Supporting Information

Investigation of Hydrophilic Auristatin Derivatives for Use in Antibody Drug Conjugates

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Chemical Synthesis

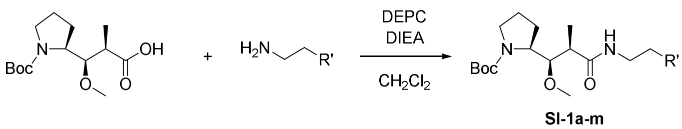
General Methodology. Reactions were carried out at ambient temperature with exposure to air, unless otherwise noted. All reagents and solvents were purchased from commercial sources and used as received. Flash chromatography was carried out on a Yamazen purification system using pre-packed Yamazen Universal columns. Preparatory HPLC was carried out using a Phenomenex Gemini-NX 10µm, C18 110 Å column (150 x 30 mm) using a 5 to 100% gradient of acetonitrile/0.05% aqueous trifluoroacetic acid mixture over 13 minutes unless another column or solvent system is noted. Drug compounds purified by preparatory HPLC (**12** – **28**) were assumed to be salts containing one molecule of TFA. NMR spectra were obtained on a Bruker 400 or 500 MHz Avance NMR at 25 °C. The NMR spectra obtained were referenced by setting the residual solvent peak to reported values.[1] LC-MS analysis was carried out on Waters Acquity UPLCs equipped with Single-Quad mass spectrometry detectors using a 5 to 100% gradient of acetonitrile in 0.05% aqueous trifluoroacetic acid mixture over 2 minutes with a Waters BEH C18 1.7 µm (2.1 x 50 mm) column. High resolution mass spectrometry samples were injected without column on to a Dionex 3000-Orbitrap Velos LC-MS.

Abbreviation

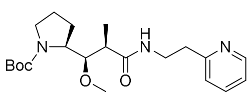
ADC, Antibody Drug Conjugate; DAR, Drug to Antibody Ratio; DEPC, Diethyl cyanophosphonate; DIEA, diisopropylethylamine; HIC, Hydrophobic Interaction

Chromatography; mc, maleimidocaproyl; mcVCP, maleimidocaproyl-Valine-Citrulline-Paba; MMAE, monomethyl auristatin E; MMAF, monomethyl auristatin F; MMAPYE, monomethyl auristatin PYE; SEC, size exclusion chromatography; TCEP, tris(2-carboxyethyl)phosphine.

General Procedure for the Synthesis of P4-P5 Unit

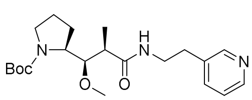


To a stirred room temperature suspension of Boc-Dap-OH dicyclohexamine salt (1 equiv) and P5 amine (1.1 equiv) in CH_2Cl_2 (0.5 M) was added DIEA (2 equiv), followed by DEPC (1.5 equiv). LCMS analysis indicated completion of reaction after 8 h. Compounds **SI-1a-m** were isolated by flash chromatography using 2% to 10% MeOH/1% NEt_3 in CH_2Cl_2 as the eluent.



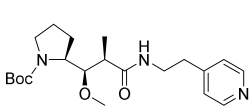
SI-1a (5 main text)

Boc-Dap-2-(2-aminoethyl)pyridine (**SI-1a, 5 main text**) (0.796 g, 2.03 mmol) was obtained in 95% yield. ESI MS: m/z = 414.2 (M + Na^+), 392.3 (M + H), 336.2, 292.2 ((M + H) – Boc).



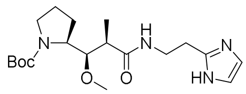
SI-1b

Boc-Dap-3-(2-aminoethyl)pyridine (**SI-1b**) (1.29 g, 3.30 mmol) was obtained in 94% yield. ESI MS: m/z = 414.2 (M + Na^+), 392.3 (M + H), 292.2 ((M + H) – Boc).

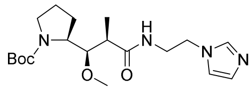


SI-1c

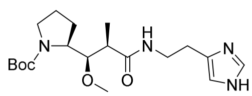
Boc-Dap-4-(2-aminoethyl)pyridine (**SI-1c**) (2.92 g, 7.46 mmol) was obtained in 91% yield. ESI MS: m/z = 414.2 (M + Na^+), 392.2 (M + H), 292.2 ((M + H) – Boc).



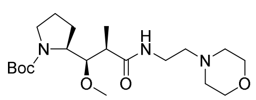
SI-1d **Boc-Dap-[2-(1*H*-imidazol-2-yl)ethyl]amine** (**SI-1d**) (1.48 g, 3.89 mmol) was obtained in 72% yield. ESI MS: *m/z* 381.3 (M + H), 325.3, 281.2 ((M + H) – Boc).



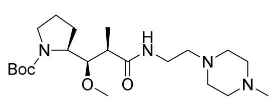
SI-1e **Boc-Dap-2-(1*H*-imidazole-1-yl)-ethylamine** (**SI-1e**) (1.64 g, 4.31 mmol) was obtained in 79% yield. ESI MS: *m/z* 381.7 (M + H), 325.2, 281.2 ((M + H) – Boc).



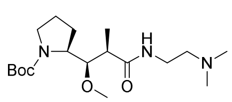
SI-1f **Boc-Dap-[2-(1*H*-imidazol-4-yl)ethyl]amine** (**SI-1f**) (1.77 g) was obtained in 109% yield. The excess mass was attributed to dicyclohexylamine, however the material was used without further purification. ESI MS: *m/z* 403.3 (M + Na^+), 381.3 (M + H), 281.2 ((M + H) – Boc).



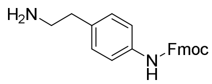
SI-1g **Boc-Dap-4-(2-aminoethyl)morpholine** (**SI-1g**) (3.43 g) was obtained in 134% yield. The excess mass was attributed to dicyclohexylamine, however the material was used without further purification. ESI MS: *m/z* 400.4 (M + H), 422.3 (M + Na^+).



SI-1h **Boc-Dap-2-(4-methyl-piperazin-1-yl)-ethylamine** (**SI-1h**) (1.69 g, 4.10 mmol) was obtained in 59% yield. ESI MS: *m/z* 413.2 (M + H), 357.3.

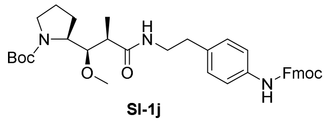


SI-1i **Boc-Dap-*N,N*-dimethylethylenediamine** (**SI-1i**) (1.54 g, 4.31 mmol) was obtained in 67% yield. ESI MS: *m/z* 358.3 (M + H), 302.3.



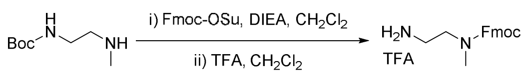
SI-2

Synthesized according to Perron, V. et al.[2] **Fmoc-4-(2-aminoethyl)aniline•HCl** (**SI-2**) (6.57 g, 16.6 mmol) was obtained in 84% yield.



SI-1j

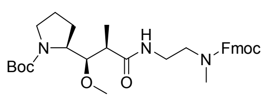
Boc-Dap-4-(2-aminoethyl)aniline-Fmoc (**SI-1j**) (1.25 g, 1.99 mmol) was obtained in 93% yield. ESI MS: *m/z* 628.4 (M + H), 650.4 (M + Na^+), 528.4 ((M + H) - Boc).



SI-3 SI-4

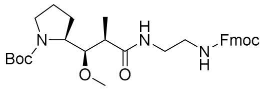
***N*-Fmoc-*N*-methylaminoethylamine (SI-4).** To a stirred room temperature solution of *N'*-Boc-*N*-methylaminoethylamine **SI-3** (1.00 g, 4.75 mmol) in CH_2Cl_2 (10 mL) was added Fmoc-OSu (1.76 g, 5.22 mmol) and DIEA (1.69 g, 9.49 mmol). After 1 h, analysis by LC-MS showed the reaction was complete. The solvent was removed in vacuo and the viscous mixture was dissolved in EtOAc (100 mL), washed with 0.1 M HCl (50 mL), sat. $NaHCO_3$ (50 mL), and brine (50 mL). The organic fraction was dried over magnesium sulfate, filtered, and concentrated in vacuo. The crude *N'*-Boc-*N*-Fmoc-*N*-methylaminoethylamine was used without further purification.

To a stirred room temperature solution of crude *N'*-Boc-*N*-Fmoc-*N*-methylaminoethylamine in CH_2Cl_2 (5 mL) was added TFA (2 mL). After 10 h, analysis by LC-MS showed the reaction was complete. The solvent was removed in vacuo and the crude product was purified by flash chromatography using MeOH:CH_2Cl_2 as the eluent. *N*-Fmoc-*N*-methylaminoethylamine•TFA (**SI-4**) (2.04 g, 3.89 mmol) was obtained in 79% yield. ESI MS: *m/z* 297.2 (M + H).



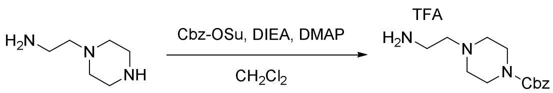
SI-1k

Boc-Dap-*N*-Fmoc-*N*-methylaminoethylamine (**SI-1k**) (0.617 g, 1.09 mmol) was obtained in 58% yield. ESI MS: *m/z* 566.6 (M + H), 466.4 ((M + H) – Boc).

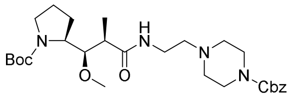


SI-1l

Boc-Dap-N-Fmoc-N-ethylenediamine (**SI-1l**) (1.38 g, 2.50 mmol) was obtained in 39% yield. ESI MS: *m/z* 552.4 (M + H), 574.4 (M + Na^+), 452.3 ((M + H) – Boc).



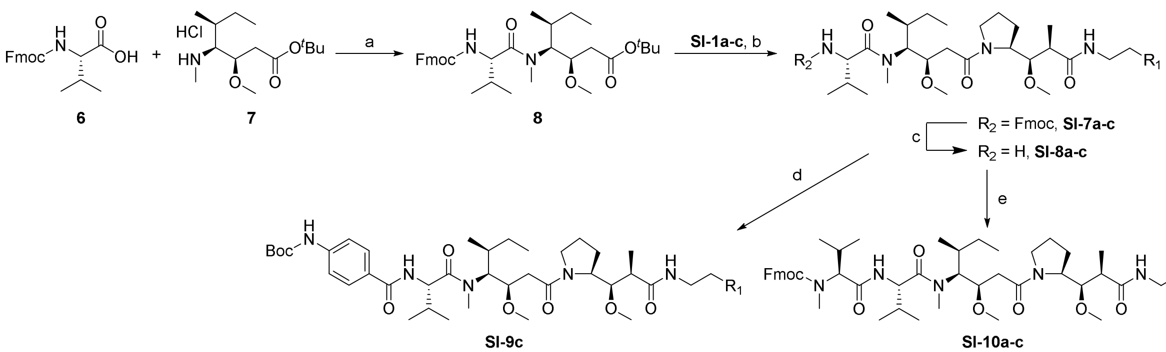
2-(4-Cbz-piperazin-1-yl)ethanamine•TFA (SI-6). To a stirred room temperature solution of 1-(2-aminoethyl)piperazine **SI-5** (4.00 g, 31.0 mmol) in CH_2Cl_2 (170 mL) was added Cbz-OSu (7.73 g, 31.0 g) followed by DIEA (5.40 mL, 31.0 mmol) and DMAP (10 mg, 0.08 mmol). After 24 h, the slightly yellow reaction mixture was washed with 0.1 M HCl (150 mL), sat. $NaHCO_3$ (100 mL), and brine (100 mL). The organic fraction was dried over magnesium sulfate, filtered, and concentrated in vacuo. The crude product was isolated by flash chromatography using 2% – 10% MeOH:CH_2Cl_2, followed by further purification by preparatory HPLC. 2-(4-Cbz-piperazin-1-yl)ethanamine•TFA **SI-6** (0.862 g, 2.28 mmol) was obtained in 7% yield.



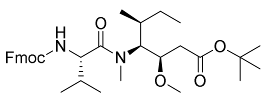
SI-1m

Boc-Dap-2-(4-Cbz-piperazin-1-yl)ethanamine (SI-1m) (0.96 g, 1.80 mmol) was obtained in 79% yield. ESI MS: *m/z* 533.2 (M + H), 1065.9 (2M + H).

Scheme SI1. Synthesis of Pyridine-Containing Derivatives[1]

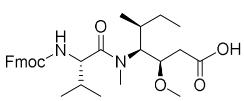


[1](a) CMPI, DIEA, EtOAc, 0 °C; (b) (*i*) TFA, CH_2Cl_2; (*ii*) DEPC, DIEA, EtOAc; (c) piperidine, CH_2Cl_2; (d) Boc-Abz-OH, DEPC, DIEA, EtOAc; (e) Fmoc-NH-MeVal-OH, DEPC, DIEA, CH_2Cl_2;



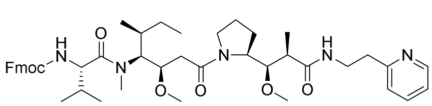
8

Fmoc-Val-Dil-O^{t}Bu (8). To a solution of Dil-O^{t}Bu hydrochloride **7** (0.60 g, 2.03 mmol) and Fmoc-Val-OH **6** (0.829 g, 2.44 mmol) in EtOAc (3 mL) was added DIEA (0.65 mL, 3.65 mmol). The reaction was cooled to 0 °C and stirred for 20 min, followed by the addition of a further portion of DIEA (0.65 mL, 3.65 mmol). The reaction mixture was cooled for another 20 min, followed by addition of CMPI (0.83 g, 3.65 mmol). After 8 h, the reaction mixture was extracted sequentially with 1M HCl (25 mL x 2) and brine (50 mL). The organic phase was dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The product was purified by flash chromatography (18% – 55% EtOAc:hexanes, then 90% EtOAc:hexanes). Fmoc-Val-Dil-O^{t}Bu (**8**) (1.10 g, 1.89 mmol) was obtained in 93% yield. ESI MS: m/z = 581.6 (M + H), 525.4 ((M + 1) – ^{t}Bu).



SI-11

Fmoc-Val-Dil-OH (SI-11). Fmoc-Val-Dil-OtBu (**8**) (10.0 g, 17.2 mmol) was dissolved in 4 M HCl/dioxane (30 mL, 120 mmol) and the mixture was stirred at 23 °C. After 24 h, the mixture was concentrated under reduced pressure to yield Fmoc-Val-Dil-OH **SI-11** (9.0 g, 17 mmol) as a pale yellow foam in 99% yield. ESI MS: m/z = 525.4 (M + H).

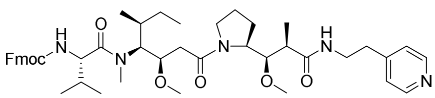


SI-7a (9 main text)

Fmoc-Val-Dil-Dap-2-(2-aminoethyl)pyridine (SI-7a, 9 main text). To a room temperature suspension of Fmoc-Val-Dil-O^{t}Bu (**8**) (0.883 g, 1.52 mmol) and Boc-Dap-2-(2-aminoethyl)pyridine (**SI-1a, 5 main text**) (0.451 g, 1.52 mmol) in CH_2Cl_2 (5 mL) was added TFA (2 mL). LC-MS analysis indicated complete reaction after 8 h. Volatile organics were evaporated under reduced pressure and the residue was used as-is. To a room temperature suspension of this mixture of H-Dap-2-(2-pyridyl)ethylamine•TFA and Fmoc-Val-Dil-OH in EtOAc (2 mL) was added DIEA (1.1 mL, 6.08 mmol), followed by DEPC (0.92 mL, 6.08 mmol). After 15 h, sat. $NaHCO_3$ (50 mL) was added and the mixture was extracted with EtOAc (3 x 25 mL). The combined organics were dried over magnesium sulfate and concentrated in vacuo. The resulting viscous oil was dissolved in a minimal amount of CH_2Cl_2

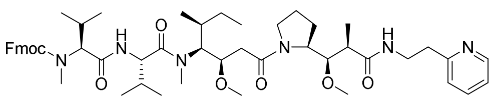
and purified by flash chromatography (5% – 10% MeOH:CH_2Cl_2). Fmoc-Val-Dil-Dap-2-(2-aminoethyl)pyridine (**SI-7a, 9 main text**) (0.888 g, 1.11 mmol) was obtained in 73% yield. ESI MS: *m/z* = 798.5 (M + H).

Compound **SI-7c** was synthesized in an analogous fashion:



SI-7c

Fmoc-Val-Dil-Dap-4-(2-aminoethyl)pyridine (**SI-7c**) (2.51 g, 3.15 mmol) was obtained in 53% yield. ESI MS: *m/z* = 799 (M + H).

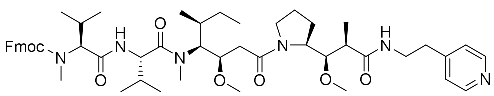


SI-10a, 11 main text

Fmoc-MeVal-Val-Dil-Dap-2-(2-aminoethyl)pyridine (SI-10a, main text 11). To a stirred room temperature solution of Fmoc-Val-Dil-Dap-2-(2-aminoethyl)pyridine (**SI-7a, 9 main text**) (1.75 g, 2.19 mmol) in CH_2Cl_2 (5 mL) was added piperidine (5 mL). After 8 h, analysis by LC-MS showed the reaction was complete. Volatile organics were evaporated in vacuo to yield crude H-Val-Dil-Dap-2-(2-pyridyl)ethylamine (**SI-8a, 10 main text**) that was used without further purification.

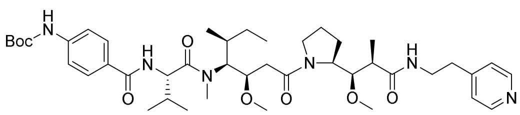
To a stirred room temperature suspension of crude H-Val-Dil-Dap-2-(2-aminoethyl)pyridine (**SI-8a, 10 main text**) and Fmoc-MeVal-OH (1.55 g, 4.38 mmol) in CH_2Cl_2 (7 mL) was added DIEA (1.56 mL, 8.76 mmol), followed by DEPC (1.32 mL, 8.76 mmol). After 18 h, a further portion of DIEA (1 mL, 5.61 mmol) and DEPC (1 mL, 6.57 mmol) was added. Once analysis by LC-MS showed the reaction was complete, the solvent was removed in vacuo, the crude mixture was dissolved in EtOAc (100 mL) and washed with sat. $NaHCO_3$ (50 mL), and water (50 mL x 2). The organic fraction was dried over magnesium sulfate and concentrated in vacuo. The resulting oil was purified by flash chromatography using 5% – 10% MeOH:CH_2Cl_2 as the eluent. Fmoc-MeVal-Val-Dil-Dap-2-(2-pyridyl)ethylamine **SI-10a, 11 main text** (1.07 g, 1.17 mmol) was obtained in 59% yield. ESI MS: *m/z* = 912.7 (M + H).

Compound **SI-10c** was synthesized in an analogous fashion:



SI-10c

Fmoc-MeVal-Val-Dil-Dap-4-(2-aminoethyl)pyridine (SI-10c) (1.95 g, 2.15 mmol) was obtained in 30% yield after purification by flash chromatography. ESI MS: *m/z* 911.2 (M + H).



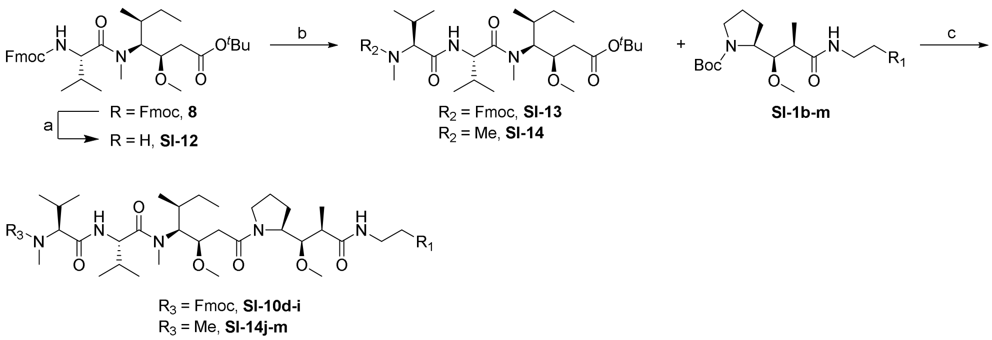
SI-9c

***N*-Boc-4-Abz-Val-Dil-Dap-4-(2-aminoethyl)pyridine (SI-9c)**. To a stirred room temperature solution of Fmoc-Val-Dil-Dap-4-(2-aminoethyl)pyridine (**SI-7a, 9 main text**) (3.46 g, 4.34 mmol) in CH_2Cl_2 (5 mL) was added piperidine (2 mL). After 8 h, volatile organics were evaporated in vacuo to yield crude H-Val-Dil-Dap-4-(2-aminoethyl)pyridine (**SI-8a, 10 main text**) that was used without further purification.

To a stirred room temperature suspension of crude H-Val-Dil-Dap-4-(2-aminoethyl)pyridine (**SI-8a, 10 main text**) and *N*-Boc-4-Abz-OH (2.06 g, 8.68 mmol) in CH_2Cl_2 (7 mL) was added DIEA (3.10 mL, 17.4 mmol), followed by DEPC (2.62 mL, 17.4 mmol). After 18 h, analysis by LC-MS showed the reaction was complete. The solvent was removed in vacuo and the crude product was dissolved in EtOAc (20 mL), washed with sat. $NaHCO_3$ (50 mL), water (50 mL x 2), dried over magnesium sulfate and concentrated in vacuo. The resulting viscous oil was purified by flash chromatography using 5% – 10% MeOH:CH_2Cl_2 as the eluent. *N*-Boc-4-Abz-Val-Dil-Dap-4-(2-aminoethyl)pyridine (**SI-9c**) (2.09 g, 2.63 mmol) was obtained in 61% yield. ESI MS: *m/z* 795.5 (M + H).

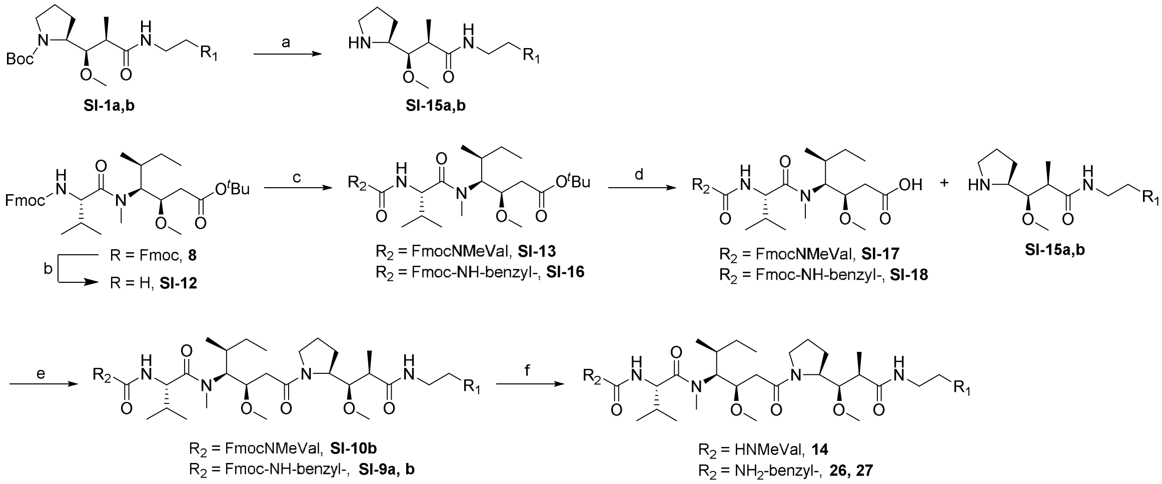
The remaining examples were synthesized by coupling P1-P3 with P4-P5:

Scheme SI2. Synthesis of *N*-MeVal Derivatives through P1-P3 coupling

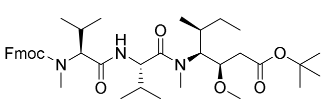


(a) piperidine, CH_2Cl_2; (b) Fmoc-MeVal *or* Me_2N-Val (Dov), DEPC, DIEA, EtOAc; (c) (*i*) TFA, CH_2Cl_2 (*ii*) HATU, DIEA, HOBt, DMF;

Scheme SI3. Alternate synthesis of some compounds through P1-P3 coupling



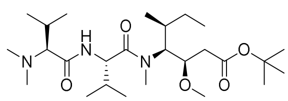
(a) 4M HCl/dioxane; (b) $HNEt_2$, EtOAc; (c) CMPI, DIPEA, EtOAc; (d) 4M HCl/dioxane; (e) EDCI, HOBt, CH_2Cl_2, DMF; (f) $HNEt_2$ *or* dodecyl mercaptan, DBU then $HNEt_2$.



SI-13

Fmoc-MeVal-Val-Dil-O^{t}Bu (**SI-12**). To a stirred room temperature solution of Fmoc-Val-Dil-O^{t}Bu (**8**) (5.00 g, 8.61 mmol) in CH_2Cl_2 (10 mL) was added piperidine (5 mL). After 8 h, analysis by LC-MS showed the reaction was complete. Volatile organics were evaporated in vacuo to yield crude H-Val-Dil-O^{t}Bu (**9**) that was used without further purification.

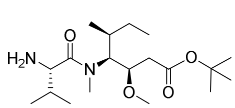
To a stirred room temperature suspension of crude H-Val-Dil-O^{t}Bu (**9**) and Fmoc-MeVal-OH (9.13 g, 25.8 mmol) in EtOAc (10 mL) was added DIEA (6.14 mL, 34.4 mmol), followed by DEPC (5.19 mL, 34.4 mmol). After 12 h, analysis by LC-MS showed the reaction was complete. The reaction mixture was washed with 1 M HCl (150 mL x 2) and brine (150 mL). The organic fraction was dried over magnesium sulfate and concentrated in vacuo. The resulting viscous oil was purified by flash chromatography using 18% – 90% EtOAc:hexanes as the eluent. Fmoc-MeVal-Val-Dil-O^{t}Bu (**SI-13**) (5.54 g, 7.98 mmol) was obtained in 93% yield. ESI MS: *m/z* 694.6 (M + H), 716.5 (M + Na$^+$), 638.5 ((M + H) – ^{t}Bu).



SI-14

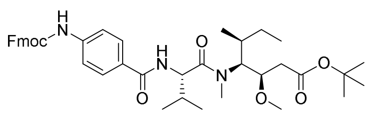
Dov-Val-Dil-O^{t}Bu•TFA (**SI-14**). To a stirred room temperature solution of crude Fmoc-Val-Dil-O^{t}Bu (**8**) (13.3 g, 22.8 mmol) in CH_2Cl_2 (20 mL) was added piperidine (15 mL). After 8 h, analysis by LC-MS showed the reaction was complete. Volatile organics were evaporated in vacuo to yield crude H-Val-Dil-O^{t}Bu that was used without further purification.

To a stirred room temperature suspension of crude H-Val-Dil-O^{t}Bu (**9**) and Dov (6.63 g, 45.7 mmol) in CH_2Cl_2 (20 mL) was added DIEA (12.2 mL, 68.5 mmol), followed by DEPC (10.3 mL, 68.5 mmol). After 12 h, analysis by LC-MS showed the reaction was complete. The solvent was evaporated in vacuo*,* the crude mixture was dissolved in EtOAc (50 mL), washed with water (50 mL x 2), and brine (50 mL). The organic fraction was dried over magnesium sulfate and concentrated in vacuo. The resulting viscous oil was purified by preparatory HPLC. Dov-Val-Dil-O^{t}Bu•TFA (**SI-14**) (9.58 g, 15.2 mmol) was obtained in 67% yield. ESI MS: *m/z* 508.4 (M + Na$^+$), 486.3 (M + H), 430.3 ((M + H) – ^{t}Bu).



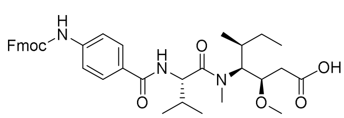
SI-12

Val-Dil-O^{t}Bu (**SI-12).** To a stirred solution of Fmoc-Val-Dil-O^{t}Bu (**8**) (143 g, 246 mmol) in EtOAc (200 mL) was added diethylamine (200 mL, 1.9 mol). After 1 h, the crude mixture was concentrated under reduced pressure. Ethyl acetate was added (2 x 200 mL) and the mixture was concentrated under reduced pressure. Toluene (50 mL) was then added and the mixture was concentrated under reduced pressure. To the residue was added hexanes (1000 mL) and 0.5 M HCl (1000 mL). The layers were separated and the organic layer was extracted with 0.1 M HCl (2 x 500 mL). The combined aqueous layers were then washed with hexanes (2 x 500 mL). The pH of the aqueous layer was adjusted to >10 using K_2CO_3, and was extracted with three portions of EtOAc. The combined organic layers were washed with brine (500 mL), dried over $MgSO_4$ and concentrated under reduced pressure. Val-Dil-O^{t}Bu, **SI-12** (66.8 g, 186 mmol) was obtained as a pink oil in 76% yield. ESI MS: *m/z* 359.4 (M + H).



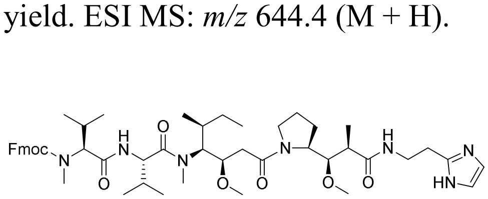
SI-16

Fmoc-4-aminobenzyl-Val-Dil-O^{t}Bu (**SI-16).** To a stirred suspension of Val-Dil-O^{t}Bu (**SI-12**) (2.05 g, 5.72 mmol), Fmoc-4-aminobenzoic acid (2.24 g, 6.23 mmol) and CMPI (2.25 g, 8.81 mmol) in EtOAc (20 mL) was added DIPEA (4.0 mL, 23.0 mmol). After 18 h, the mixture was filtered, and diluted with EtOAc. The solution was washed with two portions of 1 M HCl, sat. $NaHCO_3$ and brine. The organic layer was passed through a phase separator and concentrated under reduced pressure. Fmoc-4-aminobenzyl-Val-Dil-O^{t}Bu (**SI-16**) (2.50 g, 3.57 mmol) was obtained as an off-white solid in 62% yield. ESI MS: *m/z* 700.4 (M + H).



SI-18

Fmoc-4-aminobenzyl-Val-Dil-O^{t}Bu (**SI-18**). To Fmoc-4-aminobenzyl-Val-Dil-O^{t}Bu (**SI-16**) (2.50 g, 3.57 mmol) was added 4 M HCl in dioxane (9.0 mL, 36.0 mmol) and the mixture was stirred 16 h. The crude solution was concentrated under reduced pressure. The residue was dissolved in MeCN and H_2O and lyophilized. Fmoc-4-aminobenzyl-Val-Dil-O^{t}Bu (**SI-18**) (2.12 g, 3.29 mmol) was obtained as a brown solid in 92% yield. ESI MS: *m/z* 644.4 (M + H).



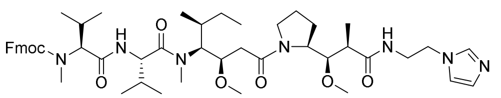
SI-10d

Fmoc-MeVal-Val-Dil-Dap-[2-(1H-imidazol-2-yl)ethyl]amine•TFA (**SI-10d**). Fmoc-MeVal-Val-Dil-O^{t}Bu (**SI-13**) (1.20 g, 1.73 mmol) and

Boc-Dap-[2-(1*H*-imidazol-2-yl)ethyl]amine (**SI-1d**) (0.60 g, 1.58 mmol) were suspended in CH_2Cl_2 (5 mL), and TFA (4 mL) was added. After 8 h, analysis by LCMS showed the reaction was complete. The volatile organics were evaporated in vacuo to yield crude Fmoc-MeVal-Val-Dil-OH and H-Dap-[2-(1*H*-imidazol-2-yl)ethyl]amine•TFA, which were used without further purification.

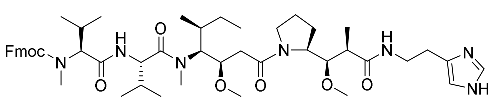
To a stirred room temperature suspension of this mixture in DMF (6 mL) was added DIEA (1.41 mL, 7.90 mmol), followed by HATU (1.80 g, 4.74 mmol) and HOBt (0.48 g, 3.16 mmol). After 12 h, analysis by LC-MS showed the reaction was complete. Sat. $NaHCO_3$ (10 mL) was added, followed by extraction with EtOAc (4 x 10 mL). The combined organic extract was washed with brine (20 mL), dried over magnesium sulfate, filtered, and concentrated in vacuo. Fmoc-MeVal-Val-Dil-Dap-[2-(1H-imidazol-2-yl)ethyl]amine•TFA (**SI-10d**) (0.578 g, 0.553 mmol) was obtained in 35% yield after purification by preparatory HPLC. ESI MS: *m/z* 900.7 (M + H).

Compounds **SI-10e-i** were synthesized in an analogous fashion:



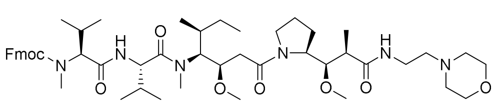
SI-10e

Fmoc-MeVal-Val-Dil-Dap-2-(1*H*-imidazole-1-yl)-ethylamine•TFA (**SI-10e**) (0.211 g, 0.202 mmol) was obtained in 3% yield after purification by preparatory HPLC. ESI MS: *m/z* 900.2 (M + H).



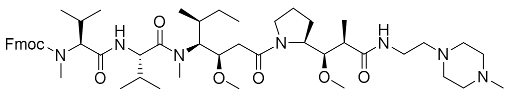
SI-10f

Fmoc-MeVal-Val-Dil-Dap-[2-(1*H*-imidazol-4-yl)ethyl]amine•TFA (**SI-10f**) (60 mg, 0.057 mmol) was obtained in 1% yield after purification by preparatory HPLC. ESI MS: *m/z* 900.8 (M + H).



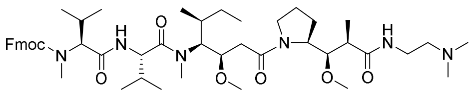
SI-10g

Fmoc-MeVal-Val-Dil-Dap-4-(2-aminoethyl)morpholine•TFA (**SI-10g**) (0.578 g, 0.544 mmol) was obtained in 7% yield after purification by preparatory HPLC. ESI MS: *m/z* 919.9 (M + H).



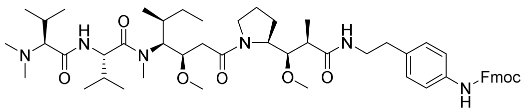
SI-10h

Fmoc-MeVal-Val-Dil-Dap-2-(4-methyl-piperazin-1-yl)-ethylamine•TFA (**SI-10h**) (0.533 g, 0.495 mmol) was obtained in 10% yield after purification by preparatory HPLC. ESI MS: *m/z* 932.9 (M + H).



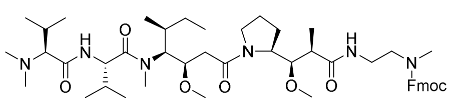
SI-10i

Fmoc-MeVal-Val-Dil-Dap-*N,N*-dimethylethylenediamine•TFA (**SI-10i**) (3.75 g, 3.67 mmol) was obtained in 52% yield after purification by preparatory HPLC. ESI MS: *m/z* 877.8 (M + H).



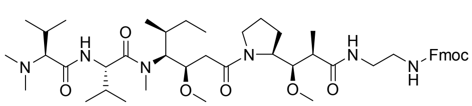
SI-14j

Dov-Val-Dil-Dap-4-(2-aminoethyl)aniline-Fmoc (**SI-14j**) (2.63 g) was obtained in 141% yield after purification by flash chromatography using 5% – 10% MeOH:CH_2Cl_2 as the eluent. Excess mass was attributed to unknown impurities, however material was used without further purification. ESI MS: *m/z* 939.7 (M + H), 961.7 (M + Na^+).



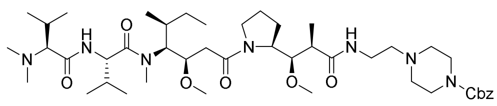
SI-14k

Dov-Val-Dil-Dap-*N*-Fmoc-*N*-methylaminoethylamine•TFA (**SI-14k**) (0.437 g, 0.428 mmol) was obtained in 39% yield after purification by preparatory HPLC. ESI MS: *m/z* 877.8 (M + H), 899.7 (M + Na^+).



SI-14l

Dov-Val-Dil-Dap-ethylenediamine-Fmoc (**SI-14l**) (1.04 g, 1.20 mmol) was obtained in 48% yield after purification by flash chromatography using 5% – 10% MeOH:CH_2Cl_2 as the eluent. ESI MS: *m/z* 863.7 (M + H), 885.7 (M + Na^+).

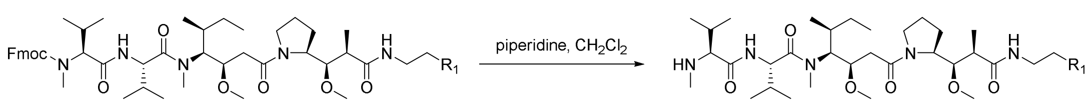


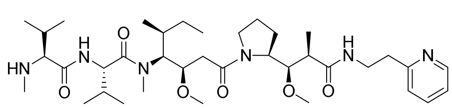
SI-14m

Dov-Val-Dil-Dap-2-(4-Cbz-piperazin-1-yl)ethanamine•TFA (SI-14m). To a stirred room temperature suspension of Boc-Dap-2-(4-Cbz-piperazin-1-yl)ethanamine **SI-1m** (0.31 g, 0.59 mmol) and Dov-Val-Dil-OtBu **SI-13** (0.314 g, 0.65 mmol) in CH_2Cl_2 (5 mL) was added TFA (4 mL). After 8 h, analysis by LC-MS showed the reaction was complete. The volatile organics were evaporated in vacuo to yield crude Dov-Val-Dil-OH•TFA and H-Dap-2-(4-Cbz-piperazin-1-yl)ethanamine•TFA that were used without further purification.

To a stirred room temperature suspension of crude Dov-Val-Dil-OH•TFA and H-Dap-2-(4-Cbz-piperazin-1-yl)ethanamine•TFA in DMF (5 mL) was added DIEA (0.42 mL, 2.35 mmol), followed by HATU (0.45g, 1.18 mmol) and HOBt (0.18 g, 1.18 mmol). After 10 h, the reaction was terminated by the addition of sat. $NaHCO_3$ (20 mL), followed by extraction with EtOAc (4 x 20 mL). The combined organic extract was washed with brine (50 mL), dried over magnesium sulfate, and concentrated in vacuo. The resulting viscous oil was purified by preparatory HPLC. Dov-Val-Dil-Dap-2-(4-Cbz-piperazin-1-yl)ethanamine•TFA **SI-14m** (0.029 g, 0.029 mmol) was obtained in 5% yield. ESI MS: *m/z* 844.9 (M + H), 866.8 (M + Na^+), 422.9 $(M + 2H)^{2+}$.

Scheme SI4. Deprotection of Fmoc-NH-MeVal



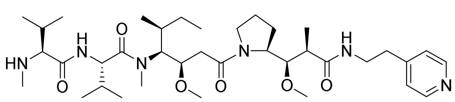


12

***N*-MeVal-Val-Dil-Dap-2-(2-aminoethyl)pyridine (12).** To a stirred solution of Fmoc-MeVal-Val-Dil-Dap-2-(2-aminoethyl)pyridine (**SI-10a**, **11 main text**) (1.07 g, 1.17 mmol) in CH_2Cl_2 (5 mL) was added piperidine (5 mL). After 10 h, the crude reaction mixture was diluted with DMSO and purified directly by preparatory HPLC. Compound **12** (624 mg, 0.78 mmol) was obtained as the TFA salt in 66% yield. ^1H NMR (400 MHz, DMSO-d_6) - a complex spectrum was observed, likely due to conformational isomers - δ

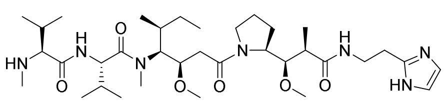
8.83 (s), 8.79 (t, J = 8 Hz), 8.49 (dt, J = 5.2, 0.8 Hz), 8.08 (t, J = 5.4 Hz), 7.86 (t, J = 5.5 Hz), 7.77 – 7.58 (m), 7.26 – 7.17 (m), 4.69 – 4.78 (m), 4.67 – 4.54 (m), 4.04 – 3.95 (m), 3.86 – 3.76 (m), 3.74 – 3.63 (m), 3.63 – 3.52 (m), 3.53 – 3.44 (m), 3.32 (s), 3.30 (s), 3.26 (s), 3.32 – 3.22 (m), 3.21 (s), 3.20 – 3.17 (m), 3.14 (s), 3.15 – 3.07 (m), 2.99 (s), 3.02 – 2.95 (m), 2.95 – 2.83 (m), 2.73 – 2.63 (m), 2.68 (s), 2.52 – 2.39 (m), 2.29 (dd, J = 15.8, 9.2 Hz), 2.24 – 2.13 (m), 2.13 – 1.92 (m), 1.91 – 1.72 (m), 1.69 – 1.50 (m), 1.38 – 1.20 (m), 1.12 – 1.00 (m), 1.01 – 0.82 (m), 0.82 – 0.69 (m), 0.64 (d, J = 6.9 Hz). ^{13}C NMR (125 MHz, DMSO-d_6) signals corresponding to TFA were not included δ 173.5, 173.1, 172.9, 172.12 (br), 172.07, 171.9, 171.7, 169.1, 168.9, 166.02, 166.00, 159.1, 158.9, 149.1, 148.9, 136.5, 136.4, 123.2, 123.1, 121.5, 89.3, 85.7, 81.8, 77.9, 77.7, 73.7, 69.4, 65.58, 65.54, 64.49, 61.1, 60.3, 58.7, 58.5, 57.3, 57.1, 55.9 (br), 55.4, 55.2, 54.66, 54.63, 47.2, 46.20, 46.18, 43.7, 43.4, 40.9, 38.2, 37.9, 37.4, 37.1, 37.0, 36.9, 35.2, 33.8, 32.0, 31.8, 31.6, 31.54, 31.47, 30.6, 30.1, 30.0, 29.5, 27.2, 26.3, 25.5, 25.4, 25.1, 24.5, 24.3, 23.2, 18.61, 18.55, 18.4, 18.33, 18.29, 17.6, 15.6, 15.44, 15.38, 14.9, 13.6, 12.4, 12.1, 10.4, 10.2. ESI-MS: m/z 689.8 (M + H)$^+$; 711.6 (M + Na)$^+$; ESI HRMS: m/z calc'd for $[C_{37}H_{64}N_6O_6+H]^+$ 689.4966, found 689.4966.

The following compounds were synthesized in an analogous fashion:



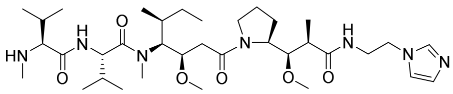
15

Compound **15** (0.963 g, 1.16 mmol) was obtained as the TFA salt in 54% yield. ESI-MS m/z 689.5 (M + H)$^+$, 711.4 (M + Na)$^+$; ESI HRMS m/z calc'd for $[C_{37}H_{64}N_6O_6+H]^+$ 689.4966, found 689.4966.



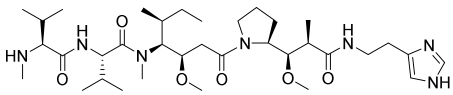
16

Compound **16** (0.111 g, 0.135 mmol) was obtained as the TFA salt in 24% yield. ESI-MS: m/z 678.7 (M + H)$^+$, 339.9 (M + 2H)$^{2+}$; ESI HRMS: m/z calc'd for $[C_{35}H_{63}N_7O_6+H]^+$ 678.4918, found 678.4918.



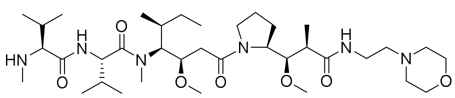
17

Compound **17** (100 mg, 0.122 mmol) was obtained as the TFA salt in 60% yield. ESI-MS: *m/z* 678.5 $(M + H)^+$; ESI HRMS: *m/z* calc'd for $[C_{35}H_{63}N_7O_6+H]^+$ 678.4918, found 678.4918.



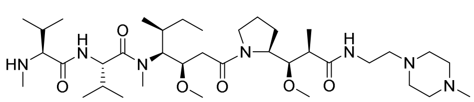
18

Compound **18** (24 mg, 0.029 mmol) was obtained as the TFA salt in 51% yield. ESI-MS: *m/z* 678.7 $(M + H)^+$; 700.6 $(M + Na)^+$, 340.0 $(M + 2H)^{2+}$; ESI HRMS: *m/z* calc'd for $[C_{35}H_{63}N_7O_6+H]^+$ 678.4918, found 678.4918.



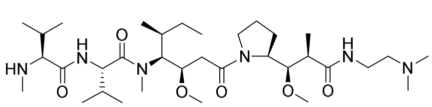
19

Compound **19** (0.246 g, 0.293 mmol) was obtained as the TFA salt in 54% yield. ESI-MS: *m/z* 697.7 $(M + H)^+$, 349.3 $(M + 2H)^{2+}$; ESI HRMS: *m/z* calc'd for $[C_{36}H_{68}N_6O_7+H]^+$ 697.5228, found 697.5228.



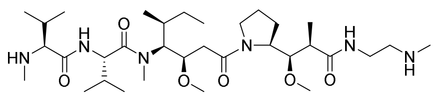
20

Compound **20** (0.268 g, 0.314 mmol) was obtained as the TFA salt in 63% yield. ESI-MS: *m/z* 710.8 $(M + H)^+$, 356.1 $(M + 2H)^{2+}$; ESI HRMS: *m/z* calc'd for $[C_{37}H_{71}N_7O_6+H]^+$ 710.5544, found 710.5544.



21

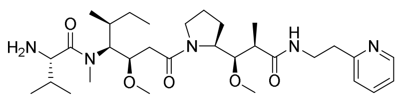
Compound **21** (0.963 g, 1.21 mmol) was obtained as the TFA salt in 62% yield. ESI-MS: *m/z* 655.8 $(M + H)^+$, 328.1 $(M + 2H)^{2+}$; ESI HRMS: *m/z* calc'd for $[C_{34}H_{66}N_6O_6+H]^+$ 655.5122, found 655.5122.



24

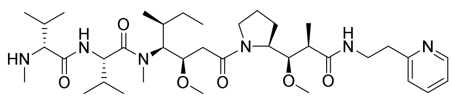
Compound **24** (0.212 g, 0.265 mmol) was obtained as the TFA salt in 62% yield. ESI-MS *m/z* 655.8 $(M + H)^+$, 328.5 $(M + 2H)^{2+}$; ESI HRMS: *m/z* calc'd for $[C_{34}H_{66}N_6O_6+H]^+$ 655.5117, found 655.5122.

The remaining compounds were synthesized with slight variations:



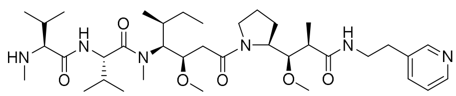
SI-8a

Val-Dil-Dap-2-(2-ethylamino)pyridine•TFA (SI-8a). To a stirred 23 °C suspension of **SI-1a** (0.35 g, 0.67 mmol), Fmoc-Val-Dil-OH **SI-11** (0.353 g, 0.67 mmol) in DMF (5 mL) was added HATU (0.384 g, 1.0 mmol) and DIEA (0.48 mL, 2.7 mmol). After 0.5 h, piperidine (50 uL, 0.51 mmol) was added. After stirring a further 1 h, the mixture was concentrated under reduced pressure, the residue was re-dissolved in CH_2Cl_2 (5 mL) and precipitated in diethyl ether (40 mL). After the solvent was decanted, the precipitate was purified by preparatory HPLC. Val-Dil-Dap-2-(2-ethylamino)pyridine•TFA **SI-8a** (0.296 g, 0.37 mmol) was obtained in 55% yield as a white solid. ESI MS: *m/z* 575.4 (M + H), 227.1 $(M + 2H)^{2+}$.



13

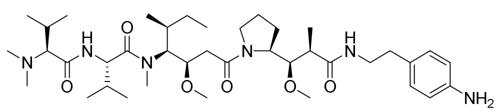
Compound **13**. To a stirred 23 °C mixture of Val-Dil-Dap-2-(2-ethylamino)pyridine•TFA **SI-8a** (0.105 g, 0.17 mmol) and Boc-D-MeVal-OH (0.048 g, 0.21 mmol) in DMF (1 mL) was added HATU (0.098 g, 0.26 mmol), followed by DIEA (0.12 mL, 0.69 mmol). After 0.2 h, the DMF was removed under reduced pressure. The residue was redissolved in CH_2Cl_2 (1 mL) and TFA (1 mL). After stirring a further 0.2 h, the solvent was removed under reduced pressure. The residue was purified by preparatory HPLC to yield compound **13** as the TFA salt (0.042 g, 0.05 mmol) in 31% yield as a white powder. ESI-MS *m/z* 688.5 (M + H), 344.7 $(M + 2H)^{2+}$. HRMS: *m/z* calc'd for $[C_{37}H_{64}N_6O_6+H]^+$ 689.4957, found 689.4967.



14

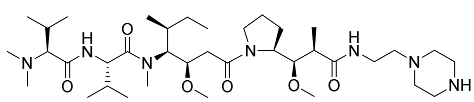
Compound **14**. To a mixture of Fmoc-MeVal-Val-Dil-OH (**SI-17**), HOBt (95 mg, 0.62 mmol) and EDCI (163 mg, 0.92 mmol) was added a solution of Dap-3-(2-ethylamino)pyridine (**SI-1b**) in CH_2Cl_2 (2 mL) and DMF (1 mL), followed by DIPEA (423 µL, 2.43 mmol). After 18 h, the crude solution was concentrated under reduced pressure to remove CH_2Cl_2. The remaining solution was diluted with DMF and was purified by preparatory HPLC.

To a solution of Fmoc-MeVal-Val-Dil-Dap-3-(2-ethylamino)pyridine (**SI-10b**) in EtOAc (10 mL) was added dodecyl mercaptan (220 µL, 0.92 mmol), followed by DBU (18 µL, 0.12 mmol). After 5 h, diethylamine (1.0 mL, 0.97 mmol) was added. After a further 18 h (23 h total), the crude solution was concentrated under reduced pressure. The resulting oil was dissolved in DMF and purified by preparatory HPLC. Compound **14** (160 mg, 0.02 mmol) was obtained as a colourless, glassy solid in 33% yield. ESI-MS: *m/z* 689.4 (M + H), 345.4 $(M + 2H)^{2+}$. ESI HRMS: *m/z* calc'd for $[C_{37}H_{64}N_6O_6+H]^+$ 689.4966, found 689.4957.



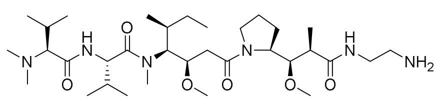
22

Compound **22.** To a stirred room temperature suspension of impure Dov-Val-Dil-Dap-4-(2-aminoethyl)aniline-Fmoc **SI-14j** (1.75 g) in CH_2Cl_2 (5 mL) was added piperidine (2 mL). After 10 h, analysis by LC-MS showed the reaction was complete. The solvent was removed in vacuo and the product was purified by preparatory HPLC. Compound **22** (0.759 g, 0.91 mmol) was obtained as the TFA salt in 47% yield. ESI-MS: *m/z* 717.7 $(M + H)^+$; 740.7 $(M + Na)^+$, 359.5 $(M + 2H)^{2+}$; ESI HRMS: *m/z* calc'd for $[C_{39}H_{68}N_6O_6+H]^+$ 717.5279, found 717.5278.



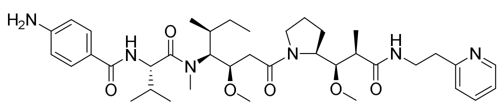
23

Compound **23**. To a stirred room temperature suspension of Dov-Val-Dil-Dap-2-(4-Cbz-piperazin-1-yl)ethanamine•TFA **SI-14m** (29 mg, 0.029 mmol) and ammonium formate (3 mg, 0.051 mmol) in DMF (3 mL) and water (0.5 mL) was added 10% Pd/C (17 mg). After stirring 48 h at 25 °C, the reaction mixture was filtered through a pad of diatomaceous earth and the filtrate was concentrated to give 18 mg of **23** as the formate salt. The product was purified by preparatory HPLC, to yield compound **23**•TFA (0.015 g, 0.018 mmol) in 79% yield. ESI-MS *m/z* 710.8 $(M + H)^+$, 356.1 $(M + 2H)^{2+}$; HRMS *m/z* calc'd for $[C_{37}H_{71}N_7O_6+H]^+$ 710.5544, found 710.5544.



25

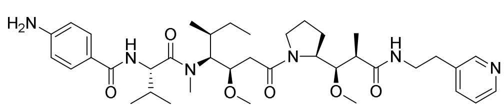
Compound **25.** To a stirred room temperature suspension of Dov-Val-Dil-Dap-ethylenediamine-Fmoc **SI-14l** (1.04 g, 1.20 mmol) in CH_2Cl_2 (5 mL) was added piperidine (2 mL). After 10 h, analysis by LC-MS showed the reaction was complete. The solvent was removed and the product was purified by preparatory HPLC. Compound **25** (0.716 g, 0.82 mmol) was obtained as the TFA salt in 76% yield. ESI HRMS: *m/z* calc'd for $[C_{33}H_{64}N_6O_6+H]^+$ 641.4966, found 641.4966.



26

Compound **26**. To a solution of Fmoc-4-aminobenzyl-Val-Dil-OH (**SI-18**) (0.20 g, 0.30 mmol), Dap-2-(2-ethylamino)pyridine **SI-1a** (0.14 g, 0.35 mmol), HOBt (52 mg, 0.34 mmol) and EDCI (89 mg, 0.50 mmol) in CH_2Cl_2 (2 mL) was added DIPEA (211 µL, 1.2 mmol). After 20 h, the crude mixture was diluted with DMF and purified directly by preparatory HPLC.

To a solution of the crude Fmoc-4-aminobenzyl-Val-Dil-Dap-2-(2-ethylamino)pyridine **SI-9a** in EtOAc (10 mL) was added diethylamine (1.0 mL, 9.22 mmol). After 6 h, the crude mixture was concentrated under reduced pressure and purified by preparatory HPLC. Compound **26** (8.0 mg, 0.009 mmol) was obtained in 3% yield. ESI MS: *m/z* 695.4 (M + H). ESI HRMS: *m/z* calc'd for $[C_{38}H_{58}N_6O_6+H]^+$ 695.4496, found 695.4496.

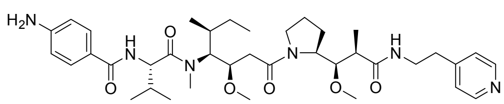


27

Compound **27**. To a mixture of Fmoc-4-aminobenzyl-Val-Dil-OH (**SI-18**) (0.40 g, 0.61 mmol), HOBt (95 mg, 0.62 mmol), and EDCI (0.166 g, 0.93 mmol) was added a solution of Dap-3-(2-ethylamino)pyridine **SI-1b** (0.22 g, 0.60 mmol) in

CH_2Cl_2 (2 mL) and DMF (1 mL). To this mixture was added DIPEA (423 µL, 2.43 mmol). After 18 h, the crude solution was concentrated under reduced pressure to remove CH_2Cl_2. The remaining solution was diluted with DMF and purified directly by preparatory HPLC.

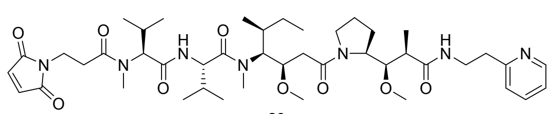
To the resulting Fmoc-4-aminobenzyl-Val-Dil-Dap-3-(2-ethylamino)pyridine **SI-9b** dissolved in EtOAc (10 mL) was added dodecyl mercaptan (197 µL, 0.82 mmol), followed by DBU (18 µL, 0.12 mmol). After 5 h, diethylamine (1.0 mL, 9.67 mmol) was added. After a further 18 h (23 h total), the crude solution was concentrated under reduced pressure. The crude oil was dissolved in DMF and purified directly by preparatory HPLC. After concentration, compound **27** (29.0 mg, 0.03 mmol) was obtained an off-white solid in 5% yield. ESI MS: *m/z* 695.4 (M + H). ESI HRMS *m/z* calc'd for $[C_{38}H_{58}N_6O_6+H]^+$ 695.4496, found 695.4496.



28

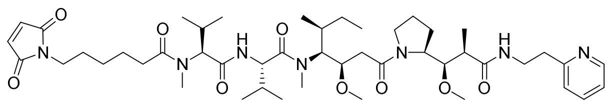
Compound **28**. To a stirred room temperature solution of Boc-4-Abz-Val-Dil-Dap-4-(2-aminoethyl)pyridine **SI-9c** (2.09 g, 2.63 mmol) in CH_2Cl_2 (10 mL) was added TFA (2 mL). After 10 h, analysis by LC-MS showed the reaction was complete. The product was purified by preparatory HPLC. Compound **28** (0.633 g, 0.755 mmol) was obtained as the TFA salt in 29% yield. ESI-MS: *m/z* 695.5 $(M + H)^+$; 717.5 $(M + Na)^+$; ESI HRMS *m/z* calc'd for $[C_{38}H_{58}N_6O_6+H]^+$ 695.4496, found 695.4496.

Drug-Linker Synthesis.



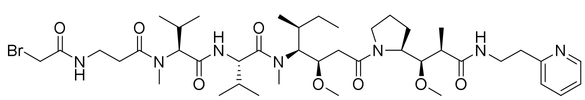
29

Maleimidopropyl-12 (29). To a stirred solution of compound **12**•TFA (0.0247 g, 0.031 mmol) in EtOAc (0.5 mL) was added DIEA (0.02 mL, 0.11 mmol) and the mixture was stirred at 0 °C for 20 min. A further portion of DIEA (0.02 mL, 0.11 mmol) was added, followed by maleimidopropyl hydroxysuccinimide (0.029 g, 0.11 mmol) and the mixture was warmed to 23 °C. After 16 h a further portion of DIEA (0.02 mL, 0.11 mmol) and maleimidopropyl hydroxysuccinimide (0.029 mg, 0.11 mmol) was added. After a further 3 h, the mixture was concentrated in vacuo and the residue was purified directly by preparatory HPLC. Maleimidopropyl-**12** (**29**) (9 mg, 0.009 mmol) was obtained as the TFA salt in 31% yield. ESI MS: *m/z* 840.5 (M + H). (**SDB-1501-71**)



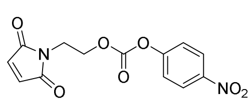
30

Maleimidocaproyl-12 (30). To a stirred solution of compound **12**•TFA (0.024 g, 0.030 mmol) and maleimidohexanoic acid (0.022 g, 0.11 mmol) in EtOAc (0.5 mL) was added DIEA (0.02 mL, 0.11 mmol). The mixture was stirred at 0 °C for 20 min, after which a further portion of DIEA (0.02, 0.11 mmol) was added. CMPI (0.027 g, 0.11 mmol) was added and the mixture was stirred at 23 °C for 16 h. The resulting mixture was concentrated in vacuo and the residue was purified directly by preparatory HPLC. Maleimidocaproyl-**12** (18 mg, 0.018 mmol) was obtained as the TFA salt in 60% yield. ESI MS: *m/z* 882.6 (M + H).



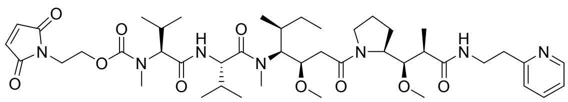
31

Bromoacetamido-(β-Ala)-12 (31). To a stirred solution of compound **12**•TFA (18.8 mg, 0.023 mmol) in DMF (0.4 mL) was added DIEA (15 µL, 0.082 mmol), followed by succinimidyl-3-(bromoacetamide)propionate (25 mg, 0.082 mmol). After the reaction was deemed complete by LC-MS, the crude reaction mixture was purified directly by preparatory HPLC. Bromoacetamido-(β-Ala)-**12** (**31**) (9 mg, 0.009 mmol) was obtained as the TFA salt in 39% yield. ESI MS: *m/z* 880.3, 882.6 (M + H).



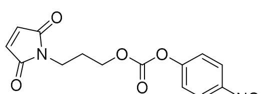
SI-19

Maleimidoethyl-4-nitrophenyl carbamate (SI-19). To a stirred solution of ethanolmaleimide (500 mg, 3.54 mmol) and 4-nitrophenylcarbonate (2.15 g, 7.08 mmol) in DMF (10 mL) was added DIEA (0.92 mL, 5.31 mmol). After 1 h, EtOAc and H_2O were added. The organic layer was concentrated under reduced pressure. The oily residue was triturated with diethyl ether and the precipitate formed was filtered and dried. ^{1}H NMR analysis shows the presence of the product, mixed with 4-nitrophenol. The mixture was used without further purification. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.28 (d, *J* = 9.2 Hz, 2H), 7.43 – 7.37 (m, 2H), 6.78 (s, 2H), 4.44 – 4.38 (m, 2H), 3.97 – 3.93 (m, 2H).



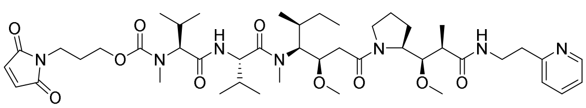
32

Maleimidoethylcarbamyl-12 (32). To a stirred solution of compound **12**•TFA (10 mg, 0.012 mmol) and maleimidoethyl-4-nitrophenyl carbamate **SI-19** (10 mg, 0.014 mmol) in DMF (0.3 mL) was added DIEA (3.3 uL, 0.019 mmol) and HOBt (1.9 mg, 0.014 mmol). After 16 h, the mixture was diluted with DMSO and purified directly by preparatory HPLC. Maleimidoethylcarbamyl-**12** (**32**) (3.4 mg, 0.004 mmol) was obtained in 32% yield. ESI MS: *m/z* 854.8 (M + H), 876.8 (M + Na^+).



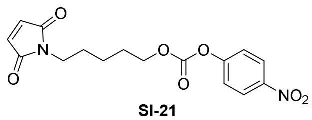
SI-20

Maleimidopropyl-4-nitrophenyl carbamate (SI-20). To a stirred solution of propanolmaleimide (500 mg, 3.22 mmol) and 4-nitrophenyl carbonate (2.0 g, 6.44 mmol) in DMF (10 mL) was added DIEA (0.92 mL, 4.84 mmol). After 1 h, EtOAc and H_2O were added. The organic layer was concentrated under reduced pressure. The oily residue was triturated with diethyl ether and the precipitate formed was filtered and dried. ^{1}H NMR shows the presence of the product mixed with 4-nitrophenol. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.34 – 8.22 (m, 2H), 7.47 – 7.36 (m, 2H), 6.52 (t, J = 0.9 Hz, 2H), 4.26 (t, J = 6.1 Hz, 2H), 3.67 (t, J = 6.6 Hz, 2H), 2.16 – 1.89 (m, 2H).



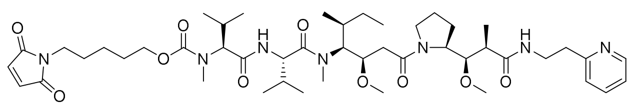
33

Maleimidopropylcarbamyl-12 (33). To a stirred solution of compound **12**•TFA (10 mg, 0.012 mmol), maleimidopropyl-4-nitrophenyl carbamate **SI-20** (25.6 mg, 0.080 mmol) and HOBt (3.4 mg, 0.025 mmol) in DMF (0.1 mL) was added DIEA (5 μL, 0.025 mmol). After 16 h, the reaction was complete. The crude reaction mixture was purified directly by preparatory HPLC. Maleimidopropyl carbamyl-**12** (**33**) (6.8 mg, 0.008 mmol) was obtained in 63% yield. ESI MS: *m/z* 870.8 (M + H), 892.8 (M + Na^+).



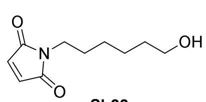
SI-21

Maleimidopentyl-4-nitrophenyl carbonate (SI-21). To a stirred solution of pentanolmaleimide (500 mg, 2.54 mmol) and 4-nitrophenyl carbonate (1.83 g, 6.00 mmol) in DMF (3 mL) was added DIEA (0.78 mL, 4.50 mmol). After 16 h, the reaction mixture was diluted with EtOAc, and washed with sat $NaHCO_3$, H_2O and brine. The crude residue was purified by flash chromatography using CH_2Cl_2:EtOAc. Maleimidopentyl-4-nitrophenyl carbonate **SI-21** (126 mg, 0.36 mmol) was obtained in 13% yield.



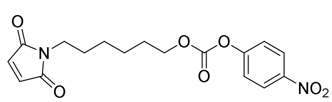
34

Maleimidopentylcarbamyl-12 (34). To a stirred solution of compound **12**•TFA (3.2 mg, 0.004 mmol), maleimidopentyl-4-nitrophenyl carbonate **SI-21** (2.8 mg, 0.008 mmol) and HOBt (1.1 mg, 0.008 mmol) in DMF (0.1 mL) was added DIEA (1.4 µL, 0.008 mmol). After 16 h, the reaction was complete. The crude mixture was diluted with DMSO and purified directly by preparatory HPLC. Maleimidopentylcarbamyl-**12, 34** (2.1 mg, 0.002 mmol) was obtained in 59% yield. ESI MS: *m/z* 898.8 (M + H), 920.7 (M + Na^+).



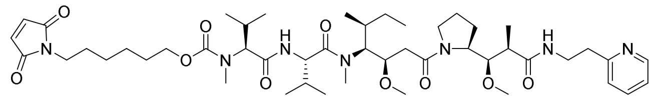
SI-22

Maleimido-1-hexanol (SI-22). Synthesized according to He, F. et al.[3]



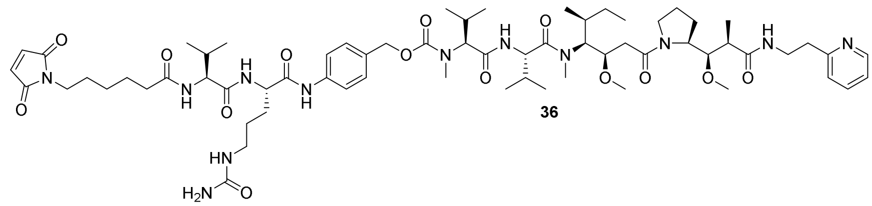
SI-23

Maleimidohexyl-4-nitrophenyl carbonate (SI-23). To a stirred solution of hexanolmaleimide **SI-22** (600 mg, 3.04 mmol) and 4-nitrophenyl carbonate (1.82 g, 6.00 mmol) in DMF (3 mL) was added DIEA (0.78 mL, 4.50 mmol). After 16 h, the mixture was diluted with EtOAc and washed with sat. $NaHCO_3$, H_2O and brine. The crude solid obtained was purified by flash chromatography using CH_2Cl_2:EtOAc. Maleimidohexyl-4-nitrophenyl carbonate **SI-23** (454 mg, 1.25 mmol) was obtained in 41% yield.



35

Maleimidohexylcarbamyl-12 (35). To a stirred solution of compound **12**•TFA (7.2 mg, 0.009 mmol), maleimidohexyl-4-nitrophenyl carbonate **SI-23** (4.7 mg, 0.013 mmol) and HOBt (2.4 mg, 0.018 mmol) in DMF (0.1 mL) was added DIEA (3.1 μL, 0.018 mmol). After 16 h, the reaction mixture was purified directly by preparatory HPLC using neutral H_2O (without TFA)/MeCN as the mobile phase. Maleimidohexylcarbamyl-**12, 35** (5.2 mg, 0.006 mmol) was obtained in 64% yield. ESI MS: *m/z* 912.6 (M + H), 934.8 ($M + Na^+$).



36

MC-Val-Cit-Paba-12 (36). To a stirred solution of compound **12**•TFA (23 mg, 0.03 mmol), MC-Val-Cit-Paba-PNP (Purchased from Concortis) (46.70 mg, 0.06 mmol) and in DMF (0.2 mL) was added DIEA (23 μL, 0.13 mmol). After 16 h, the reaction mixture was purified directly by preparatory HPLC. Mc-Val-Cit-Paba-**12** (17.3 mg, 0.01 mmol) was obtained in 44% yield as the TFA salt. ESI MS: *m/z* 1287.9 (M + H), 644.7 $(M + 2H)^{2+}$

Hydrophobic Interaction Chromatography (HIC) was performed to separate conjugated antibody species on the basis of drug load. A TSKGel Butyl NPR 4.6 ID x 3.5cm, 2.5 μm column (Tosoh®) was used with 1.5 M ammonium sulfate in 25 mM potassium phosphate pH 7.0 (mobile phase A) and 25 mM potassium phosphate pH 7.0 containing 25% isopropanol (mobile phase B) run at a flow rate of 0.8 mL/min over a 12 minute linear gradient with UV monitoring which is following precisely the procedure in the literature.[4] The retention time of trastuzumab showed very similar retention time.

Biological Testing Data

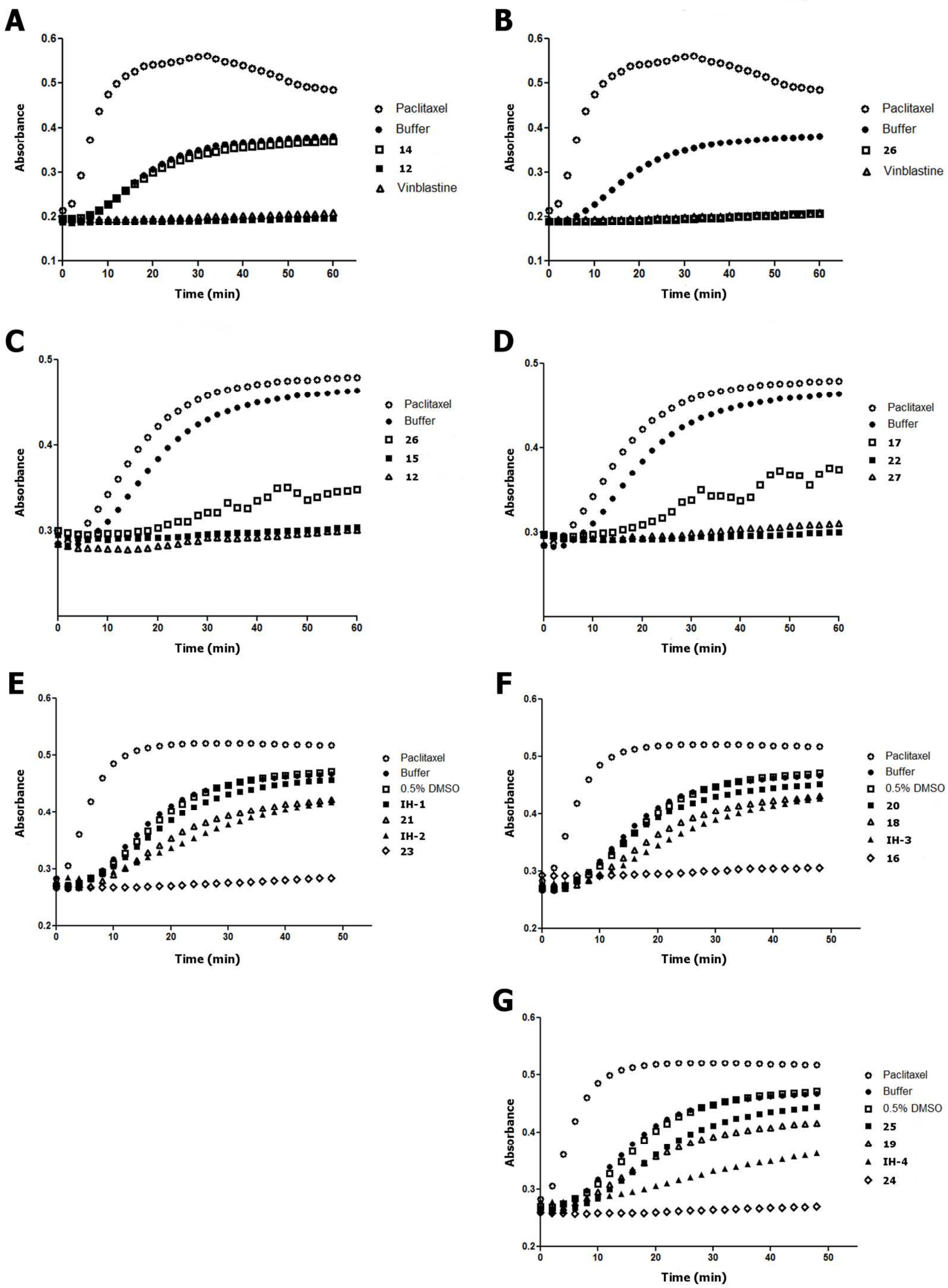


Figure SI1. Tubulin polymerization curves, at 10 μM of payload. **IH-** denotes In-House comparator.

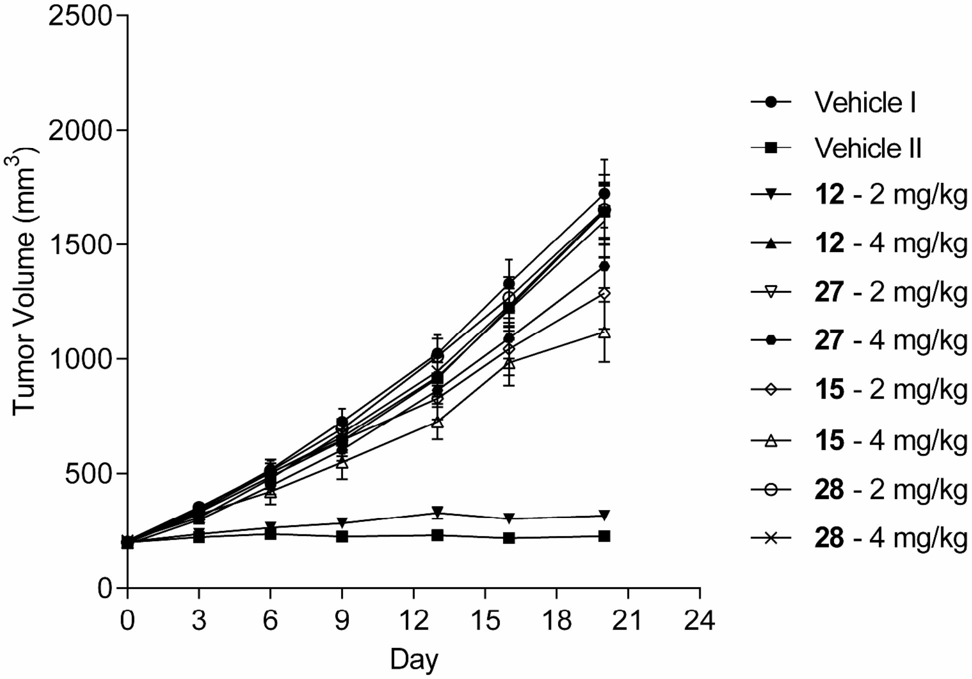


Figure SI2. Efficacy of Pyridine-containing Free Drugs (corresponds to Figure 4A in the main text).

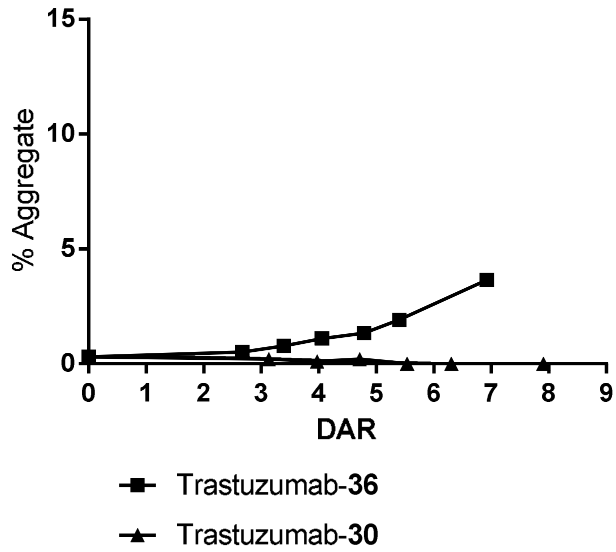


Figure SI3. Soluble Aggregate vs. DAR for Trastuzumab (IgG1) ADCs.

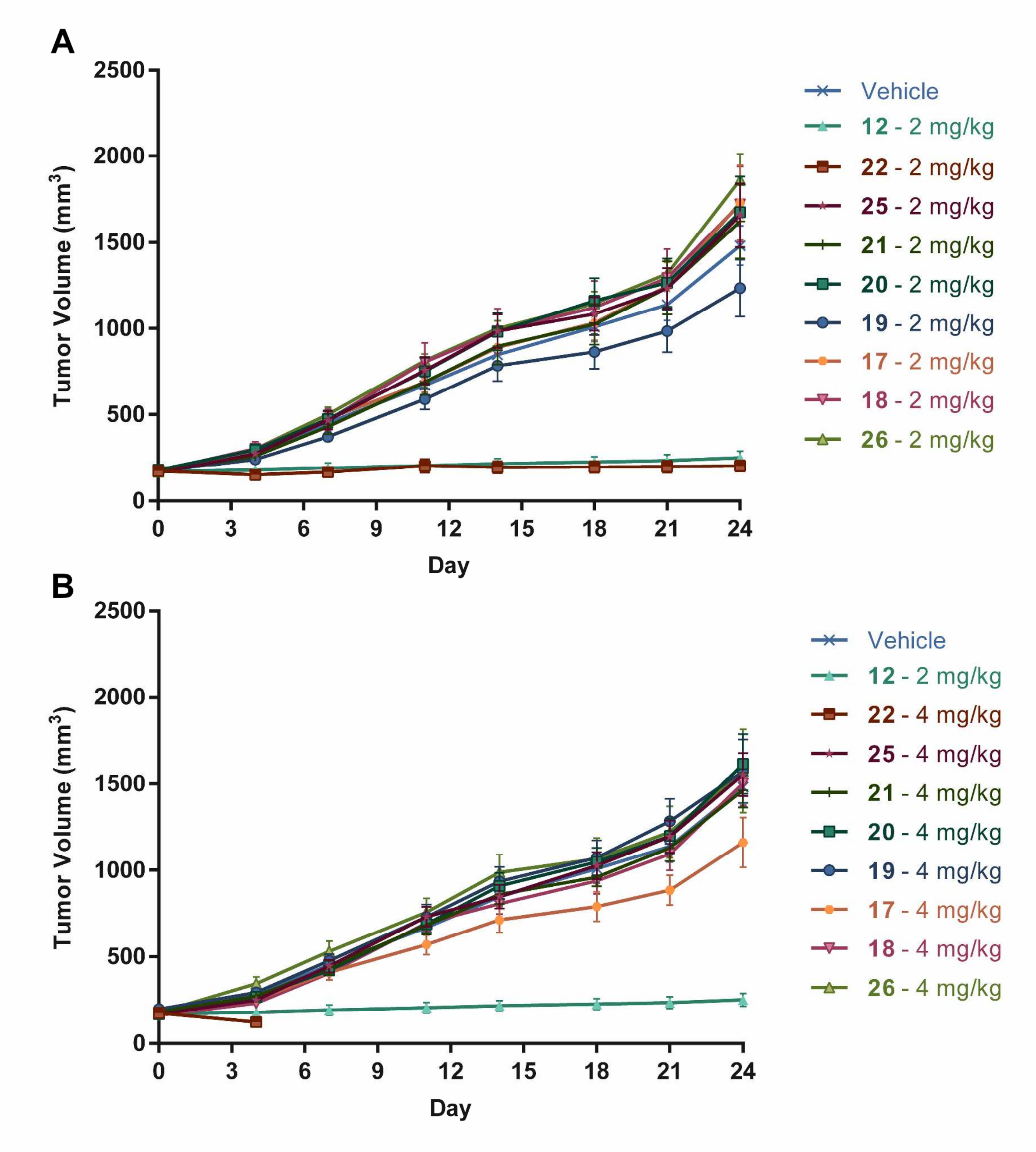


Figure SI4. Plot of Efficacy of Free Drugs at both 2 mg/kg and 4 mg/kg (corresponds to main text Figure 4B). Male ICR/SCID mice bearing subcutaneously implanted human bladder cancer SW780 xenografts were dosed intravenously at either 2 or 4 mg/kg. Groups dosed at 2 mg/kg (A) and 4 mg/kg (B) are presented separately for clarity. Vehicle: 20 mM Histidine pH 6.0/5% sucrose with 9% DMSO. Doses were given on days 0, 6 and 13. Each group consisted of 8 animals. The group treated with compound **22** at 4 mg/kg was terminated early due to animal weight loss. The mean tumor volume in each group was plotted over time with the standard error.

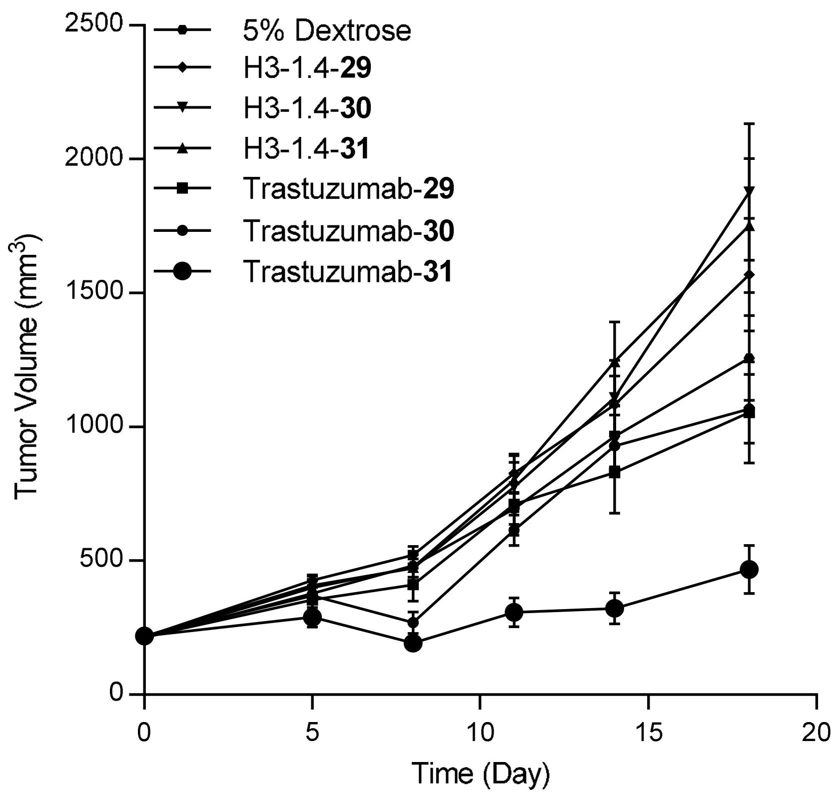


Figure SI5. In vivo efficacy of non-cleavable ADCs (corresponds to main text Figure 7A). Female ICR/SCID mice bearing size-matched HCC1954 human breast cancer xenograft of 200 mm^3 in volume were dosed intravenously at 10 mg/kg as a single dose on day 0. Each group consisted of 10 animals. The mean tumor volume is plotted overt time with the standard error.

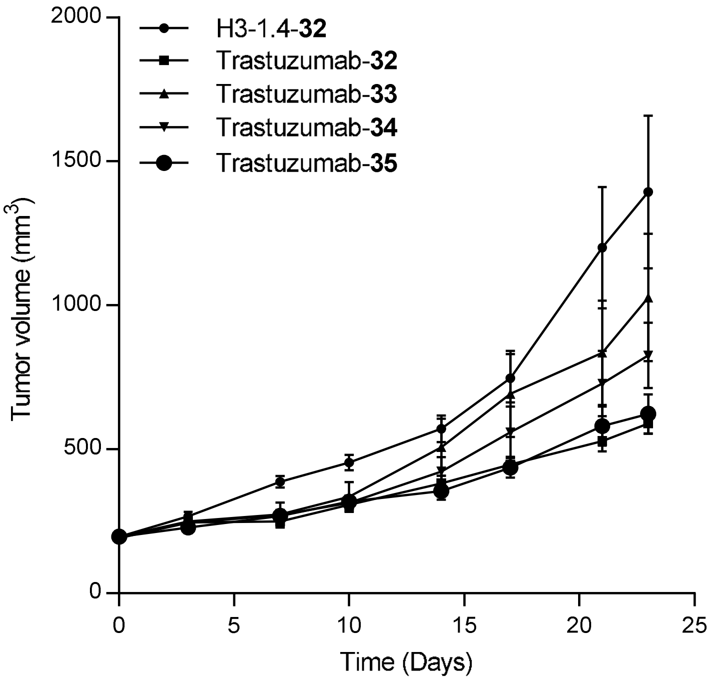
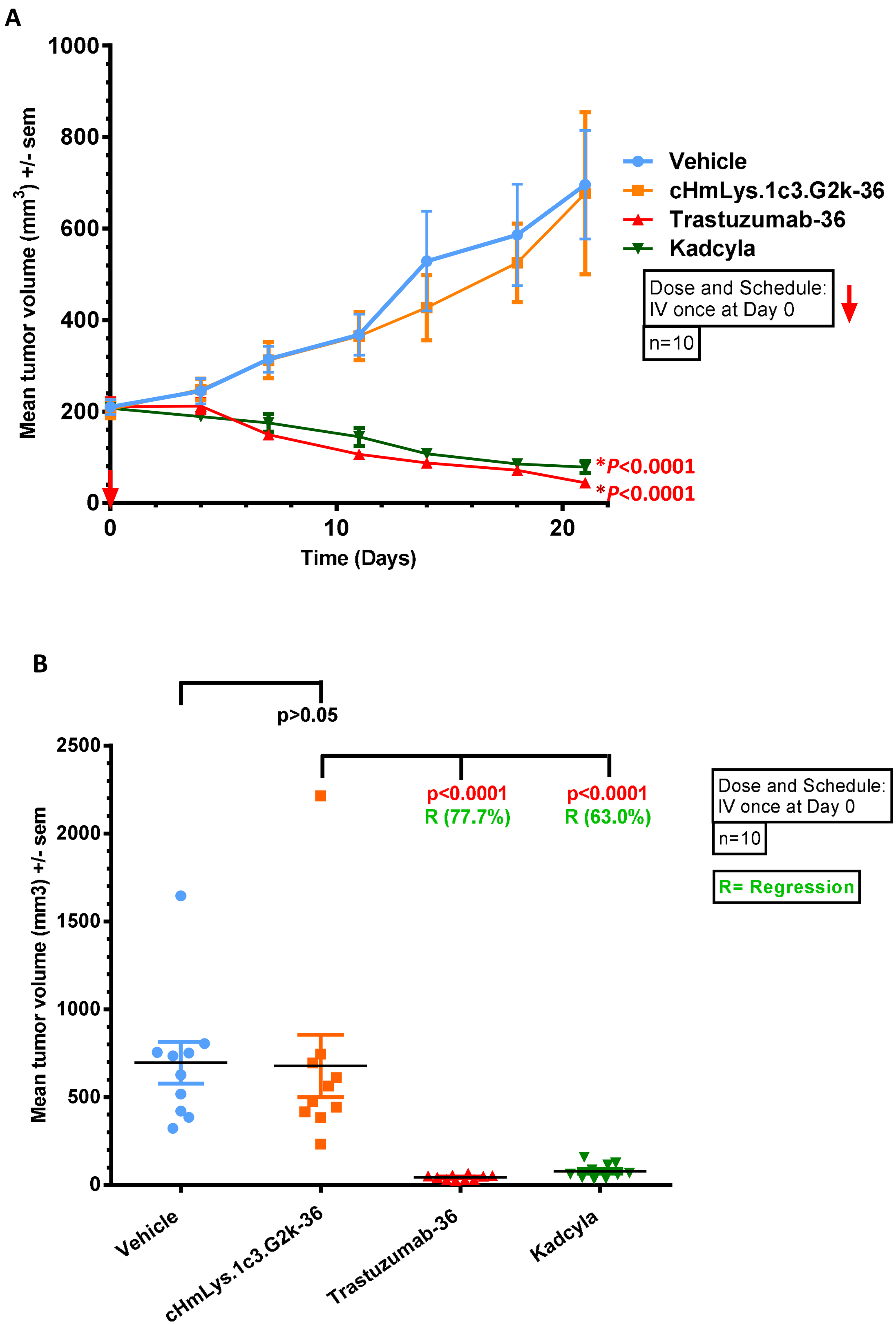


Figure SI6. In vivo efficacy of carbamate ADCs (corresponds to Figure 7B main text). Female ICR/SCID mice bearing size-matched HCC1954 human breast cancer xenograft of 200 mm^3 in volume were dosed intravenously at 10 mg/kg as a single dose on day 0. Each group consisted of 6 animals. The mean tumor volume is plotted over time with the standard error.



A
Mean tumor volume (mm3) +/- sem
1000
800
600
400
200
0
0
10
20
Time (Days)
Vehicle
cHmLys.1c3.G2k-36
Trastuzumab-36
Kadcyla
Dose and Schedule:
IV once at Day 0
n=10
*P<0.0001
*P<0.0001
B
p>0.05
Mean tumor volume (mm3) +/- sem
2500
2000
1500
1000
500
0
p<0.0001
R (77.7%)
p<0.0001
R (63.0%)
Dose and Schedule:
IV once at Day 0
n=10
R= Regression
Vehicle
cHmLys.1c3.G2k-36
Trastuzumab-36
Kadcyla

Figure SI7. In vivo efficacy of a cleavable ADC corresponds to Figure 8 in main text. Human breast carcinoma HCC1954 cells (3 x 10^6 cells per mouse) were implanted into the mammary fatpad of female CB17/SCID mice. Treatment with Trastuzumab-**36** or Kadcyla™ at 5 mg/kg as a single dose was started when tumors reached 200 mm^3 (n=10). cHmLys.1c3.G2k-**36** is the non-binding Control ADC and the Vehicle is 20 mM Histidine / 5% Trehalose, pH 5.2. **A.** Graph shows mean tumor volume over time with standard error for each cohort. **B.** Individual tumor volumes on day 21 in each cohort with mean and standard error.

Compound Characterization Data:

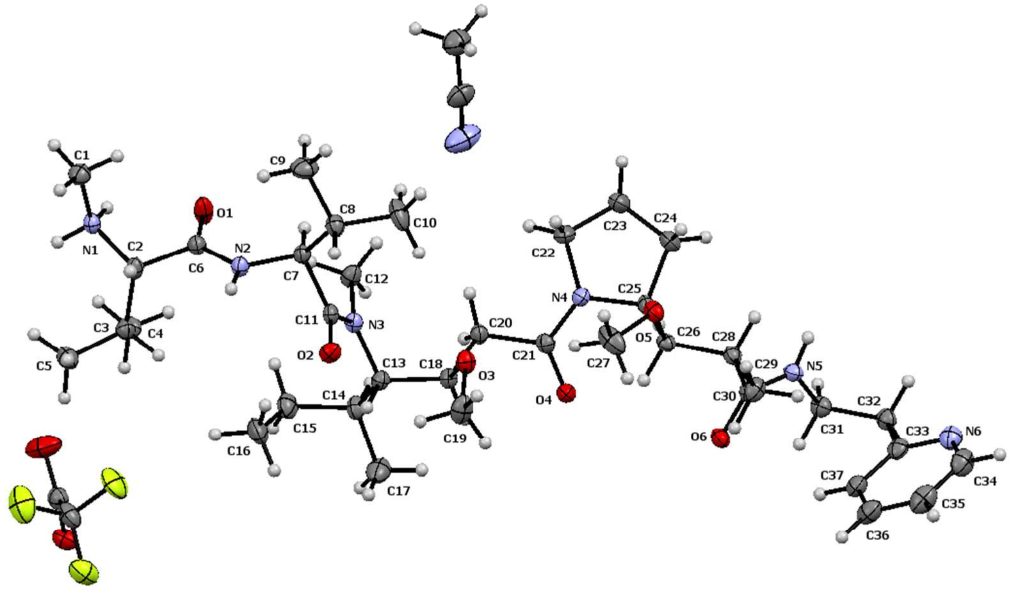


Figure SI8. ORTEP Drawing of **12** with 50% thermal ellipsoids. Crystals were grown in acetonitrile. The structure was refined as a two-component twin.

Table SI1. Summary of Crystallographic Information

Formula	$C_{37}H_{65}N_6O_6$, $C_2F_3O_2$, C_2H_3N
Formula weight	688.96, 114.02, 41.05
Crystal system	monoclinic
Space group	P 2_1
Color of crystal	colorless
Cell Lengths (Å)	**a** 9.4629(4) **b** 15.2084(7) **c** 15.6029(6)
Cell Angles (°)	**α** 90 **β** 93.466(3) **γ** 90
Cell Volume ($Å^3$)	2241.39
Z, Z’	**Z**: 2 **Z’**: 0
Temperature of Data Collection (K)	100 (2)
Reflections Collected	18345
R-Factor (%)	3.12
Goodness of Fit	1.048

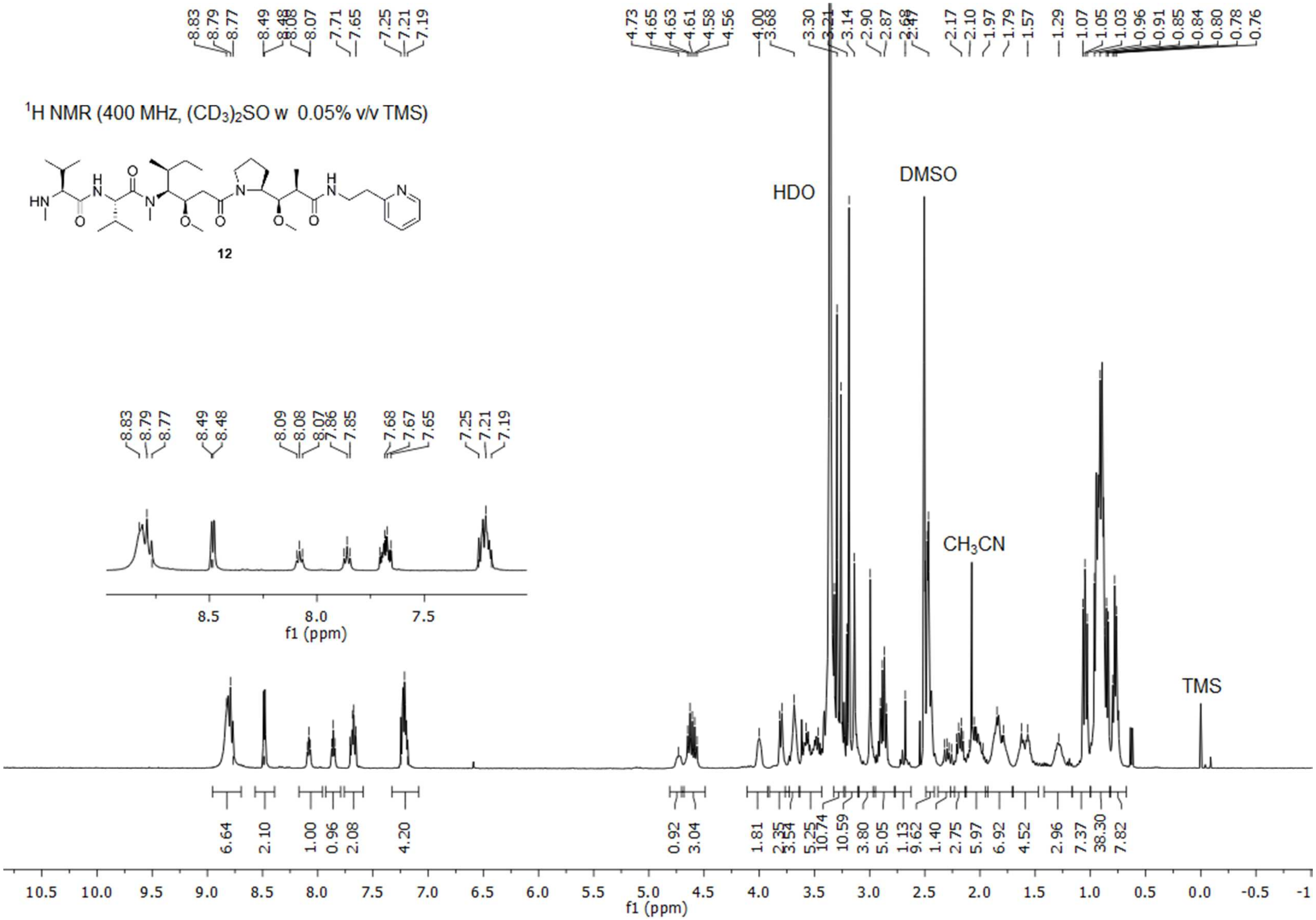


Figure SI9. ^{1}H NMR of compound **12** (full spectrum)

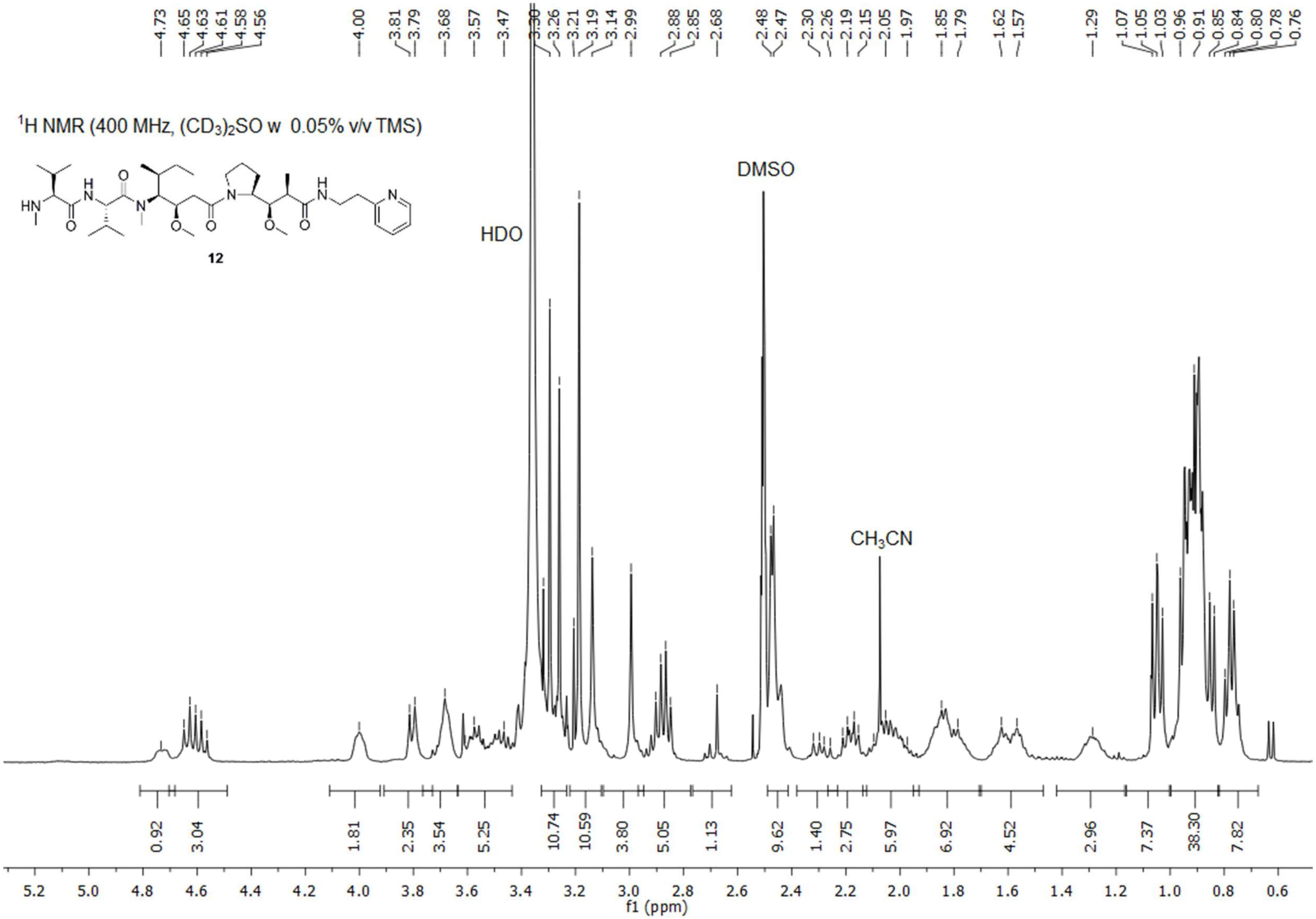


Figure SI10. ^{1}H NMR for compound **12** (aliphatic region expansion)

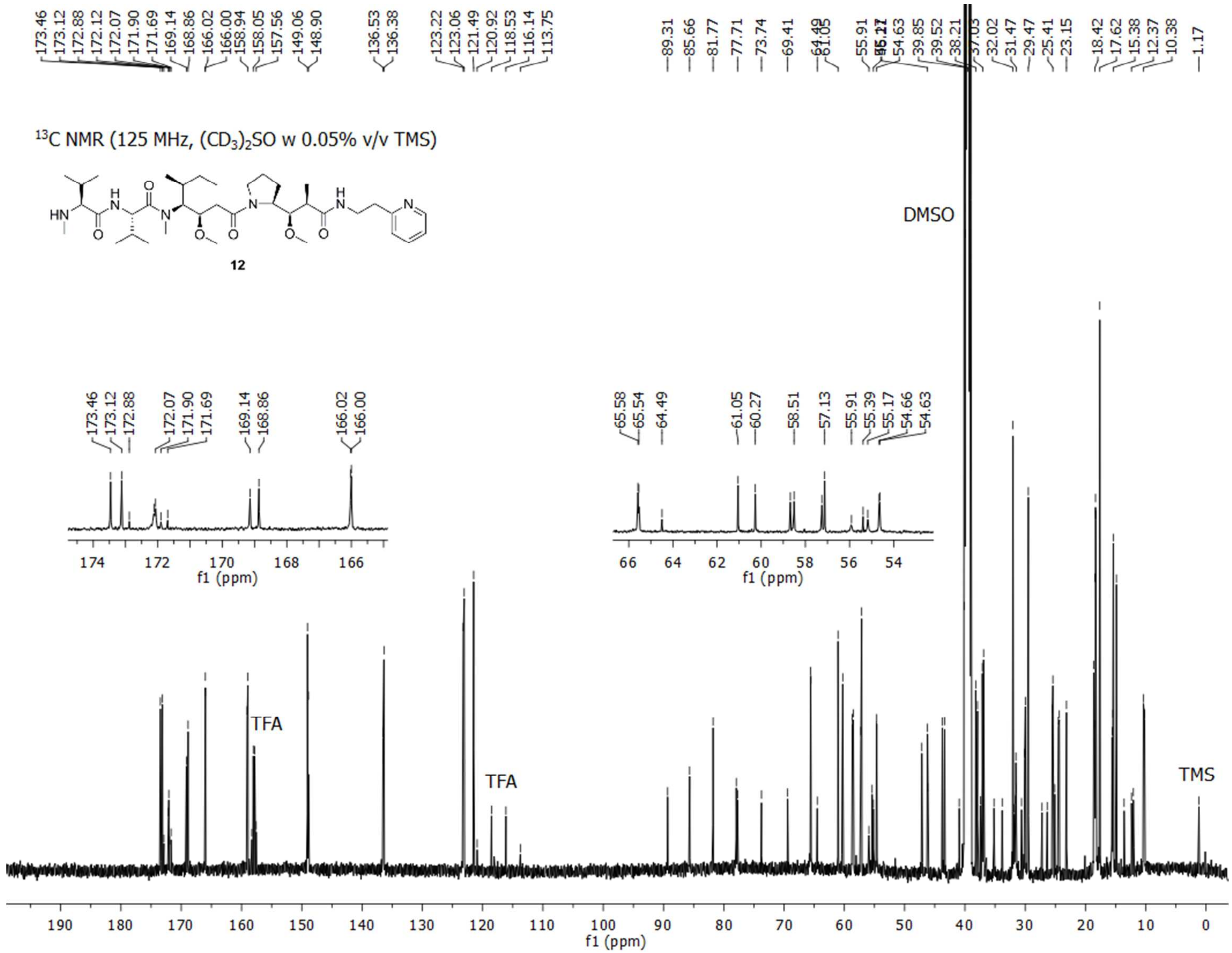


Figure SI11. ^{13}C NMR for compound **12**

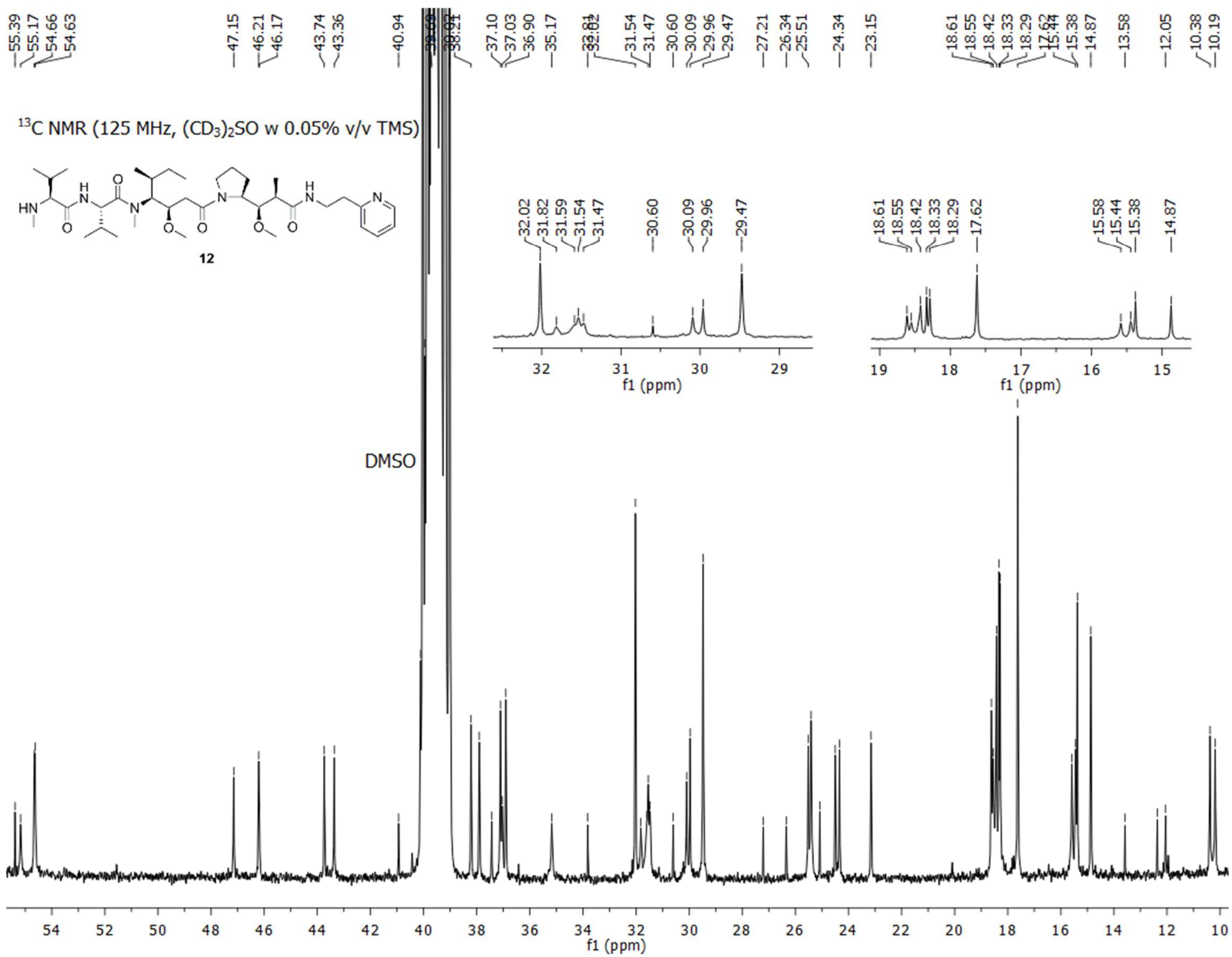


Figure SI12. ^{13}C NMR of compound **12** (aliphatic region expansion)

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