

# Supporting Information

## Nicotinamide Phosphoribosyltransferase Inhibitor as a Novel Payload for Antibody-Drug Conjugates

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- 1. EXPERIMENTAL PROCEDURES FOR THE SYNTHESIS OF PAYLOADS AND LINKER-PAYLOADS**

All reagents and solvents were purchased from commercial suppliers and used without further purification or were prepared according to published procedures. Unless otherwise noted, all reactions were performed under inert conditions. <sup>1</sup>H NMR spectra were recorded on a Bruker

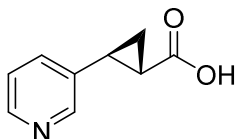
400 MHz or a Bruker 600 MHz NMR spectrometer. Chemical shifts are reported in parts per million (ppm) relative to an internal solvent reference. Significant peaks are tabulated in the order multiplicity (s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad), coupling constants (Hz), and number of protons. Final compounds were purified to  $\geq 95\%$  purity as assessed by analytical UPLC-MS with the following methods.

*Method A:* Waters Acquity UPLC with Waters SQ detector; Column: Acquity HSS T3 1.8  $\mu\text{m}$  2.1 x 50 mm, (flow: 1 ml/min, column temperature: 60  $^{\circ}\text{C}$ , solvent A: water + 0.05% formic acid + 3.75 mM ammonium acetate, solvent B: acetonitrile + 0.04% formic acid, gradient: from 5 to 98% B in 1.4 min)

*Method B:* Waters Acquity UPLC with Waters SQ detector; Column: Acquity HSS T3 1.8  $\mu\text{m}$  2.1 x 50 mm, (flow: 1 ml/min, column temperature: 60  $^{\circ}\text{C}$ , solvent A: water + 0.05% formic acid + 3.75 mM ammonium acetate, solvent B: acetonitrile + 0.04% formic acid, gradient: from 5 to 98% B in 9.4 min)

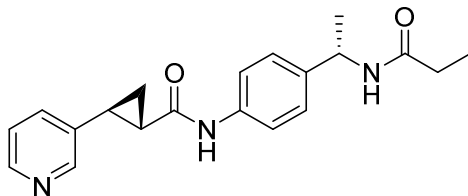
Mass spectrometry results are reported as the ratio of mass over charge. Silica gel chromatography was performed using a Combi Flash Rf 200 system (Teledyne ISCO) and prepacked silica gel columns. Preparative reverse phase purification was performed using Waters Autopurification-MS system. Supercritical fluid chromatography SFC purification was performed using Waters Preparative SFC-100-MS system.

**(1S,2S)-2-(Pyridin-3-yl)cyclopropanecarboxylic acid**



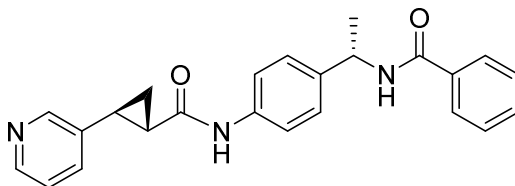
Prepared as previously described.<sup>1</sup>

**(1S,2S)-N-(4-((S)-1-Propionamidoethyl)phenyl)-2-(pyridin-3-yl)cyclopropane-1-carboxamide**



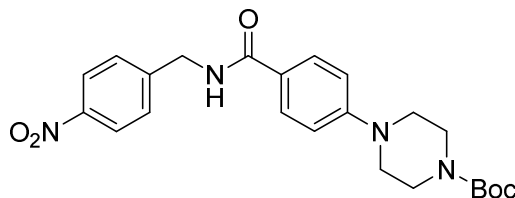
Prepared as previously described.<sup>1</sup>

**N-((S)-1-(4-((1S,2S)-2-(Pyridin-3-yl)cyclopropane-1-carboxamido)phenyl)ethyl)benzamide**



Prepared as previously described.<sup>1</sup>

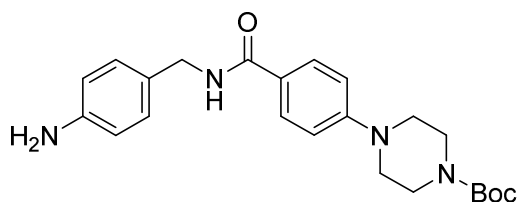
**tert-Butyl 4-(4-((4-nitrobenzyl)carbamoyl)phenyl)piperazine-1-carboxylate**



To a solution of 4-(4-(tert-butoxycarbonyl)piperazin-1-yl)benzoic acid (2 g, 6.53 mmol) in DMF (Volume: 36.3 mL) was added (4-nitrophenyl)methanamine (1.231 g, 6.53 mmol), DIPEA (4.56

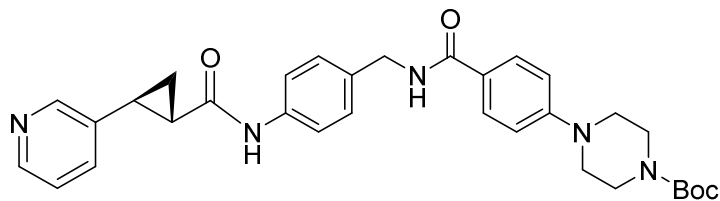
mL, 26.1 mmol), and HATU (2.98 g, 7.83 mmol). The reaction mixture was stirred at room temperature for 6 h, concentrated *in-vacuo* and absorbed onto Isolute. Purification by flash chromatography (40 g, silica gel) eluting with heptane/EA afforded the product (996 mg, 1.696 mmol, 26% yield, about 75% pure, taken to the next step without further purification). UPLC-MS [M+H]<sup>+</sup> Calcd for C<sub>23</sub>H<sub>29</sub>N<sub>4</sub>O<sub>5</sub> 441.2; Found 441.3, R<sub>t</sub> = 1.14 min (method A).

**tert-Butyl 4-(4-((4-aminobenzyl)carbamoyl)phenyl)piperazine-1-carboxylate**



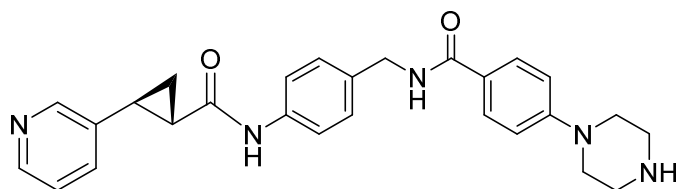
An ice-cooled solution of 4-(4-((4-nitrobenzyl)carbamoyl)phenyl)piperazine-1-carboxylate (996 mg, 1.696 mmol, 75% pure) in MeOH (Volume: 11.3 mL) and EA (Volume: 11.3 mL) was purged with argon, and Pd/C (10 wt% on activated carbon) (108 mg, 0.102 mmol) added. The argon atmosphere was then exchanged for hydrogen. The reaction mixture was stirred at room temperature for 5 h and filtered through a pad of celite. The solvents were removed *in-vacuo* to afford the crude product (761 mg, 1.613 mmol, 71% yield, 87% pure, taken to the next step without further purification) UPLC-MS [M+H]<sup>+</sup> Calcd for C<sub>23</sub>H<sub>31</sub>N<sub>4</sub>O<sub>3</sub> 411.2; Found 411.2, R<sub>t</sub> = 0.92 min (method A).

**tert-Butyl 4-(4-((4-((1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxamido)benzyl)carbamoyl)phenyl)piperazine-1-carboxylate**



To a solution of (1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxylic acid hydrochloride (170 mg, 0.853 mmol) in DMF (Volume: 5684  $\mu$ l) was added HATU (421 mg, 1,108 mmol), DIPEA (596  $\mu$ l, 3.41 mmol), and tert-butyl 4-(4-((4-aminobenzyl)carbamoyl)phenyl)piperazine-1-carboxylate (315 mg, 0.742 mmol). The reaction mixture was stirred at room temperature for 1 h, concentrated *in-vacuo* and absorbed onto Isolute. Purification by flash chromatography (24 g, silica gel) eluting with DCM/(DCM:MeOH 4:1) afforded the target compound (217 mg, 0.371 mmol, 44% yield, 95% pure). UPLC-MS  $[M+H]^+$  Calcd for  $C_{32}H_{38}N_5O_4$  556.3; Found 556.2,  $R_t$  = 0.96 min (method A).

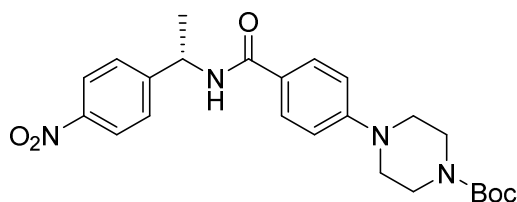
**4-(Piperazin-1-yl)-N-(4-((1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxamido)benzyl)benzamide (compound 5)**



To a solution of tert-butyl 4-(4-((4-((1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxamido)benzyl)carbamoyl)phenyl)piperazine-1-carboxylate (90 mg, 0.162 mmol) in ACN (Volume: 1.3 mL) was added TFA (0.936 mL, 12.15 mmol). The reaction mixture was stirred at room temperature for 4 h. The target product **5** was obtained after purification by reverse phase chromatography eluting with ACN/water (0.1% TFA) (66 mg,

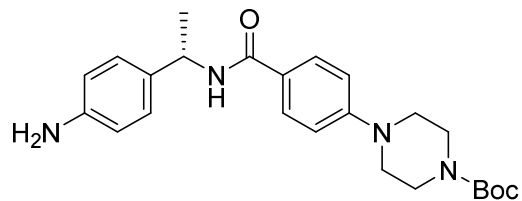
0.115 mmol, 71% yield, 99% pure, TFA salt) as a colorless solid. <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>) δ ppm 10.33 (s, 1H), 8.97 (s, 2H), 8.80 (t, *J* = 5.9 Hz, 1H), 8.72 (s, 1H), 8.61 (d, *J* = 4.7 Hz, 1H), 7.97 (d, *J* = 7.9 Hz, 1H), 7.83 (d, *J* = 8.8 Hz, 2H), 7.72 – 7.61 (m, 1H), 7.55 (d, *J* = 8.4 Hz, 2H), 7.24 (d, *J* = 8.4 Hz, 2H), 7.04 (d, *J* = 8.9 Hz, 2H), 4.41 (d, *J* = 5.8 Hz, 2H), 3.57 – 3.43 (m, 4H), 3.25 (s, 4H), 2.58 – 2.39 (m, 1H), 2.23 (m, 1H), 1.63 – 1.48 (m, 2H). UPLC-MS [M+H]<sup>+</sup> Calcd for C<sub>27</sub>H<sub>30</sub>N<sub>5</sub>O<sub>2</sub> 456.2; Found 456.3, R<sub>t</sub> = 0.46 min (Method A).

**(S)-tert-Butyl 4-(4-((1-(4-nitrophenyl)ethyl)carbamoyl)phenyl)piperazine-1-carboxylate**



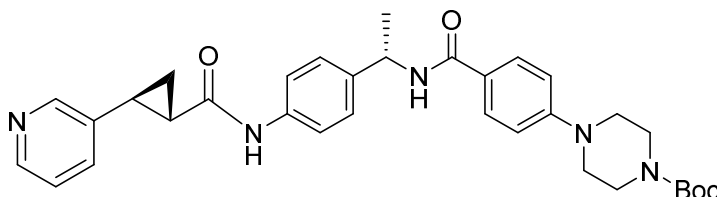
To a solution of (4-(tert-butoxycarbonyl)piperazin-1-yl)benzoic acid (454 mg, 1.480 mmol) in DMF (Volume: 10 ml) was added (S)-1-(4-nitrophenyl)ethanamine hydrochloride (250 mg, 1.234 mmol), DIPEA (1.077 ml, 6.17 mmol), and HATU (610 mg, 1.604 mmol). The reaction mixture was stirred at room temperature for 1 h, diluted with ethyl acetate and washed with HCl (1M), NaHCO<sub>3</sub> (sat.), and brine. After extraction, the organics were combined, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, concentrated *in-vacuo* and absorbed onto Isolute. Purification by flash chromatography (40 g, silica gel) eluting with heptane/EA furnished the target product (580 mg, 1.148 mmol, 93% yield, 90% pure) as a yellow oil. UPLC-MS [M+H]<sup>+</sup> Calcd for C<sub>24</sub>H<sub>31</sub>N<sub>4</sub>O<sub>5</sub> 455.2; Found 455.2, R<sub>t</sub> = 1.13 min (method A).

**(S)-tert-Butyl 4-(4-((1-(4-aminophenyl)ethyl)carbamoyl)phenyl)piperazine-1-carboxylate**



A solution of (S)-tert-butyl 4-(4-((1-(4-nitrophenyl)ethyl)carbamoyl)phenyl)piperazine-1-carboxylate (580 mg, 1.238 mmol) in MeOH (Volume: 15 ml) and EA (Volume: 15.00 ml) was purged with argon, and then ammonium formate (1561 mg, 24.76 mmol) and Pd/C (5 wt% on activated carbon) (263 mg, 0.124 mmol) added. The resulting black suspension was stirred under an argon atmosphere at 55 °C for 1 h. The reaction mixture was filtered through a short pad of celite. The filter cake was then washed with MeOH/EA (1:1) (4x50ml). The filtrate was concentrated *in-vacuo* and the crude product partitioned between water and EA. After extraction, the organics were combined, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated *in-vacuo* to give the target product (520 mg, 1.225 mmol, 99% yield, 95% pure) as a beige oil which was used without further purification. UPLC-MS [M+H]<sup>+</sup> Calcd for C<sub>24</sub>H<sub>33</sub>N<sub>4</sub>O<sub>3</sub> 425.3; Found 425.3, R<sub>t</sub> = 0.97 min (method A).

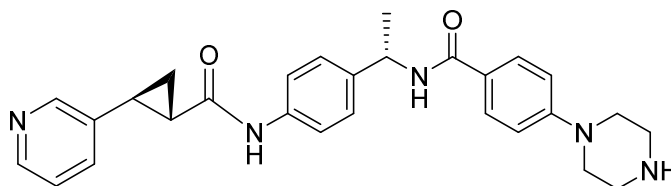
**tert-Butyl 4-(4-(((S)-1-(4-((1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxamido)phenyl)ethyl)carbamoyl)phenyl)piperazine-1-carboxylate**





To a solution of (1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxylic acid hydrochloride (93 mg, 0.466 mmol) in DMF (Volume: 5 ml) was added (S)-tert-butyl 4-(4-((1-(4-aminophenyl)ethyl)carbamoyl)phenyl)piperazine-1-carboxylate (180 mg, 0.424 mmol), DIPEA (0.296 ml, 1.696 mmol), and HATU (210 mg, 0.551 mmol). The reaction mixture was stirred at room temperature for 1 h, diluted with DCM and washed with brine. After extraction, the organics were combined, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, concentrated *in-vacuo* and absorbed onto Isolute. Purification by flash chromatography (24 g, silica gel) eluting with DCM/ (DCM:MeOH 4:1) furnished the product (300 mg, 0.421 mmol, 99% yield, 80% pure) as a colorless solid. UPLC-MS [M+H]<sup>+</sup> Calcd for C<sub>33</sub>H<sub>40</sub>N<sub>5</sub>O<sub>4</sub> 570.3; Found 570.5, R<sub>t</sub> = 1.01 min (method A).

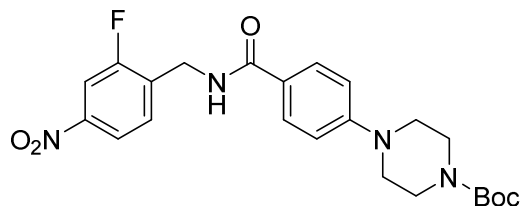
**4-(Piperazin-1-yl)-N-((S)-1-(4-((1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxamido)phenyl)ethyl)benzamide (compound 3)**



To a solution of tert-butyl 4-(4-(((S)-1-(4-((1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxamido)phenyl)ethyl)carbamoyl)phenyl)piperazine-1-carboxylate (34 mg, 0.048 mmol, 80% pure) in ACN (Volume: 1.5 mL) was added HCl (6M) (0.398 mL, 2.387 mmol). The reaction mixture was stirred at room temperature for 4 h. The product **3** was obtained after purification by reverse phase chromatography eluting with ACN/water (0.1% TFA) as a colorless solid (18 mg, 0.026 mmol, 54% yield, 99% pure, TFA salt). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ ppm 10.24 (s, 1H), 8.73 (br s, 2H), 8.59 (s, 1H), 8.50 (d, *J* = 8.1 Hz, 2H), 7.87 – 7.80 (m, 2H), 7.74 – 7.69 (m, 1H), 7.56 – 7.49 (m, 2H), 7.50 – 7.43 (m, 1H), 7.34 – 7.27

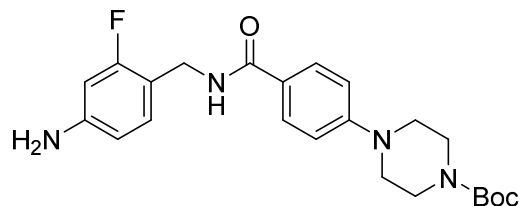
(m, 2H), 7.12 – 6.99 (m, 2H), 5.19 – 5.06 (m, 1H), 3.48 – 3.43 (m, 4H), 3.29 – 3.19 (m, 4H), 2.47 – 2.41 (m, 1H), 2.20 – 2.11 (m, 1H), 1.60 – 1.42 (m, 5H). UPLC-MS  $[M+H]^+$  Calcd for  $C_{28}H_{32}N_5O_2$  470.3; Found 470.2,  $R_t = 1.40$  min (method B).

**tert-Butyl 4-(4-((2-fluoro-4-nitrobenzyl)carbamoyl)phenyl)piperazine-1-carboxylate**



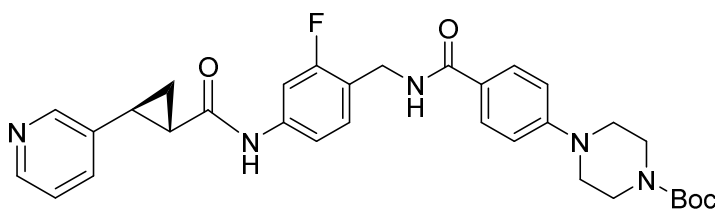
To a solution of 4-(4-(tert-butoxycarbonyl)piperazin-1-yl)benzoic acid (1.48 g, 4.84 mmol) in DMF (Volume: 32.3 ml) was added DIPEA (4.23 ml, 24.20 mmol), HATU (2.393 g, 6.29 mmol), and (2-fluoro-4-nitrophenyl)methanamine hydrochloride (1.00 g, 4.84 mmol). The reaction mixture was stirred at room temperature for 48 h, diluted with EA and washed with HCl (1M),  $NaHCO_3$  (sat.), and brine. After extraction, the organics were combined, dried over  $Na_2SO_4$ , filtered and concentrated *in-vacuo* to afford the crude product (3.6 g, 97% yield, about 60% pure, taken to the next step without further purification) as a yellow oil. UPLC-MS  $[M+H]^+$  Calcd for  $C_{23}H_{28}FN_4O_5$  459.2; Found 459.2,  $R_t = 1.13$  min (method A).

**tert-Butyl 4-(4-((4-amino-2-fluorobenzyl)carbamoyl)phenyl)piperazine-1-carboxylate**



A solution of tert-butyl 4-(4-((2-fluoro-4-nitrobenzyl)carbamoyl)phenyl)piperazine-1-carboxylate (3.6 g, 4.71 mmol, 60% pure) in MeOH (Volume: 100 ml) was purged with argon, and then ammonium formate (5.94 g, 94 mmol) and Pd/C (5 wt% on activated carbon) (0.501 g, 0.236 mmol) added. The resulting black suspension was stirred under an argon atmosphere at 55 °C for 2.5 h. The reaction mixture was filtered through a short pad of celite. The filter cake was then washed with methanol/EA (1:1) (4x50ml). The filtrate was concentrated *in-vacuo* and the crude product partitioned between water and ethyl acetate. After extraction, the organics were washed with sat. brine (3\*50mL), dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated *in-vacuo* to give the crude product as a yellow foam (1.94 g, 83% yield, 80% pure, taken to the next step without further purification). UPLC-MS [M+H]<sup>+</sup> Calcd for C<sub>23</sub>H<sub>30</sub>FN<sub>4</sub>O<sub>3</sub> 429.2; Found 429.3, R<sub>t</sub> = 1.00 min (method A).

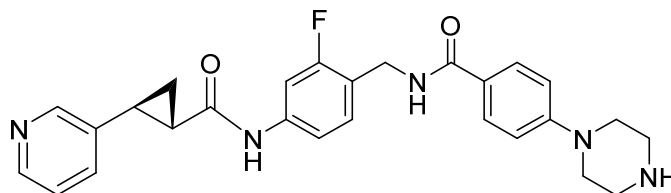
**tert-Butyl 4-(4-((2-fluoro-4-((1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxamido)benzyl)carbamoyl)phenyl)piperazine-1-carboxylate**



To a solution of (1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxylic acid hydrochloride (128 mg, 0.639 mmol) in DMF (Volume: 5 mL) was added HATU (287 mg, 0.755 mmol). After stirring at RT for 5 min, DIPEA (0.406 mL, 2.324 mmol) and tert-butyl 4-(4-((4-amino-2-fluorobenzyl)carbamoyl)phenyl)piperazine-1-carboxylate (300 mg, 0.560 mmol, 80% pure) were added and stirring continued for 2 h. The reaction mixture was then diluted with EA and washed

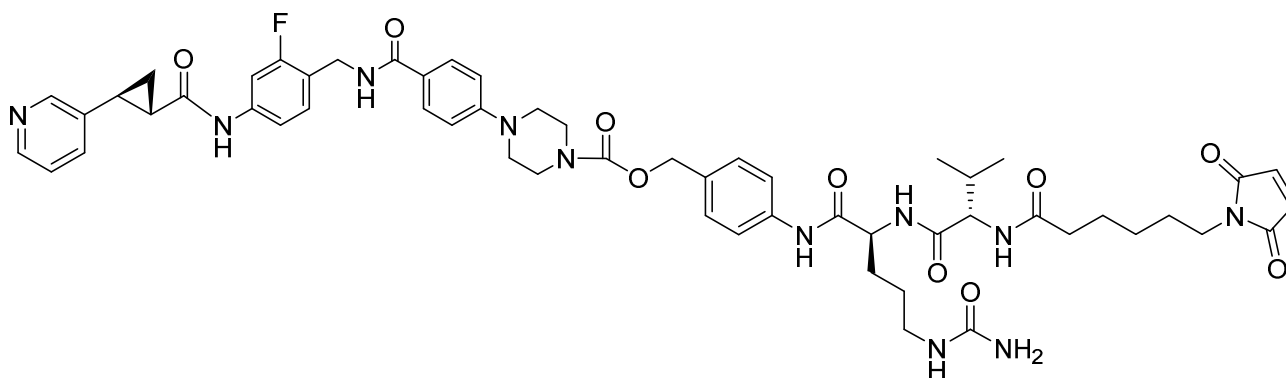
with NaHCO<sub>3</sub> (sat.) and brine. After extraction, the organics were combined, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, concentrated *in-vacuo* and absorbed onto Isolute. Purification by flash chromatography (24 g, silica gel) eluting with DCM/(DCM:MeOH 4:1) furnished the target product (275 mg, 0.455 mmol, 78% yield, 95% pure) as a colorless solid. UPLC-MS [M+H]<sup>+</sup> Calcd for C<sub>32</sub>H<sub>37</sub>FN<sub>5</sub>O<sub>4</sub> 574.3; Found 574.2, R<sub>t</sub> = 1.01 min (method A).

**N-(2-Fluoro-4-((1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxamido)benzyl)-4-(piperazin-1-yl)benzamide (compound 4)**



A solution of tert-butyl 4-(4-((2-fluoro-4-((1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxamido)benzyl)carbamoyl)phenyl)piperazine-1-carboxylate (20 mg, 0.035 mmol) in DCM (Volume: 1 mL) was cooled to 0 °C and TFA (0.134 mL, 1.743 mmol) added. The reaction mixture was stirred at room temperature for 2 h. Product **4** was obtained as a colorless solid after purification by reverse phase chromatography eluting with ACN/ water (0.1% TFA) (10 mg, 39% yield, 95% pure, TFA salt). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ ppm 10.48 (s, 1H), 8.78-8.70 (m, 2H), 8.62 (d, *J* = 2.1 Hz, 1H), 8.53 (dd, *J* = 5.0, 1.4 Hz, 1H), 7.87 – 7.76 (m, 3H), 7.60 (dd, *J* = 12.6, 1.9 Hz, 1H), 7.52 (dd, *J* = 8.0, 5.0 Hz, 1H), 7.32 – 7.18 (m, 2H), 7.06 – 7.02 (m, 2H), 4.43 (d, *J* = 5.6 Hz, 2H), 3.51 – 3.43 (m, 4H), 3.29 – 3.19 (m, 4H), 2.20 – 2.11 (m, 1H), 1.62 – 1.47 (m, 2H). The missing signal of 1 proton is under solvent peak of DMSO at 2.5 ppm. UPLC-MS [M+H]<sup>+</sup> Calcd for C<sub>27</sub>H<sub>29</sub>FN<sub>5</sub>O<sub>2</sub> 474.2; Found 474.4, R<sub>t</sub> = 1.37 min (method B).

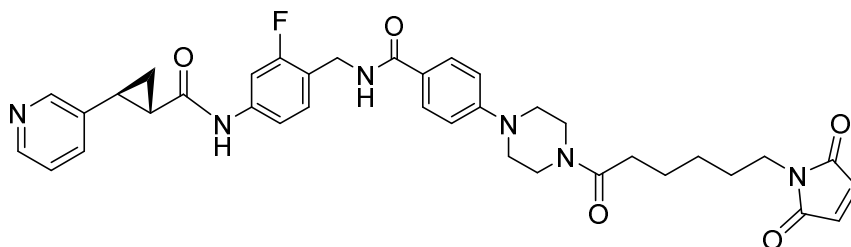
**4-((S)-2-((S)-2-(6-(2,5-Dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)-3-methylbutanamido)-5-ureidopentanamido)benzyl 4-(4-((2-fluoro-4-((1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxamido)benzyl)carbamoyl)phenyl)piperazine-1-carboxylate (LP1)**



To a solution of compound **4** (25 mg, 0.042 mmol, 79% pure) in DMF (Volume: 1.5 mL) was added DIPEA (0.036 mL, 0.209 mmol), and 4-((S)-2-((S)-2-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)-3-methylbutanamido)-5-ureidopentanamido)benzyl (4-nitrophenyl) carbonate (46.2 mg, 0.063 mmol). The reaction mixture was stirred at room temperature for 16 h. Purification by reverse phase chromatography eluting with ACN/water (0.1% TFA) afforded the desired **LP1** (19 mg, 0.016 mmol, 36% yield, 95% pure) as a colorless solid. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ ppm 10.48 (s, 1H), 10.00 (s, 1H), 8.72-8.67 (m, 2H), 8.58 (d, *J* = 4.4 Hz, 1H), 8.08 (d, *J* = 7.5 Hz, 1H), 7.97 – 7.90 (m, 1H), 7.85 – 7.75 (m, 3H), 7.68 – 7.57 (m, 4H), 7.36 – 7.16 (m, 4H), 7.00 (s, 2H), 6.99 - 6.94 (m, 2H), 5.98 (s, 1H), 5.55 – 5.30 (m, 2H), 5.04 (s, 2H), 4.45 - 4.35 (m, 3H), 4.22 – 4.16 (m, 1H), 3.56-3.49 (m, 4H), 3.37 (t, *J* = 7.1 Hz, 2H), 3.31 – 3.23 (m, 4H), 3.08-2.90 (m, 2H), 2.60-2.53 (m, 1H), 2.25 – 2.06 (m, 3H), 2.04-1.90 (m, 1H),

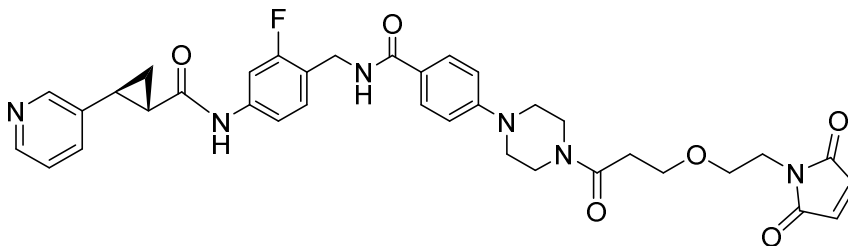
1.77 – 1.30 (m, 10H), 1.25-1.12 (m, 2H), 0.88-0.80 (m, 6H). UPLC-MS  $[M+H]^+$  Calcd for  $C_{56}H_{67}FN_{11}O_{10}$  1072.5; Found 1072.7,  $R_t = 3.61$  min (method B).

**4-(4-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl)piperazin-1-yl)-N-(2-fluoro-4-((1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxamido)benzyl)benzamide (LP2)**



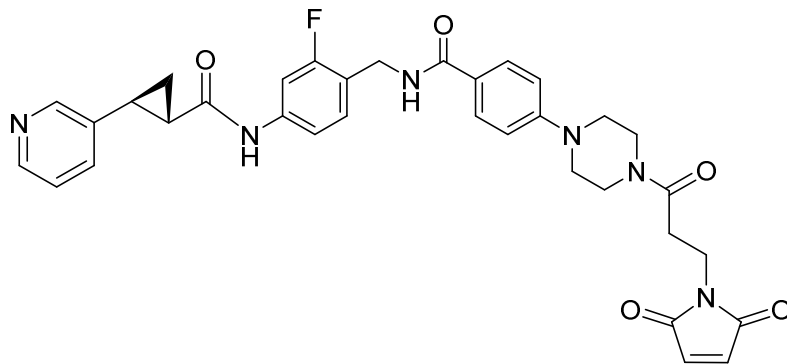
To a solution of compound **4** (27 mg, 0.044 mmol, 79% pure) and 6-Maleimidohexanoic acid (10.30 mg, 0.049 mmol) in DMF (Volume: 1.5 ml) was added DIPEA (0.031 ml, 0.177 mmol), and HATU (21.92 mg, 0.058 mmol). The reaction mixture was stirred at room temperature for 3 h. Purification by supercritical fluid chromatography (column: 250x30 Reprosil DiAmino 100A 5  $\mu$ m-FLOW100) afforded the desired **LP2** (12 mg, 0.018 mmol, 41% yield) as a colorless solid.  $^1H$  NMR (400 MHz,  $DMSO-d_6$ )  $\delta$  ppm 10.37 (s, 1H), 8.62 (t,  $J = 5.8$  Hz, 1H), 8.43 (d,  $J = 2.2$  Hz, 1H), 8.34 (dd,  $J = 4.7, 1.5$  Hz, 1H), 7.75-7.67 (m, 2H), 7.58 – 7.44 (m, 2H), 7.29 – 7.09 (m, 3H), 6.93 (s, 2H), 6.92-6.86 (m, 2H), 4.35 (d,  $J = 5.7$  Hz, 2H), 3.55 – 3.47 (m, 4H), 3.32 (t,  $J = 7.0$  Hz, 2H), 3.23 – 3.12 (m, 4H), 2.40 – 2.30 (m, 1H), 2.26 (t,  $J = 7.4$  Hz, 2H), 2.08 – 1.99 (m, 1H), 1.51 – 1.33 (m, 6H), 1.23 – 1.10 (m, 2H). UPLC-MS  $[M+H]^+$  Calcd for  $C_{37}H_{40}FN_6O_5$  667.3; Found 667.4,  $R_t = 3.21$  min (method B).

**4-(4-(3-(2-(2,5-Dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethoxy)propanoyl)piperazin-1-yl)-N-(2-fluoro-4-((1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxamido)benzyl)benzamide (LP3)**



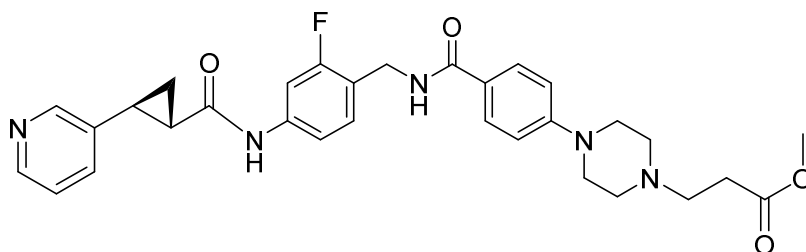
To a solution of compound **4** (27 mg, 0.044 mmol, 79% pure) and 3-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethoxy)propanoic acid (10.40 mg, 0.049 mmol) in DMF (Volume: 1.5 ml) was added DIPEA (0.031 ml, 0.177 mmol), and HATU (21.92 mg, 0.058 mmol). The reaction mixture was stirred at room temperature for 1.5 h. Purification by supercritical fluid chromatography (column: 250x30 Reprosil DiAmino 100A 5  $\mu$ m-FLOW100) afforded the desired **LP3** (4.5 mg, 6.73  $\mu$ mol, 15% yield, purity 99%) as a colorless solid.  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  ppm 10.37 (s, 1H), 8.62 (t,  $J = 5.8$  Hz, 1H), 8.43 (d,  $J = 2.3$  Hz, 1H), 8.34 (dd,  $J = 4.8, 1.5$  Hz, 1H), 7.78-7.67 (m, 2H), 7.59 – 7.44 (m, 2H), 7.29 – 7.07 (m, 3H), 6.94 (s, 2H), 6.91-6.86 (m, 2H), 4.35 (d,  $J = 5.7$  Hz, 2H), 3.59 – 3.40 (m, 10H), 3.21 – 3.13 (m, 4H), 2.48 (t,  $J = 6.5$  Hz, 2H), 2.39 – 2.31 (m, 1H), 2.09 – 1.99 (m, 1H), 1.53 – 1.33 (m, 2H). UPLC-MS  $[\text{M}+\text{H}]^+$  Calcd for  $\text{C}_{36}\text{H}_{38}\text{FN}_6\text{O}_6$  669.3; Found 669.4,  $R_t = 2.70$  min (method B).

**4-(4-(3-(2,5-Dioxo-2,5-dihydro-1H-pyrrol-1-yl)propanoyl)piperazin-1-yl)-N-(2-fluoro-4-((1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxamido)benzyl)benzamide (LP4)**



To a solution of compound **4** (23 mg, 0.038 mmol, 79% pure) and 2,5-dioxopyrrolidin-1-yl 3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)propanoate (15.32 mg, 0.058 mmol) in DMF (Volume: 1.5 ml) was added DIPEA (0.034 ml, 0.192 mmol). The reaction mixture was stirred at room temperature for 3 h. Purification by reverse phase chromatography eluting with ACN/ water (0.1% TFA) afforded the desired **LP4** (12 mg, 0.016 mmol, 41% yield, 99% pure) as a light yellow solid. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  ppm 10.48 (s, 1H), 8.74 – 8.66 (m, 2H), 8.61 – 8.53 (m, 1H), 7.91 (d, *J* = 8.2 Hz, 1H), 7.83 – 7.75 (m, 2H), 7.66 – 7.57 (m, 2H), 7.32 – 7.17 (m, 2H), 7.03 (s, 2H), 7.01 – 6.95 (m, 2H), 4.43 (d, *J* = 5.7 Hz, 2H), 3.69-3.53 (m, 6H), 3.34 – 3.22 (m, 4H), 2.69 – 2.63 (m, 2H), 2.58 – 2.53 (m, 1H), 2.20 – 2.14 (m, 1H), 1.62 – 1.50 (m, 2H). UPLC-MS [M+H]<sup>+</sup> Calcd for C<sub>34</sub>H<sub>34</sub>FN<sub>6</sub>O<sub>5</sub> 625.3; Found 625.3, R<sub>t</sub> = 2.62 min (method B).

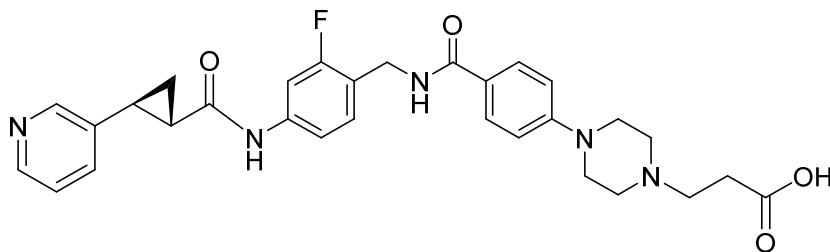
**Methyl 3-(4-(4-((2-fluoro-4-((1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxamido)benzyl)carbamoyl)phenyl)piperazin-1-yl)propanoate**





To a solution of N-(2-fluoro-4-((1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxamido)benzyl)-4-(piperazin-1-yl)benzamide (120 mg, 0.200 mmol, 79% pure) in MeOH (Volume: 3 mL) was added methyl acrylate (0.114 mL, 1.267 mmol), and triethylamine (0.177 mL, 1.267 mmol). The reaction mixture was stirred at 55 °C for 16 h and concentrated *in-vacuo*. Purification by supercritical fluid chromatography (column: 250x30 Princeton PPU 100A 5um) afforded the desired product (70 mg, 0.125 mmol, 63% yield). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ ppm 10.45 (s, 1H), 8.67 (t, *J* = 5.8 Hz, 1H), 8.53 – 8.47 (m, 1H), 8.42 (dd, *J* = 4.7, 1.6 Hz, 1H), 7.77 (d, *J* = 8.9 Hz, 2H), 7.66 - 7.52 (m, 2H), 7.36 – 7.16 (m, 3H), 6.95 (d, *J* = 8.9 Hz, 2H), 4.42 (d, *J* = 6.0 Hz, 2H), 3.61 (s, 3H), 3.26 – 3.20 (m, 4H), 2.67 – 2.58 (m, 2H), 2.57 – 2.52 (m, 6H), 2.48 – 2.38 (m, 1H), 2.16 – 2.07 (m, 1H), 1.58 – 1.41 (m, 2H). UPLC-MS [M+H]<sup>+</sup> Calcd for C<sub>31</sub>H<sub>35</sub>FN<sub>5</sub>O<sub>4</sub> 560.3; Found 560.2, R<sub>t</sub> = 0.57 min (method A).

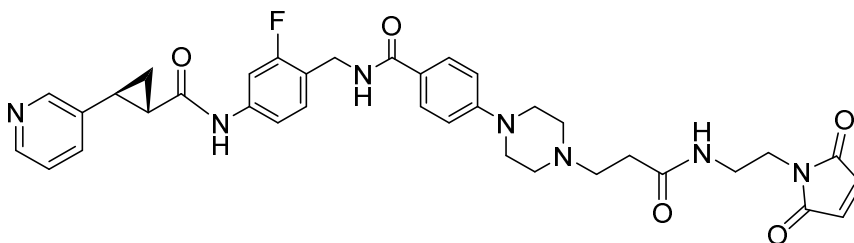
**3-(4-(4-((2-Fluoro-4-((1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxamido)benzyl)carbamoyl)phenyl)piperazin-1-yl)propanoic acid**



To a solution of methyl 3-(4-(4-((2-fluoro-4-((1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxamido)benzyl)carbamoyl)phenyl)piperazin-1-yl)propanoate (19 mg, 0.034 mmol) in MeOH (Volume: 1 ml) and THF (Volume: 1 ml) was added a solution of LiOH.H<sub>2</sub>O

(4.27 mg, 0.102 mmol) in Water (Volume: 0.50 ml). The reaction mixture was stirred at room temperature for 14 h. The mixture was then concentrated *in-vacuo*, and diluted with 4 mL of 1:1 ACN/water. The solution was cooled to 0 °C, acidified with HCl (1 M) (0.136 ml, 0.136 mmol) and freeze-dried to afford the crude product (28 mg, 0.034 mmol, 100% yield, purity 66%). UPLC-MS [M+H]<sup>+</sup> Calcd for C<sub>30</sub>H<sub>33</sub>FN<sub>5</sub>O<sub>4</sub> 546.2; Found 546.3, R<sub>t</sub> = 0.53 min (method A).

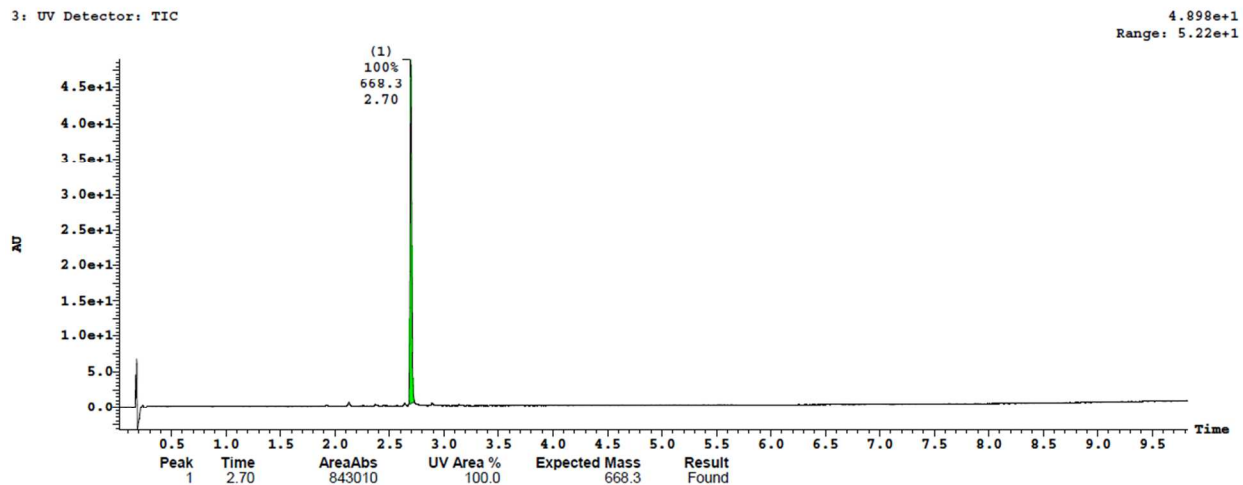
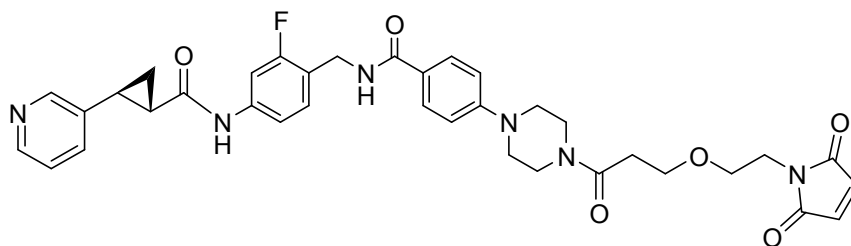
**4-(4-(3-((2-(2,5-Dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)amino)-3-oxopropyl)piperazin-1-yl)-N-(2-fluoro-4-((1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxamido)benzyl)benzamide (LP5)**



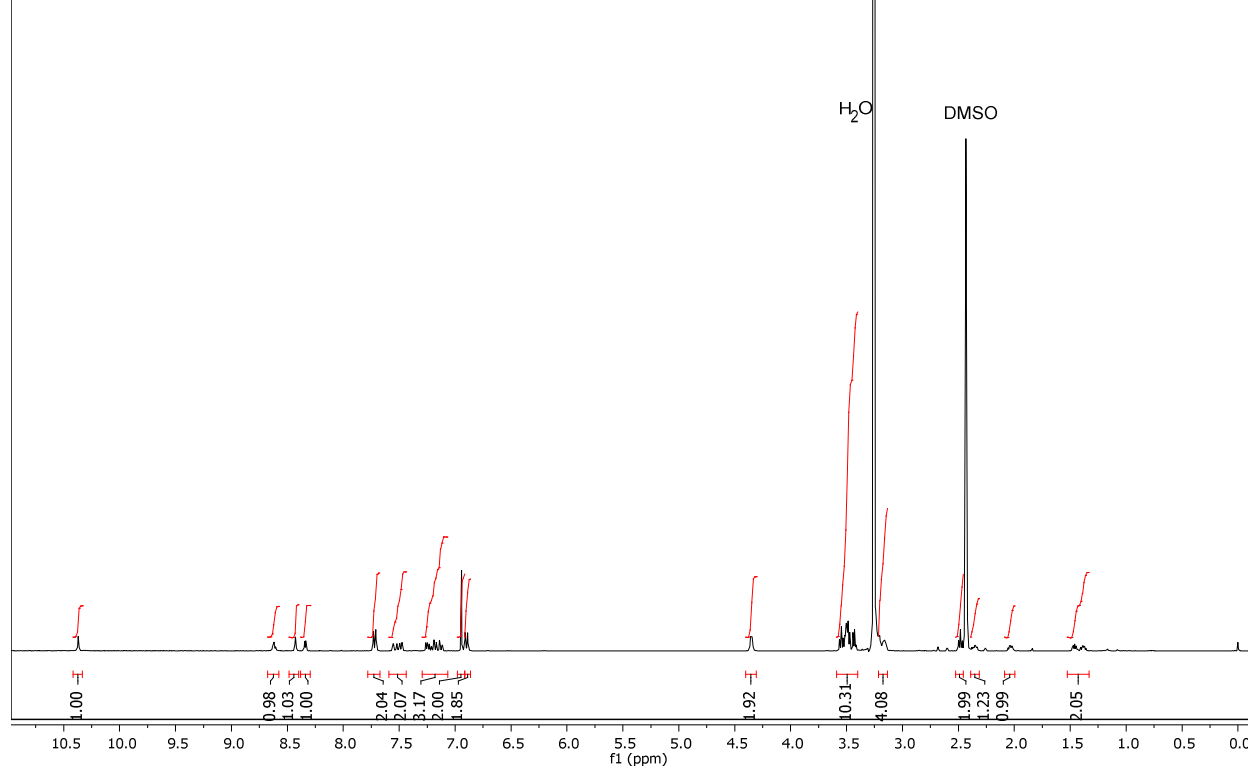
To a solution of 3-(4-(4-((2-fluoro-4-((1S,2S)-2-(pyridin-3-yl)cyclopropanecarboxamido)benzyl)carbamoyl)phenyl)piperazin-1-yl)propanoic acid (28 mg, 0.034 mmol, 66% pure) in DMF (Volume: 1 ml) was added DIPEA (0.030 ml, 0.169 mmol), and HATU (19.32 mg, 0.051 mmol). The reaction mixture was stirred at room temperature for 10 min, then N-(2-aminoethyl)maleimide (17.22 mg, 0.068 mmol) added and stirring continued for 16 h. Purification by reverse phase chromatography eluting with ACN/water (0.1% TFA) afforded the desired **LP5** (7 mg, 7.74 μmol, 23% yield, TFA salt) as a colorless solid. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ ppm 10.48 (s, 1H), 9.53 (br s, 1H), 8.75 (t, *J* = 5.8 Hz, 1H), 8.62 (d, *J* =

2.2 Hz, 1H), 8.53 (dd,  $J = 5.0, 1.5$  Hz, 1H), 8.27 (t,  $J = 6.1$  Hz, 1H), 7.87 – 7.76 (m, 3H), 7.60 (dd,  $J = 12.6, 1.9$  Hz, 1H), 7.52 (dd,  $J = 8.0, 5.0$  Hz, 1H), 7.32 – 7.18 (m, 2H), 7.09 – 7.01 (m, 4H), 4.43 (d,  $J = 5.7$  Hz, 2H), 4.05 – 3.96 (m, 2H), 3.59-3.47 (m, 4H), 3.37 (t,  $J = 7.2$  Hz, 2H), 3.28-3.21 (m, 2H), 3.17 – 3.05 (m, 4H), 2.59 – 2.52 (m, 3H), 2.20 – 2.09 (m, 1H), 1.62 – 1.47 (m, 2H). UPLC-MS  $[M+H]^+$  Calcd for  $C_{36}H_{39}FN_7O_5$  668.3; Found 668.3,  $R_t = 1.66$  min (method B).

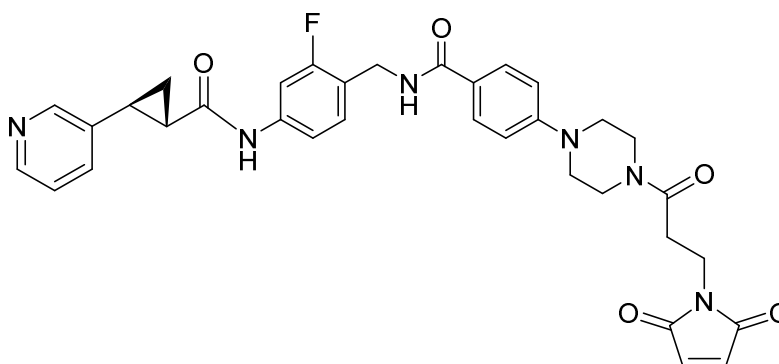
## 2. UPLC-MS AND $^1H$ NMR SPECTRA OF LP3



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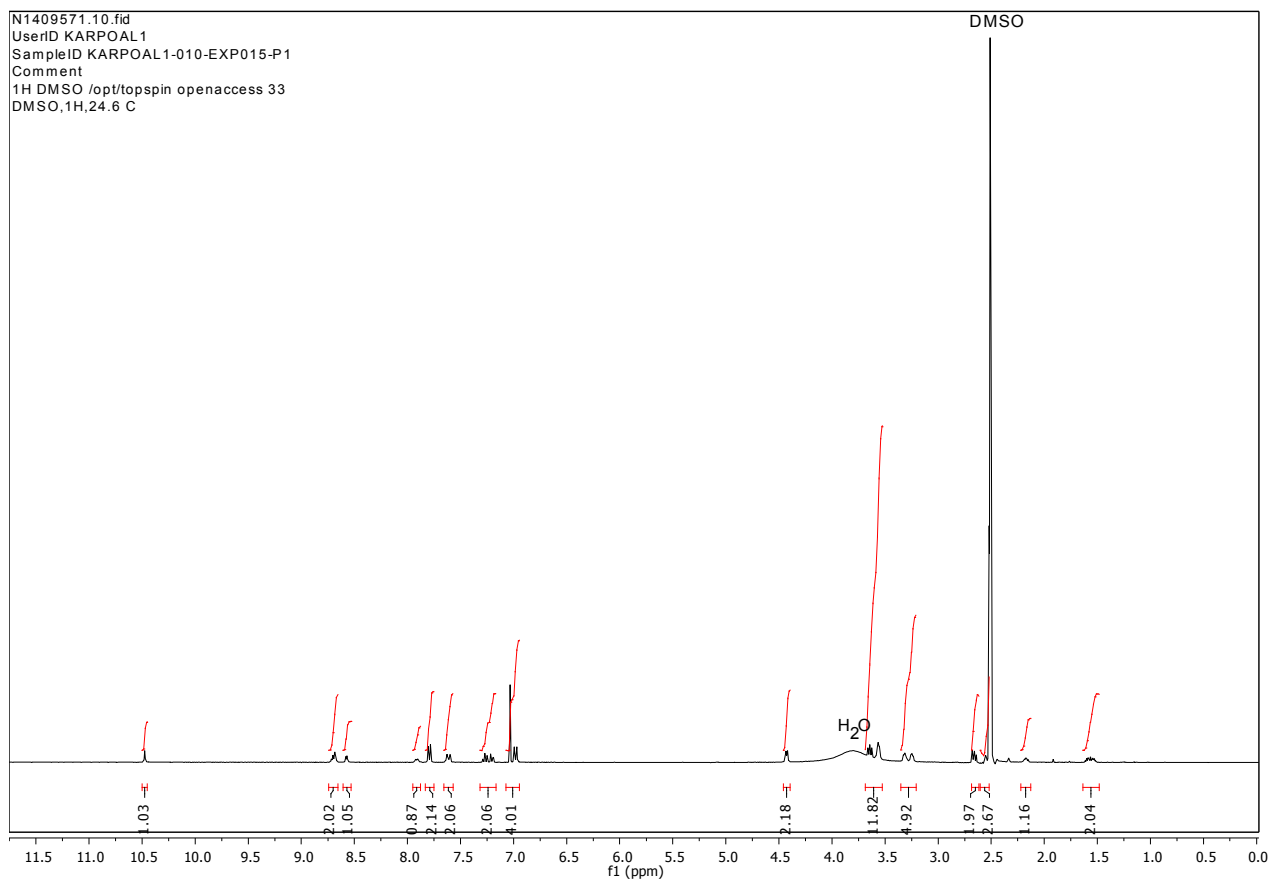
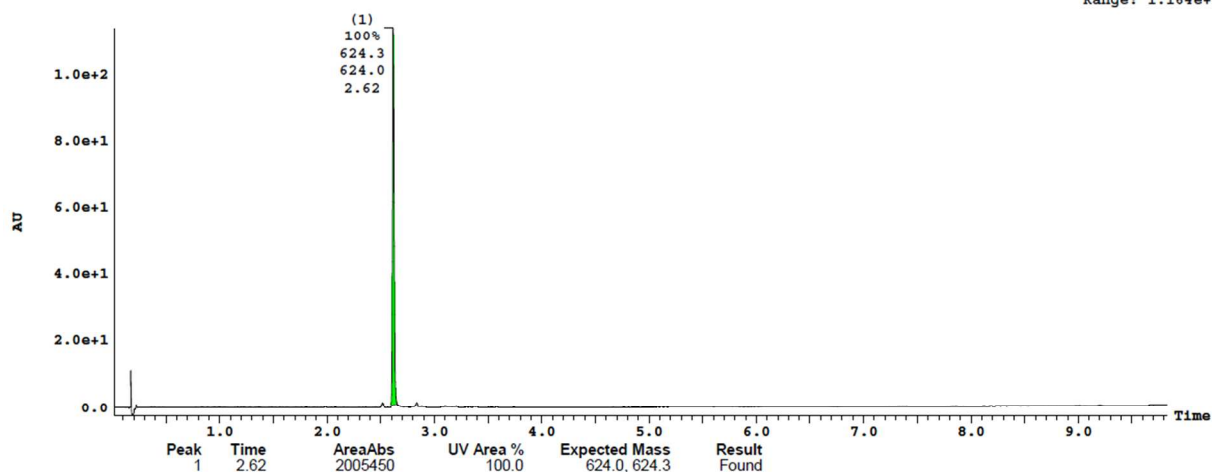


## UPLC-MS AND 1H NMR SPECTRA OF LP4



3: UV Detector: TIC

1.14e+2  
Range: 1.164e+2



### 3. BIOCONJUGATION METHODS

Conjugation to antibodies (e.g., anti-c-Kit, TBS or non-binding IgG) at partially reduced hinge and inter-chains disulfides was performed in a two step process. The antibody, at a concentration of 5-10 mg/ml in PBS containing 2 mM EDTA, was first partially reduced for 1.5 h at 37 °C with 50 mM mercaptoethylamine hydrochloride (added as a solid). After desalting and addition of 1% w/v PS-20 detergent, the partially reduced antibody (1-2 mg/ml) was reacted overnight at 4 °C with the Linker-Payload, dissolved at 10 mg/ml in DMSO per 10 mg antibody. The ADC was purified by Protein A chromatography (GE Healthcare). After base-line washing with PBS, the conjugate is eluted with 50 mM citrate, pH 2.7, 140 mM NaCl, neutralized and sterile filtered. This procedure yields an average drug loading (DAR) of 2-4 molecules of linker-payload per antibody. The number of conjugated drug molecules per monoclonal antibody (mAb) was quantified by UPLC-MS analysis of the reduced and deglycosylated samples (30 uL of the 1 mg/mL ADC treated with 1.5 uL PGNase F from BioLabs (P0705L) and 1.5 uL of DTT (1M in water) in eppendorf tube at 37 °C for 1 h) with the following method:

Waters Acquity UPLC with Waters UPLC/XEVO G2-S quadrupole time-of-flight (Q-TOF) detector; Column: Acquity UPLC BEH C4, 2.1 x 100mm Column, 1.7 µm (Waters), (flow: 0.5 ml/min, column temperature: 80 °C, solvent A: water + 0.05% TFA, solvent B: acetonitrile + 0.04% TFA, gradient: from 5 to 60% B in 28.0 min)

Aggregation was measured by analytical size exclusion chromatography (Superdex 200 5/150 GL run in PBS).

#### **4. CELLULAR IN-VITRO CYTOTOXICITY ASSAY**

The cell lines NCI-N87, NCI-H526 and MDA-MB-453 were obtained from the American Type Culture Collection (ATCC) and maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Invitrogen) and 2mM L-glutamine. The Gastrointestinal stromal tumor (GIST-T1) line

was licensed from Kochi University, Japan and maintained in DMEM (ThermoFisher Scientific, Catalog no: 11965084) supplemented with 10% fetal bovine serum (Gibco). Cell lines were seeded at 1,000 cells per well in 40µl of complete media in 384-well flat clear-bottom white polystyrene tissue-culture-treated microplates (Corning). Antibody–drug conjugates (2 mg/ml in 20 mM histidine acetate, 240 mM sucrose, 0.02% PS20 pH 5.5 buffer) or small molecules (10mM in DMSO) were transferred to cells seeded in 384-well plates using an ECHO acoustic dispenser (Labcyte) or Tecan EVO liquid handler (Tecan) to create a ten-point dose–response curve in triplicate starting from 20 µg/ml for ADCs or 1µM for small molecules with a 1:3 serial dilution. Cells were cultured in a humidified incubator set at 37 °C and maintained at an atmosphere of 5% CO<sub>2</sub>. On Day 4 cells were equilibrated to room temperature and 40 µl per well of Cell Titer-Glo II reagent (Promega) was added. The plates were shaken for 10 min and then incubated for 30 min at room temperature in the dark after which luminescence was read using an EnVision 2101 Multilabel Reader (PerkinElmer). Normalized luminescence intensity data was analyzed using GraphPad Prism 6, and subsequent IC<sub>50</sub> values were calculated using a four-parameter sigmoidal fit.

## **5. NAD-NADH GLO ASSAY (FIGURE 3)**

The Gastrointestinal stromal tumor (GIST-T1) line was licensed from Kochi University, Japan. The cell line was maintained in DMEM (ThermoFisher Scientific, Catalog no: 11965084) supplemented with 10% fetal bovine serum (Gibco). On Day 0, GIST-T1 cells were seeded in a Corning® 384 Well Flat Clear Bottom White Polystyrene TC-Treated Microplates (Product #3707) at 500cells/well in 36ul complete growth media (DMEM+10% FBS) and cultured in a humidified incubator set at 37 °C and maintained at an atmosphere of 5% CO<sub>2</sub>. On Day 1, the seeded cells were treated with 4ul of 10X nM **ADC-4** or **ADC-6** to create a six-point dose–response curve in duplicate starting from 10 nM with 10X serial dilution (few seeded wells were

left untreated as a control). The plate was returned back to the incubator after adding the treatment. 24 hours following treatment, NAD-GloAssay reagents were prepared as per manufacturer's protocol (NAD/NADH-Glo™, Promega, Cat no: G9071) and 40ul of the reagent was added to each well. The plate was then incubated in the dark at room temperature for 30 minutes and luminescence was recorded on EnVision 2101 Multilabel reader. Average luminescence units were plotted using the Spotfire package (Tibco).

## **6. CELL CYCLE ANALYSIS (FIGURE 4A)**

GIST-T1 cells were plated at 10,000 cells/well in a 96 well plate polystyrene, flat bottom (Corning® Cat #: Z736732) in complete growth media (DMEM+10% FBS) and cultured in a humidified incubator set at 37 °C and maintained at an atmosphere of 5% CO<sub>2</sub>. The following day, the seeded cells were treated with either s-me-DM1 or **ADC-4**, starting from 10nM with 10X serial dilution up to six concentrations lower. The plate was returned back to the incubator after adding treatment. 48 hours post treatment, the cells were harvested using ACCUTASE (Stemcell Technologies, Cat no: 07920) and transferred to a 96 Well Clear Round Bottom TC-Treated Microplate (Corning, Cat no: 3799). The cells were washed once at 1400 rpm for 5 min and then fixed using 200ul ice-cold 70% ethanol for 1hr at 4 °C. The cells were washed again at 1400rpm for 5 min, stained with Guava Cell Cycle Reagent for Flow Cytometry (Millipore Sigma, Cat no: 4500-0220) for 30 min in dark at room temperature and analyzed using Cell cycle Assay module (CytoSoft 5.3) on Guava® easyCyte Flow Cytometer.

## **7. REAL-TIME GLO (FIGURE 4B)**

On Day 0, GIST-T1 cells were seeded at 4000 cells/well in a Corning® 96 Well Flat Clear Bottom Black Polystyrene TC-Treated Microplates (Cat no: 3904) in complete growth media



(DMEM+10% FBS). The viability reagents (RealTime-Glo™ MT Cell Viability Assay, Promega, Cat no: G9712) were added at the time of cell seeding by including 1X MT Cell Viability Substrate and 1X NanoLuc Enzyme in the cell culture suspension. On Day 1, the seeded cells were treated with either s-me-DM1 or **ADC-4** to create a six-point dose–response curve in duplicate starting from 10 nM with 10X serial dilution. Cells were cultured in a humidified incubator set at 37 °C and maintained at an atmosphere of 5% CO<sub>2</sub>. Post treatment, luminescence was recorded in real time on EnVision 2101 Multilabel reader at 24 h, 48 h, 72 h, 96 h and 144 h. Percent growth inhibition was calculated based on treated wells versus untreated controls and plotted using the Spotfire package (Tibco).

## 8. IN-VIVO STUDY

All in-vivo studies conducted were performed in strict accordance with the Novartis animal welfare policies. Female scid-beige (SCID bg) mice were used in each study and were obtained from Charles River Laboratories. Tumor volume for all in-vivo studies was measured using the formula:  $(\text{length} \times \text{width}^2) \times (\pi/6)$ , and results were depicted as means of tumor volume  $\pm$  SEM. Body weights were depicted as percent change of body weight over time.

For the c-Kit positive GIST-T1 gastric xenograft studies, mice were subcutaneously inoculated with 10 million cells in a 1:1 ratio of HBSS and Matrigel®. When tumors reached approximately 200 mm<sup>3</sup> (n=7/group), mice were randomly assigned to receive a single intravenous dose of the treatments. Mice were treated with PBS, isotype control **ADC-6** at 17.5 mg/kg, anti-c-Kit **ADC-3** or **ADC-4** at 20 mg/kg (dose levels adjusted to DAR-match to that of the highest DAR).

## 9. ABBREVIATIONS

ACN, acetonitrile; ADC, Antibody-Drug Conjugate; Boc, *tert*-butoxycarbonyl; BSA, Bovine Serum Albumin; DAR, drug-to-antibody ratio; DCM, dichloromethane; DIPEA,

diisopropylethylamine; DMEM, Dulbecco's modified Eagle's medium; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DIPEA, diisopropylethylamine; DTT, Dithiothreitol; EA, ethylacetate; EDTA, Ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HATU, 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate; HBSS, Hank's Based Salt Solution; LP, linker-payload; MeOH, methanol; NAD<sup>+</sup>, nicotinamide adenine dinucleotide (oxidized); NADH, nicotinamide adenine dinucleotide (reduced); NMP, N-methylpyrrolidone; PBS, phosphate-buffered saline; RT, room temperature; SEM, standard error of the mean; TFA, trifluoroacetic acid; UPLC-MS, ultra performance liquid chromatography mass spectrometry.

#### SUPPLEMENTAL REFERENCES

- (1) Palacios, D. S.; Meredith, E.; Kawanami, T.; Adams, C.; Chen, X.; Darsigny, V.; Geno, E.; Palermo, M.; Baird, D.; Boynton, G.; Busby, S. A.; George, E.L.; Guy, C.; Hewett, J.; Tierney, L.; Thigale, S. Structure Based Design of Nicotinamide Phosphoribosyltransferase (NAMPT) Inhibitors from a Phenotypic Screen. *Bioorg. Med. Chem. Lett.* **2018**, 28, 365-370.