Supplementary Information

DNA model-based design and execution of some fused benzodiazepine hybrid payloads for Antibody-Drug Conjugate modality

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Chemistry

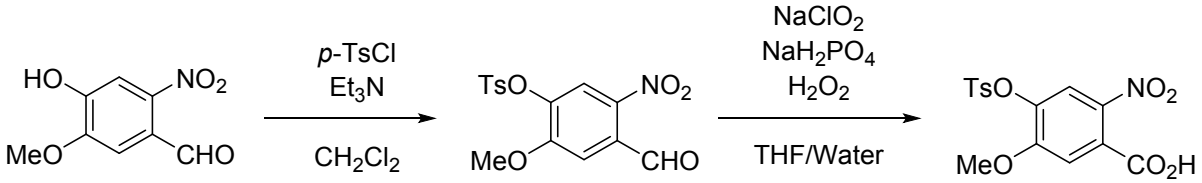
General Information

Commercially available anhydrous solvents were used without further purification. All anhydrous reactions were performed under a N_2 atmosphere. Analytical thin layer chromatography was performed on silica gel 60 F254 aluminum sheets (EMD Chemicals, Gibbstown, NJ). 1H NMR spectra were recorded on Bruker 400 MHz spectrometer. All spectra were determined in the solvents indicated. Multiplicity patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad; dd, doublet of doublets; dt, doublet of triplets. Preparative HPLC was performed on Waters instrument using the following conditions: column: XBridge C18, 200 mm x 19 mm, 5-µm particles; Mobile Phase A: 5:95 acetonitrile: water with 0.05% formic acid; Mobile Phase B: 95:5 acetonitrile: water with 0.05% formic acid; Gradient: a 0-minute hold at 5% B, 5-45% B over 20 minutes, then a 0-minute hold at 100% B; Flow Rate: 20 mL/min; Column Temperature: 25 °C. HPLC fractions with desired product mass were filtered under gravity through a cartridge containing PL-HCO_3 MP-resin (Agilent), washed with acetonitrile and lyophilized. Liquid chromatography (LC) data were recorded

on a Shimadzu LC-10AS liquid chromatograph using a SPD-10AV UV−vis detector with mass spectrometry (MS) data determined using a Micromass Platform for LC in electrospray mode. Purities of final compounds were determined by HPLC and were greater than 95%.

All final compounds were assumed to be highly potent cytotoxics for safety reasons. As such, these should only be handled by experienced practitioners, with extra precautions taken in the final steps, including additional personal protective equipment, dedicated waste streams and use of a designated laboratory.

Synthesis and characterization of **4-32.**



33 **34** **35**

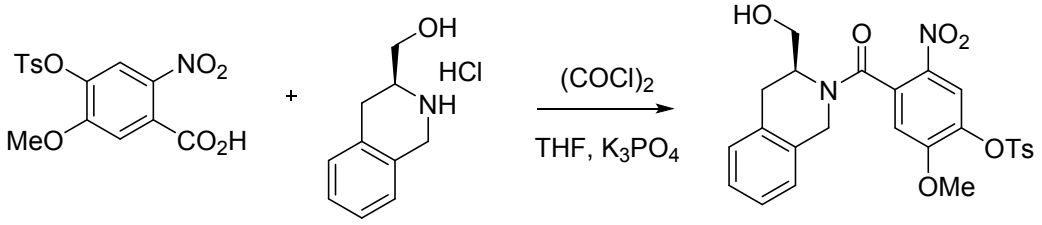
5-Methoxy-2-nitro-4-(tosyloxy)benzoic acid (35):

To a round bottomed flask equipped with a mechanical stirrer and thermocouple was added the aldehyde **33** (1.00 equiv.) and dichloromethane (5 mL/g). The slurry was cooled to an internal temperature of 10 °C with an ice water bath and triethylamine (1.30 equiv.) was added slowly at a rate such that the internal temperature did not exceed 15 °C. *p*-Toluenesulfonyl chloride (1.10 equiv.) was added in portions at a rate such that the internal temperature did not exceed 15 °C. The reaction was complete 30 minutes after the end of the addition. The reaction mixture was washed with an aqueous solution of citric acid (10 wt%, 1 x 5 mL/g, then 1 x 2.5 mL/g), then an aqueous solution of tribasic potassium phosphate (5 mL/g). The aqueous potassium phosphate solution was extracted with dichloromethane (2.5 mL/g). The combined organic layers were combined and washed with brine (2.5 mL/g), dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo* to afford a yellow-beige solid. The solid was washed with a solution of methyl *tert*-butyl ether in heptane (50 v%, 5 mL/g) and the solid was dried in a vacuum oven (40 °C) to constant weight. The product was used directly in the following step without further purification (approximate yield is 94%).

To a round bottomed flask equipped with a mechanical stirrer, addition funnel and thermocouple was added sodium chlorite (1.50 equiv.), monobasic sodium phosphate (2.00 equiv.) and water (5 mL/g). The mixture was stirred vigorously to afford a light yellow solution, then cooled to an internal temperature of 8 °C with an ice water bath and placed under a heavy nitrogen purge. A solution of the tosyl aldehyde **34** (1.0 equiv.) in tetrahydrofuran (5 mL/g) was charged to the addition

funnel and added slowly to the aqueous solution at a rate such that the internal temperature did not exceed 10 °C. After the addition, an aqueous solution of hydrogen peroxide (30 wt%, 1.30 equiv.) was added drop wise at a rate such that the internal temperature did not exceed 10 °C and the reaction was stirred for a further 1 hour. The mixture was cooled to an internal temperature of 3 °C and quenched by the slow addition of a freshly prepared saturated, aqueous solution of sodium bisulfite (3 mL/g), and then the mixture was warmed to room temperature. The presence of peroxides was tested and was negative. The pH was lowered to <1 with concentrated hydrochloric acid (~0.1 mL/g) and ethyl acetate (5 mL/g) was added. The layers were separated, and the aqueous layer was washed with a saturated, aqueous solution of sodium chloride (5 mL/g). The water content of the organic layer was ~3.5 wt% by Karl Fischer titration. The stream was dried to <0.5 wt% via constant-volume distillation, maintained at ~6.5 mL/g, with ethyl acetate (~20 mL/g) causing precipitation of residual salts. The slurry was polish filtered to a reactor preheated to 55 °C and concentrated down to a final volume of 5 mL/g. The solvent was exchanged to toluene maintaining a constant volume causing the acid to crystallize. The slurry was cooled to an internal temperature of approximately 2-3 °C, and was held for 1 hour. The crystals were collected by Buchner filtration, washed with toluene (1 mL/g), and dried in a vacuum oven at 45 °C until constant weight was achieved. The acid was obtained as sandy, white crystals. The typical yield is 92%.

^{1}H NMR (400 MHz, DMSO-d_6) δ: 14.02 (br s, 1H), 7.83 (s, 1H), 7.78 (d, J = 8.3 HZ, 2H), 7.50 (d, J = 8.2 Hz, 2H), 7.40 (s, 1H), 3.67 (s, 3H), 2.44 (s, 3H).



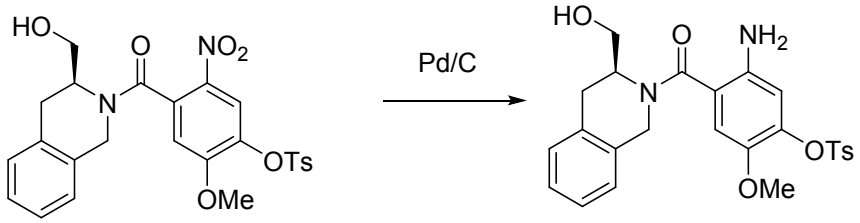
35 **36** **37**

(*S*)-4-(3-(hydroxymethyl)-1,2,3,4-tetrahydroisoquinoline-2-carbonyl)-2-methoxy-5-nitrophenyl 4-methylbenzenesulfonate (37):

To a round bottomed flask equipped with a stir bar and a nitrogen purge line terminating into a saturated aqueous solution of sodium bicarbonate was added the acid **35** (1.00 equiv.) and tetrahydrofuran (5 mL/g). *N*,*N*-dimethylformamide (0.10 equiv.) was added and oxalyl choride (1.10 equiv.) was added drop wise. The reaction was complete 15 minutes after the addition. The crude acid chloride was added to a vigorously stirring solution containing the tetrahydroisoquinoline **36** (1.20 equiv.), tribasic potassium phosphate (4.00 equiv.) and water (10 mL/g). The reaction was complete 30 minutes after the addition. The layers were separated and the organic layer was diluted with methyl

tert-butyl ether (5 mL/g) then washed with an aqueous solution of citric acid (10 wt%, 2 x 5.0 mL/g). The combined aqueous layers were extracted with methyl *tert*-butyl ether (5.0 mL/g). The combined organic layers were concentrated *in vacuo*, then diluted with toluene (10 mL/g) and concentrated *in vacuo* to afford a residue that was mostly dissolved in tetrahydrofuran (3 mL/g) at 40 °C. The solids were removed by filtration into a reactor preheated to 55 °C and the solvent was exchanged to toluene maintaining a constant volume (5 mL/g) causing the amide to precipitate. The solids, contaminated with citric acid, were collected by Buchner filtration, then dissolved in 2-methyltetrahydrofuran (10 mL/g) and washed with an aqueous solution of potassium phosphate (10 wt%, 2 x 10 mL/g), and then a saturated, aqueous solution of sodium chloride (10 mL/g). The solvent was concentrated *in vacuo*, then the material was recrystallized from methanol and water. The typical yield is 82%.

LRMS (m/*z*): Calculated for $C_{25}H_{25}N_2O_8S$ $[M+H]^+$ 513.1, found 513.2.

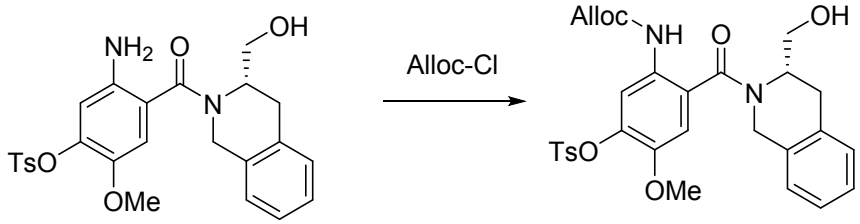


37 **SI-1**

(*S*)-5-amino-4-(3-(hydroxymethyl)-1,2,3,4-tetrahydroisoquinoline-2-carbonyl)-2-methoxyphenyl 4-methylbenzenesulfonate (SI-1):

To a high-pressure reactor was charged the nitro compound **37** (1.00 equiv.) and 5 wt% palladium on carbon doped with 1.0 wt% iron (5% uncorrected, gross wt/wt%, the catalyst was 62.68 wt% water wet). Methanol was added (10 mL/g) and reactor was sealed. The atmosphere was exchanged for hydrogen though three purge cycles, then pressurized with hydrogen to 35 psig and stirred for 6.5 hours whereupon hydrogen uptake ceased. The atmosphere was exchanged for nitrogen and dichloromethane (5 mL/g) was added. The mixture was filtered through a polypropylene filter cartridge to remove the catalyst and the material was concentrated *in vacuo*. The typical yield is 99%.

LRMS (m/*z*): Calculated for $C_{25}H_{27}N_2O_6S$ $[M+H]^+$ 483.2, found 483.0.

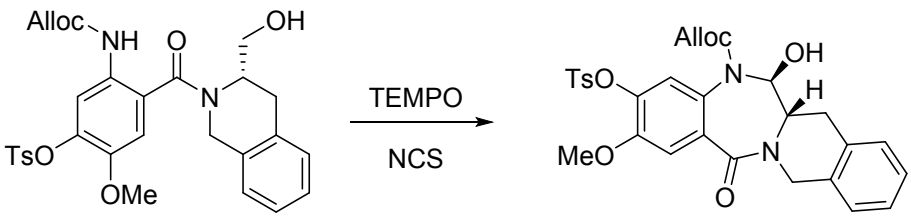


SI-1 **38**

(*S*)-5-(Allyloxycarbonylamino)-4-(3-(hydroxymethyl)-1,2,3,4-tetrahydroisoquinoline-2-carbonyl)-2-methoxyphenyl 4-methylbenzenesulfonate (38):

Note!! Alloc-Cl is freshly distilled prior to use. Unpurified Alloc-Cl can lead to the formation of urea-like impurities. To a mechanically stirred solution of sodium bicarbonate (2.00 equiv.) in water (5 mL/g) was added a solution of the aniline **SI-1** (1.00 equiv.) in tetrahydrofuran (5 mL/g). The purified alloc-Cl (1.05 equiv.) was added drop wise and the mixture was stirred for an additional 1 hour after the addition was ended. 2-Methyltetrahydrofuran (10 mL/g) was added and the layers were separated. The organic layer was washed with an aqueous solution of hydrochloric acid (1.0 M, 5 mL/g), a saturated, aqueous solution of sodium chloride (5 mL/g) then concentrated *in vacuo* to a final volume of 2 mL/g. Methanol (3.0 mL/g) was added, then water (10 mL/g) was added to crystallize the product. The solids were collected by Buchner filtration and dried in a vacuum oven to constant weight. The typical yield is 98%.

LRMS (m/*z*): Calculated for $C_{29}H_{31}N_2O_8S$ $[M+H]^+$ 567.2, found 567.1.



38 **39**

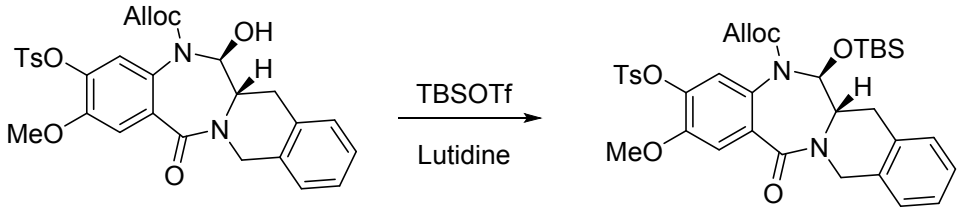
Allyl (6*S*,6a*S*)-6-hydroxy-2-methoxy-14-oxo-3-(tosyloxy)-6,6a,7,12-tetrahydrobenzo[5,6][1,4]diazepino[1,2-*b*]isoquinoline-5(14*H*)-carboxylate Toluene Solvate (39):

To a Morton flask was added the alcohol **38** (1.0 equiv.), TEMPO (0.05 equiv.), tetrabutylammonium chloride (0.10 equiv.) and dichloromethane (10 mL/g). An aqueous carbonate buffer (10 mL/g), prepared by mixing sodium bicarbonate (42 g/L) and potassium carbonate (6.91 g/L) was added and the biphasic mixture was vigorously stirred with a mechanical stirrer. *N*-Chlorosuccinimide (1.50 equiv.) was added and the mixture was stirred for 5 hours. An additional charge of *N*-chlorosuccinimide (0.10 equiv.) was added and the mixture was stirred for 2 hours. After reaction completion, an aqueous solution of sodium bisulfite (10 wt%. 10 mL/g) was added and the layers were separated. The organic layer was washed with a saturated aqueous solution of sodium chloride (10 mL/g), dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo* to afford a foam. The foam was charged with toluene (5 mL/g) and heated to an internal temperature of 80 °C, then cooled to room temperature forming a heavy slurry. The crystals were isolated by filtration and

washed with toluene (5 mL/g), then dried in a vacuum oven to constant weight. The mother liquor was concentrated *in vacuo* and the residue was purified by flash column chromatography over silica gel (0 to 20% ethyl acetate in dichloromethane gradient) to afford a semi-pure product that was dissolved in toluene (2 mL/g of crude residue) at 80 °C. Cooling and isolating as above afforded an additional crop of material. The product contains 0.67 mol% toluene. The total yield is typically 86%.

^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.72 (d, J = 8.2 Hz, 2H), 7.45 (d, J = 8.2 Hz, 2H), 7.29-7.38 (m, 3H), 7.23-7.29 (m, 2.4H), 7.12-7.21 (m, 3H), 7.02 (br s, 1H), 6.58-6.73 (br m, 1H), 5.78 (ddt, J = 16.0, 10.4, 5.1 Hz, 1H), 4.93-5.23 (m, 3H), 4.71 (d, J = 15.4 Hz, 1H), 4.37-4.60 (m, 3H), 3.59 (s, 3H), 3.37-3.47 (m, 1H), 2.98-3.13 (m, 2H), 2.42 (s, 3H), 2.30 (s, 2H). Note: 0.67 mol% toluene present.

LRMS (m/z): Calculated for $C_{29}H_{29}N_2O_8S$ $[M+H]^+$ 565.2, found 565.2.



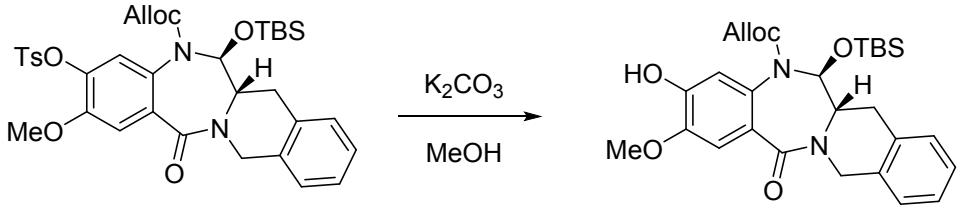
39 **SI-2**

Allyl (6*S*,6a*S*)-6-(*tert*-butyldimethylsilyloxy)-2-methoxy-14-oxo-3-(tosyloxy)-6,6a,7,12-tetrahydrobenzo[5,6][1,4]diazepino[1,2-*b*]isoquinoline-5(14*H*)-carboxylate (SI-2):

To a round bottomed flask was added the aminal **39** (1.0 equiv.) and dichloromethane (10 mL/g) then placed under an atmosphere of nitrogen. 2,6-lutidine (3.00 equiv.) was added and the solution was cooled to an internal temperature of 0 °C with an ice water bath. TBSOTf (2.00 equiv.) was added drop wise at a rate such that the internal temperature did not exceed 2 °C. The mixture was warmed to room temperature and stirred for 1 hour. Upon reaction completion, the mixture was washed with an aqueous solution of citric acid (10 wt%, 10 mL/g), then a saturated, aqueous solution of sodium bicarbonate (10 mL/g), dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo* to give a brown gel. The gel was purified by flash column chromatography over silica gel (0 to 50% ethyl acetate in hexanes gradient) to afford the product as a white foam. The typical yield is 89%.

^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.70 (d, J = 8.3 Hz, 2H), 7.47 (d, J = 8.3 Hz, 2H), 7.23-7.28 (m, 5H), 6.84 (s, 1H), 5.75 (ddt, J = 16.4, 10.5, 5.0 Hz, 1H), 5.21 (app d, J = 9.4 Hz, 1H), 5.12 (d, J = 10.8 Hz, 1H), 5.06 (d, J = 17.0 Hz, 1H), 4.77 (br d, J = 15.7, 1H), 4.47-4.63 (m, 1H), 4.27-4.45 (m, 2H), 3.63 (s, 3H), 3.44-3.52 (m, 1H), 3.11 (br dd, J = 15.0, 5.8 Hz, 1H), 2.92 (br dd, J = 15.4, 1.8 Hz, 1H), 2.41 (s, 3H), 0.86 (s, 9H), 0.09 (s, 3H), 0.00 (s, 3H).

LRMS (m/z): Calculated for $C_{35}H_{43}N_2O_8SSi$ $[M+H]^+$ 679.3, found 679.2.



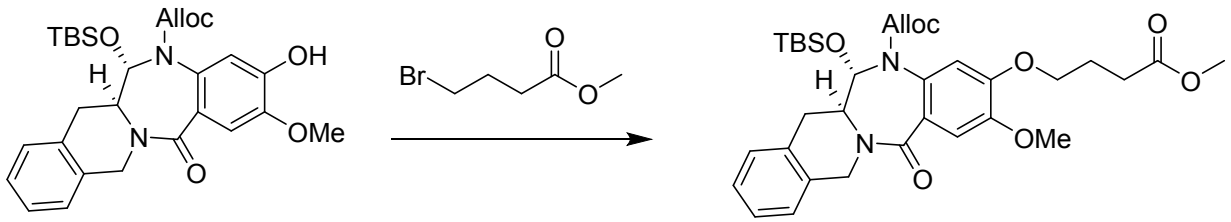
SI-2 **40**

Allyl (6*S*,6a*S*)-6-(*tert*-butyldimethylsilyloxy)-3-hydroxy-2-methoxy-14-oxo-6,6a,7,12-tetrahydrobenzo[5,6][1,4]diazepino[1,2-*b*]isoquinoline-5(14*H*)-carboxylate (40):

A suspension of compound **SI-2** (1.00 equiv.) and potassium carbonate (2.00 equiv.) in methanol (10 mL/g) was stirred at room temperature for 8 hours, then concentrated *in vacuo* to afford a residue that was partitioned between 1 N HCl (15 mL/g) and ethyl acetate (15 mL/g). The layers were separated and the organic layer was washed with an aqueous solution of a pH 7 sodium phosphate buffer (15 mL/g), dried over anhydrous sodium sulfate, filtered then concentrated *in vacuo*. The crude product was purified by flash column chromatography over silica gel (5 to 65% ethyl acetate in hexanes gradient) to afford the product as a white foam. The typical yield is 82%.

^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.87 (s, 1H), 7.36 (br d, J = 6.8 Hz, 1H), 7.23-7.34 (m, 3H), 7.11 (s, 1H), 6.60 (s, 1H), 5.72 (ddt, J = 15.4, 10.0, 4.8 HZ, 1H), 5.21 (d, J = 9.4 Hz, 1H), 4.96-5.12 (m, 2H), 4.72 (br d, J = 15.5 Hz, 1H), 4.51 (br dd, J = 14.2, 4.1 Hz, 1H), 4.26-4.37 (m, 2H), 3.83 (s, 3H), 3.09 (br dd, J = 15.2, 5.5 Hz, 1H), 2.91 (br d, J = 13.8 Hz, 1H), 0.88 (s, 9H), 0.10 (s, 3H), 0.08 (s, 3H).

LRMS (m/z): Calculated for $C_{28}H_{37}N_2O_6Si$ $[M+H]^+$ 525.2, found 525.2.

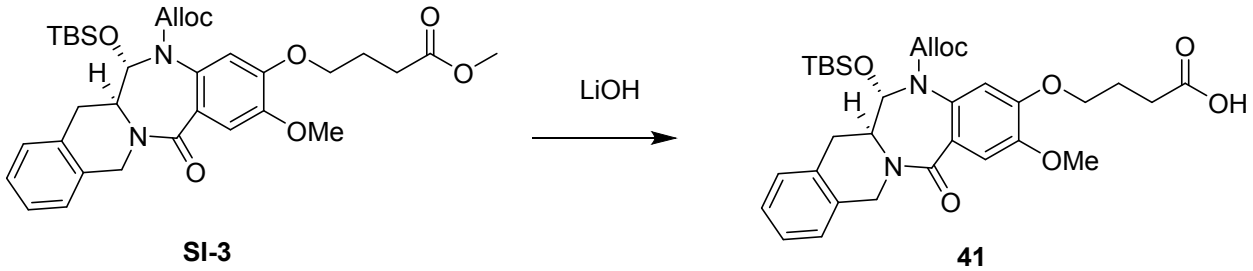


40 **SI-3**

Allyl (6S,6aS)-6-((tert-butyldimethylsilyl)oxy)-2-methoxy-3-(4-methoxy-4-oxobutoxy)-14-oxo-6,6a,7,12-tetrahydrobenzo[5,6][1,4]diazepino[1,2-b]isoquinoline-5(14H)-carboxylate (SI-3):

A suspension of phenol **40**, methyl 4-bromobutanoate (1000 mg, 5.52 mmol) and cesium carbonate (2000 mg, 6.14 mmol) in DMF (5 mL) was stirred at ambient temperature for 1hour. The mixture was quenched with 0.1 M citric acid and extracted thrice with ethyl acetate. The combined organics were washed with brine and dried over sodium sulfate, filtered and evaporated. The mixture

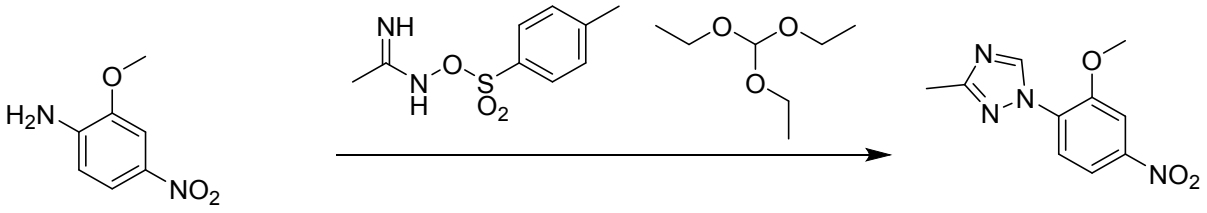
was purified by silica gel chromatography, eluting with a gradient from 30-80% ethyl acetate in hexanes to afford ester **SI-3** (1710 mg, 2.74 mmol, 96% yield) LRMS (m/*z*): Calculated for $C_{33}H_{44}N_2O_8Si$ $[M+H]^+$ 626.0, found 625.3.



SI-3 **41**

4-(((6S,6aS)-5-((allyloxy)carbonyl)-6-((tert-butyldimethylsilyl)oxy)-2-methoxy-14-oxo-5,6,6a,7,12,14-hexahydrobenzo[5,6][1,4]diazepino[1,2-b]isoquinolin-3-yl)oxy)butanoic acid (41):

Ester **SI-3** (1710 mg, 2.74 mmol) was dissolved in MeOH (40 mL) and water (10 mL) was added. To this solution was added lithium hydroxide hydrate (655 mg, 27.4 mmol). After stirring 1hour, the mixture was quenched with 0.1 M citric acid and extracted thrice with ethyl acetate. The combined organics were washed with brine and dried over sodium sulfate, filtered and evaporated to afford acid **41**, which was used without further purification (1.54 g, 5.52 mmol, 92% yield). LRMS (m/*z*): Calculated for $C_{32}H_{42}N_2O_8Si$ $[M+H]^+$ 611.3, found 611.2.

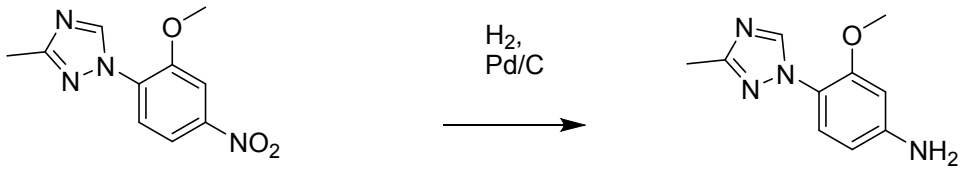


SI-4 **SI-5**

1-(2-methoxy-4-nitrophenyl)-3-methyl-1H-1,2,4-triazole (SI-5):

N-(tosyloxy)acetimidamide (4.89 g, 21.41 mmol, prepared according to EP 0795551 A1) and triethoxymethane (4.45 ml, 26.8 mmol) were added to a solution of 2-methoxy-4-nitroaniline **SI-4** (3 g, 17.84 mmol) in THF (30 mL). The mixture was heated at 60 °C overnight, cooled to room temperature and concentrated. The residue was taken up in methylene chloride, washed with 40 mL of a 2:1 mixture of saturated sodium bicarbonate:1 N NaOH and extracted a second time with methylene chloride. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered and concentrated. The residue was taken up in 50 mL of hot EtOAc and 25 mL of hexane was added. The mixture was heated

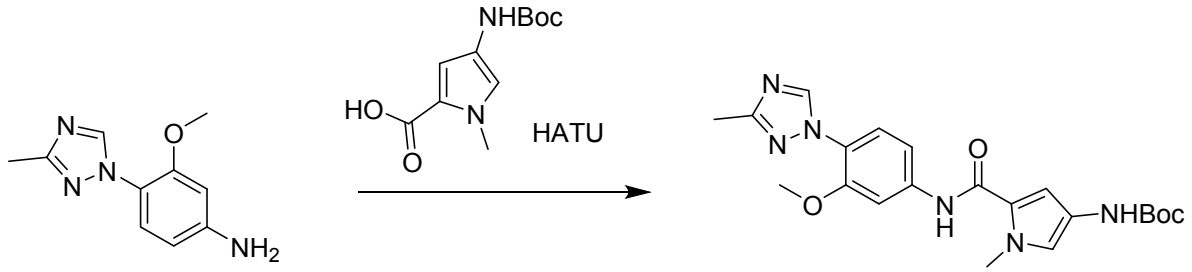
to boiling, cooled and filtered to give **SI-5** a red brown solid (2.6 g, 62 %). LRMS (m/*z*): Calculated for $C_{10}H_{10}N_4O_3$ $[M+H]^+$ 235.1, found 235.1.



SI-5 **SI-6**

3-methoxy-4-(3-methyl-1H-1,2,4-triazol-1-yl)aniline (SI-6):

A dried 25 mL, two-necked, round-bottom flask was equipped with a magnetic stirring bar, an adaptor with an N_2 inlet, and with an cooled condenser, was charged with triazole **SI-5** (0.5 g, 2.135 mmol) in MeOH (20 mL). To this was added Pd/C (0.045 g, 0.427 mmol), and the reaction mixture was fitted with a hydrogen balloon and stirred for 4 hours. The reaction mixture was filtered through a celite bed and concentrated under reduced pressure. The residue was dissolved in DCM (10 mL), and silica gel (5 g) was added. The resultant slurry of the compound on silica was subjected to flash chromatography using a Teledyne Isco instrument (40 g Redi*Sep* silica column, 50 % ethyl acetate in petroleum ether) to afford aniline **SI-6** as a light green solid (0.4 g, 92 %). LRMS (m/*z*): Calculated for $C_{10}H_{12}N_4O$ $[M+H]^+$ 205.1, found 205.2.

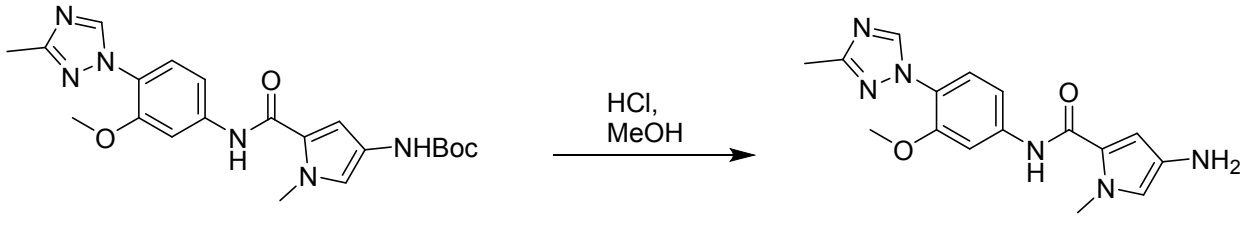


SI-6 **SI-7**

tert-butyl (5-((3-methoxy-4-(3-methyl-1H-1,2,4-triazol-1-yl)phenyl)carbamoyl)-1-methyl-1H-pyrrol-3-yl)carbamate (SI-7):

A mixture of 4-((tert-butoxycarbonyl)amino)-1-methyl-1H-pyrrole-2-carboxylic acid (0.5 g, 2.081 mmol) and 2-(3H-[1,2,3]triazolo[4,5-b]pyridin-3-yl)-1,1,3,3-tetramethylisouronium hexafluorophosphate (V) (HATU, 0.870 g, 2.289 mmol) in DMF (2.5 mL) was stirred for 10 minutes at room temperature. N-ethyl-N-isopropylpropan-2-amine (DIPEA, 1.090 mL, 6.24 mmol) was added followed by **SI-6** (0.425 g, 2.081 mmol). The reaction mixture was stirred at room temperature for 3 hours, and then heated at 97 ^oC for 12 hours. The reaction mixture was diluted with DCM and purified

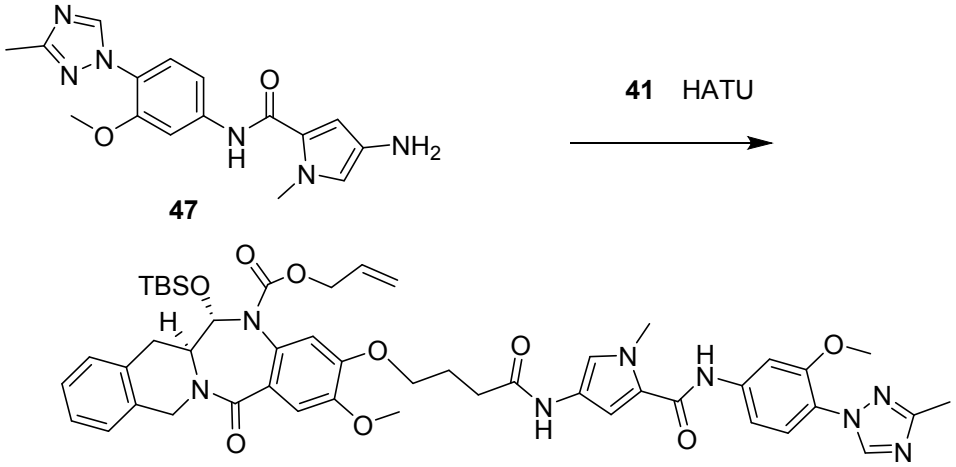
on silica gel (120 g isco column) using 0-10 % methanol in ethyl acetate. The desired fractions were concentrated to give **SI-7** as a beige solid (0.69 g, 78 %). LRMS (m/*z*): Calculated for $C_{21}H_{26}N_6O_4$ $[M+H]^+$ 427.2, found 427.1.



SI-7 **47**

4-amino-N-(3-methoxy-4-(3-methyl-1H-1,2,4-triazol-1-yl)phenyl)-1-methyl-1H-pyrrole-2-carboxamide (47):

HCl (4.04 mL, 16.18 mmol, 4 N in dioxane) was added to a solution of **SI-7** (0.69 g, 1.618 mmol) in DCM (4 mL). The reaction mixture was stirred at room temperature 24 hours. The reaction mixture (suspension) was diluted with ether, filtered, suction dried and dried under vacuum to give **47** as a beige solid (0.53 g, 90 %). LRMS (m/*z*): Calculated for $C_{16}H_{28}N_6O_2$ $[M+H]^+$ 327.2, found 327.0.



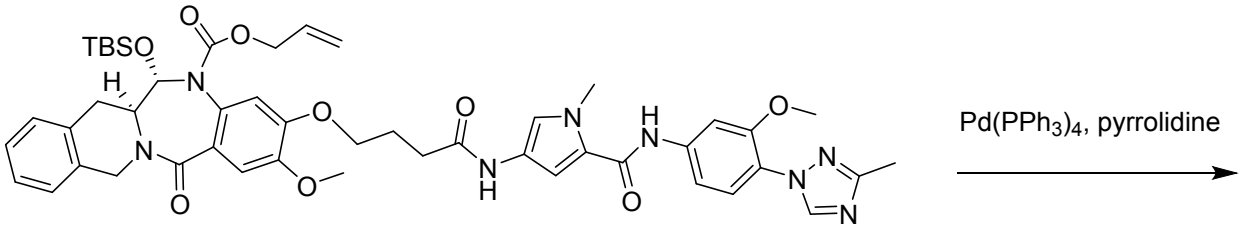
47

SI-8

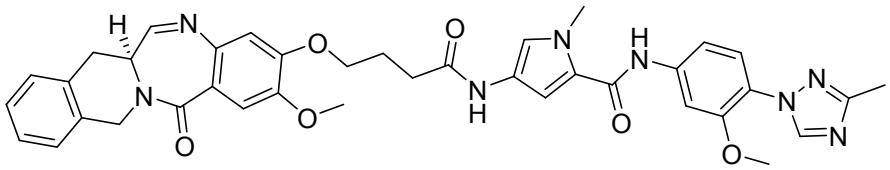
allyl (6S,6aS)-6-((tert-butyldimethylsilyl)oxy)-2-methoxy-3-(4-((5-((3-methoxy-4-(3-methyl-1H-1,2,4-triazol-1-yl)phenyl)carbamoyl)-1-methyl-1H-pyrrol-3-yl)amino)-4-oxobutoxy)-14-oxo-6,6a,7,12-tetrahydrobenzo[5,6][1,4]diazepino[1,2-b]isoquinoline-5(14H)-carboxylate (SI-8):

HATU (0.037 g, 0.098 mmol) was added to a solution of **41** (0.05 g, 0.082 mmol) in DMF (0.5 mL). The mixture was stirred for 10 minutes, DIPEA (0.057 mL, 0.327 mmol) was added, followed by the addition of **47** (0.030 g, 0.082 mmol) and stirring was continued at room temperature for 16 hours. The reaction mixture was diluted with DCM (1 mL) and purified on silica gel (24 g isco column) using

25-100 % ethyl acetate in hexanes. The desired fractions were concentrated to give **SI-8** as an off-white solid (0.056 g, 74 %). LRMS (m/*z*): Calculated for $C_{48}H_{58}N_8O_9Si$ $[M+H]^+$ 919.4, found 919.3.



SI-8

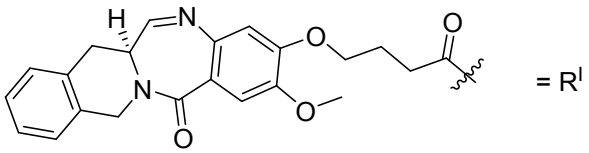


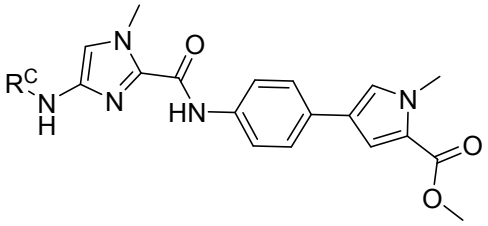
13

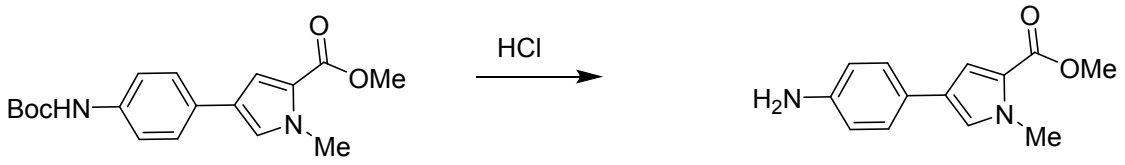
(S)-4-(4-((2-methoxy-14-oxo-6a,7,12,14-tetrahydrobenzo[5,6][1,4]diazepino[1,2-b]isoquinolin-3-yl)oxy)butanamido)-N-(3-methoxy-4-(3-methyl-1H-1,2,4-triazol-1-yl)phenyl)-1-methyl-1H-pyrrole-2-carboxamide (13):

Pyrrolidine (0.042M in DCM) (3.63 mL, 0.152 mmol) was added to **SI-8** (0.056 g, 0.061 mmol), followed by tetrakis(triphenylphosphine)palladium(0) (6.27 mg, 5.42 μmol). The mixture was stirred for 1 hour and concentrated under a stream of nitrogen. The residue was purified on a C18 biotage column (55 g) using 20-100 % of B (95 % acetonitrile / 5 % water / 0.05 % Formic acid) in A (5 % acetonitrile / 95 % water /0.05 % Formic acid) over 20 column volumes. The desired fractions were lyophilized to give **13** as an off-white solid (15 mg, 33%). LRMS (m/*z*): Calculated for $C_{38}H_{38}N_8O_6$ $[M+H]^+$ 703.3, found 703.40.

The following examples were prepared according to methods analogous to those used for **13**, starting with the appropriate heterocyclic amine in place of **47**.



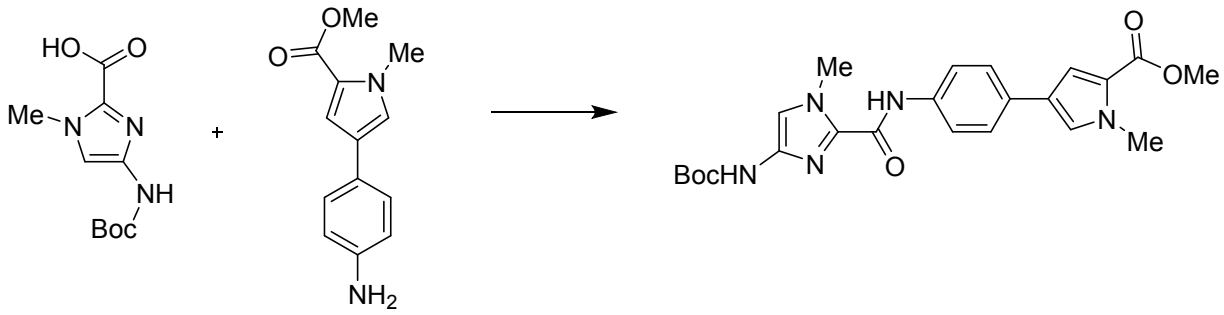
Ex.	Starting material (R^C=H) Product (R^C=R^I)	Starting material Source/Reference	Formula	Calculated [M+H]+	Product LCMS [M+H]
5		**SI-9**	$C_{40}H_{39}N_7O_7$	730.3	730.4



SI-10 **SI-11**

<u>methyl 4-(4-aminophenyl)-1-methyl-1H-pyrrole-2-carboxylate (SI-11):</u>

To a solution of **SI-10** (0.46 g, 1.39 mmol, 1 equiv) in MeOH (3.5 mL) was added 4 N HCl in dioxane (3.5 mL). After 2 hours, the solution was slowly added to ether. The white solid was filtered to deliver the HCl salt of the **SI-11** (0.30 g, 81%). LRMS (m/*z*): Calculated for $C_{13}H_{14}N_2O_2$ $[M+H]^+$ 231.1, found 231.00.

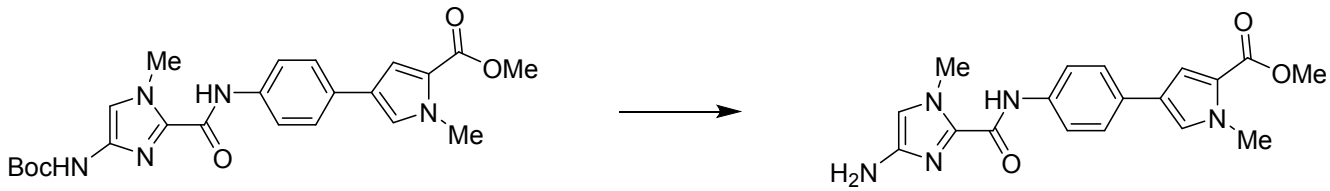


SI-12 **SI-11** **SI-13**

<u>methyl 4-(4-(4-((tert-butoxycarbonyl)amino)-1-methyl-1H-imidazole-2-carboxamido)phenyl)-1-methyl-1H-pyrrole-2-carboxylate (SI-13):</u>

To a solution of **SI-12** (0.16 g, 0.600 mmol, 1 equiv) and DIPEA (0.42 mL, 2.40 mmol, 4 equiv) in DMF (6 mL) was added HATU (0.32 g, 0.84 mmol, 1.4 equiv). After 5 minutes, **SI-11** (0.20 g, 0.84 mmol, 1.4 equiv) was added. The reaction was stirred for 2 hours and then diluted with EtOAc. The EtOAc solution was washed with water then brine. The EtOAc layer was dried (Na_2SO_4) and

concentrated *in vacuo*. The crude product was purified by flash column silica gel chromatography (0-100% EtOAc/hexane) to provide **SI-13** (0.22 g. 81%) as a yellow foam. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.97 (s, 1H), 7.63 (d, *J* = 8.5 Hz, 2H), 7.48 (d, *J* = 8.5 Hz, 2H), 7.21 (d, *J* = 1.8 Hz, 1H), 7.18 (br. s., 1H), 7.09 (s, 1H), 6.82 (br. s., 1H), 4.07 (s, 3H), 3.98 (s, 3H), 3.86 (s, 3H), 1.53 (s, 9H); LRMS (m/*z*): Calculated for $C_{23}H_{27}N_5O_5$ $[M+H]^+$ 454.2, found 454.2.

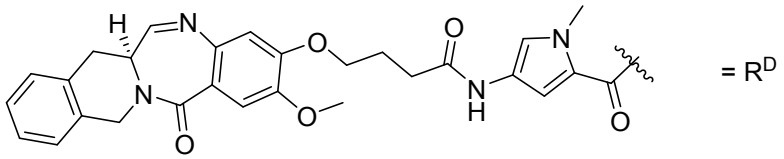


SI-13 **SI-9**

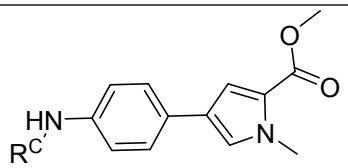
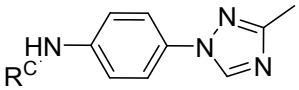
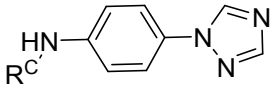
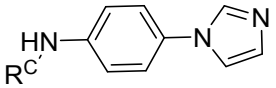
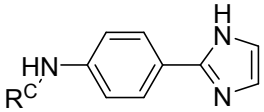
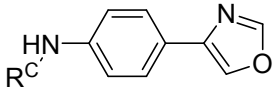
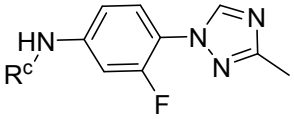
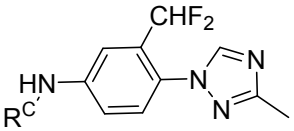
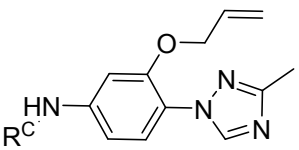
methyl 4-(4-(4-amino-1-methyl-1H-imidazole-2-carboxamido)phenyl)-1-methyl-1H-pyrrole-2-carboxylate (SI-9):

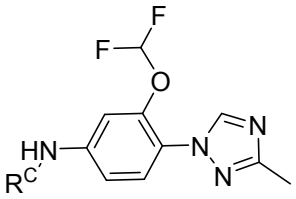
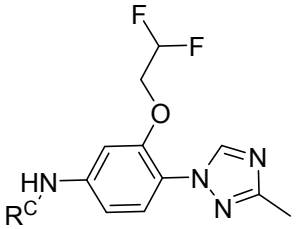
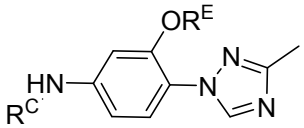
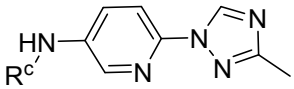
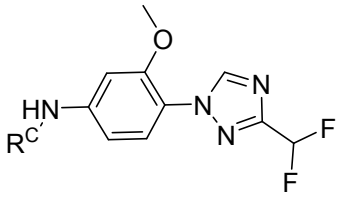
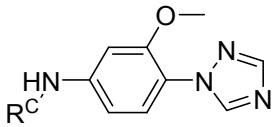
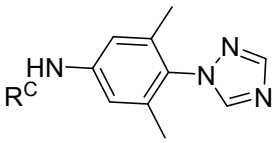
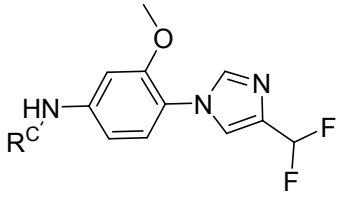
To a slurry of **SI-13** (0.22 g, 0.485 mmol, 1 equiv) in MeOH (2.4 mL) was added 4 N HCl in dioxane (2.4 mL). After 6 hours, concentrate *in vacuo* to provide the HCl salt of **SI-9** (0.18 g, 95%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.18 (br. s., 1H), 7.74 (d, *J*=8.5 Hz, 2H), 7.61 - 7.52 (m, 4H), 7.28 - 7.23 (m, 1H), 7.22 (s, 2H), 7.14 (br. s., 1H), 3.98 (s, 3H), 3.89 (s, 3H), 3.76 (s, 3H); LRMS (m/*z*): Calculated for $C_{18}H_{19}N_5O_3$ $[M+H]^+$ 354.2, found 354.1.

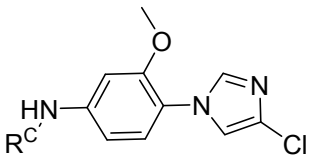
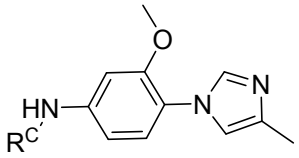
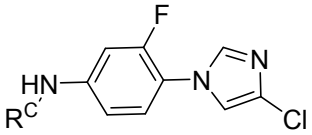
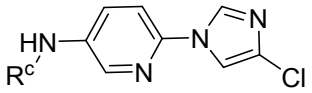
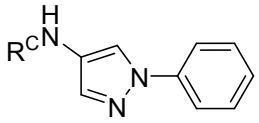
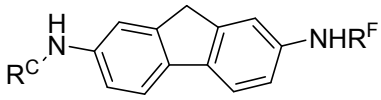
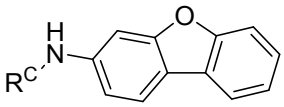
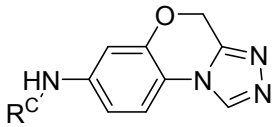
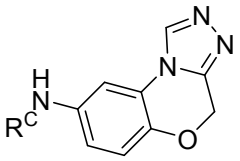
The following examples were prepared according to methods analogous to those used for **13**, starting with the appropriate heterocyclic amine in place of **SI-6**.

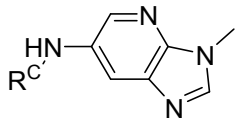


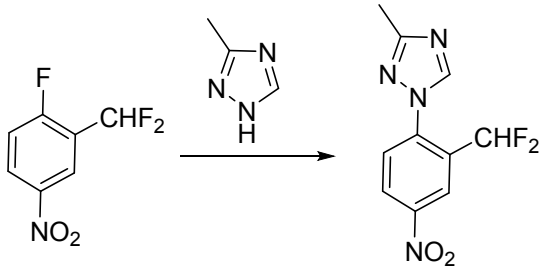
Ex.	Starting material (R^C=H) Product (R^C=R^D)	Starting material Source/Reference	Formula	Calculated [M+H]+	Product LCMS [M+H]

4		J. Med. Chem. 2013, p 2911-2935	$C_{41}H_{40}N_6O_7$	729.3	729.4
6		WO 2009/032861	$C_{37}H_{36}N_8O_5$	673.3	673.4
7		WO 2016/073652	$C_{36}H_{34}N_8O_5$	659.3	659.4
8		Alfa	$C_{36}H_{35}N_7O_5$	658.3	658.4
9		GreenChem	$C_{36}H_{35}N_7O_5$	658.3	658.4
10		Synquest	$C_{36}H_{35}N_6O_6$	659.3	659.4
11		US 2012/00289994	$C_{37}H_{35}FN_8O_5$	691.3	691.4
12		**SI-16**	$C_{38}H_{36}F_2N_8O_5$	723.3	723.2
14		WO 2012/009309	$C_{40}H_{40}N_8O_6$	729.3	729.5

15		WO 2015/153709	$C_{38}H_{36}F_2N_8O_6$	739.3	739.4
16		**SI-19**	$C_{39}H_{38}F_2N_8O_6$	753.3	753.4
17	 Starting material: R^E=allyl Product: R^E=H	Second product obtained from same starting material as Example 14	$C_{37}H_{36}N_8O_6$	689.3	689.4
18		WO 2012/094647	$C_{36}H_{35}N_9O_5$	674.3	674.4
19		US 2012/00289994	$C_{38}H_{36}F_2N_8O_6$	739.3	739.4
20		**SI-22**	$C_{37}H_{36}N_8O_6$	689.3	689.40
21		**SI-25**	$C_{38}H_{38}N_8O_5$	687.3	687.40
22		US 2012/00289994	$C_{39}H_{37}F_2N_7O_6$	738.3	738.4

23		US 2012/00289994	$C_{38}H_{36}ClN_7O_6$	722.2	722.4
24		US 2012/00289994	$C_{39}H_{39}N_7O_6$	702.3	702.4
25		**SI-28**	$C_{37}H_{33}FClN_7O_5$	710.2	710.35
26		WO 2015/115673	$C_{36}H_{33}ClN_8O_5$	693.2	693.3
27		ZelinskyBB	$C_{37}H_{35}N_7O_5$	658.3	658.4
28	 Starting material: R^F=alloc Product: R^F=H	Combi-Blocks	$C_{41}H_{38}N_6O_5$	695.3	695.4
29		Sigma	$C_{40}H_{35}N_5O_6$	682.3	682.4
30		WO 1999/9940094	$C_{37}H_{34}N_8O_6$	687.3	687.4
31		**SI-33**	$C_{37}H_{34}N_8O_6$	687.3	687.4

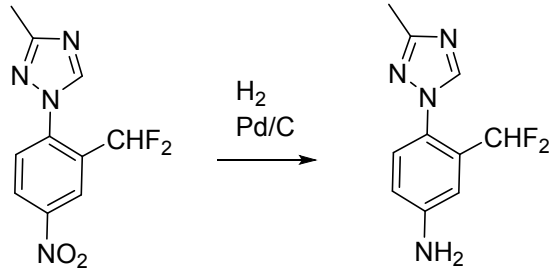
32		Combi-Blocks	$C_{35}H_{34}N_8O_5$	647.3	647.4



SI-14 **SI-15**

1-(2-(difluoromethyl)-4-nitrophenyl)-3-methyl-1H-1,2,4-triazole (SI-15):

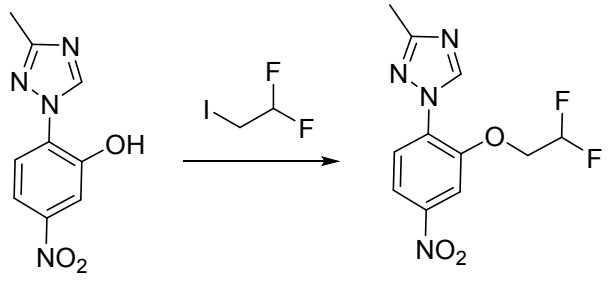
To a magnetically stirred solution of 2-(difluoromethyl)-1-fluoro-4-nitrobenzene (**SI-14**, 2 g, 10.47 mmol) in DMSO (10 mL) at 0 °C were added K_2CO_3 (1.446 g, 10.47 mmol) and 3-methyl-1H-1,2,4-triazole (0.870 g, 10.47 mmol). The reaction mixture was allowed to warm to room temperature and stirred overnight, poured into ice and extracted with ethyl acetate (50 mL, twice). The combined organic layer was washed with brine, dried over sodium sulphate, and concentrated under reduced pressure. The residue was purified on biotage using 0-20 % ethyl acetate in petroleum ether. Two sets of fractions with desired product mass were isolated one of which was confirmed to be the desired product **SI-15** by X-ray crystal analysis. ^{1}H NMR (400 MHz, CHLOROFORM-d) δ 400 MHz: 8.74-8.73 (1H, d, J=2.1 Hz), 8.51-8.47 (1H, m), 8.39 (1H, s), 7.70-7.67 (1H, d, J=8.4), 7.39-7.02 (1H, t, J=54), 2.53 (3H, s). LRMS (m/*z*): Calculated for $C_{10}H_8N_4O_2$ $[M+H]^+$ 255.1, found 255.2.



SI-15 **SI-16**

3-(difluoromethyl)-4-(3-methyl-1H-1,2,4-triazol-1-yl)aniline (SI-16):

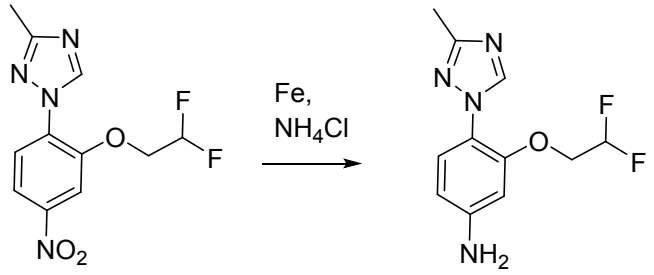
A dried 250 mL, two-necked, round-bottom flask was equipped with a magnetic stirring bar and an adaptor with an N_2 inlet, with an cooled condenser was charged with **SI-15** (0.1 g, 0.393 mmol) in MeOH (50 mL) to this added Pd/C (8.37 mg, 0.079 mmol), stirred it at room temperature under hydrogen pressure for 6 hours. The reaction mixture was filtered through celite bed and concentrated under reduced pressure. The residue was dissolved in DCM (10 mL), silica (5 g). The resultant slurry of the compound on silica (4 g Redi*Sep* silica column, 50 % ethyl acetate in petroleum ether) to get the title compound **SI-16** as a light green solid (0.07 g, 79 %). ^{1}H NMR (400 MHz, CHLOROFORM-d) δ 8.11 (1H, s), 7.17-7.14 (1H, d, J=8.4 Hz), 7.02-7.01 (1H , d, J=2.0 Hz), 6.80-6.78 (1H, m), 6.78-6.51 (1H, t, J=51 Hz), 4.02 (2H, bs), 2.47 (3H, s). LRMS (m/*z*): Calculated for $C_{10}H_{10}F_2N_4$ $[M+H]^+$ 225.2, found 225.2.



SI-17 **SI-18**

1-(2-(2,2-difluoroethoxy)-4-nitrophenyl)-3-methyl-1H-1,2,4-triazole (SI-18):

A mixture of **SI-17** (1 g, 4.54 mmol),1,1-difluoro-2-iodoethane (0.872 g, 4.54 mmol) was dissolved in DMF (5 mL) to that was added K_2CO_3 (0.753 g, 5.45 mmol) was refluxed at 80 °C for overnight. Then the reaction mixture was concentrated under reduced pressure. Then the crude mixture was quenched with ice cold water (15 ml) to get precipitate which was filtered and dried overnight to get **SI-18** (1 g, 77 %) as a brown solid. LRMS (m/*z*): Calculated for $C_{11}H_{10}F_2N_4O_3$ $[M+H]^+$ 285.1, found 285.3.

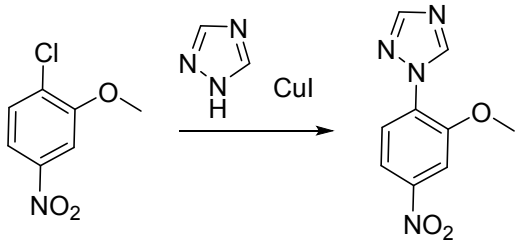


SI-18 **SI-19**

3-(2,2-difluoroethoxy)-4-(3-methyl-1H-1,2,4-triazol-1-yl)aniline (SI-19):

SI-18 (1 g, 3.5 mmol) was dissolved in Ethanol (30mL) and water (15mL) under a nitrogen atmosphere. Ammonium chloride (0.94g, 17.6 mmol) and iron powder (.98g, 17.6 mmol) were added

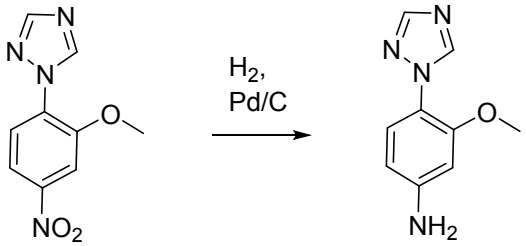
and the mixture was stirred and heated to 80 °C overnight. The mixture was then cooled to room temperature and filtered through celite. The filtrate was concentrated *in vacuo*, then 50 mL ice cold water was added and the mixture stirred for 5 minutes at which point the precipitated product was collected by filtration and dried on the filter overnight to afford **SI-19** (0.8g, 89 %) as a brown solid. LRMS (m/*z*): Calculated for $C_{11}H_{12}F_2N_4O$ $[M+H]^+$ 255.1, found 255.2.



SI-20 **SI-21**

1-(2-methoxy-4-nitrophenyl)-1H-1,2,4-triazole (SI-21):

Copper(I) iodide (24.62 mg, 0.129 mmol) and L-Histidine (40.1 mg, 0.259 mmol) in DMSO (8 mL) were stirred at 100 °C for 30 minutes. To this mixture were added 1-bromo-2-methoxy-4-nitrobenzene (**SI-20**, 300 mg, 1.293 mmol), 1H-1,2,4-triazole (107 mg, 1.552 mmol) and K_2CO_3 (357 mg, 2.59 mmol) and the mixture was stirred at 100 °C for 48 hours. The reaction mixture was transferred to a separatory funnel containing $NaHCO_3$ solution and extracted with EtOAc (3x). The combined organic extract was concentrated *in vacuo* and the residue was purified by biotage (10 % MeOH in CH_2Cl_2) to afford **SI-21** (70 mg, 0.318 mmol, 25 %) as a yellow solid. LRMS (m/*z*): Calculated for $C_9H_8N_4O_3$ $[M+H]^+$ 221.1, found 221.10.

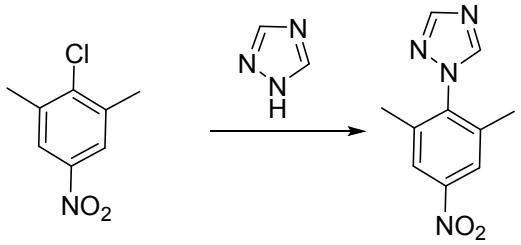


SI-21 **SI-22**

3-methoxy-4-(1H-1,2,4-triazol-1-yl)aniline (SI-22):

SI-21 (70 mg, 0.318 mmol) was dissolved in ethanol (6 mL) in a Parr bottle. Pd/C (67.7 mg, 0.064 mmol) was added and the mixture was placed on the Parr shaker under H_2 at 40 psi for 1.5 hours. The catalyst was removed by filtration and the filtrate was concentrated to afford **SI-22** as a grey solid (50 mg, 0.263 mmol, 83 %). ^{1}H NMR (400 MHz, CHLOROFORM-d) δ ppm 8.50 (1 H, s), 8.05 (1 H,

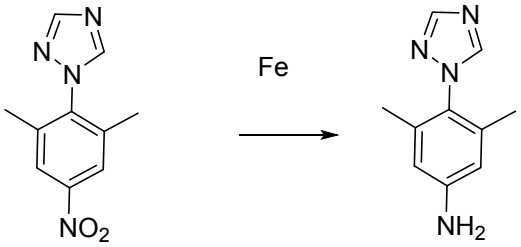
s), 7.34 - 7.49 (1 H, m), 6.32 - 6.39 (2 H, m), 3.83 (3 H, s). LRMS (m/*z*): Calculated for $C_9H_{10}N_4O$ $[M+H]^+$ 191.1, found 191.1.



SI-23 **SI-24**

1-(2,6-dimethyl-4-nitrophenyl)-1H-1,2,4-triazole (SI-24):

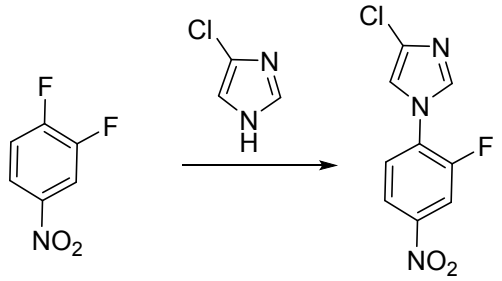
A mixture of 2-chloro-1,3-dimethyl-5-nitrobenzene (**SI-23**, 1 g, 5.39 mmol), 1H-1,2,4-triazole (0.372 g, 5.39 mmol) and cesium carbonate (1.931 g, 5.93 mmol) in DMSO (10 mL) was stirred at 90 °C for 16 hours then cooled to room temperature (LCMS showed minor product formation). The reaction mixture was poured into water and the mixture was stirred overnight and filtered. The dark brown filter cake was washed with water and suction-dried, and further dried on the vacuum pump to give **SI-24** (1.04 g, 88 %, used as is). LRMS (m/*z*): Calculated for $C_{10}H_{10}N_4O_2$ $[M+H]^+$ 219.1, found 219.1.



SI-24 **SI-25**

3,5-dimethyl-4-(1H-1,2,4-triazol-1-yl)aniline (SI-25):

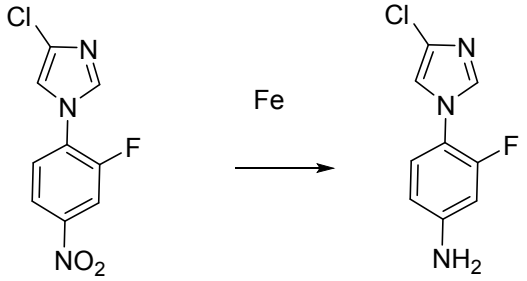
SI-24 (1 g, 4.58 mmol) was taken up in ethanol/water (2:1, 12 mL), ammonium chloride (1.961 g, 36.7 mmol) and iron (1.024 g, 18.33 mmol) were added successively. The mixture was heated at 70 °C for 1 hour and cooled to room temperature. The mixture was then treated with aqueous ammonium hydroxide (10 mL) and ethyl acetate (100 mL). Mixture was stirred for 10 minutes then filtered through celite. Filter cake was washed successively with water and ethyl acetate. Filtrate layers were partitioned. The organic phases were dried over $MgSO_4$, filtered and concentrated to give **SI-25** as a golden yellow solid (0.85 g, 99 %). LRMS (m/*z*): Calculated for $C_{10}H_{12}N_4$ $[M+H]^+$ 189.1, found 189.0.



SI-26 **SI-27**

4-chloro-1-(2-fluoro-4-nitrophenyl)-1H-imidazole (SI-27):

A magnetically stirred mixture of 4-chloro-1H-imidazole (15.0 g, 146 mmol), 1,2-difluoro-4-nitrobenzene (**SI-26**, 30 g, 189 mmol), and K_2CO_3 (20.22 g, 146 mmol) in DMSO (50 mL) was heated at 80 °C for 4 hours. The resulting dark black solution was allowed to cool to room temperature. The reaction contents was poured into 800 mL water and vigorously stirred for 15 minutes. An orange precipitate immediately crashed out and was collected to give **SI-27** (34.6 g, 98 %). ^{1}H NMR (500 MHz, CHLOROFORM-d) δ 8.23 - 8.16 (m, 2H), 7.81 - 7.78 (m, 1H), 7.61 - 7.56 (m, 1H), 7.26 - 7.25 (m, 1H). LRMS (m/*z*): Calculated for $C_9H_5ClFN_3O_2$ $[M+H]^+$ 242.0, found 242.1.

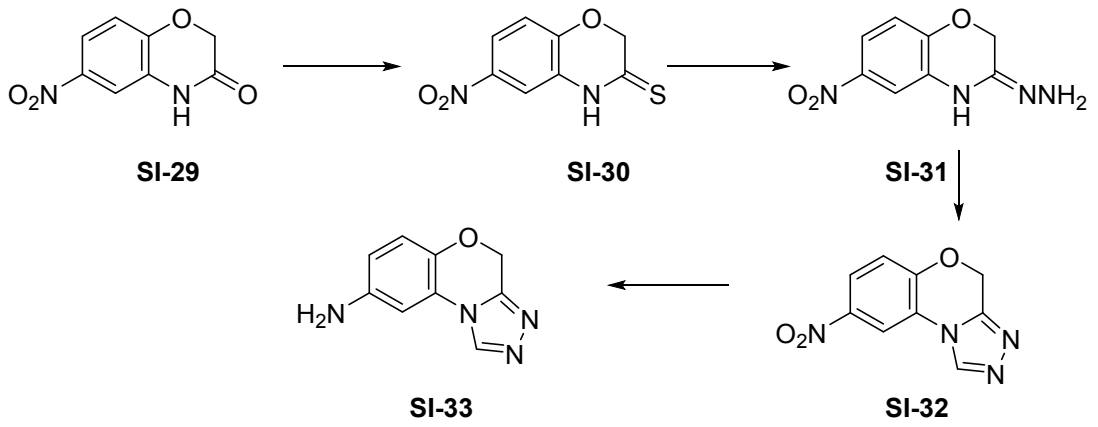


SI-27 **SI-28**

4-(4-chloro-1H-imidazol-1-yl)-3-fluoroaniline (SI-28):

Iron powder (4.62 g, 83 mmol) was added to a 250 mL round bottom flask charged with a mixture of **SI-27** (10 g, 41.4 mmol), acetic acid (40 mL, 699 mmol), and ethanol (100 mL). A water cooled reflux condenser was attached to the flask and the heterogeneous mixture was heated to 100 ºC with vigorous stirring for 30 minutes and cooled to room temperature. The crude reaction mixture was filtered over celite and washed with ethyl acetate. The organic layer was chilled in an ice bath and neutralized with 5 M NaOH. The resulting solution was poured into separatory funnel and extracted with EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated *in vacuo* to afford a solid. The crude product was analyzed by LCMS and the major peak had an ion consistent with the desired product **SI-28** (5.5 g, 68 %, used as is). LRMS (m/*z*): Calculated for $C_9H_7ClFN_3$ $[M+H]^+$ 212.0, found 212.1.

Synthesis of 4H-benzo[b][1,2,4]triazolo[4,3-d][1,4]oxazin-8-amine (SI-33)

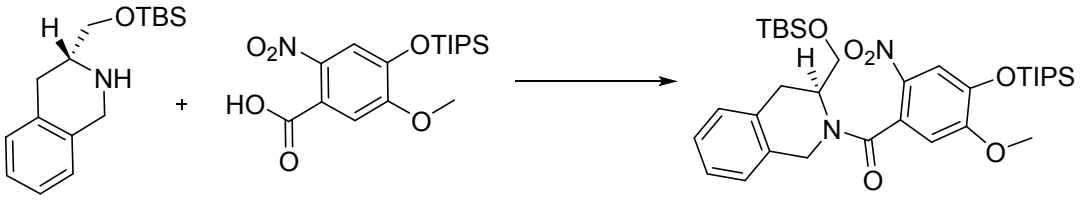


Lawesson's reagent (6.5 g, 15.75 mmol) was added to a brown suspension of 6-nitro-2H-benzo[b][1,4]oxazin-3(4H)-one (CAS#[81912-93-8], 6.15 g, 31.68 mmol) in THF (160 mL) and the heterogeneous mixture was stirred at room temperature for 18 hours. The resultant clear golden yellow solution was diluted with water (1300 mL), and the yellow suspension was filtered. The filter cake was washed with water, taken up in ethyl acetate, washed with brine, then concentrated. The residue was purified on silica using 1-5 % ethyl acetate in DCM. The desired fractions were concentrated to give a yellow solid (5.45 g, 82 %). LRMS (m/*z*): Calculated for $C_8H_6N_2O_3S$ $[M+H]^+$ 211.0, found 211.3.

Hydrazine monhydrate (0.46 mL, 9.28 mmol) was added to a pale yellow solution of 6-nitro-2H-benzo[b][1,4]oxazine-3(4H)-thione **SI-30** (1.5 g, 7.14 mmol) in ethanol (100 mL) and the resultant bright yellow suspension was stirred at room temperature overnight and concentrated. The residue was taken up in water and the suspension was filtered, the filter cake was washed with water and suction-dried to give a bright yellow solid **SI-31** (1.3 g, 87 %). LRMS (m/*z*): Calculated for $C_8H_8N_4O_3$ $[M+H]^+$ 209.1, found 209.4.

A mixture of (E)-3-hydrazono-6-nitro-3,4-dihydro-2H-benzo[b][1,4]oxazine (0.5 g, 2.4 mmol) and triethyl orthoformate (8.15 mL, 48 mmol) was heated to 150 °C for 3 hours and concentrated. The bright yellow residue was taken up in methanol (18 mL), filtered, filter cake washed with methanol and suction-dried to give a yellow solid (0.46 g, 88 %). LRMS (m/*z*): Calculated for $C_9H_6N_4O_3$ $[M+H]^+$ 219.0, found 219.2.

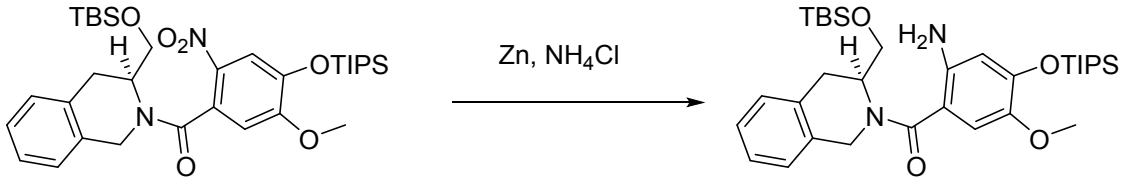
A suspension of 8-nitro-4H-benzo[b][1,2,4]triazolo[4,3-d][1,4]oxazine (0.46 g, 2.1 mmol) in THF/MeOH (1:1, 100 mL) was subjected to hydrogenation at 1 atm using 10 % Pd/C (0.1 g) for 18 hours. The reaction mixture was filtered through Celite and concentrated. The residue was purified on silica using 2-7 % methanol in DCM. The desired fractions were concentrated to give a pale yellow solid **SI-33** (0.29 g, 73 %). LRMS (m/*z*): Calculated for $C_9H_8N_4O$ $[M+H]^+$ 189.1, found 189.3.



42 **43** **44**

(S)-(3-(((tert-butyldimethylsilyl)oxy)methyl)-3,4-dihydroisoquinolin-2(1H)-yl)(5-methoxy-2-nitro-4-((triisopropylsilyl)oxy)phenyl)methanone (44):

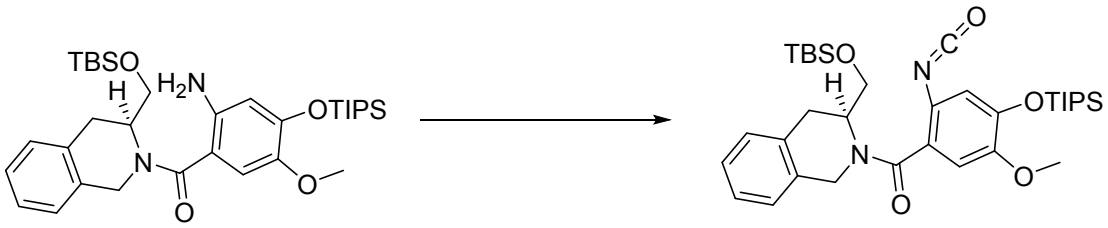
A flask was charged with 5-methoxy-2-nitro-4-((triisopropylsilyl)oxy)benzoic acid **43** (CAS Reg. No. 1430738-03-6, 9.0 g, 24.36 mmol) and N,N,N′,N′-tetramethyl-O-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate (HATU, 10.19 g, 26.8 mmol) in DCM (100 mL) at 0 °C. The reaction mixture was stirred for 10 minutes and treated with N.N-diisopropylethyl amine (DIEA or DIPEA, 4.68 mL, 26.8 mmol) and isoquinoline **42** (CAS Reg. No. 215928-81-7, 7.43 g, 26.8 mmol). The reaction was maintained at 0 °C for 3 hours and then stirred at room temperature for 24 hours. The reaction mixture was poured into saturated NH_4Cl and DCM. The organic phase was collected and concentrated to a residue. The residue was further purified by silica gel chromatography (Biotage) eluting with 10%-30% EtOAc in hexanes. The product was collected and concentrated to afford amide **44** as a light tan oil (10.15g, 66% yield). LRMS (m/*z*): Calculated for $C_{33}H_{52}N_2O_6Si_2$ $[M+H]^+$ 629.3, found 629.7.



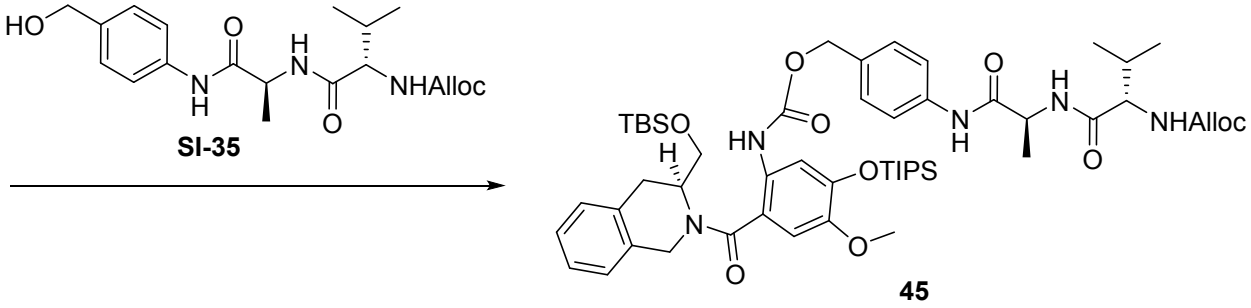
44 **SI-33**

(S)-(2-amino-5-methoxy-4-((triisopropylsilyl)oxy)phenyl)(3-(((tert-butyldimethylsilyl)oxy)methyl)-3,4-dihydroisoquinolin-2(1H)-yl)methanone (SI-33):

A solution of amide **44** (10.1 g, 16.06 mmol) in MeOH (200 mL) was cooled to 0 °C and NH_4Cl (4.29 g, 80 mmol) and zinc dust (5.25 g, 80 mmol) were added. The resulting green suspension was stirred at 0 °C for 45 minutes, then allowed to warm to room temperature overnight. The reaction mixture was filtered through a CELITE™ pad (washing with MeOH) and the filtrate was concentrated to a residue. The residue was taken up in DCM and loaded onto silica gel pad. This was flushed with 50% EtOAc and hexanes to afford aniline **SI-33** (8.02g, 83% yield). LRMS (m/*z*): Calculated for $C_{33}H_{54}N_2O_4Si_2$ $[M+H]^+$ 599.4, found 599.4.



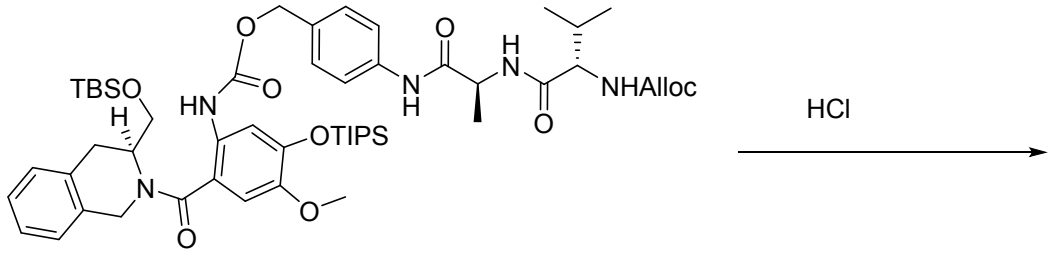
SI-33 **SI-34**



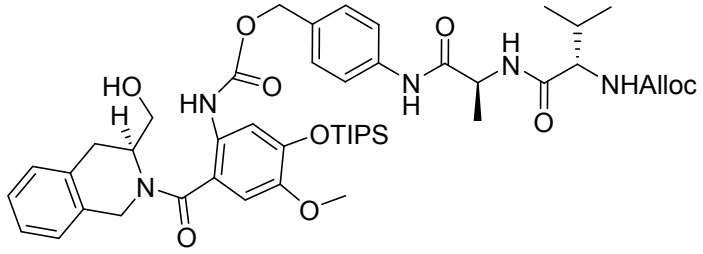
SI-35 **45**

4-**((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl (2-((S)-3-(((tert-butyldimethylsilyl)oxy)methyl)-1,2,3,4-tetrahydroisoquinoline-2-carbonyl)-4-methoxy-5-((triisopropylsilyl)oxy)phenyl)carbamate (45):**

Aniline **SI-33** (2.1g, 3.51 mmol) was dissolved in DCM (30 mL) and pyridine (0.3 mL, 3.71 mmol) was added. The mixture was cooled to 0 °C. Nitrophenyl carbonochloridate **SI-34** (0.707 g, 3.51 mmol) was added and the mixture aged for 7 minutes at the same temperature. A solution of compound **SI-35** (CAS Reg. No. 1343407-91-9, 1.323 g, 3.51 mmol) and DIEA (0.750 mL, 4.29 mmol) in DMF (3 mL) was added. The mixture was placed on a rotary evaporator at room temperature to remove the DCM. After 20 minutes, the DMF was evaporated under a stream of nitrogen and then the residue was purified by silica gel chromatography (Biotage) eluting with 10-100% EtOAc in hexanes to afford compound **45** (1.579 g, 1.575 mmol, 44.9% yield). LRMS (m/*z*): Calculated for $C_{53}H_{79}N_5O_{10}Si_2$ $[M+H]^+$ 1002.4, found 1002.5.



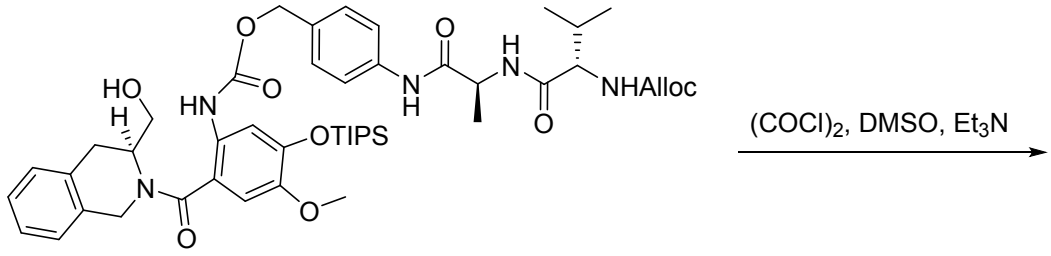
45



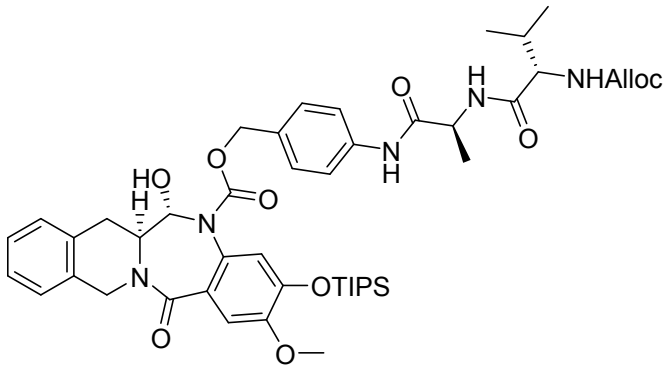
SI-36

4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl (2-((S)-3-(hydroxymethyl)-1,2,3,4-tetrahydroisoquinoline-2-carbonyl)-4-methoxy-5-((triisopropylsilyl)oxy)phenyl)carbamate (SI-36):

A solution of compound **45** (1.579g, 1.575 mmol) in MeOH (14.4 ml) was treated with 10% concentrated HCl in MeOH (1.6 ml, 5.27 mmol). The mixture was aged 30 minutes, quenched with saturated $NaHCO_3$, and extracted with chloroform (3x). The combined organic phases were dried over Na_2SO_4, filtered and evaporated to leave a residue. The residue was combined with another batch of the same reaction (starting with 0.816 g of compound **45**) for purification. The combined crude residues were purified by silica gel chromatography (Biotage) eluting with 20-100% EtOAc/Hexanes to afford carbamate **SI-36** (1.7412 g, 1.961 mmol, 82% yield). LRMS (m/*z*): Calculated for $C_{47}H_{65}N_5O_{10}Si$ $[M+H]^+$ 888.5, found 888.3.



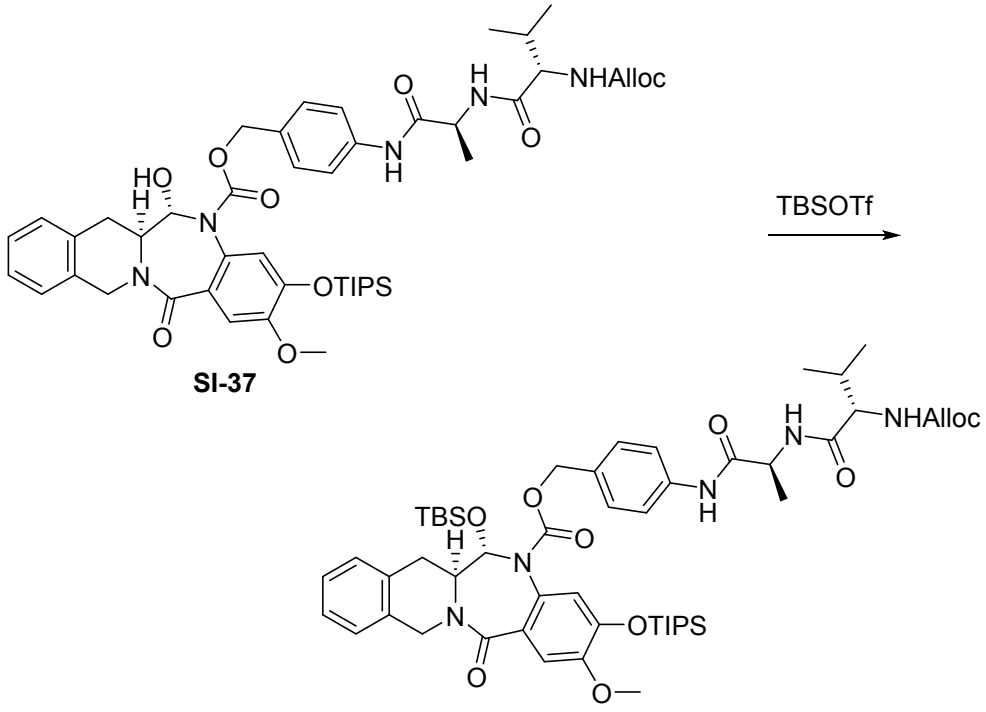
SI-36



SI-37

4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl (6S,6aS)-6-hydroxy-2-methoxy-14-oxo-3-((triisopropylsilyl)oxy)-6,6a,7,12-tetrahydrobenzo[5,6][1,4]diazepino[1,2-b]isoquinoline-5(14H)-carboxylate (SI-37):

A solution of oxalyl chloride (2.0M, 1.00 mL, 2.000 mmol) in 10 mL DCM was cooled to -78 ^{o}C. A solution of DMSO (0.348 mL, 4.90 mmol) in 5mL DCM was added drop wise and the mixture aged at the same temperature for 10 minutes. A solution of carbamate **SI-36** (1741.2 mg, 1.961 mmol) in 5mL DCM was added drop wise and the mixture was again aged for 15 minutes. NEt_3 (1.366 mL, 9.80 mmol) was added drop wise; the mixture was aged at the same temperature for 5 minutes and then the cold bath was removed and the mixture was allowed to warm to room temperature. The mixture was quenched with NH_4Cl solution and extracted twice with DCM. The combined organic phases were washed with water and brine, dried over Na_2SO_4, filtered, and evaporated. The residue was purified by silica gel chromatography (Biotage) eluting with 50-80% EtOAc/Hexanes to afford compound **SI-37** (1376.7 mg, 1.554 mmol, 79% yield). LRMS (m/*z*): Calculated for $C_{47}H_{63}N_5O_{10}Si$ $[M+H]^+$ 886.4, found 886.3.

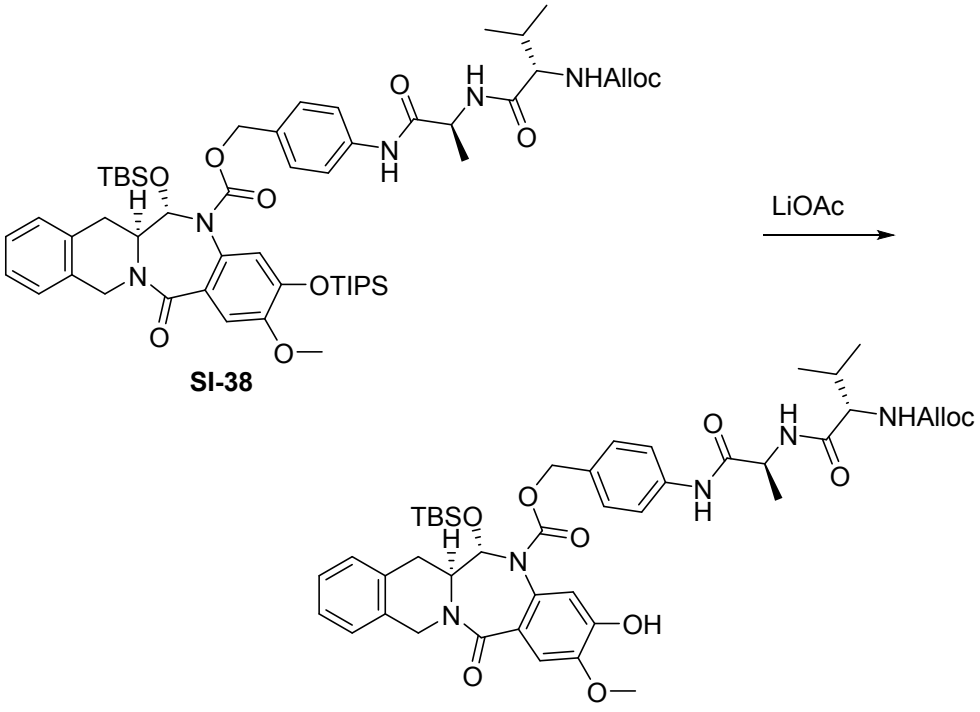


SI-37

SI-38

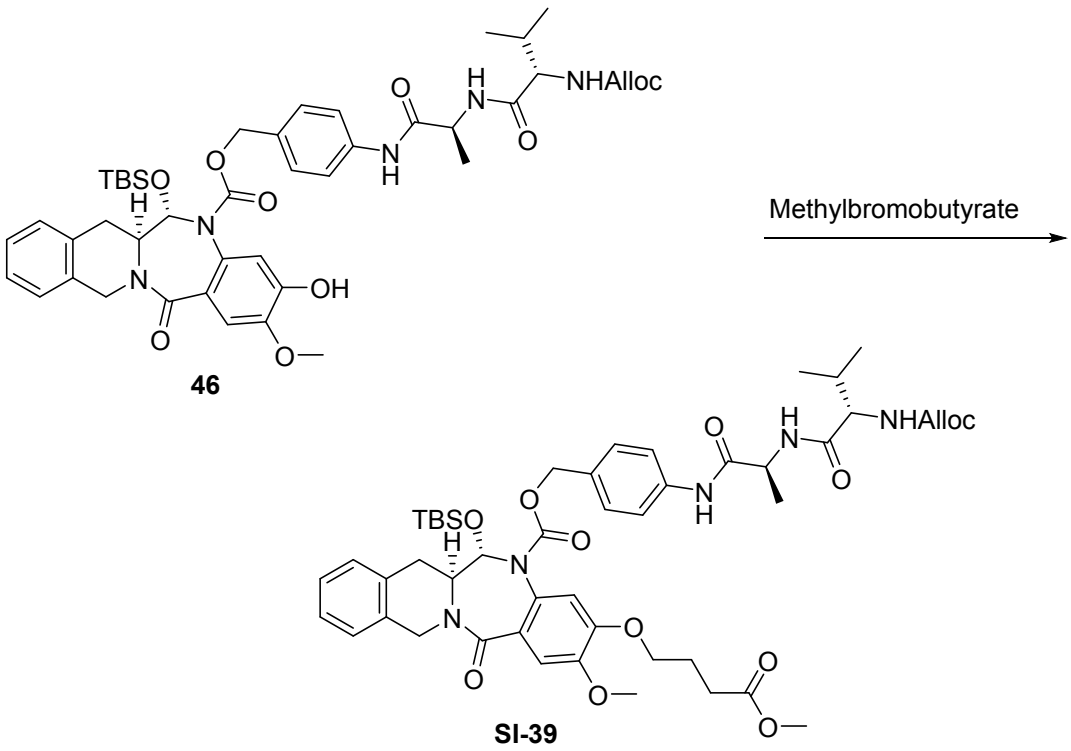
4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl (6S,6aS)-6-((tert-butyldimethylsilyl)oxy)-2-methoxy-14-oxo-3-((triisopropylsilyl)oxy)-6,6a,7,12-tetrahydrobenzo[5,6][1,4]diazepino[1,2-b]isoquinoline-5(14H)-carboxylate (SI-38):

Compound **SI-37** (1045 mg, 1.179 mmol) was dissolved in DCM (10 ml) and 2,6-lutidine (0.549 ml, 4.72 mmol) was added. The mixture was cooled on an ice bath and tertbutyldimethylsilyltrifluoromethanesulfonate (0.813 ml, 3.54 mmol) was added. After 1 hour, the mixture was diluted with DCM, washed with saturated $NaHCO_3$ and brine, dried over Na_2SO_4, filtered and evaporated. The residue was purified by silica gel chromatography (Biotage) eluting 20-100% EtOAc/Hexanes. Some mixed fractions were obtained, which were repurified by silica gel chromatography (Biotage) eluting with 50% EtOAc/Hexanes (isocratic). The pure fractions were combined to afford compound **SI-38** (676.9 mg, 0.677 mmol, 57.4% yield). LRMS (m/*z*): Calculated for $C_{53}H_{77}N_5O_{10}Si_2$ $[M+H]^+$ 1000.5, found 1000.3.



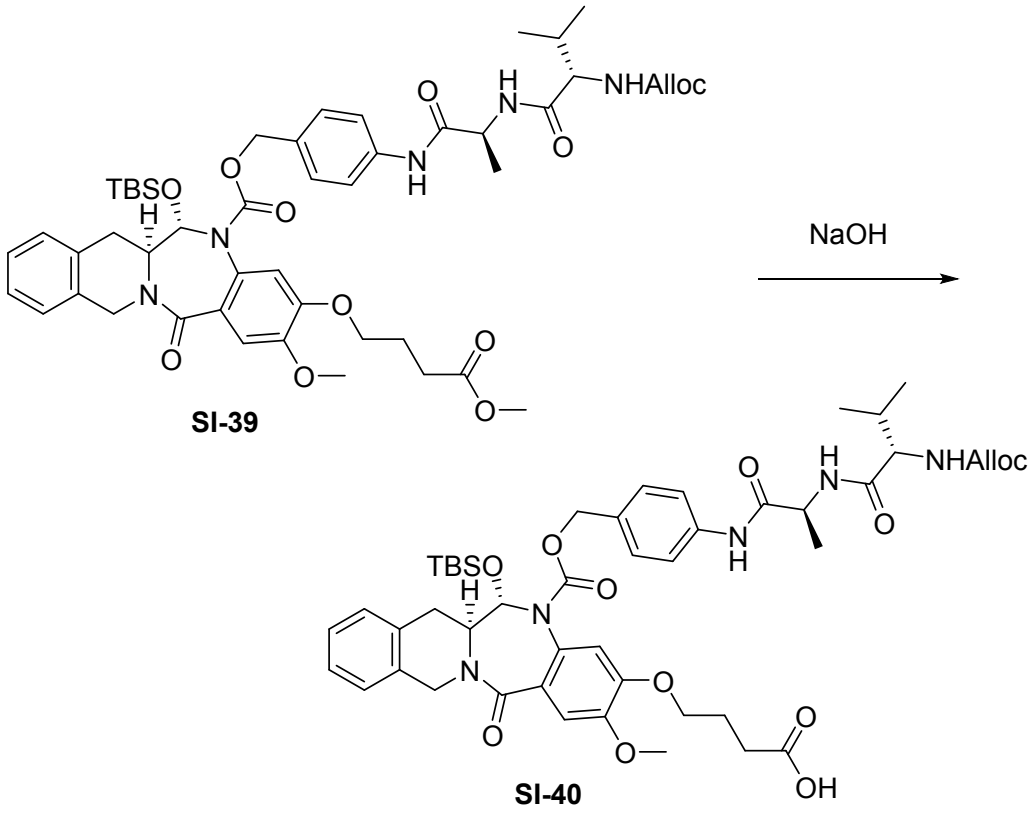
4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl (6S,6aS)-6-((tert-butyldimethylsilyl)oxy)-3-hydroxy-2-methoxy-14-oxo-6,6a,7,12-tetrahydrobenzo[5,6][1,4]diazepino[1,2-b]isoquinoline-5(14H)-carboxylate (46):

A solution of compound **SI-38** (676 mg, 0.676 mmol) in DMF (5 mL) and water (0.1mL) was treated with LiOAc (44.6 mg, 0.676 mmol). The mixture was aged overnight, and the solvent was evaporated under a stream of nitrogen. The residue was partitioned between EtOAc and 0.1M citric acid. The phases were separated and the organic phases were washed twice with 0.1M citric acid, once with brine and then dried over Na_2SO_4, filtered and evaporated. The residue was purified by silica gel chromatography (Biotage) eluting with 50-100% EtOAc/Hexanes to afford compound **46** (543.6 mg, 0.644 mmol, 95% yield). LRMS (m/*z*): Calculated for $C_{44}H_{57}N_5O_{10}Si$ $[M+H]^+$ 844.4, found 844.4.



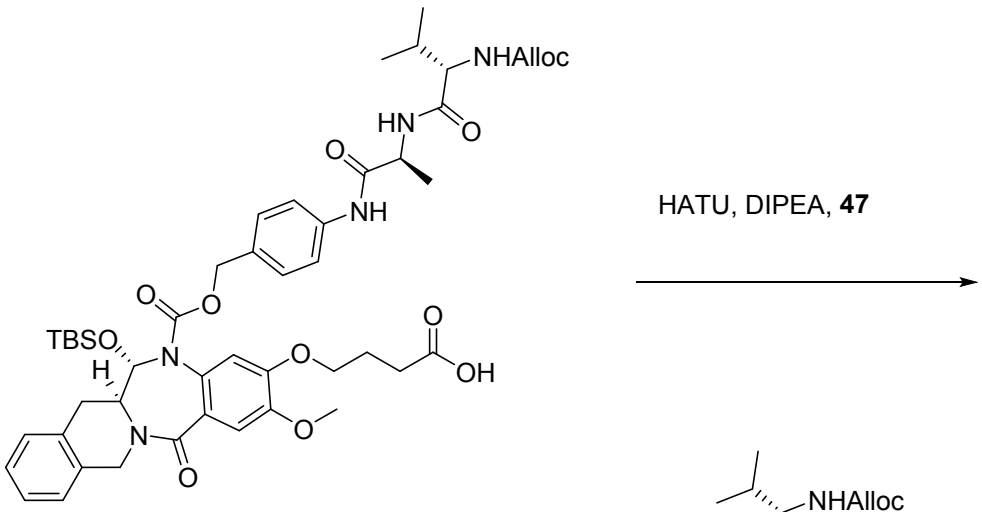
4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl (6S,6aS)-6-((tert-butyldimethylsilyl)oxy)-2-methoxy-3-(4-methoxy-4-oxobutoxy)-14-oxo-6,6a,7,12-tetrahydrobenzo[5,6][1,4]diazepino[1,2-b]isoquinoline-5(14H)-carboxylate (SI-39):

To a solution of phenol **46** (0.53 g, 0.628 mmol) in DMF (6.28 mL) was added methylbromobutyrate (0.167 mL, 1.256 mmol) and cesium carbonate (0.45 g, 1.381 mmol). This mixture was stirred for 18 hours, diluted with EtOAc and washed successively with saturated solutions of sodium bicarbonate and brine. The organic phase was dried over sodium sulfate, filtered and evaporated. The mixture was purified on silica gel using 30-100 % ethyl acetate in hexanes. The desired fractions were concentrated to give **SI-39** as a white solid (0.48 g, 81 %). LRMS (m/*z*): Calculated for $C_{49}H_{65}N_5O_{12}Si$ $[M+H]^+$ 944.4, found 944.3.



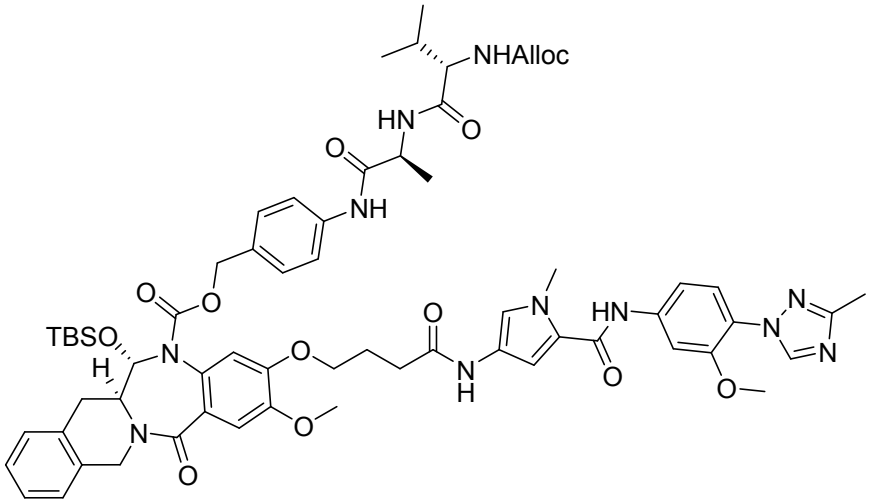
4-(((6S,6aS)-5-(((4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl)oxy)carbonyl)-6-((tert-butyldimethylsilyl)oxy)-2-methoxy-14-oxo-5,6,6a,7,12,14-hexahydrobenzo[5,6][1,4]diazepino[1,2-b]isoquinolin-3-yl)oxy)butanoic acid (SI-40):

Ester **SI-39** (0.46 g, 0.487 mmol) was dissolved in THF (19.49 mL) and a 1M solution of NaOH (4.87 mL, 4,87 mmol) was added. The homogeneous solution was aged with stirring for 7 hours, and then quenched by the addition into 5% citric acid. The mixture was extracted thrice with DCM and dried over sodium sulfate. Filtration and evaporation of the solvent afforded acid **SI-40** as a tan solid (0.46g, 71 %, ~70% purity). This material was approximately 70% pure and was used as-is in subsequent transformations. LRMS (m/*z*): Calculated for $C_{48}H_{63}N_5O_{12}Si$ $[M+H]^+$ 930.4, found 930.4.



HATU, DIPEA, **47**

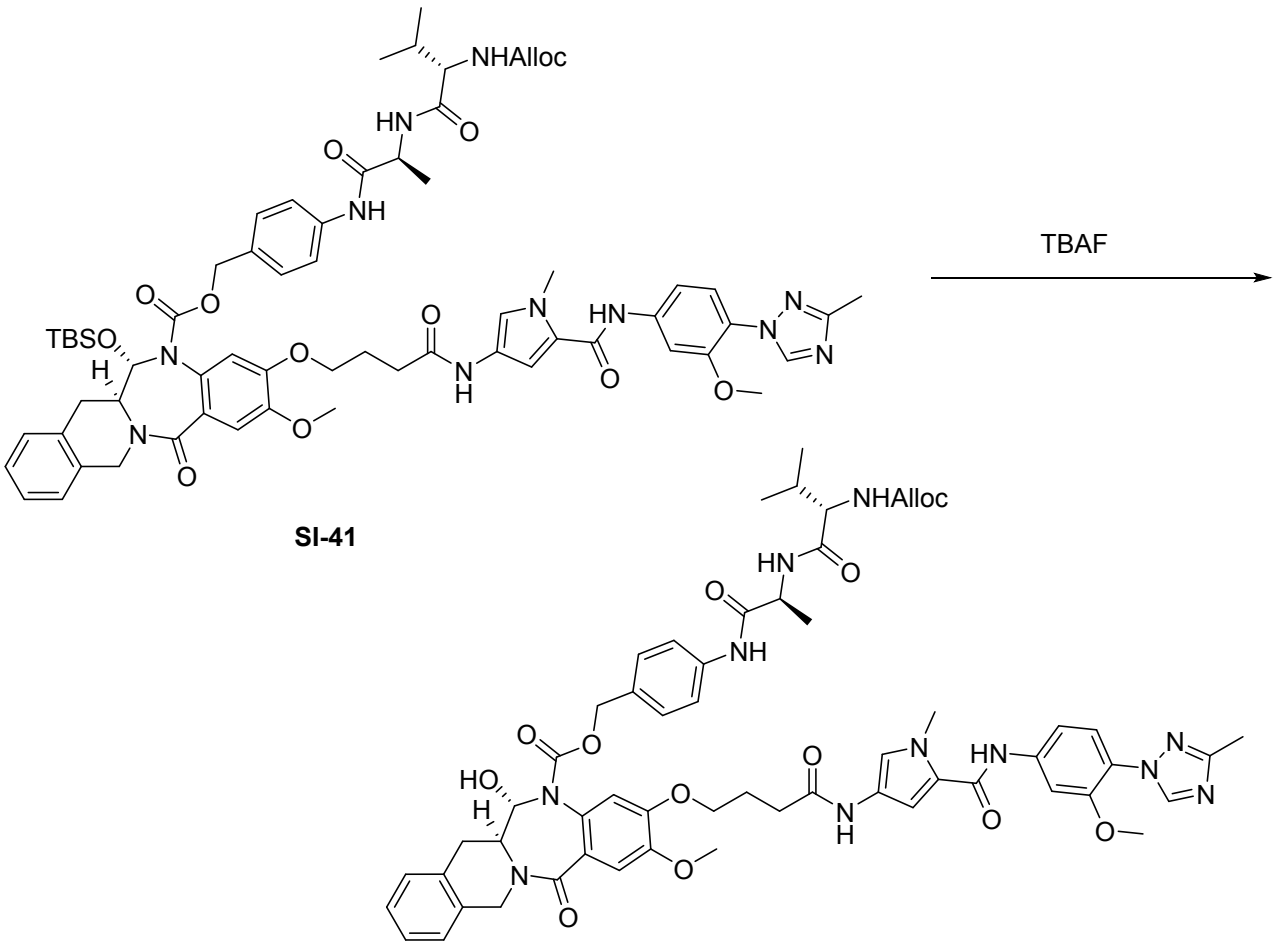
SI-40



SI-41

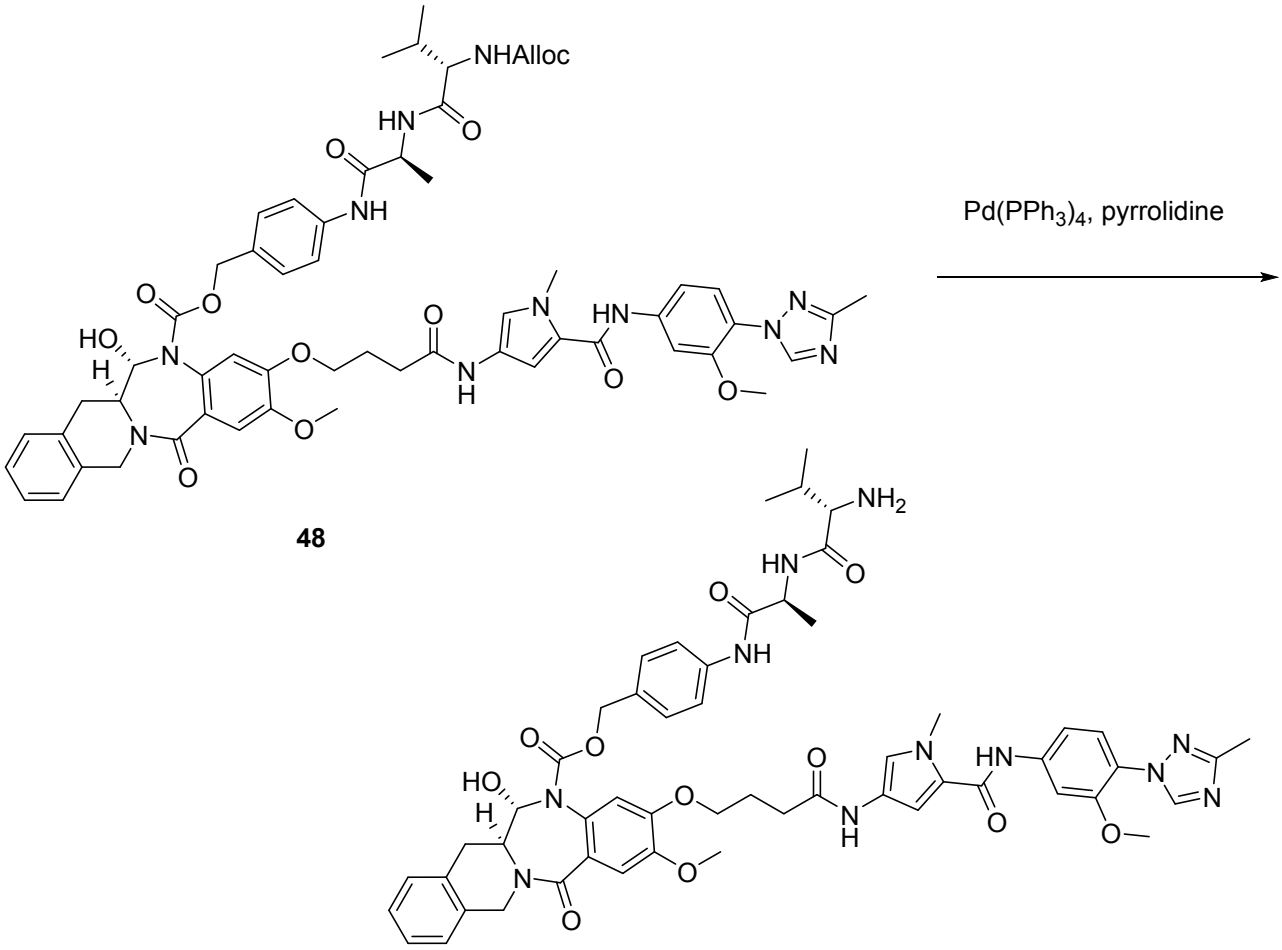
4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl (6S,6aS)-6-((tert-butyldimethylsilyl)oxy)-2-methoxy-3-(4-((5-((3-methoxy-4-(3-methyl-1H-1,2,4-triazol-1-yl)phenyl)carbamoyl)-1-methyl-1H-pyrrol-3-yl)amino)-4-oxobutoxy)-14-oxo-6,6a,7,12-tetrahydrobenzo[5,6][1,4]diazepino[1,2-b]isoquinoline-5(14H)-carboxylate (SI-41):

HATU (0.032 g, 0.084 mmol) was added to a solution of acid **SI-40** (0.065 g, 0.070 mmol) in DMF (1 mL). The mixture was stirred for 10 min, DIPEA (0.049 mL, 0.280 mmol) was added, followed by the addition of **47** (0.028 g, 0.077 mmol) and stirring was continued at room temperature for 16 hours. The reaction mixture was diluted with DCM (1 mL) and purified on silica gel (24 g isco column) using 25-100% ethyl acetate in hexanes, then 5-10% methanol in ethyl acetate. The desired fractions were concentrated to give **SI-41** as a beige solid (0.074 g, 85 %). LRMS (m/*z*): Calculated for $C_{64}H_{79}N_{11}O_{13}Si$ $[M+H]^+$ 1238.6, found 1238.4.



4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl (6S,6aS)-6-hydroxy-2-methoxy-3-(4-((5-((3-methoxy-4-(3-methyl-1H-1,2,4-triazol-1-yl)phenyl)carbamoyl)-1-methyl-1H-pyrrol-3-yl)amino)-4-oxobutoxy)-14-oxo-6,6a,7,12-tetrahydrobenzo[5,6][1,4]diazepino[1,2-b]isoquinoline-5(14H)-carboxylate (48):

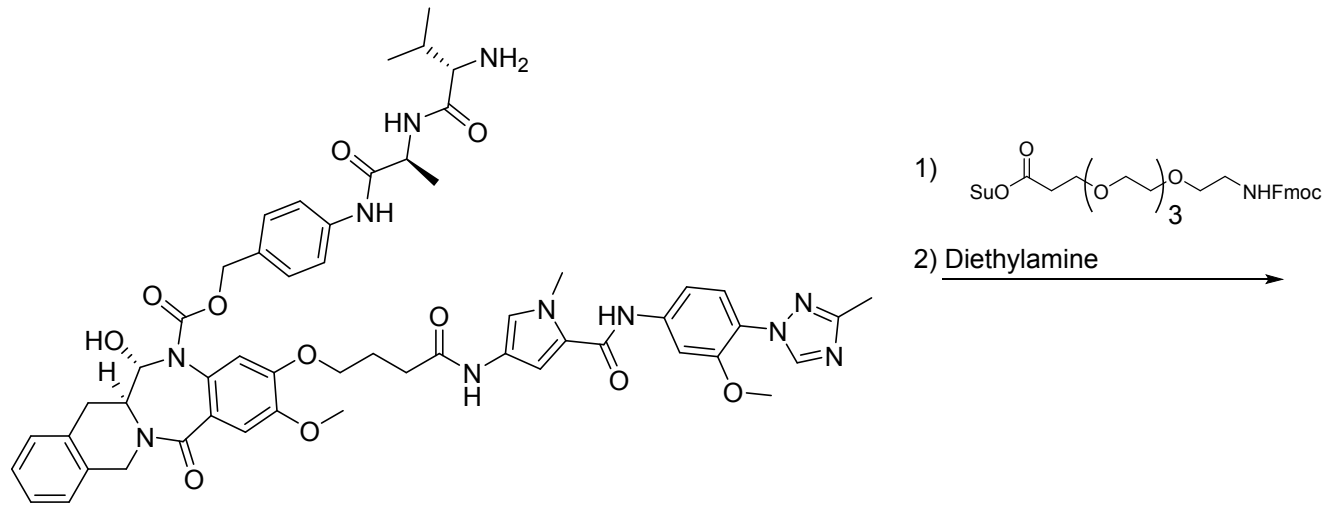
Tetrabutylammonium fluoride (0.119 mL, 0.119 mmol) was added to **SI-41** (0.0736 g, 0.059 mmol) in THF. The mixture was stirred for 30 minutes. The reaction mixture was diluted with ethyl acetate (40 mL), wash with water, saturated sodium bicarbonate, and brine, dried over Na_2SO_4, concentrated and purified on silica gel (40 g isco column) using 1-10 % methanol in ethyl acetate. The desired fractions were concentrated to give **48** as a pale yellow solid (0.0935 g, 88 %). LRMS (m/*z*): Calculated for $C_{58}H_{65}N_{11}O_{13}$ $[M+H]^+$ 1124.5, found 1124.4.



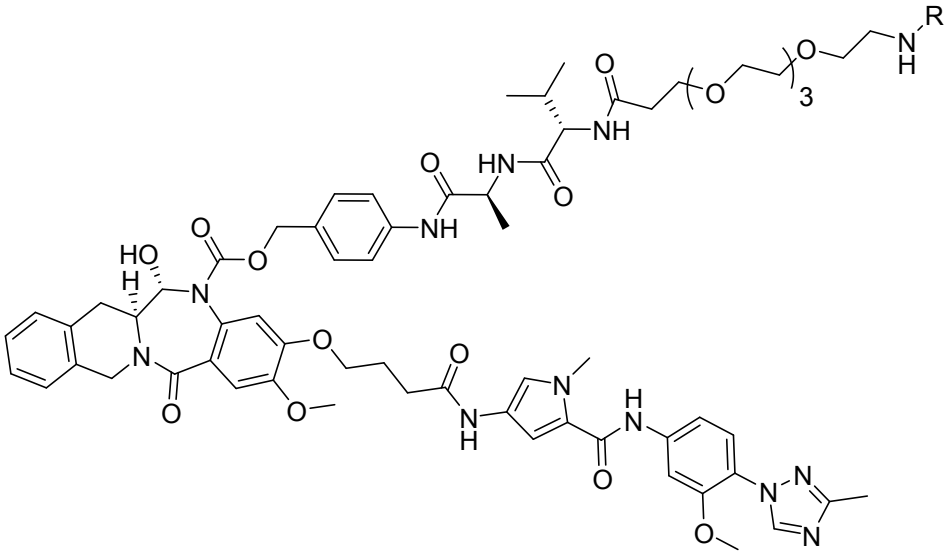
48 **SI-42**

4-((S)-2-((S)-2-amino-3-methylbutanamido)propanamido)benzyl (6S,6aS)-6-hydroxy-2-methoxy-3-(4-((5-((3-methoxy-4-(3-methyl-1H-1,2,4-triazol-1-yl)phenyl)carbamoyl)-1-methyl-1H-pyrrol-3-yl)amino)-4-oxobutoxy)-14-oxo-6,6a,7,12-tetrahydrobenzo[5,6][1,4]diazepino[1,2-b]isoquinoline-5(14H)-carboxylate (SI-42):

Pyrrolidine (4.95 mL, 0.208 mmol, 0.042 M in DCM) was added to **48** (0.0935 g, 0.083 mmol), followed by tetrakis(triphenylphosphine)palladium(0) (8.55 mg, 7.40 μmol). The mixture was stirred for 30 minutes, diluted with DCM, washed with saturated NH_4Cl and brine. The organic layer was dried over Na_2SO_4 and concentrated to give **SI-42** as a yellow solid (0.087 g, 101 %, used as is). LRMS (m/*z*): Calculated for $C_{54}H_{61}N_{11}O_{11}$ $[M+H]^+$ 1040.5, found 1040.6.



SI-42



R=Fmoc : SI-43
R =H: 49

(6S,6aS)-4-((21S,24S)-1-(9H-fluoren-9-yl)-21-isopropyl-24-methyl-3,19,22-trioxo-2,7,10,13,16-pentaoxa-4,20,23-triazapentacosanamido)benzyl 6-hydroxy-2-methoxy-3-(4-((5-((3-methoxy-4-(3-methyl-1H-1,2,4-triazol-1-yl)phenyl)carbamoyl)-1-methyl-1H-pyrrol-3-yl)amino)-4-oxobutoxy)-14-oxo-6a,7,12,14-tetrahydrobenzo[5,6][1,4]diazepino[1,2-b]isoquinoline-5(6H)-carboxylate (SI-43):

Diisopropylethylamine (2.0 mL, 0.100 mmol, 0.05M in DMF) was added to a mixture of 2,5-dioxopyrrolidin-1-yl 1-(9H-fluoren-9-yl)-3-oxo-2,7,10,13,16-pentaoxa-4-azanonadecan-19-oate (0.073 g, 0.125 mmol) and **SI-42** (0.087 g, 0.0.084 mmol) and stirred at room

temperature overnight. The mixture was purified on a MPLC C18 column (Biotage) eluting with a gradient of 15-100%B (95% acetonitrile/ 5% water / 0.05% Formic acid) in A (5% acetonitrile / 95% water /0.05% Formic acid) over 20 column volumes. The desired fractions concentrated to give **SI-43** as a yellow oily film. LRMS (m/*z*): Calculated for $C_{80}H_{92}N_{12}O_{18}$ $[M+H]^+$ 1509.7, found 1509.9.

(6S,6aS)-4-((17S,20S)-1-amino-17-isopropyl-20-methyl-15,18-dioxo-3,6,9,12-tetraoxa-16,19-diazahenicosanamido)benzyl 6-hydroxy-2-methoxy-3-(4-((5-((3-methoxy-4-(3-methyl-1H-1,2,4-triazol-1-yl)phenyl)carbamoyl)-1-methyl-1H-pyrrol-3-yl)amino)-4-oxobutoxy)-14-oxo-6a,7,12,14-tetrahydrobenzo[5,6][1,4]diazepino[1,2-b]isoquinoline-5(6H)-carboxylate (49):

Diethylamine (0.021 mL, 0.205 mmol) was added to a solution of **SI-43** (0.031 g, 0.021 mmol) in DMF and the mixture was stirred for 30 minutes and concentrated to remove diethylamine. The residue was purified on a C18 biotage column (55 g) using 15-100% of B (95% acetonitrile / 5% water / 0.05% Formic acid) in A (5% acetonitrile / 95% water / 0.05% Formic acid) over 20 column volumes. The desired fractions were lyophilized to give an off-white solid. HRMS: Calculated for $C_{65}H_{82}N_{12}O_{16}[M+H]^+$ 1287.6045, found 1287.5993.

Site-Specific Conjugation

Antibody at 5 mg/mL, in 20 mM Tris, 50 mM NaCl, pH 8.0, was reacted with recombinant bacterial transglutaminase (1/5 mole equivalent) and 10 mole equivalents of linker-payload **49**. The reaction was carried out at 37 °C overnight. The ADC was purified on a Protein A (GE Health sciences MabSelect Sure) column pre-equilibrated with PBS, pH 7.4. A wash step with PBS containing 15% acetonitrile was carried out to ensure the removal of any unreacted payload-linker. The ADC was eluted with 10 mM succinic acid, 20 mM glycine, pH 3.1. The elution fractions were immediately neutralized with 1 M Tris-HCl, pH 8.0 (4:1 ratio of elution buffer: neutralizing buffer). The purified ADC was formulated via tangential flow filtration in 20 mM Histidine, 10% Sucrose, pH 6.

Serum Stability of ADC

ADC was spiked into BALB/c mouse serum to achieve a final concentration of 0.1 mg/mL. The samples were incubated at 37 °C, and aliquots were taken at 0, 24, 48 and 96 h post incubation and immediately frozen at –20 °C. The samples were processed and analyzed by affinity capture LC-MS method previous reported with some modifications.[1] Briefly, the ADC was captured from serum using streptavidin magnetic beads coated with a biotinylated anti human Fc capture reagent. The unbound serum components were washed away and the ADC was eluted from beads using 1% formic acid in 30% isopropanol. The inter-chain disulfide bonds on the eluted ADC was reduced with TCEP and the reduced elute with the LC and HC+Drug mixture was then analyzed using LC-MS on a Waters G2-XS TOF instrument.

1. Kotapati, S.; Passmore, D.; Yamazoe, S.; Sanku, R.K.K.; Cong, Q.; Poudel, Y.B.; Chowdari, N.S.; Gangwar, S.; Rao, C.; Rangan, V.S.; Cardarelli, P.M.; Deshpande, S.; Strop, P.; Dollinger, G, Rajpal, A. Universal affinity capture liquid chromatography-mass spectrometry assay for evaluation of biotransformation of site-specific Antibody Drug Conjugates in preclinical studies, *Anal. Chem.* **2020,** *92*, 2065-2073.

Cathepsin B enzyme digestion of ADC

ADC samples were diluted with 25 mM MES, 0.5 mM EDTA (pH 5.5) buffer to achieve a final concentration of 0.1 mg/ml. The disulfide bonds in ADCs were then reduced by addition of 10 mM of Cysteamine HCl and incubated at 37 ^{o}C for 30 minutes. After reduction, 0.3 units of human liver Cathepsin B enzyme (Millipore Sigma) was added and the samples were incubated at 37 ^{o}C for an additional 4 hours. A negative control was included for each of the samples where incubations were carried out without the addition of the enzyme. After 4 hours, a 20 µl aliquot was taken and the digestion was stopped by addition of 80 µl of 0.1% formic acid in 3:1 methanol:acetonitrile. The samples were then held at -20 ^{o}C for 1 hour to ensure protein precipitation and centrifuged at 14000 rpm for 15 minutes. The supernatant was transferred and analyzed by LC-MS/MS. The % payload cleaved by Cathepsin B is accurately determined using peak area of payload spiked in ADC sample at

concentration equivalent to 0.1 mg/mL ADC but without addition of Cathepsin B, followed by the same sample preparation steps as described above.

LC-MS/MS analysis was done on a Shimadzu Nexera UPLC connected to a AB Sciex API 4000 Triple Quadrupole MS system. 0.5 µl of the supernatant was injected onto a Acquity UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm) held at 60 ^{o}C. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (Solvent B). The flow rate was 0.5 ml/min with the following gradient: 0−1 min, held at 20%B; 1−3 min, linear ramp to 75%B; 3−3.1 min, fast ramp to 95%B; 3.1−4.1 min, held at 95%B; 4.1−4.5 min, ramp down to 20% B; 4.5−6 min, equilibration at 20%B. The MS parameters were as follows: curtain gas = 25 psi, ion source gas 1 = 30 psi, ion source gas 2 = 40 psi, Ion Spray Voltage = 5000 V, Source Temperature = 500 ^{o}C. MRM transition for the payload was *m/z* 703.45 → 309.3 (DP = 126 V, CXP = 8 V, EP = 10 V, CE = 47 V) with a dwell time of 150 ms.

***In vivo* efficacy**

The efficacy of Mesothelin-ADC **50** or FucGM1-ADC **50** was evaluated in a xenograft model of N87 human gastric tumors (from ATCC). SCID (C.B.-17) mice were implanted with N87 cells (2.5e6 cells/mouse) and dosed via intravenous route 11 days post-implantation. Body weight and tumor volume (LWH*0.5) was measured twice a week.

Mice were maintained at Bristol Myers Squibb (BMS) Co., animal facility. All animal experiments and procedures were performed in accordance with protocols approved by the BMS Institutional Animal Care and Use Committee (IACUC).

Molecular Modeling

DNA models were built and optimized using the program Quanta from Polygen Corporation. Payloads were hand docked into the DNA models and optimized using Maestro[2]. Binding model stability was checked using molecular dynamics simulations performed using Desmond[3].

2. Schrödinger: Maestro, Schrödinger, LLC, New York, NY.

3. Schrödinger: Desmond Molecular Dynamics System, D. E. Shaw Research, New York, NY.