

A solution of Trastuzumab antibody TPP-1015 in PBS buffer (0.5 mL, 10 mg/mL) was supplemented with Compound 4g (0.17 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) and the mixture was stirred for 1 hour at room temperature. Compound 4g (0.17 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) was added. After stirring for an additional hour at room temperature the mixture was diluted to 2.5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 1.80 mg/mL

DAR: 6.4

ADCs 6h and 7h



Anti-TWEAKR ADC with TPP-7007 (Compound 6h):

A solution of TWEAKR antibody TPP-7007 in PBS buffer (0.5 mL, 10 mg/mL) was supplemented with Compound 4h (0.185 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) and the mixture was stirred for 1 hour at room temperature. Compound 4h (0.18 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) was added. After stirring for an additional hour at room temperature the mixture was diluted to 2.5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 2.63 mg/mL

DAR: 4.3

Anti-HER2 ADC with TPP-1015 (Compound 7h):

A solution of Trastuzumab antibody TPP-1015 in PBS buffer (0.5 mL, 10 mg/mL) was supplemented with Compound 4h (0.18 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) and the mixture was stirred for 1 hour at room temperature. Compound hg (0.18 mg, 0.17 μ mol) dissolved in DMSO (50 μ L) was added. After stirring for an additional hour at room temperature the mixture was diluted to 2.5 mL with PBS buffer and then applied to PBS-equilibrated PD 10 columns (Sephadex[®] G-25, GE Healthcare) and eluted with PBS buffer. The ADC solution was concentrated by ultracentrifugation and rediluted with PBS buffer (pH = 7.2).

Protein concentration: 2.27 mg/mL

DAR: 6.0

6 Analytical characterization of ADCs

a. Analysis of aggregation by SEC-HPLC and determination of DAR and concentration

The monomer content of all ADCs was determined by size exclusion chromatography (SEC). Here, 50 µL of aliquots the ADCs were analyzed using an Agilent 1260 HPLC system with detection at 280 nm and 260 nm. A Superdex 200 10/300 GL column from GE Healthcare (Lot No: 10194037; 10x310 mm, 13 µm particle size) was used at a flow rate of 1 mL/min using isocratic condition. The mobile phase consisted of PBS buffer (pH = 7.2). For the determination of the monomer content, the % peak area of the monomer peak at 280 nm was calculated. The observed side products were dimers or aggregates. All ADCs showed a monomer content of ≥ 94 %.

The toxophore load (DAR) was determined by UV absorption during SEC. The ratio R of the peak area of the monomer peak at 260 nm and at 280 nm was determined and the DAR was calculated as:

$$DAR = \frac{\epsilon_{Ab}^{\lambda_{drug}} - R \cdot \epsilon_{Ab}^{280}}{R \cdot \epsilon_D^{280} - \epsilon_D^{\lambda_{drug}}}$$

where ϵ stands for the molar extinction coefficients of the antibody (Ab) and the drug (D), and λ_{Drug} stands for 260 nm.

The extinction coefficients of the antibody at a wavelength of 280 nm and of the drug at a wavelength 260 nm were determined experimentally. Mean values of different antibodies were used for calculation. The following wavelengths and extinction coefficients were determined and used for the DAR calculations:

	$\epsilon(280 \text{ nm})$ [1/µM]	$\epsilon(260 \text{ nm})$ [1/µM]
Antibody	0.2284	0.1163
Toxophore (KSPi)	0.010	0.014

The concentration of ADCs was determined by measuring the absorption at 280 nm. The concentration was calculated using the absorption coefficient of the respective antibody. To take into account the absorbance of the toxophore at 280 nm, the concentration was corrected using the following equation:

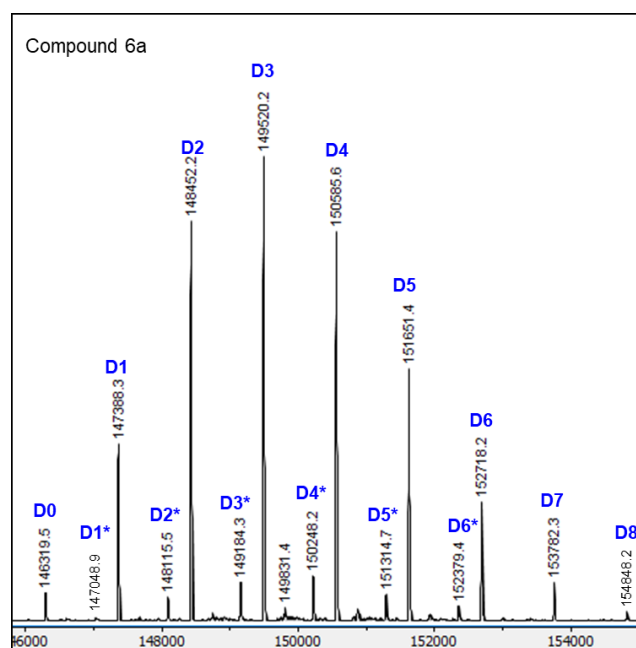
$$\text{concentration} = \text{preliminary concentration} / [1 + \text{DAR}_{UV} * (\epsilon_{\text{Toxophore } 280\text{nm}} / \epsilon_{\text{Antibody } 280\text{nm}})]$$

where “preliminary concentration” is the concentration being calculated only using the extinction coefficient of the antibody, DAR_{UV} is the drug load of the respective ADC determined by UV absorption, and $\epsilon_{Toxophore\ 280nm}$ and $\epsilon_{Antibody\ 280nm}$ are the extinction coefficients at 280 nm of the toxophore and the antibody, respectively.

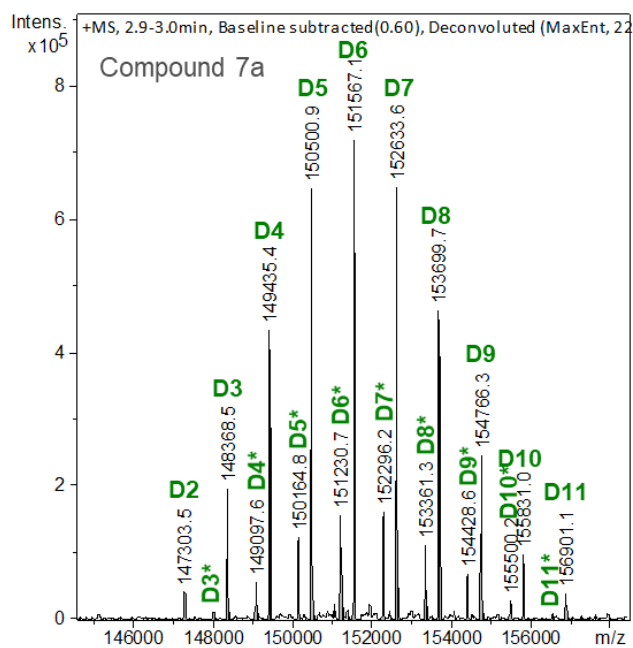
b. Distribution of the payload on ADC, analytical method and data

The determination of the distribution of the payload on the ADC was carried out by mass spectrometry. After deglycosylation with PNGaseF at 37°C over night, the ADC samples were acidified and diluted to a concentration of 1pmol/μL. After HPLC desalting, distribution of the payload was analyzed by mass spectrometry using an Impact II ESI-Q/ToF (Bruker Daltonics). All spectra over the signal in the TIC (Total Ion Chromatogram) were added and the molecular weight of the individual conjugated species were calculated based on MaxEnt deconvolution (additional signals marked with * explained as mass spectrometry derived ionization fragments).

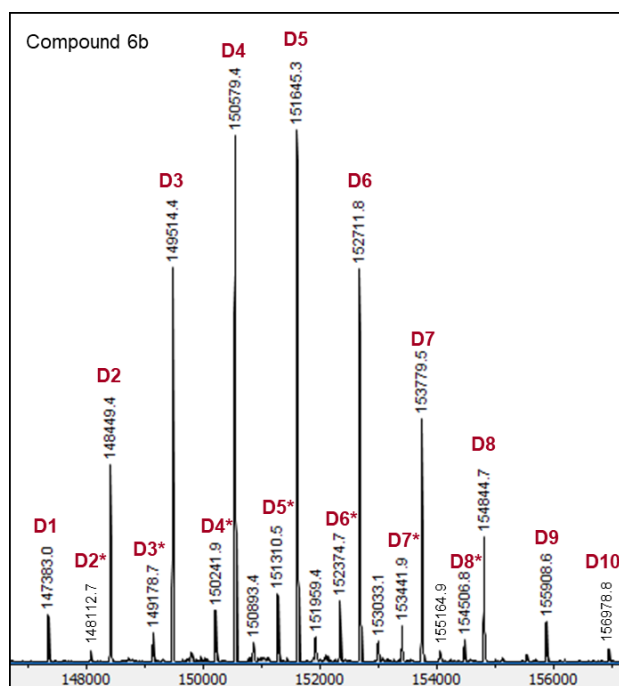
Anti-TWEAKR ADC with TPP-7007 (Compound 6a):



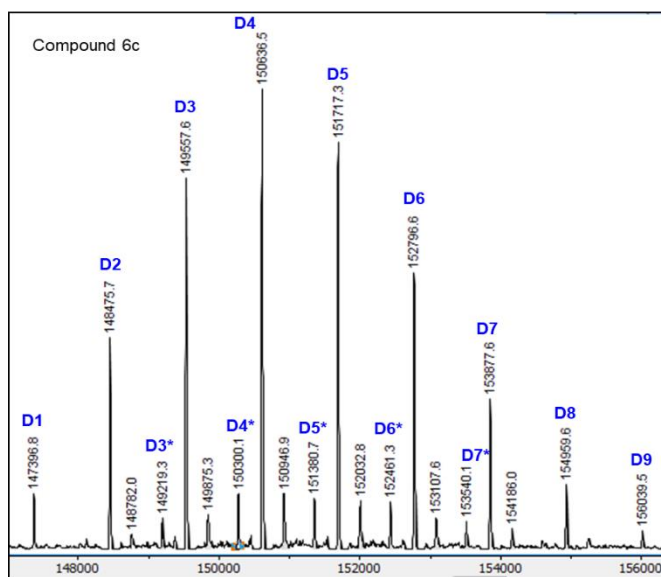
Anti-HER2 ADC with TPP-1015 (Compound 7a):



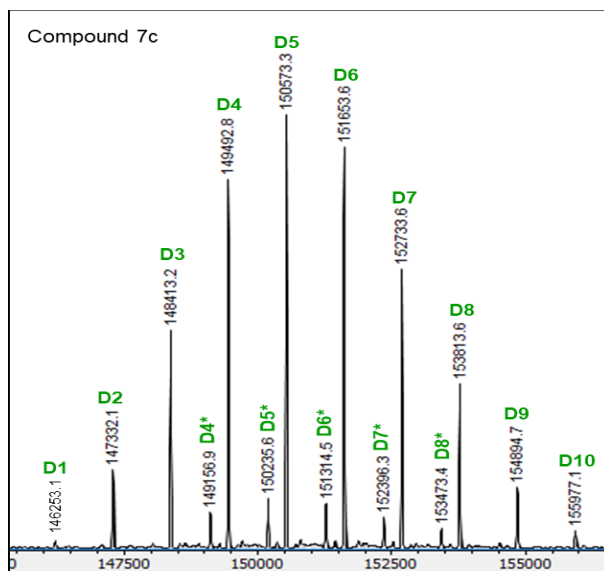
Anti-TWEAKR ADC with TPP-7007 (Compound 6b):



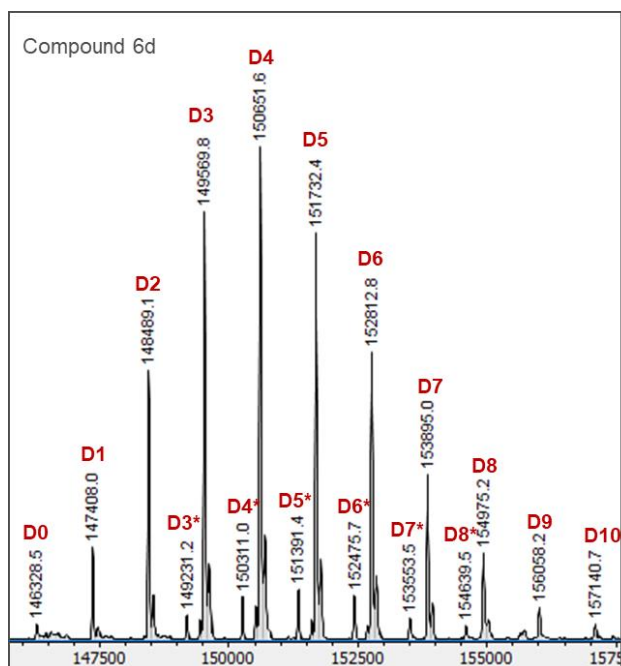
Anti-TWEAKR ADC with TPP-7007 (Compound 6c):



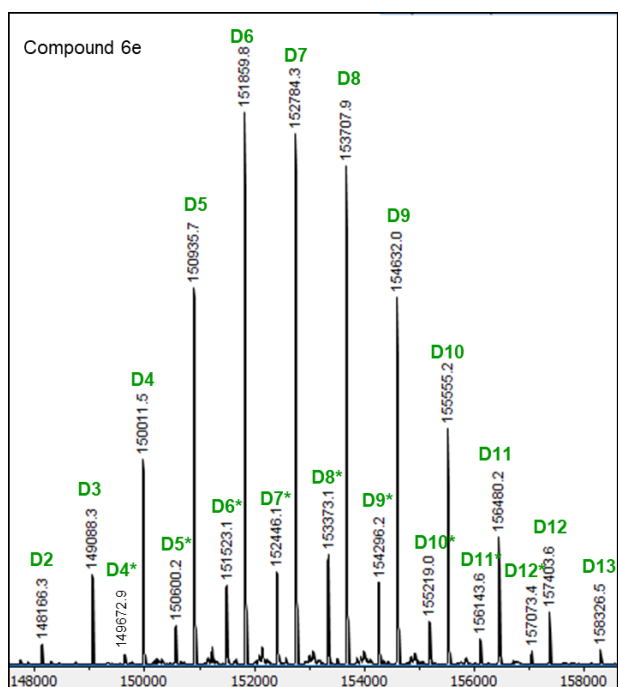
Anti-HER2 ADC with TPP-1015 (Compound 7c):



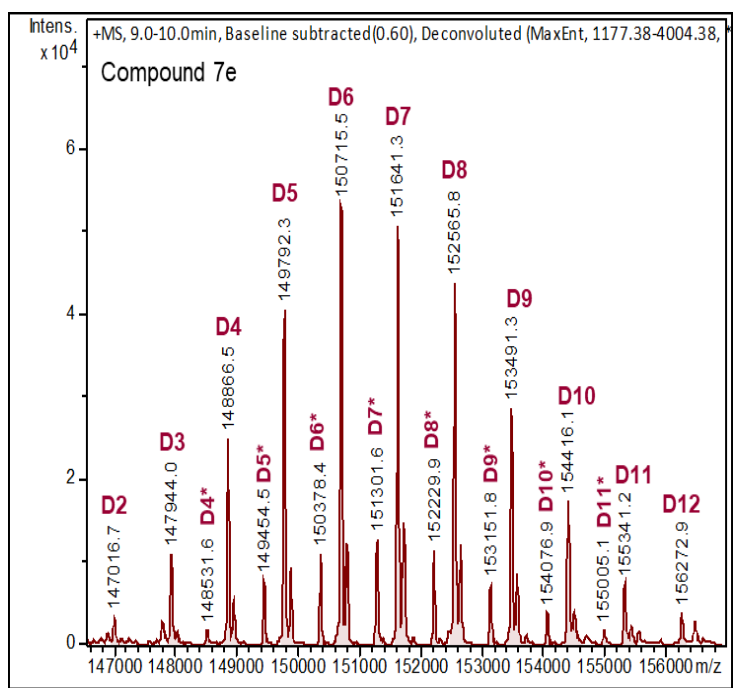
Anti-TWEAKR ADC with TPP-7007 (Compound 6d)



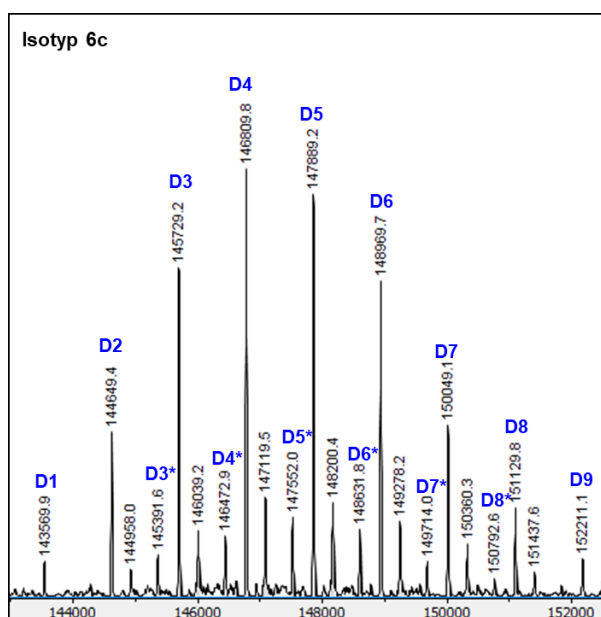
Anti-TWEAKR ADC with TPP-7007 (Compound 6e):



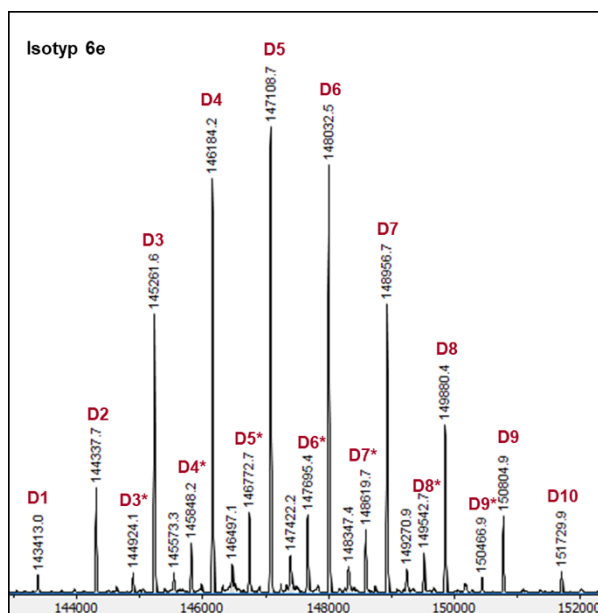
Anti-HER2 ADC (TPP-1015) (Compound 7e):



Isotype control ADC with TPP-5657 (Isotyp 6c):



Isotype control ADC with TPP-5657 (Isotype 6e):



7. In vitro assays

Biophysical evaluation and cellular characterization of the purified antibodies was performed before using them for conjugation.

a. Surface plasmon resonance (SPR)

Binding assays were performed on a Biacore T200 instrument at 25 °C with a CM5 sensor chip and assay buffer HBS-EP+. IgGs were captured via an amine coupled anti-Fc capture Ab and recombinant human target molecule was used as analyte at concentrations ranging from 1.56 - 200 nM. Data were fit to a 1:1 Langmuir binding model.

b. Flow cytometry

Binding of antibodies to target expressing cells was analyzed by flow cytometry. Additionally, TWEAKR expression in tumor cell lines was assessed by quantitative flow cytometry with the QIFI kit (Dako, K0078) using the anti-TWEAKR mouse IgG2a (BAY-039) and the FITC-conjugated goat anti-mIgG detection antibody.

c. Internalization

Internalization was monitored via fluorescent labelling of specific anti-TWEAKR antibodies and an isotype control antibody. The fluorescent dye was conjugated to lysines of the antibody. Then, cancer cells were incubated with the labelled antibody. The fluorescence measurement was

carried out using the InCell Analyser 1000 (GE Healthcare). This was followed by kinetic evaluation via measurement of the parameters granule counts/cell and total granule intensity/cell.

Table S1: Summary of the biophysical and cellular data of anti-TWEAKR antibodies in NCI-H292 cells. SPR analysis was performed with recombinant human TWEAKR as antigen/analyte for the anti-TWEAKR antibodies.

Antibody	Surface Plasmon Resonance: K_d [M]	Binding to NCI-H292 cells: EC_{50} [M]	Internalization in NCI-H292 cells: Granule count/cell
TPP-2658 Agonistic anti-TWEAKR Ab	1.3E-08	4.3E-10	22
TPP-7007 Antagonistic anti-TWEAKR Ab	1.4E-09	8.0E-10	28
Isotype control Ab	n.a	no binding	0-0.3

d. Cell proliferation assays

Determination of IC_{50} by CellTiterGlo assay:

The cytotoxicity of anti-TWEAKR and corresponding isotype control ADCs, SMOL KSP inhibitors, and payload metabolites was determined in TWEAKR-positive NCI-H292, BxPC3, and LoVo cancer cell lines. Cells were treated with indicated compounds for 72 h and the CellTiter Glo (CTG) cell viability assay was performed according to manufacturer's instructions (Promega, Madison, WI).

Determination of IC_{50} by MTT assay:

The cytotoxicity of anti-HER2 and corresponding isotype control ADCs, KSP inhibitors, and payload metabolites was determined in the HER2-positive breast cancer cell line KPL-4. Cells were treated with the indicated compounds of interest for 96 h and the MTT cell proliferation assay was performed according to manufacturer's instructions (ATCC).

e. Legumain cleavage assay

Activation buffer: 50 mM sodium acetate buffer/ 100 mM NaCl, pH 4.0

Assay buffer: 50 mM MES buffer/ 250 mM NaCl, pH 5.0

10 µg of recombinant human legumain (R&D systems) was added to 100 µL of activation buffer and incubated for 2 h at 37°C, before 4.9 mL of assay buffer was added (final concentration of recombinant human legumain: 2 µg/mL).

For each test sample, a 500 µL aliquot of the compound solution (6 µM) was added to 500 µL of legumain solution. The solution was incubated at 37°C. At different time points (5 min, 15 min, 30 min, 45 min, 90 min, 3 h and 24 h) a 50 µL sample was taken and added to 100 µL of ice-cold methanol. For the untreated control (0 h), ice-cold methanol was pipetted in a tube before the legumain and compound solutions were added. Samples were stored at -20°C or analyzed directly by HPLC or LC-MS. The metabolite (product) was measured and the kinetic evaluation was performed.

f. Elastase cleavage assay

Assay buffer: H₂O

For each test sample, Elastase (Sigma E8140-1U) solution (3 µg/mL final concentration) was added to the compound solution (final concentration of 100 nM). For ADCs, the payload concentration was calculated based on the respective DAR and set to a final concentration of 100 nM. The samples were incubated at 37°C with shaking at 600 rpm. At different time points (4 h, 24 h and 48 h), a 50 µL sample was taken. Immediately afterwards, the enzymatic activity was stopped by adding 100 µL of ice-cold methanol. For the untreated control (0 h), ice-cold methanol was pipetted in a tube before the enzyme and compound solutions were added. Samples were stored at -20°C or analyzed directly by HPLC or LC-MS. The metabolite (product) was measured and the kinetic evaluation was performed.

g. Cathepsin B cleavage assay

Assay buffer: 50 mM Natriumphosphate buffer / 2 mM DTT

For each test sample, Cathepsin B (Sigma C8571-25UG) solution (2.8 µg/mL final concentration) was added to the compound solution (final concentration of 32 µM). The concentration of the ADC solution was calculated based on the respective DAR. The samples were incubated at 40°C with shaking at 600 rpm. At different time points (4 h, 24 h and 48 h), a 50 µL sample was taken. Immediately afterwards, the enzymatic activity was stopped by adding 100 µL of ice-cold methanol. For the untreated control (0 h), ice-cold methanol was pipetted in a tube before the enzyme and compound solutions were added. Samples were stored at -20°C or analyzed directly by HPLC or LC-MS. The metabolite (product) was measured and the kinetic evaluation was performed.

h. Quantification of metabolite formation from ADCs

For quantification of the ADC metabolites released from ADCs, cancer cells were incubated with ADCs 6b*, 6c* and 6e* for up to 72 h. Cell lysates and corresponding supernatants were collected at various time points and the active payload metabolite was quantified by LC-MS. For calibration, the cell homogenate (for cell lysate) or cell culture medium (for supernatant), was spiked with 0.6–1000 µg/L of active metabolite 8. Samples were analyzed using HPLC coupled to a triple-quadrupole mass spectrometer API 4500 (AB Sciex).

i. Preparation of rat lysosome extracts

The preparation of lysosomes from rat liver was performed based on published protocols of Lardeux *et al.*^{S5} and Graham *et al.*^{S6} and modified as described. Briefly, fresh liver was isolated from male Wistar rats and homogenized in homogenization buffer containing 0.25 M sucrose (AppliChem), 1 mM Na₂EDTA (Sigma Aldrich) and 10 mM HEPES (Alfa Aesar) at pH 7. After centrifugation at 3,000 g for 10 min, supernatant was subjected to an ultracentrifugation step at 17,000 g for 10 min to harvest the crude lysosomal fraction. The remaining pellet was immediately resuspended in 5 mL lysis buffer (25 mM HEPES, 150 mM NaCl, 0.1% Triton X-100, pH 5.0) and kept frozen at -80°C until use for lysosomal stability assay.

j. Lysosomal stability assay

The lysosomal extract was mixed 1:10 with citrate buffer (0.09 M, Sigma Aldrich) and ADCs 6a-c and 6e were added at a final concentration of 50 µg/mL to investigate lysosomal stability. As a control, a cathepsin-cleavable ADC was incubated under the same conditions. Incubation at 37°C was stopped after 0, 1, 2, 6, 24 and 48 hours by adding 150 µL of acetonitrile to a 50 µL sample. After acetonitrile precipitation, samples were analyzed for the formation of metabolite 8 or corresponding metabolite of the control ADC using HPLC coupled to a triple-quadrupole mass spectrometer API 4500 (AB Sciex). Results are expressed as % formation of the metabolite. Therefore, a c_{\max} concentration of metabolite was calculated using the molar ADC concentration in the assay multiplied by the respective drug load of the ADC resulting in the maximum concentration of metabolite that can be achieved upon cleavage from the ADC (represents c_{\max} of 100%).

Time [h]	Metabolite release from ADC [% of c_{\max}]				
	6a	6b	6c	6e	control
1	0.7	0.2	0.1	0.2	8
2	1	0.4	0.2	0.2	13
6	2	0.9	0.3	0.2	23
24	11	3	0.5	0.3	55
48	18	4	0.8	0.7	85

Table S2. Formation of metabolites from the ADCs 6a-c, 6e and control ADC (cathepsin cleavable ADC 1.3 in ref. (S2) after 1, 2, 6, 24 and 48 h.

k. Caco-2 assay

The cell permeability of a substance can be investigated by means of *in vitro* testing in a flux assay using Caco-2 cells.^{S7} For this purpose, the cells were cultured for 15-16 days on 24-well filter plates. For the determination of permeation, the respective working example was applied in a HEPES buffer to the cells either apically (A) or basally (B) and incubated for 2 hours. After 0 hours and after 2 hours, samples were collected from the cis- and trans-compartments. The samples were separated by HPLC (Agilent 1200, Böblingen, Germany) using reverse phase columns. The HPLC system was coupled via a Turbo Ion Spray Interface to a Triple Quadrupol mass spectrometer API 4000 (Applied Biosystems Applera, Darmstadt, Germany). The permeability was evaluated on the basis of a Papp value, which was calculated using the formula published by Schwab *et al.*^{S8}

	SMOL KSP Inhibitor A (R= H)	Active metabolite 8
Caco-2 transporter B→A (nm/s)	213	2.7

8. In vivo studies

For the cell line-derived *in vivo* models NCI-H292 (NSCLC, ATCC) or Ku-19-19 (urothelial cancer, DSMZ), either 1×10^6 cells or 2×10^6 cells, respectively, in 100 μ L medium/ matrigel (1:1) were inoculated subcutaneously into the flank of female NMRI *nu/nu* mice (Janvier, France) with n=10 per group. Vehicle (PBS) or the anti-TWEAKR or isotype control ADCs were applied i.v. when tumors reached a size of approximately 100 mm³. Animals were treated with 5 mg/kg ADC (volume: 5 mL/kg) as a QWx2 (NCI-H292) or a QWx3 (Ku-19-19) schedule as indicated in the figure legends. Tumor size and body weight was determined two to three times per week. All animal studies were conducted in line with the German Animal Welfare Act. Statistical analysis was performed using the One-Way-ANOVA (GraphPad Prism 7) based on log-transformed data. The obtained p-values were adjusted for multiple comparisons.

Table S3. Summary of efficacy and maximum body weight loss data of NCI-H292 human non-small cell lung cancer (NSCLC) xenograft model (n=10 mice/ group).

Compound	Dose (mg/kg), Schedule	T/C ^a	Adjusted p value ^b	Max. Body weight change ^c (%)	Response rate ^d	CR	PR	SD	PD
Vehicle PBS	5 mg/kg, QWx2	1.00		-2.94	0%	0	0	0	10
TWEAKR-ADC 6b TPP-7007	5 mg/kg, QWx2	0.05	0.9999	-0.56	80%	0	8	1	0
Isotype control ADC 6b TPP-5657	5 mg/kg, QWx2	1.02	<0.0001	-1.77	0%	0	0	0	10
TWEAKR-ADC 6c TPP-7007	5 mg/kg, QWx2	0.05	0.9968	-0.31	90%	0	9	1	0
Isotype control ADC 6c TPP-5657	5 mg/kg, QWx2	0.95	<0.0001	-0.63	0%	0	0	0	10
TWEAKR-ADC 6e TPP-7007	5 mg/kg, QWx2	0.06	0.2737	-1.07	80	0	8	1	0
Isotype control ADC 6e TPP-5657	5 mg/kg, QWx2	1.35	0.9999	-2.61	0%	0	0	4	10

a) T/C= Treatment/Control (vehicle) ratio, calculated from mean tumor volumes on day 17 after start of treatment when the vehicle group was still in the experiment or from mean tumor volumes on day 14 for isotype control ADC 6e.

b) One-way ANOVA followed by Dunnett's multiple comparisons test, adjusted p value in comparison to vehicle, $p < 0.05$ was considered statistically significant.

c) Body weight change: The maximum mean body weight change on day 17 after start of treatment (day 14 for the isotype control ADC 6e) expressed as percentage of the starting weight of the animal. Weight loss greater than 20% was considered toxic.

d) Response: PD = progressive disease, the number of tumors exhibiting >20% tumor increase; SD = stable disease, the number of tumors exhibiting <30% tumor shrinkage and <20% tumor increase; PR = partial response, the number of tumors exhibiting >30% tumor shrinkage; CR = complete response, the number of non-measurable tumors at end of experiment.

Table S4. Summary of efficacy and maximum body weight loss data of Ku-19-19 human urothelial cancer xenograft model (n=10 mice/ group).

Compound	Dose (mg/kg) and schedule	T/C ^a	Adjusted p value ^b	Max. Body weight change ^c (%)	Response rate ^d	CR	PR	SD	PD
Vehicle PBS	5 mg/kg, QWx3	1.00		2.32	0%	0	0	0	10
TWEAKR-ADC 6b TPP-7007	5 mg/kg, QWx3	0.14	<0,0001	2.93	30%	0	3	1	6
Isotype control ADC 6b TPP-5657	5 mg/kg, QWx3	1.91	0,2086	-6.78	0%	0	0	0	10
TWEAKR-ADC 6c TPP-7007	5 mg/kg, QWx3	0.11	0,0016	2.71	60%	0	6	3	1
Isotype control ADC 6c TPP-5657	5 mg/kg, QWx3	2.25	0,9747	-5.00	0%	0	0	0	10
TWEAKR-ADC 6e TPP-7007	5 mg/kg, QWx3	0.13	0,0003	-4.4	40%	0	4	6	0
Isotype control ADC 6e TPP-5657	5 mg/kg, QWx3	1.53	0,9993	-1.13	0%	0	0	0	10

a) T/C= Treatment/Control (vehicle) ratio, calculated from mean tumor volumes on day 16 after start of treatment.

b) One-way ANOVA followed by Dunnett's multiple comparisons test, adjusted p value in comparison to vehicle, $p < 0.05$ was considered statistically significant.

c) Body weight change: The maximum mean body weight change on day 16 after start of treatment expressed as percentage of the starting weight of the animal. Weight loss greater than 20% was considered toxic.

d) Response: PD = progressive disease, the number of tumors exhibiting >20% tumor increase; SD = stable disease, the number of tumors exhibiting <30% tumor shrinkage and <20% tumor increase; PR = partial response, the number of tumors exhibiting >30% tumor shrinkage; CR = complete response, the number of non-measurable tumors at end of experiment.

9. Literature (Supporting Information)

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Cover page

High resolution image of living cells was used as background. Living cells were analysed using STED microscopy (Abberior Expert line). ADC was labelled with STAR580 (green) and lysosomes were stained with SIR-lysosme (red). Costaining in a fixed single cell using pH-sensitive dye as antibody label and LAMP-1 staining for lysosomal surface detection. The merged image depicted here reveals high level of co-localization of ADC in the lysosomes (yellow).

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