Characterization of specific N-α-acetyltransferase 50 (Naa50) inhibitors identified using a DNA encoded library

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Supporting Information:

Protein expression and purification: The DNA encoding the full length of human Naa50 was cloned into pET24(+) based vector with an N-terminal cleavable his tag or his-GST tag. Protein was overexpressed in BL21 (DE3) cells induced with 0.5 mM isopropyl 1-thio-β-D-glactopyranoside (IPTG). The tagged protein was captured by nickel resin in a lysis buffer consisting 25 mM HEPES, pH7.5, 300 mM NaCl, 1 mM TCEP, and 10 mM imidazole plus protease inhibitor cocktails (Roche) and washed extensively with the lysis buffer containing high salt, high pH, and then low salt buffers. After protein was eluted from nickel resin it was treated with tobacco etch virus protease (TEV) overnight at 4 °C to obtain untagged Naa50. Protein was concentrated and subjected to a Superdex 75 gel filtration column (GE Healthcare) in buffer containing 25 mM HEPES, pH7.5, 150 mM NaCl, 0.5 mM TCEP. Naa50 was further purified by SP HP column (GE Healthcare). Two major peaks were observed after the salt-gradient elution. The first peak with an A260 to A280 ratio above 1 was identified as cofactor bound Naa50 and the second peak with an A260 to A280 ratio below 0.6 was identified as apo protein. Fractions for each peak were pooled and buffer exchanged into the storage buffer (25 mM HEPES, pH7.5, 150-185 mM NaCl, 2 mM TCEP). Apo protein was concentrated to 14.3 and 18.2 mg/mL and cofactor bound protein was concentrated to 13 and 19 mg/mL. Proteins were stored in small aliquots at -80 °C until use.

Crystallization and Structure Determination: Complexes with compounds **1**, **2**, **4a,** and **4b** were obtained by co-crystallization. Complexes with compounds **3a** and **4a** were obtained by soaking compounds into pre-formed co-crystals of Naa50•CoA. Crystallization experiments were performed by sitting-drop vapor-diffusion methods. All crystals were transferred to cryoprotectant solution containing the corresponding well solution and 25% glycerol before flash cooling in liquid nitrogen.

Crystallization conditions for **1**: Naa50 apo protein (14.3 mg/ml) was incubated with **1** in a 1:3 molar ratio on ice for 60 min. Reservoir solution containing 0.2 M NH4SO4 and 30% (w/v) PEG 3K/4K was mixed 1:1 with protein•ligand complex.

Crystallization conditions for **2**: Naa50 apo protein (18.2 mg/mL) was incubated with compound **2** in a 1:5 molar ratio on ice for 60 min. Initial crystal hits were obtained from TOP96 commercial screen (Anatrace). Optimized crystals were grown by micro-seeding in reservoir solution containing 2.1 M $(NH_4)_2SO_4$, 0.2 M K/Na Tartrate, 0.1 M Na-Citrate, pH4.6 and 4.5% (w/v) 1-Ethyl-3-methylimidazolium chloride. Crystals grown at 13^{o}C.

Crystallization conditions for **4a** with **AcCoA**: Naa50 apo protein (13.0 mg/ml) was incubated with **4a** and AcCoA in a 1:3:3 molar ratio on ice for 60 min. Reservoir solution containing 0.1 M Bis_tris, pH 5.77 and 21% w/v PEG 3350 was mixed 0.2µl:0.2 µl with Naa50•**4b**•AcCoA complex. Crystals were grown at 21^{o}C.

Crystallization conditions for **4b** with **AcCoA**: Naa50 apo protein (13.0 mg/ml) was incubated with **4b** and AcCoA in a 1:3:3 molar ratio on ice for 60 min. Reservoir solution containing 0.1 M Bis_tris, pH 5.77 and 24% w/v PEG 3350 was mixed 0.2µl:0.2 µl with Naa50•**4b**•AcCoA complex. Crystals were grown at 21^{o}C.

Crystallization conditions for **CoA**: Naa50 apo protein (14.3 mg/ml) was incubated with **CoA or AcCoA** in a 1:3 molar ratio on ice for 60 min. Crystals of the complex were obtained with reservoir solution containing 0.1 M Na acetate, pH5.0, 25% (w/v) PEG 3350, 10 mM Dithiothreitol (DTT), and 0.1% Dioxane.

Crystallization conditions for **3a** and **4a**: 10 mM of **3a** solution was added directly on the protein drops containing Naa50•CoA crystals and soaked for 2 hours. Crystals of the complex with **4a** and

CoA were obtained by transferring co-crystals of Naa50•CoA to buffer consisting 0.04 mM KH2PO4, pH7.4, 24.3% PEG 3335, 20% Glycerin, then soaking in **4a** (10mM) for 2 days.

Diffraction data were collected at 98K at the IMCA-CAT beamline 17-ID at the Advanced Photon Source, (Argonne National Laboratory in Illinois, USA) using a Dectris Pilatus 6M Pixel Array. Data were processed using autoPROC[1] and programs from the CCP4 suite[2] and structures were determined either by difference fourier in autoBuster[3] or by molecular replacement using MOLREP[4] with 2Ob0 pdb or in-house structures of Naa50 as the search model. Ligands were initially fit using AFITT[5],[6] in OpenEye and refinement was carried out using autoBUSTER[3], with cycles of rebuilding in COOT[7]. The statistics of the data processing and structure refinement are listed in Supporting Information Table S1. Initial unbiased 2Fo-Fc electron density maps, calculated after the first round of refinement, before ligands were included in the model are shown in the electron density map section below. Further details of structure refinement are given in the PDB headers. All structure figures were generated using MOVIT – Pfizer proprietary software.

Table S1. Statistics for the crystallographic analysis.

Compound	**1**	**2**	**3a**	**4a**	**4a**	**4b**
Cofactor	**na**	**na**	**CoA**	**CoA**	**AcCoA**	**AcCoA**
Data Statistics						
Beamline	APS 17-ID	APS 17-ID	APS 17-ID	APS 17-ID	APS 17-ID	APS 17-ID
Space group	P21	P21	P21	P21	P21	P21
Unit Cell	a=48.55Å, b=124.17Å, c=109.08Å, β=100.92°	a= 50.94Å, b=52.34Å, c=64.12Å, β=97.38°	a= 47.83, b=102.94Å, c=67.64Å, β=106.71°	a= 48.35Å, b=102.38Å, c=67.55Å, β=106.92°	a= 34.43Å, b=62.70Å, c=38.77Å, β=111.37°	a= 47.87Å, b=102.63Å, c=67.50Å, β=106.08°
# molecules per au	6	2	3	3	1	3
Resolution (Å)[a]	53.71-2.29 (2.41-2.29)	63.59-2.04 (2.15-2.04)	64.79-2.16 (2.28-2.16)	64.63-1.87 (2.03-1.87	36.11-1.08 (1.13-1.08)	64.86-1.85 (2.07-1.85)

#unique reflections	56682 (8301)	21247 (3080)	29839 (2870)	38205 (1910)	55133 (2758)	33141 (1657)
Completeness (%)	99.4 (99.7)	98.8 (98.6)	88.5 (58.1)	91.3 (55.5)	87.4 (38.2)	90.7 (64.7)
Multiplicity	3.4 (3.3)	3.3 (3.3)	3.3 (3.3)	3.3 (2.5)	3.0 (1.7)	3.2 (2.7)
R_{merge}[b] (%)	5.7 (52.0)	12.6 (72.3)	4.4 (63.9)	5.4 (77.8)	3.8 (35.1)	6.9 (59.7)
$C/C_{1/2}$[c]	0.997 (0.785)	0.991 (0.667)	0.998 (0.761)	0.998 (0.421)	0.998 (0.789)	0.998 (0.614)
I/σ	13.0 (2.3)	7.9 (2.4)	14.1 (1.8)	11.9 (1.2)	14.7 (2.2)	9.0 (1.6)
Refinement Statistics						
Resolution (Å)	53.71-2.29	50.52-2.04	64.79-2.16	64.63-1.87	36.10-1.07	54.83-1.85
R_{cryst}[d] (%) / R_{free}	20.1/24.3	22.2/27.4	20.1/25.3	20.0/22.2	18.9/20.1	20.2/24.0
PDB entry ID	6WF3	6WF5	6WFG	6WFK	6WFN	6WFO

[a] Numbers in parentheses refer to the highest resolution shell

[b] $R_{merge} = (\Sigma| I - \langle I \rangle |/\Sigma I) \times 100$, where I is the observed intensity and $\langle I \rangle$ is the average intensity obtained from multiple observations of symmetry-related reflections after rejections.

[c] $CC_{1/2}$ = as defined by Karplus and Diederichs[8] (Karplus and Diederichs, 2012)

[d] $R_{cryst} = (\Sigma|| F_o| - |F_c||/\Sigma|F_o|) \times 100$, where F_o and F_c are the observed and calculated structure factors, respectively.

R_{free} is the same as R_{cryst}, but for 5% of the data randomly omitted from refinement[9]. (Brunger, 1997)

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Electron Density Maps for compounds 1, 2, 3a, 4a, and 4b.

Initial unbiased 2Fo-Fc electron density maps contoured at 1 sigma, with the final refined ligand shown in ball and stick representation. Figure (a) Compound **1** (Figure 1b), (b) Compound **2** (Figure S1), (c) Compound **3a** (Figure 5), (d) Compound **4b** (Figure S10), (e) Compound **4a** in AcCoA bound structure (Figure 6), (f) Compound **4a** in CoA bound structure (Figure 5).

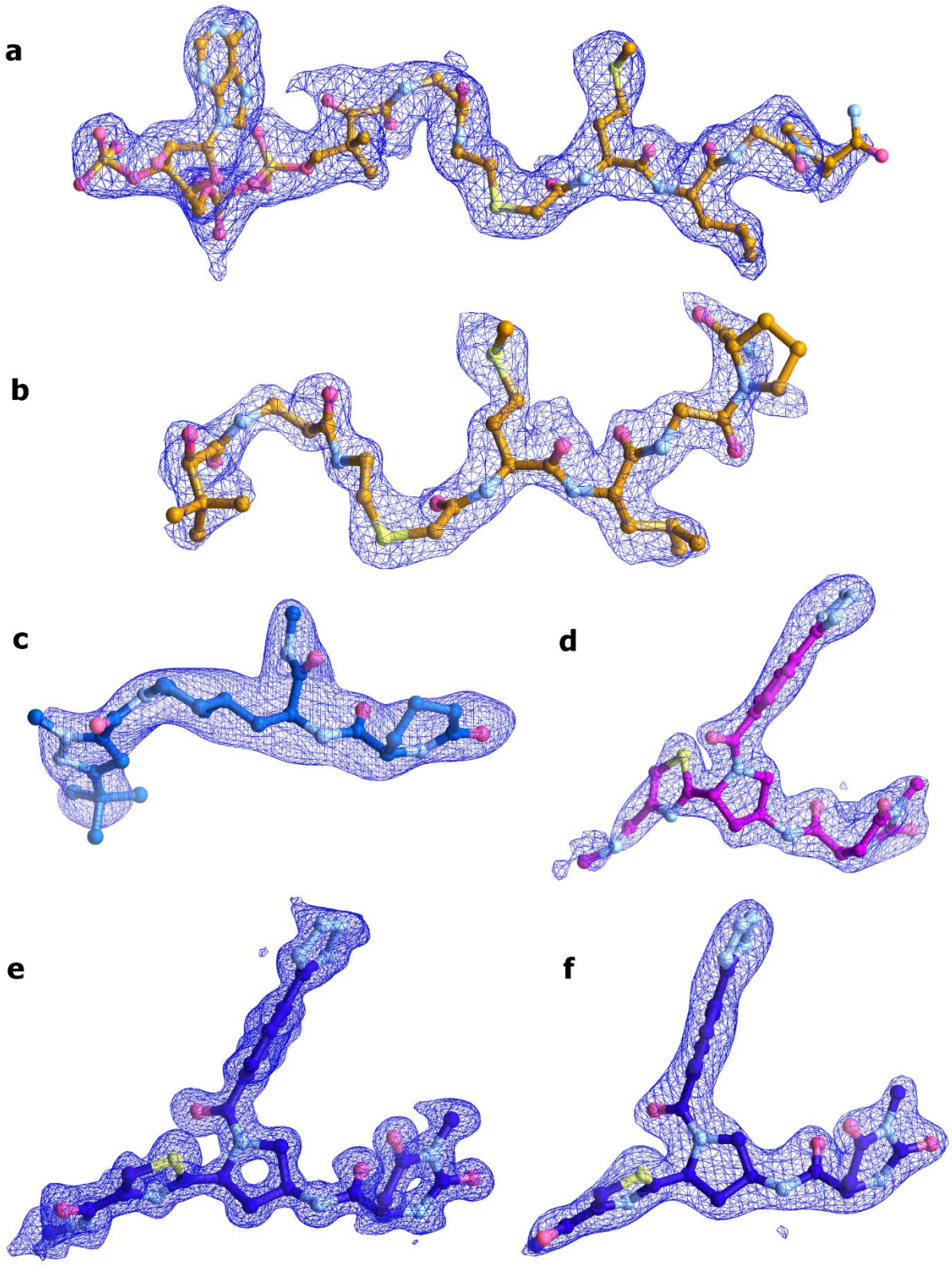
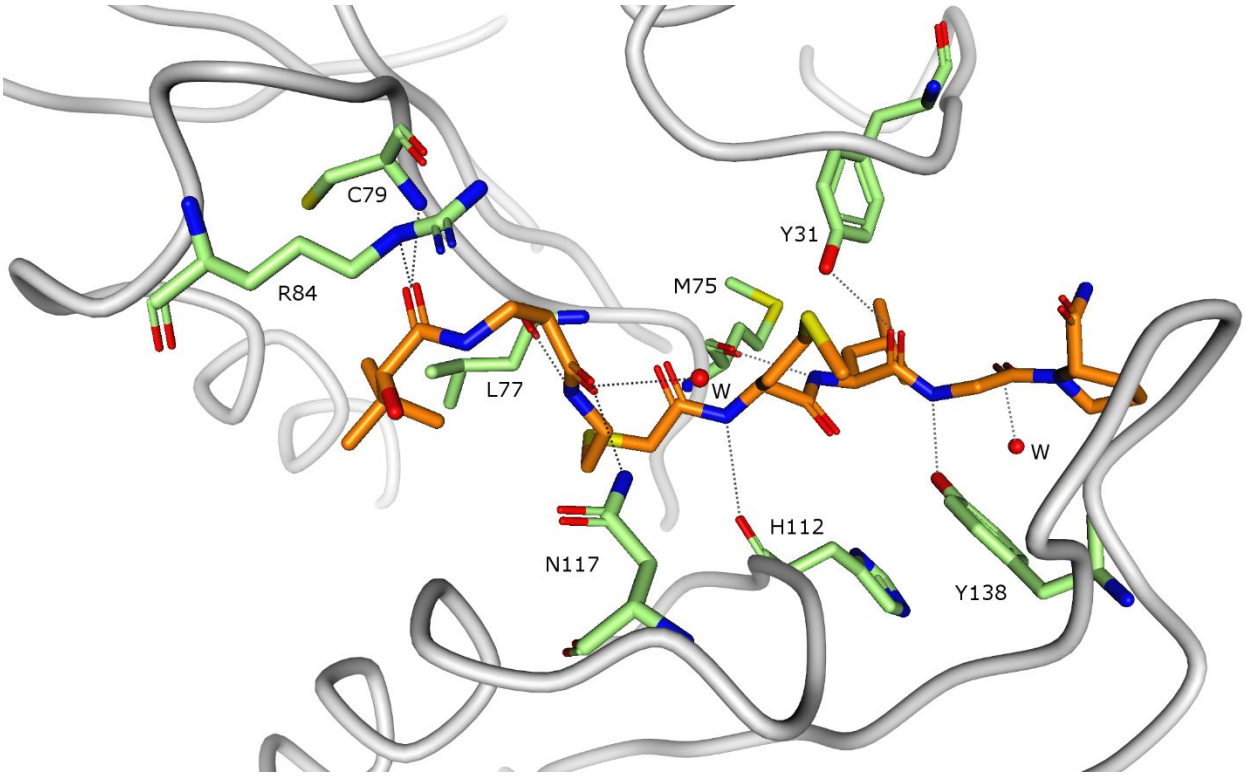

a
b
c
d
e
f

Figure S1. Co-crystal structure of 2 bound to Naa50 (2.0 Å, PDB ID 6WF5). Compound **2** (drawn as orange stick representation) binds in a very similar manner as compound 1, making similar interactions with Naa50. Hydrogen bonds are represented as dashed lines.



DEL951 library synthesis scheme (Scheme S1):

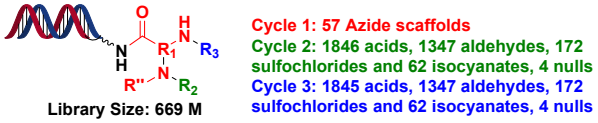
The synthesis began with the introduction of the 57 azide-containing scaffolds. Each of the azide-containing scaffolds contains a carboxylic acid moiety that can be used as a handle to connect with the DNA headpiece via an amide bond. After pooling and de-protection of the amine protecting groups of the scaffolds, the reaction was splitted into separate reaction wells. The cycle 2 building blocks (including 1846 acids, 1347 aldehydes, 172 sulfonyl chlorides and 62 isocyanates) were introduced via various on-DNA reaction settings. After standard pooling, purification via spin filtration, and splitting into different reaction wells, the azide group was reduced using triphenylphosphine and the third building blocks (including 1845 acids, 1347 aldehydes, 172

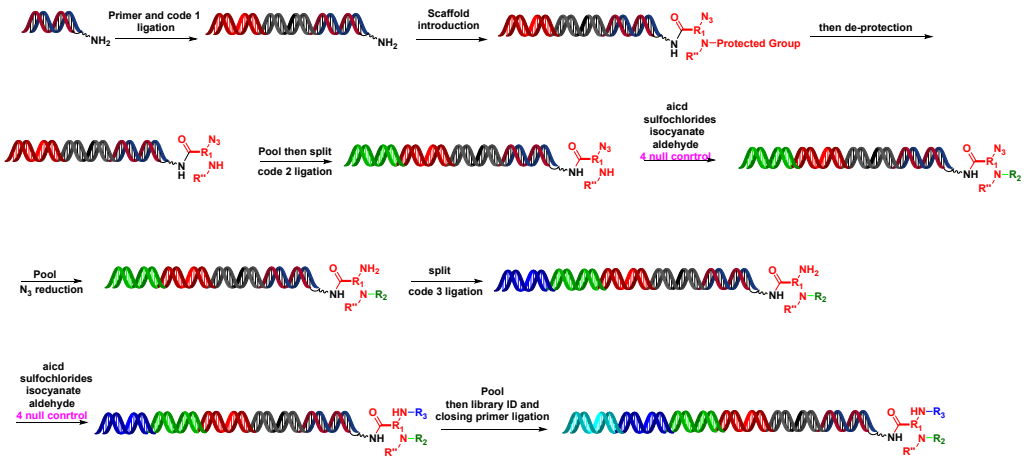
sulfonyl chlorides and 62 isocyanates) were then reacted with the amino group to provide the final library with a size of 669 M compounds.

Library affinity selection procedure:

Selection was carried out using a KingFisher Duo Prime Purification System in 96 well plate. 250 pmol His-tagged Naa50 was incubated with HitGen DELs in the presence or absence of tool compounds (CoA or compound **1**) in 100 μL selection buffer(50 mM Tris-HCl, 50 mM KCl, 1 mM DTT, 0.05% Tween 20, 10 mM imidazole, 0.3 mg/mL sheared salmon sperm DNA pH 7.5) at RT for 1h. His-tagged Naa50 along with DEL binders were captured by Ni-charged MagBeads by incubation at RT for 0.5h. Then the immobilized protein along with the beads was washed for 1 min at RT in 100 μL selection buffer to reduce weak or non-binders. Retained DEL members were recovered by heat elution in 80 μL of elution buffer (50 mM Tris-HCl, 50 mM KCl pH 7.5) at 75 °C for 15 min. After the first round of selection, another two rounds of selection were repeated. The heat eluted portion of each round was used as the input to the successive round with fresh protein. After each round, the output was quantified by qPCR. When total DNA output was brought down to 10^8 copies, the selection was completed and the output was amplified by PCR and further purified by Qiagen PCR purification kit according to the manufacture's instruction. Then sequencing was performed for PCR amplified samples on an Illumina HiSeq 2500.

Scheme S1. Synthesis of three-cycle DNA encoded library





Conditions for Cycle 1 chemistry: borate buffer (pH = 9.4, 250 mM), 80 equivalents of scaffolds (200 mM DMA stock), 80 equivalents of DMT-MM (200 mM solution in water), 25 degrees.

Conditions for deprotection of Cycle 1 products:

Removal of Boc proctection: borate buffer (250 mM, pH = 9.4) at 90 degrees, overnight.

Removal of Fmoc protection: 10% volume piperidine 1h at 25 degrees

Conditions for Cycle 2 chemistry:

Acids: BBS buffer (250 mM, pH = 9.4), acid building blocks (200 mM in DMA,100 eq.), DMT-MM (200 mM solution in water), 100 eq, 25 degrees, overnight.

Sulfonyl chlorides and isocyanates: BBS buffer (250 mM, pH = 9.4)， sulfonyl chlorides or isocyanates(0.5 mM in ACN, 100 eq.), 25 degrees, overnight.

Aldehyde: PBS buffer (150mM, pH = 5.5), aldehyde (200 mM in DMA, 100 eq.). NaCNBH3 (200 mM in DMA, 100 eq.), 60 degrees, overnight

Conditions for Reduction of azido groups:

BBS buffer (250 mM, pH = 9.4), PPh3 (60 eq, 200 mM in DMF), 60 degree, overnight

Conditions for Cycle 3 Chemistry:

Acids: BBS buffer (250 mM, pH = 9.4), acid building blocks (200 mM in DMA,100 eq.), DMT-MM (200 mM solution in water), 100 eq, 25 degrees, overnight.

Sulfonyl chlorides and isocyanates: BBS buffer (250 mM, pH = 9.4), sulfonyl chlorides or isocyanates (0.5 mM in ACN, 100 eq.), 25 degrees, overnight.

Aldehyde: PBS buffer (150mM, pH = 5.5), aldehyde (200 mM in DMA, 100 eq.). NaCNBH3 (200 mM in DMA, 100 eq.), 60 degrees, overnight

Figure S2.

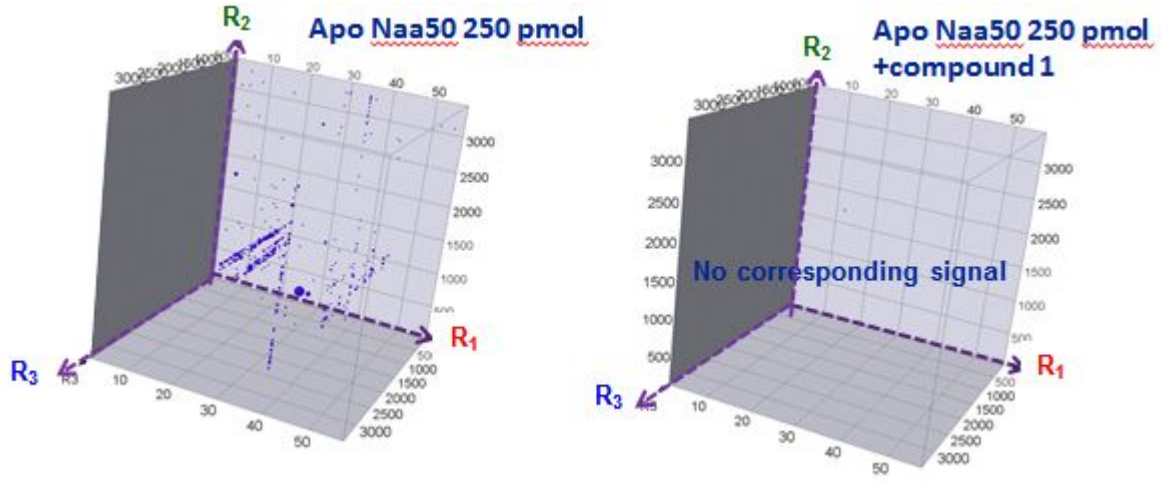


Figure S3.

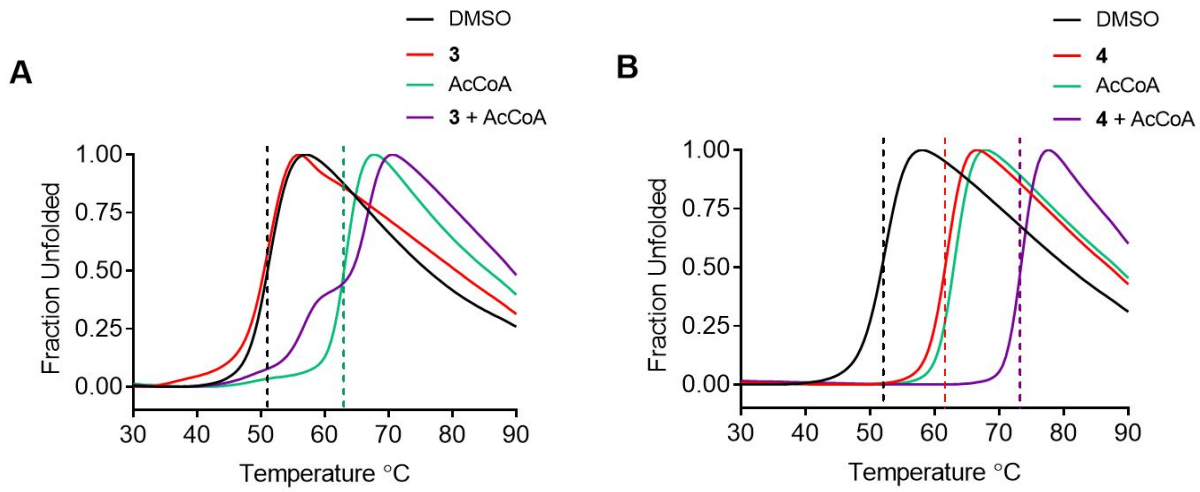
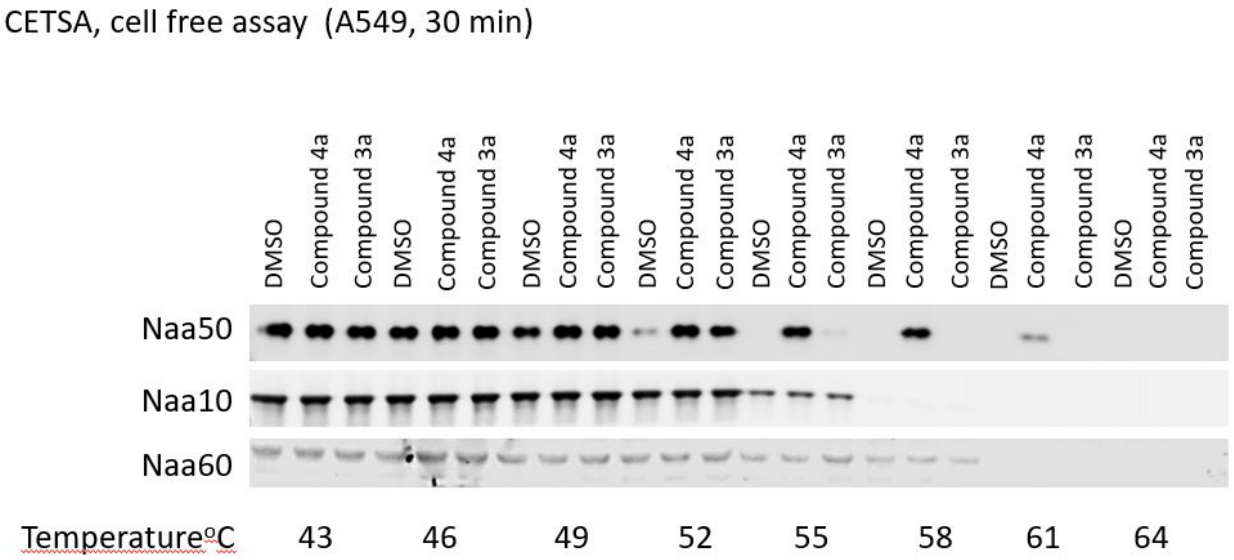


Figure S3. Thermal stability of recombinant Naa50 protein in presence of DMSO or compounds (acetyl CoA, **3** and **4**) as measured by differential scanning fluorimetry (DSF).

Differential Scanning Fluorimetry (DSF):

The protein thermal stability measurements were performed in a 384 well plate (BioRad HSP3865) using a standard thermocycler BIORAD CFX384 RT-PCR. Briefly, recombinant Naa50 protein at 0.1mg/mL concentration was incubated for 15 min with DMSO or compounds at 250 µM concentration in an assay volume of 20 µL. Sypro orange dye (5X final concentration) was added and the change in the dye fluorescence was monitored over the temperature range of 15 to 95 °C. Raw experimental data was analyzed as described previously (Huynh K. et al. *Curr. Protoc. Protein Sci.* **2015**). Areas of the curve with negative slopes (protein aggregation and precipitation phase) were excluded from analysis. The T_m values were obtained by non-linear fitting the data to a Boltzmann Sigmoidal curve. The ΔT_m values were calculated by subtracting the T_m of protein in presence of DMSO from the T_m under test conditions.

Figure S4a.



Cellular thermal shift assay (CETSA): The assay was performed based on a modified protocol for the cell lysate CETSA experiments. In brief, cultured A549 cells ($2.5x10^7$) were harvested and washed with PBS and resuspended in 500 µl PBS supplemented with complete protease inhibitor cocktail plus PMSF. The cells were lysed by three freeze-thaw cycles and clarified by centrifugation at 20,000g for 20 min at 4°C. The soluble fraction (lysate) were then treated with vehicle or 50 µM of compound 3a or compound 4a for 30 min at RT. Aliquots of 50 µl from each condition were distributed into PCR strip tubes and heated at 40, 43, 46, 49, 52, 55, 58, 61 and 64°C for 3 min before being cooled to RT for another 3 min. The supernatants were analyzed by immunoblotting after the heated lysates were centrifuged at 20,000g for 20 min at 4°C

Figure S4b.

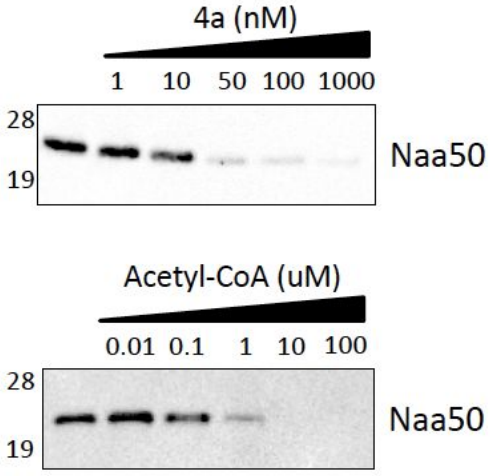


Fig. S4b. Competitive chemoproteomic profiling of **4a**. HeLa cell lysates were pre-incubated with **4a** (inhibitor), acetyl-CoA (control), or vehicle buffer for 1 h prior to capture with Lys-CoA Sepharose. Decreased band intensity indicates increased competition with the NAA50 active site by **4a** or acetyl-CoA.

Preparation of Lys-CoA Sepharose resin

Lys-CoA Sepharose (**1**) was prepared using NHS-Activated Sepharose 4 Fast Flow resin essentially according to the manufacturer's protocol (GE Healthcare Life Sciences, Instructions 71-5000-14 AD) [1]. Briefly, amine-functionalized Lys-CoA-Ahx was prepared as a 3.4 mM solution in PBS. Resin was washed with cold 1 mM HCl prior to coupling, before addition of the ligand solution at a ratio of 2:1 resin:ligand volume. The pH was adjusted to ~7-8 by addition of 20x PBS, and the mixture was then rotated at 4°C overnight. The resin was pelleted at 1400 rcf for 3 minutes, and the supernatant was discarded prior to addition of 3 resin volumes of 0.1 M Tris-HCl [pH 8.5], and the mixture was rotated for 3 hr at room temperature. Resin was washed 3x each

with alternating solutions of 0.1 M Tris-HCl [pH 8.5] and 0.1M Sodium Acetate, 0.5 M NaCl [pH 4.5] (6 washes total). Resin stored as a 33% solution in aqueous 20% EtOH at 4°C.

Procedure for Lys-CoA affinity capture, competition and Western Blot analysis

Affinity capture using Lys-CoA Sepharose was carried out essentially as previously reported[1,2]. Briefly, 33 µl of capture resin was washed once with 1 ml of PBS, prior to addition of 500 µl of clarified lysates (1.5 mg/ml, pretreated with vehicle or competitor ligand for 30 min on ice). This mixture was rotated for 1 hr at room temperature, pelleted at 1400 rcf, and supernatant discarded. Sepharose capture resins were subjected to a series of mild washes using ice cold wash buffer (50 mM Tris-HCl [pH 7.5], 5% glycerol, 1.5 mM MgCl2, 150 mM NaCl, 3 × 500 µl). Following the last wash, enriched resin was collected on top of centrifugal filters (VWR, 82031-256). For Western blotting analysis of captured proteins, enriched resin was transferred from centrifugal filters to fresh 1.7-ml tubes using 400 µl of tryptic digest buffer (50 mM Tris-HCl [pH 8.0], 1 M urea). For immunoblot analysis, proteins were eluted from resin via addition of 40 µl 1Å~ SDS sample buffer. Samples were boiled for elution from resin for 10 min at 95°C, and two elutions were performed to maximize KAT capture. Filtrates from both elutions were combined and loaded onto a 4-12% SDS-PAGE gel. After transfer to western blot, membranes were blocked, probed with anti-AT antibodies (NAA10, Genetex # NBP2-3394; NAA50, Bethyl # A305-594A-M), washed, and developed according to the manufacturer's protocol.

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Figure S5. Binding of 3a and 4a in the MLGP peptide pocket of Naa50 (6WFG and 6WFK respectively). Super position of **2** (orange stick) vs **3a** (panel a, cyan stick) and **4a** (panel b, magenta stick) in the catalytic site of Naa50 protein showing that **3a** and **4a** bind in the peptide pocket.

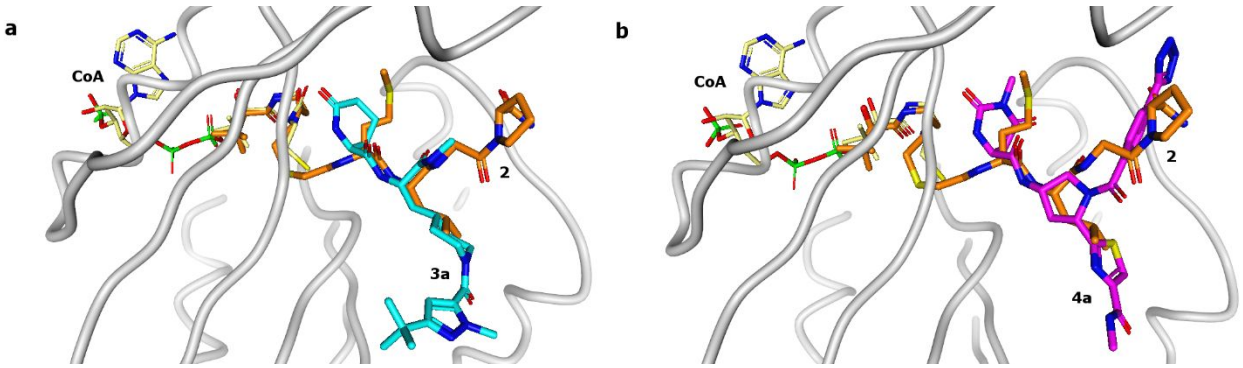
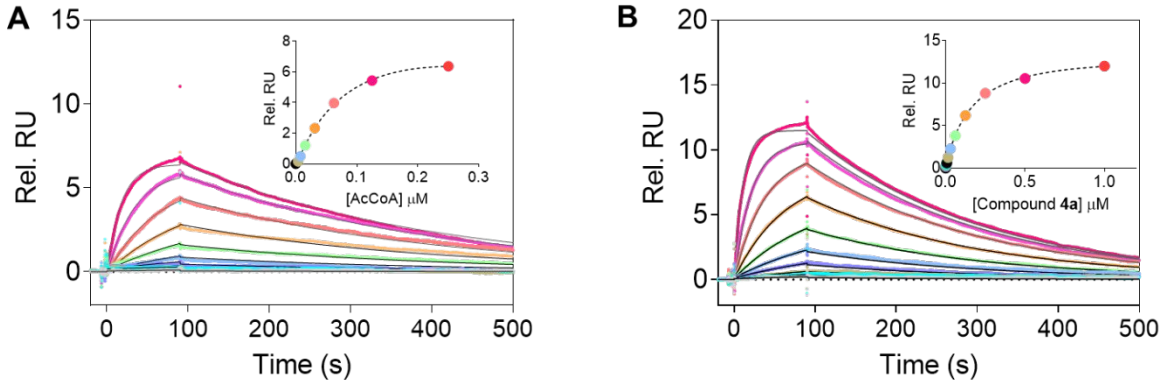


Figure S6.



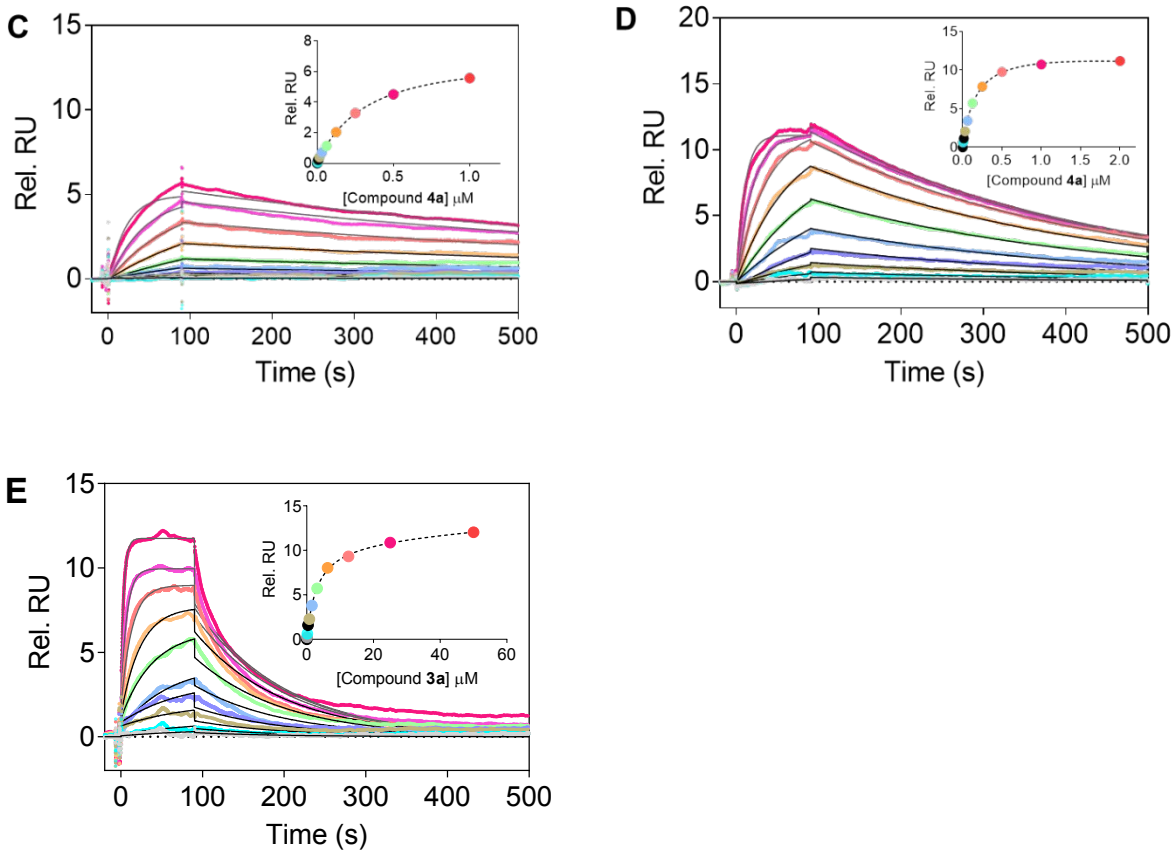


Figure S6. Measurement of binding kinetics using SPR. **A**) AcCoA binding to Naa50_apo immobilized onto SPR chip *via* amine coupling; **B**) **4a** binding to Naa50_apo immobilized onto SPR chip *via* amine coupling; **C**) **4a** binding to Naa50 complexed with AcCoA by inclusion of AcCoA (1 µM; ~60xK_D) in the SPR buffer; **D**) **4a** binding to Naa50 complexed with CoA by inclusion of CoA (10 µM; ~60xK_D) in the SPR buffer. **E**) **3a** binding to Naa50 complexed with AcCoA by inclusion of AcCoA (1 µM; ~60xK_D) in the SPR buffer

Measurement of binding kinetics using SPR: Biacore T200 or S200 instrument was desorbed and loaded with a new Series S Sensor Chip CM5. The surface of ligand and reference channel was activated at 10 ºC using a single injection of 0.4M EDC/ 0.1M NHS mixture (1:1) for 7 minutes using a flow rate of 10 µL. The recombinant Naa50 protein was diluted to 100 µg/mL with assay buffer (25 mM HEPES, 150 mM NaCl, 1 mM TCEP, 5% Glycerol, 0.02 % Tween-20, 1% DMSO, pH 7.4 with/without 1 µM Acetyl CoA or 10 µM CoA and injected into ligand channel at a flow rate of 5 µL/min and a contact time of 15 min at 10 ºC. This was followed by two 1-

minute injections of 1M ethanolamine diluted to 50% using the assay buffer. Using this procedure, ~ 34500 RU of Naa50 was captured on CM5 surface via amine coupling chemistry. The functionalized surface was then equilibrated with assay buffer containing 1 µM Acetyl CoA or 10 µM CoA for approximately 1 hour to form either Naa50•Acetyl CoA or Naa50•CoA complex. For studies with Naa50_apo, the equilibration step was performed with assay buffer without AcCoA or CoA. An un-functionalized, ethanolamine-blocked channel was used as a reference surface for binding kinetic analysis. A two-fold, 10-point serial dilution of test compounds was set-up in a 96-well microplate (Greiner; Cat # 650101). Binding kinetics was measured at 10 ºC in a multi-cycle kinetics format by injecting serial dilution of compounds onto reference and ligand channel at a flow rate of 100 µL/min and association time of 90 seconds. Compound dissociation was monitored for 600 seconds. Two buffer blanks were also run before each compound run for double referencing. No additional regeneration was used. DMSO calibration curve was obtained before and after compound analysis by injecting 0-2% of DMSO in running buffer. Data analysis was performed using Biacore T200/S200 evaluation software. The double-referenced and solvent-corrected data was fit to 1:1 Langmuir model to obtain binding constant (K_D) and binding kinetics (k_{on} and k_{off}) information.

Figure S7. Crystal structures of Naa50 bound to cofactors and the influence of 4a binding. Panel (a). Naa50•AcCoA without an inhibitor. Acetyl group of AcCoA hydrogen bonds to backbone of Gln114 and sidechain of Asn117. Terminal amide binds to the sidechain of Asn117 and is 3.5Å from the carbonyl of Leu77. Resolution = 2.85 Å (in-house structure was very similar to the PDB structure ID 2OB0). Panel (b). Naa50•CoA without an inhibitor. Terminal amide binds to the sidechain of Asn117 and the carbonyl of Leu77. Resolution = 2.43 Å (in-house structure was very similar to the PDB structure ID 2PSW). Panel (c). Naa50•AcCoA in presence

of **4a (PDB ID 6WFN)**. Acetyl group of AcCoA forms a 3.1 Å hydrogen bond to NH of Leu77. Terminal amide binds to the sidechain of Asn117 and the carbonyl of Leu77. Inhibitor **4a** hydrogen bonds to backbone NH of Gln114. Resolution = 1.07 Å. Panel (d). Naa50•CoA in presence of **4a (PDB ID 6WFK)**. Terminal amide is 3.2 Å from carbonyl of Leu77. Inhibitor **4a** hydrogen bonds to backbone NH of Gln114. Resolution = 1.87 Å.

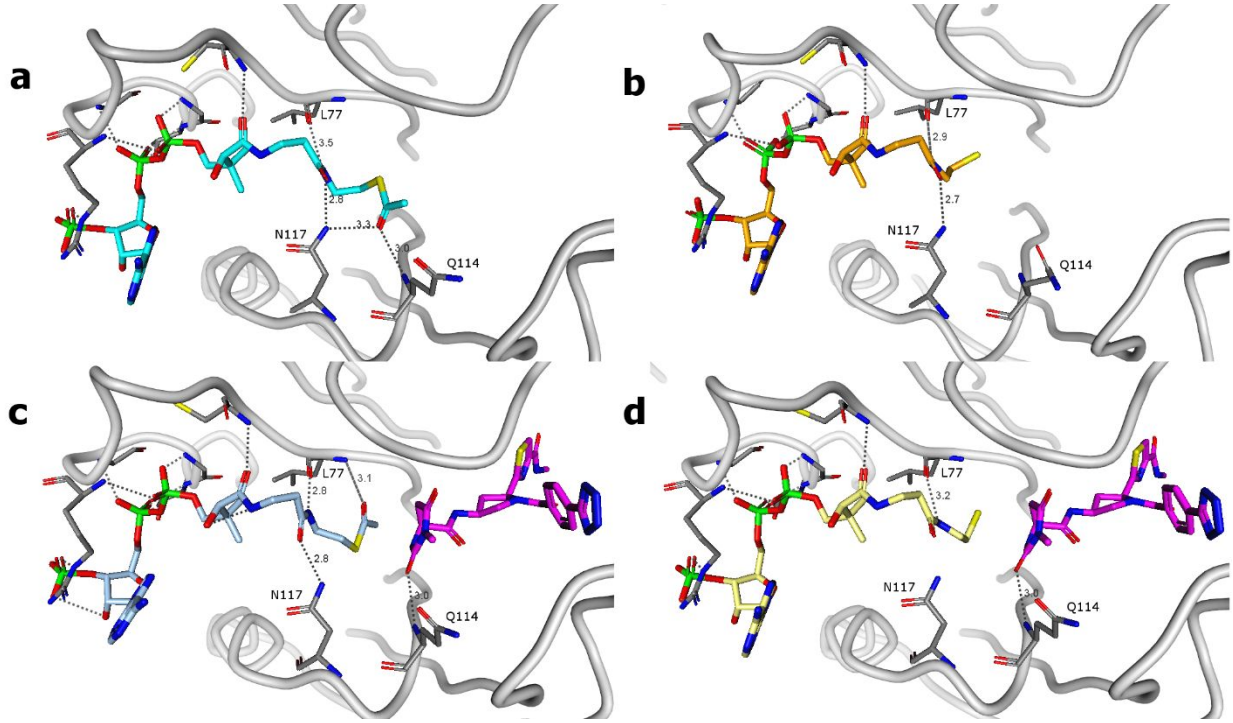
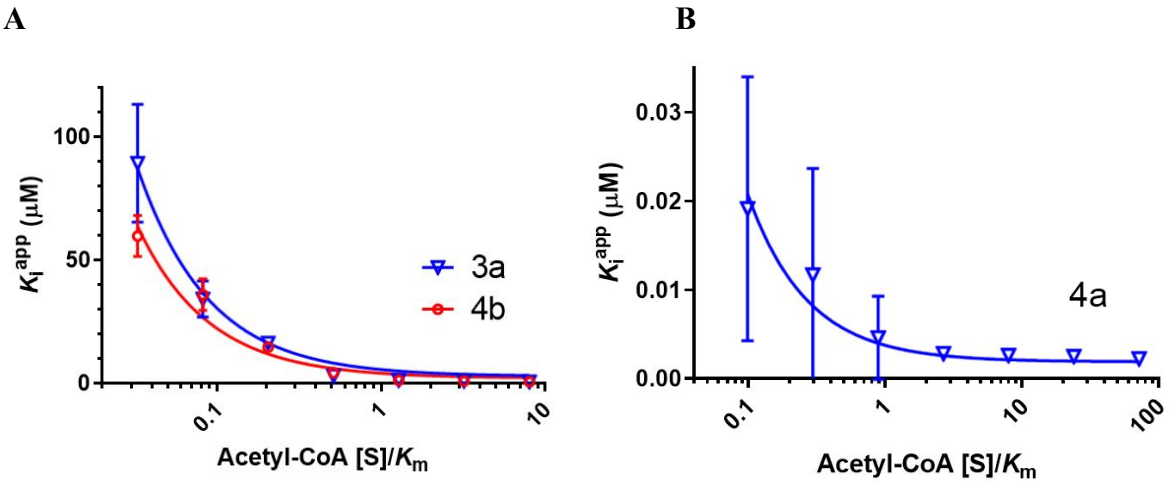


Figure S8. Mode of Naa50 inhibition by DEL inhibitors with respect to AcCoA.

(A) Naa50 apparent K_i (K_i^{app}) dependence on AcCoA with compounds **3a** and **4b**. The data were fit to an uncompetitive inhibition equation, $K_i^{app} = K_i*(1+K_m/S)$, yielding $K_i = 2.8 \pm 0.1$ and 2.0 ± 0.1 μM, respectively. (B) Naa50 K_i^{app} dependence on AcCoA with compound **4a**. The data were fit as above, yielding a $K_i = 0.0019 \pm 0.0001$ μM. (C) Mode of inhibition analysis by compound **4b** with respect to AcCoA. The data were globally fit to an uncompetitive inhibition equation as a preferred model, producing the best fit parameters, $\alpha K_i = 0.91 \pm 0.08$ and $K_m = 2.1 \pm 0.1$ μM. (D)

Mode of inhibition by compound **3a** with respect to AcCoA. The data were globally fit to an uncompetitive inhibition equation as a preferred model, producing the best fit parameters, αK_i = 0.75 ± 0.08 and K_m = 2.1 ± 0.2 μM. The experiments were performed as described in the Supplementary methods. The parameters derived by fitting the data to the appropriate equations are reported with standard errors of the fits.



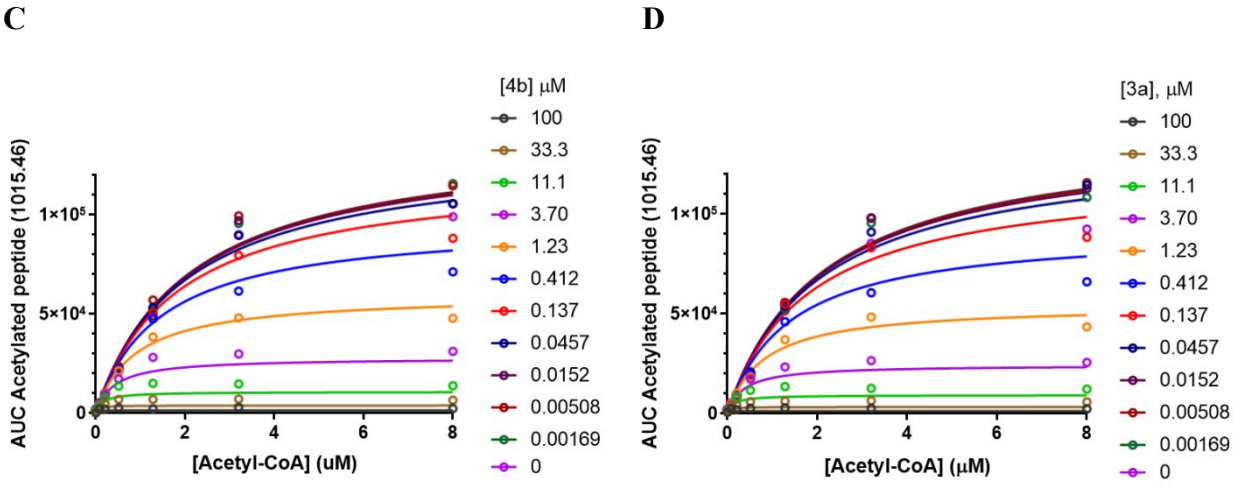
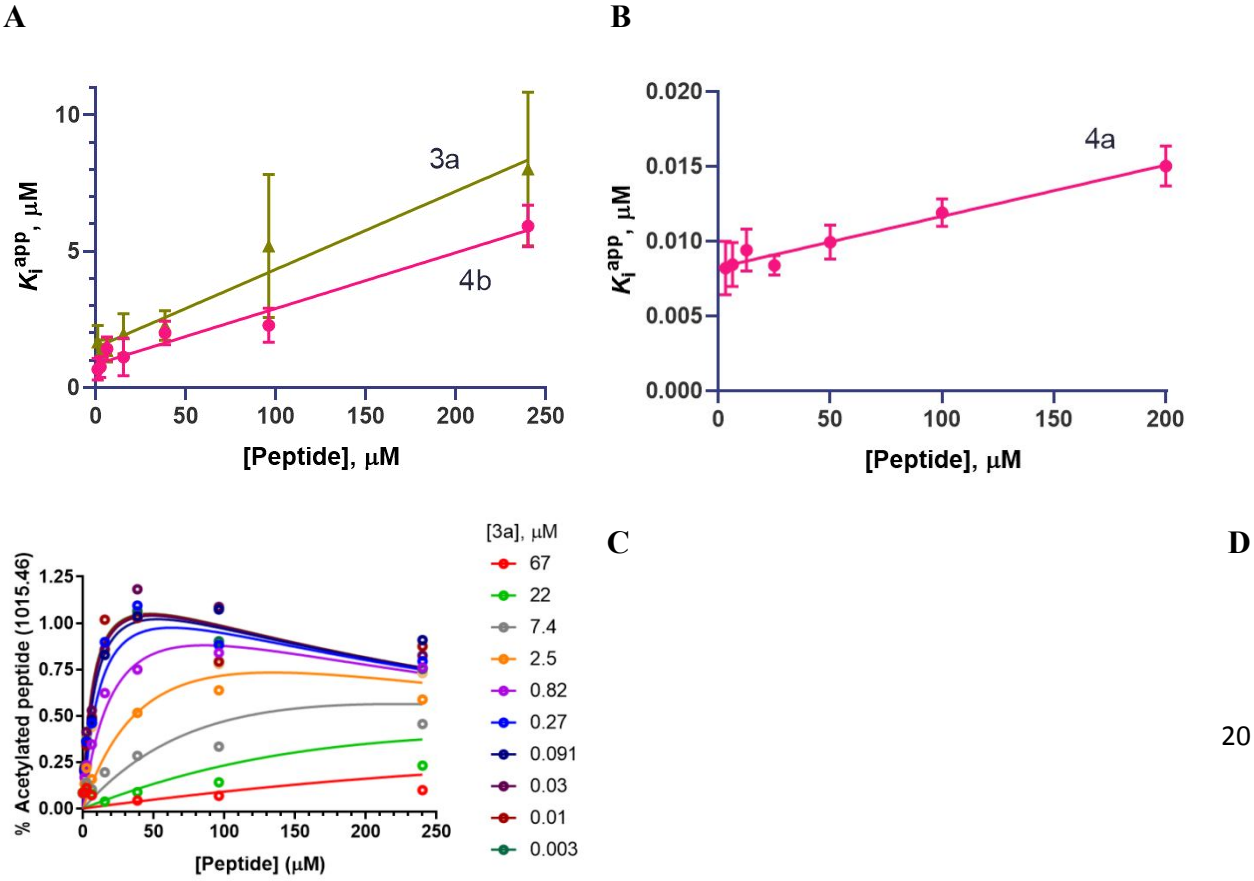


Figure S9. Mode of Naa50 inhibition by DEL inhibitors with respect to 10-mer peptide substrate.

(A) Naa50 apparent K_i (K_i^{app}) dependence on peptide substrate with compounds **3a** and **4b**. The data were fit to a linear equation, yielding *y*-intercepts 0.86 ± 0.17 and 1.48 ± 0.24 μM, and slopes 0.020 ± 0.002 and 0.029 ± 0.002, respectively. (B) Naa50 K_i^{app} dependence on peptide substrate with compound **4a**. The data were fit to a linear equation, yielding a *y*-intercept 0.0083 ± 0.0002 μM and a slope $(3.4 \pm 0.3)\times10^{-5}$. (C) Mode of inhibition analysis by compound **3a** with respect to peptide. The data were globally fit to a mixed inhibition model with substrate inhibition. Best fit parameters: α = 8.6 ± 4.2, K_m = 6.9 ± 0.9 μM, K_i = 0.81 ± 0.23 μM and K_{is} = 420 ± 80 μM. (D) Mode of inhibition analysis by compound **4b** with respect to peptide. The data were globally fit to a mixed inhibition model with substrate inhibition, as described in the Supplementary methods, to generate α = 3.7 ± 2.3, K_m = 4.4 ± 0.7 μM, K_i = 0.88 ± 0.40 μM and K_{is} = 4.5 ± 6.5 μM. The experiments were performed as described in the Supplementary methods. The parameters derived by fitting the data to the appropriate equations are reported with standard errors of the fits.



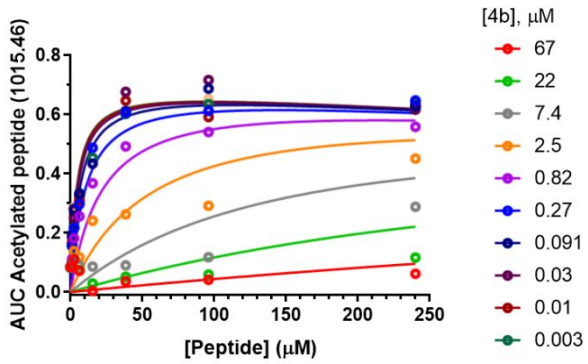


Table S2. Inhibition of Naa50 by DEL compounds 4a, 4b and 3a using variously truncated versions of the native protein substrate, hnRNP F.

DEL inhibitor	IC_{50} (μM) with hnRNP F derived substrates		
	aa 1-10	aa 1-102	aa 2-415
4a	0.010	0.014	0.020
3a	2.0	3.5	Not determined
4b	1.6	4.0	Not determined

Modes of enzyme inhibition predicted from inhibition kinetics.

Enzyme inhibition by the weaker DEL inhibitors **3a** and **4b** produced a hyperbolic dependence on AcCoA concentration, characteristic of uncompetitive inhibition (Fig. S8, A). The potent inhibitor, **4a**, produced a similar dependence, but a less reliable data fit, possibly complicated by inhibition in the tight-binding range (Fig. S8, B). The global fits of the initial rates to a competitive, non-competitive, mixed or uncompetitive inhibition models, also indicated an uncompetitive model

with respect to AcCoA to be the preferred one (Fig. S8, C-D). When concentration of peptide substrate (N-terminal 10-mer, derived from the N-terminal sequence of the native protein substrate, hnRNP F) was varied along with inhibitor concentration, an increase in apparent inhibition constant (K_i^{app}) values with peptide concentrations was observed, consistent with competitive inhibition for compounds **3a** and **4b** (Fig. S9, A) and **4a** (Fig. S9, B). A more detailed analysis was conducted for the two weaker inhibitors, by systematically varying peptide concentrations at different inhibitor concentrations and fitting the data to different equations (Fig. S9, C-D). The model discrimination analysis yielded a mixed inhibition mode as the preferred one, with the α parameter calculated to be 3.7 and 8.6 for compounds **4b** and **3a**, respectively, behaving predominantly as competitive inhibitors with respect to peptide substrate, consistent with the structure analysis of the binding site topography of these DEL inhibitors.

Since biochemical inhibition studies were conducted using a relatively short peptide substrate derived from hnRNP F, we compared inhibition data for select compounds (**4a**, **3a**, **4b**) using larger hnRNP F truncations with the amino acid boundaries 1-102 and 2-415 (end). The IC_{50} values for these inhibitors using 10-mer, 102-mer and 2-415 construct (nearly full-length protein, missing the N-terminal methionine), were within 2-fold (Table S2), suggesting that processing of and competitive inhibition of Naa50 by DEL compounds with respect to hnRNP F appears to be mostly dependent on the presence of the short N-terminal sequence.

Enzyme Assays

Recombinant human wild-type Naa50 (amino acids 1852-2082) was produced in-house using insect cell line expression, as described (see Supplemental protein expression and purification procedures). The protein was purified with most enzyme molecules loaded with Acetyl-CoA.

Enzymatic activity of Naa50 was tested with variously truncated versions of the native protein substrate, hnRNP F. The 10-mer peptide, MLGPEGGEGK (CPC Scientific, Sunnyvale, CA), derived from the N-terminal sequence of the protein was the primary construct used for both enzymatic characterization of the target, as well as screening targeted compound libraries and for DEL follow-up screening. Two truncated versions of the full-length protein, amino acids 1-102 and 2-415, were prepared via baculoviral expression and used as substrates to cross-verify IC_{50} values for select compounds. Standard screening reactions were conducted in 96-well plates in 100-µL volumes and contained 50 nM wild-type Naa50, 20 µM 10-mer peptide substrate (~2x K_m), 5 µM Acetyl-CoA (~2x K_m), 15 mM NaCl, 1 mM DTT in 50 mM Tris-HCl, pH 7.5, buffer. The reactions were incubated for 75 min at a controlled RT, stopped by the addition of 2.2% formic acid, plates were sealed and analyzed on a RapidFire-MS/MS instrument (Agilent Technologies, Santa Clara, CA), as described below under the mass-spectrometry assay. The reactions were shown to be linear with time, and initial reaction velocities were measured based on 20-25% conversion of Acetyl-CoA to CoA and 5-6% conversion of peptide substrate to acetylated peptide. For competition studies, either Acetyl-CoA or 10-mer peptide concentrations were varied in a matrix against varying concentration of the inhibitor of interest. The IC_{50} values were determined by fitting % conversion values to a four-parameter equation with GraphPad Prism (GraphPad Software, Inc., San Diego, CA). The inhibition constants (K_i) values were calculated from peptide conversion by comparing global fits for competitive inhibition, noncompetitive inhibition, uncompetitive inhibition, and mixed inhibition equations (GraphPad Prism; GraphPad Software). In the case of peptide competition studies, inhibition of enzyme by the 10-mer peptide substrate at higher peptide concentrations was accounted for, using the non-hyperbolic Michaelis-Menten equation (Eq. 1), which accounts for binding of the second, inhibitory, molecule of peptide

substrate[1]. The parameters of Eq. 1 were defined and constrained as described below for the mixed inhibition equation, Eq. 2, also known as a general noncompetitive inhibition equation (Ref. 1), used to discriminate between modes of inhibition models.

$v = V_{max}*S/(K_m + S*(1+S/K_{is}))$ (Eq. 1),

v is the reaction velocity (expressed as peptide substrate % conversion),

V_{max} is the maximum enzyme velocity in the absence of inhibitor,

S is peptide substrate concentration,

K_m is apparent Michaelis constant,

K_{is} is inhibition constant for the inhibitory substrate.

The above equation was further constrained in the presence of test compound (inhibitor) using the mixed inhibition equation:

$v = V_{max}^{app}*S/(K_m^{app} + S)$ (Eq. 2),

where $V_{max}^{app} = V_{max}/(1+I/(\alpha*K_i))$,

$K_m^{app} = K_m*(1+I/K_i)/(1+I/(\alpha*K_i))$,

V_{max}^{app} is the maximum apparent enzyme velocity in the absence of inhibitor,

K_m^{app} is apparent Michaelis constant,

I is inhibitor concentration (test compound),

K_i is inhibition constant of a test compound,

α is the factor, reflecting the effect of the inhibitor on the affinity of the enzyme for its substrate and vice versa ($0 < \alpha < 1$ indicates preference for uncompetitive inhibition, $\alpha = 1$ reflects a classic non-competitive, and $\alpha > 1$ reflects a prevalent competitive inhibition). When fitting the data to the substrate inhibition Eq. 1, the parameters $V_{max,}$ K_m, α, K_i, and K_{is} were shared between the data sets, and I was constrained to equal a data set constant.

Apparent inhibition constants (K_i^{app}) were determined by fitting initial reaction velocities of Naa50 at various inhibitor doses to a Morrison equation[2], using enzyme active site concentration as a parameter, experimentally determined by titration with compound **4a** (58% active site content in the enzyme batch used).

Mass-spectrometry assay

The Naa50 enzyme reactions, conducted as described in the enzyme assays methods, were analyzed by MS/MS following the RapidFire solid-phase extraction. An Agilent RapidFire 300 Mass Spectrometry system coupled with an Agilent 6550 iFunnel QTOF spectrometer (Agilent Technologies, Santa Clara, CA) was used to characterize enzymatic activity. For peptide detection, 42 µL reaction mix was injected onto a C18 cartridge in 0.01% trichloroacetic acid and 0.09% formic acid and eluted by buffer with 80% acetonitrile, 0.01% trichloroacetic acid and 0.09% formic acid. The RapidFire settings were as follows: aspiration time: 600 ms or until the loop is full per the sip sensor, load time: 3000 ms, elution time: 3000 ms, and re-equilibration time: 2000 ms at a flow rate of 1.25 mL/min. The Electrospray ionization multiple reaction monitoring positive polarity scan was used to detect the two desired analytes of the reaction, the substrate MLGPEGGEGK at m/z 973.45 and the product (Acetyl)-MLGPEGGEGK at m/z 1015.46. The QTOF was set to 1700mode 8/sec in Extended Dynamic range. The RapidFire Analyzer software was used to view chromatograms and manually derive peak integration for "Area Under the Curve," AUC, using RapidFire Integrator software (Agilent). For conversion of AUC to micromoles of acetylated product, standard curves were generated over a range of concentrations from 500 nM up to 50 µM. For competition studies, un-acetylated peptide was varied across Acetylated peptide standards to correct for ion suppression.

Detection of Acetyl-CoA and CoA involved injection onto an Agilent Graphite Type D cartridge with 5 mM ammonium acetate and eluted by 25% acetone, 25% acetonitrile, and 5 mM ammonium acetate. The RapidFire settings were as follows: aspiration time: 600 ms or until the loop is full per the sip sensor, load time: 3000 ms, elution time: 5000-8000 ms, and re-equilibration time: 500 ms at a flow rate of 1.5 mL/min. Acetyl-CoA and CoA were detected at m/z 809.57 and 767.54,

respectively. For conversion of AUC to pmoles of analyte, CoA standard curves were generated over a range of concentrations from 500 nM up to 5 μM.

References

1. Copeland, R.A. Evaluation of Enzyme Inhibitors in Drug Discovery, Second edition, Wiley 2013. ISBN: 978-1-118-48813-3.
2. Morrison, J.F., Kinetics of the reversible inhibition of enzyme-catalysed reactions by tight-binding inhibitors. *Biochim Biophys Acta* **1969**, *185*, 269-86.

Figure S10. Co-crystal structure of 4b with Naa50 protein (1.85 Å, PDB ID 6WFO). Panel (a**)**: Compound **4b** is shown in pink stick representation and makes similar hydrogen bond interactions with Naa50 (green stick residues with dashed lines) as was observed for compound **4a**. The cofactor CoA is drawn is yellow stick representation. Panel (b): Superposition of **4b** (pink stick) vs **4a** (magenta stick) bound to Naa50. The Connolly surface for the protein is displays in grey.

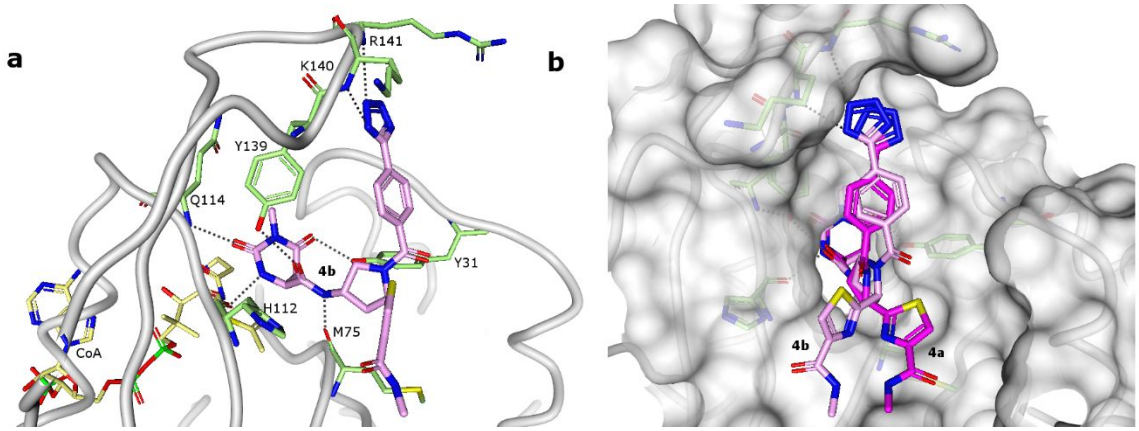
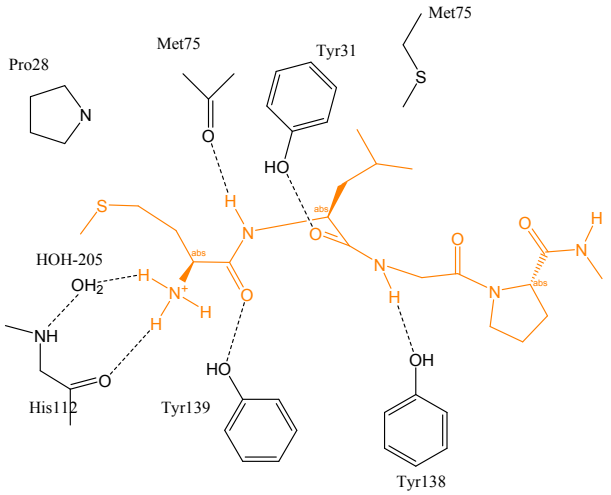


Figure S11 and S12. Comparison of 3a/4a interactions with MLGP peptide substrate interactions

The compounds **3a** (blue) and **4a** (magenta) make many of the same hydrogen bonds as the N-terminal MLGP peptide substrate (yellow, PDB 3TFY) or the MLGP portion of bi-substrate **1** (not shown). (Fig. S11). The unsubstituted carbons in the lactams in **3a** and **4a** are near the Met sidechain in the MLGP peptide (Fig. S12). The second peptide linkage is similar between the MLGP peptide and **3a** (hydrogen bonds to Tyr-31 and Tyr-138); however, the equivalent to the second peptide moiety is missing in **4a**, and in that case Tyr-31 serves as a donor to a lactam carbonyl introduced in **4a**. As far as other differences, the peptide substrate amino group forms an additional hydrogen bond to water. A lactam carbonyl in **3a** and **4a** makes a hydrogen bond to Gln-114 which does not have an equivalent in the substrate peptide. In **3a**, there is a hydrogen bond between Arg-62 and the piperidine amide. In **4a**, there is a hydrogen bond between His141 and tetrazole.



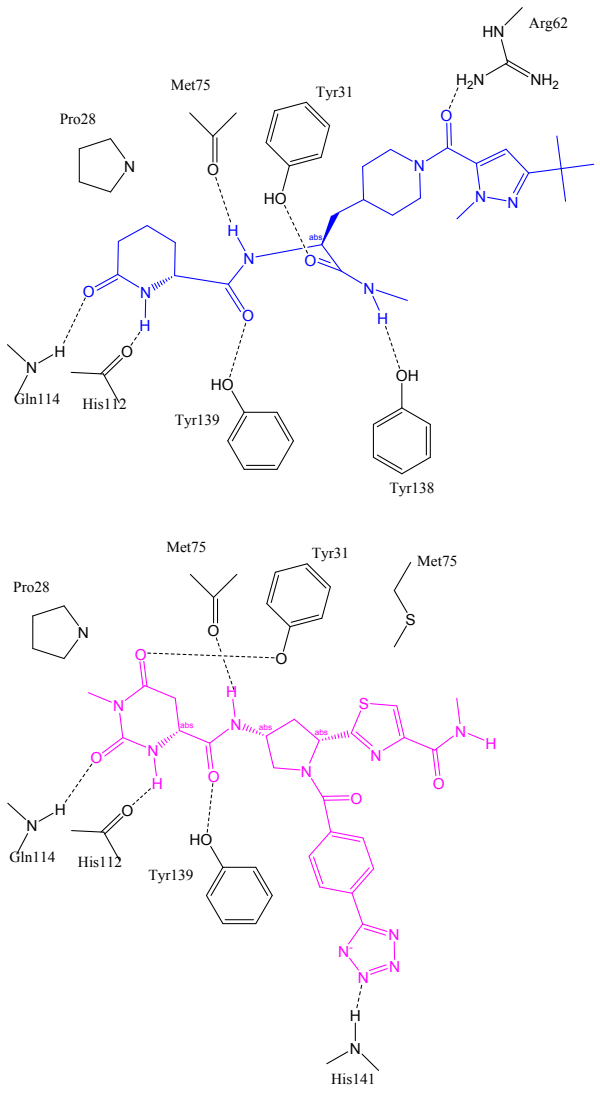


Figure S11: Schematic showing hydrogen bonds between Naa50 and the Peptide Substrate (orange), **3a** (blue) and **4a** (magenta).

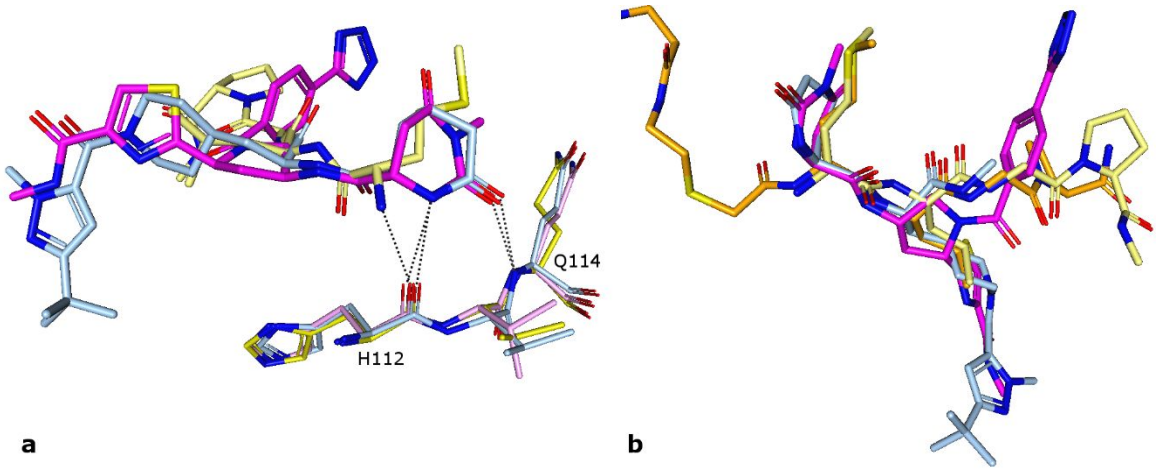


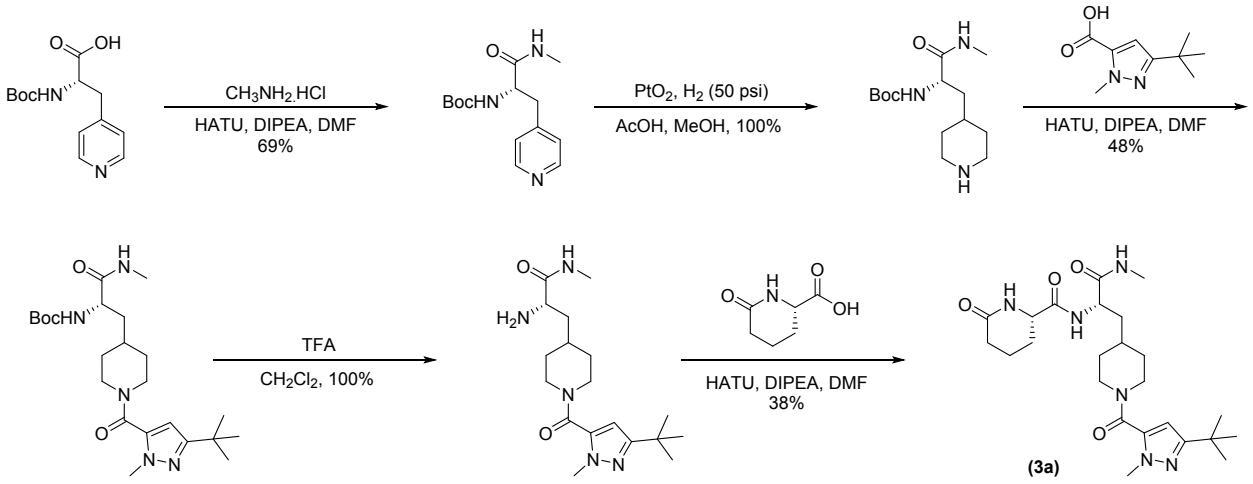
Figure S12: Naa50 co-crystal structure superposition showing relative locations of MLGP Peptide Substrate (yellow), **1** (orange), **3a** (blue) and **4a** (magenta). Panel (a): Shows the position of the lactam compared to the Met side chain and the differential hydrogen bonds to His-112 and Gln-114. The N-terminal amine is similar in location for MLGP and **1** (not shown), and for **3a** (blue) and **4a** (N-Me near the Met C_{ε} position). These amines make the hydrogen bond to His112 (stick) from different sides of the carbonyl. Panel (b): There is a common location of the first peptide linkage for 4 compounds and the second peptide linkage for 3 compounds. The glycine and proline of MLGP and **1** are oriented differently.

Chemistry – Experimental Methods

Starting materials and other reagents were purchased from commercial suppliers and were used without further purification unless otherwise indicated. All reactions were performed under a positive pressure of nitrogen, argon, or with a drying tube, at ambient temperature (unless otherwise stated), in anhydrous solvents, unless otherwise indicated. Analytical thin-layer chromatography was performed on glass-backed Silica Gel 60_F 254 plates (Analtech (0.25 mm)) and eluted with the appropriate solvent ratios (v/v). The reactions were assayed by high-performance liquid chromatography (HPLC) or thin-layer chromatography (TLC) and terminated as judged by the consumption of starting material, or as noted within the experimental write-ups. The TLC plates were visualized by UV, phosphomolybdic acid stain, or iodine stain. ^{1}H NMR spectra were recorded on a Bruker instrument operating at 400, 600 or 700 MHz. ^{1}H NMR spectra are obtained as DMSO-d_6, CD_3OD, D_2O, or $CDCl_3$ solutions as indicated (reported in ppm), using DMSO-d_6 (2.50 ppm), CD_3OD (4.78, 3.31 ppm), D_2O (4.80 ppm) or chloroform as the reference standard (7.25 ppm). Other NMR solvents were used as needed. When peak multiplicities are reported, the following abbreviations are used: s = singlet, d = doublet, t = triplet, m = multiplet, br = broadened, dd = doublet of doublets, dt = doublet of triplets. Coupling constants, when given, are reported in hertz. Many of the compounds reported herein present as mixtures of rotamers with the NMR spectra reported to reflect this. NMR spectra were processed using ACD/Labs 2017 Spectrus software. The mass spectra were obtained using liquid chromatography mass spectrometry (LC-MS) on an Agilent instrument using atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI). High resolution mass measurements were carried out on an Agilent TOF 6200 series with ESI. All test compounds showed > 95% purity as determined by combustion analysis or by high-performance liquid chromatography (HPLC). HPLC conditions

were as follows: XBridge C18 column @ 80 °C, 4.6 mm x 150 mm, 5 μm, 5%-95% MeOH/H_2O buffered with 0.2% formic acid/0.4% ammonium formate, 3 min run, flow rate 1.2 mL/min, UV detection (λ = 254, 224 nm).

Scheme S2 - Resynthesis of (3a)



tert-Butyl [(2S)-1-(methylamino)-1-oxo-3-(pyridin-4-yl)propan-2-yl]carbamate

To a suspension of *N*-(*tert*-butoxycarbonyl)-3-pyridin-4-yl-*L*-alanine (2 g, 7.51 mmol) and HATU (3.43 g, 9.01 mmol) in DMF (35 mL) was added DIPEA (6.54 mL, 37.6 mmol). The resulting mixture was stirred at room temperature for 30 minutes before methylamine hydrochloride (2.54 g, 37.6 mmol) was added. The reaction was stirred at room temperature for 72 hours after which time TLC (petroleum ether/(EtOAc/EtOH = 3:1) = 1:1) indicated that the starting material had been consumed. The reaction was poured into water (30 mL) and extracted with EtOAc (4 x 30 mL). The combined organic extracts were dried over Na_2SO_4, concentrated and the residue purified by flash chromatography (silica gel, 20 – 50% (EtOAc/EtOH = 3:1)/petroleum ether) to afford *tert*-butyl [(2*S*)-1-(methylamino)-1-oxo-3-(pyridin-4-yl)propan-2-yl]carbamate (1.45 g, 69%) as a yellow solid. MS (ESI), *m/z* 280.1 $[M+H]^+$ (calcd for C14H22N3O3 = 280.2). ^1H NMR (400 MHz,

CD_3OD) δ 8.44 (br d, *J* = 5.8 Hz, 2H), 7.34 (d, *J* = 6.0 Hz, 2H), 6.98 - 6.72 (m, 1H), 4.42 - 4.25 (m, 1H), 3.22 - 3.10 (m, 1H), 2.76 - 2.68 (m, 3H), 1.44 - 1.25 (m, 9H).

tert-Butyl [(2S)-1-(methylamino)-1-oxo-3-(piperidin-4-yl)propan-2-yl]carbamate

To a solution of *tert*-butyl [(2*S*)-1-(methylamino)-1-oxo-3-(pyridin-4-yl)propan-2-yl]carbamate (0.4 g, 1.43 mmol) in MeOH (5 mL) and AcOH (1 mL) was added PtO_2 (65 mg, 0.286 mmol) at room temperature. The mixture was degassed and refilled with N_2 twice, then degassed and refilled with H_2 twice. The mixture was stirred at room temperature for 16 hours under hydrogen pressure (50 psi). TLC (CH_2Cl_2/MeOH = 10:1) indicated the reaction was completed based on consumption of starting material. The mixture was filtered through a pad of celite, and concentrated to afford *tert*-butyl [(2*S*)-1-(methylamino)-1-oxo-3-(piperidin-4-yl)propan-2-yl]carbamate (570 mg, > 100%) as an oil, which although NMR showed still contained residual AcOH was used without further purification. MS (ESI), *m/z* 286.2 $[M+H]^+$ (calcd for C14H28N3O3 = 286.2). ^{1}H NMR (400 MHz, CD_3OD) δ 4.07 (br s, 1H), 3.44 - 3.34 (m, 2H), 2.99 - 2.89 (m, 2H), 2.98 - 2.87 (m, 2H), 2.76 - 2.69 (m, 3H), 1.77 - 1.54 (m, 4H), 1.48 - 1.32 (m, 10H).

tert-Butyl [(2S)-3-[1-(3-tert-butyl-1-methyl-1H-pyrazole-5-carbonyl)piperidin-4-yl]-1-(methylamino)-1-oxopropan-2-yl]carbamate

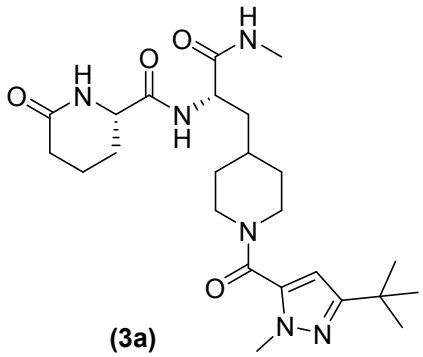
To a suspension of 3-*tert*-butyl-1-methyl-1*H*-pyrazole-5-carboxylic acid (110 mg, 0.60 mmol) and HATU (275 mg, 0.72 mmol) in DMF (2 mL) was added DIPEA (0.68 mL, 3.92 mmol). The resulting mixture was stirred at room temperature for 30 minutes before *tert*-butyl [(2*S*)-1-(methylamino)-1-oxo-3-(piperidin-4-yl)propan-2-yl]carbamate (250 mg, 0.72 mmol) was added. The reaction was allowed to stir for 16 hours after which time TLC (CH_2Cl_2/MeOH = 10:1)

indicated that the starting material had been consumed. The reaction was poured into water (20 mL) and extracted with EtOAc (2 x 20 mL). The combined organic extracts were dried over Na_2SO_4, concentrated and the residue purified by flash chromatography (silica gel, 10 – 100% EtOAc/petroleum ether) to afford *tert*-butyl [(2*S*)-1-(methylamino)-1-oxo-3-(pyridin-4-yl)propan-2-yl]carbamate (130 mg, 48%) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.09 (s, 1H), 4.99 - 4.80 (m, 1H), 4.78 - 4.50 (m, 1H), 4.01 – 3.99 (m, 1H), 3.93 - 3.81 (m, 3H), 3.02 – 2.99 (m, 1H), 2.86 - 2.69 (m, 4H), 2.00 (s, 1H), 1.98 - 1.36 (m, 15H), 1.34 - 1.28 (m, 9H).

3-[1-(3-tert-Butyl-1-methyl-1H-pyrazole-5-carbonyl)piperidin-4-yl]-N-methyl-L-alaninamide

To a solution of *tert*-butyl [(2*S*)-1-(methylamino)-1-oxo-3-(pyridin-4-yl)propan-2-yl]carbamate (130 mg, 0.29 mmol) in CH_2Cl_2 (5 mL) was added in a dropwise manner trifluoroacetic acid (1 mL) at 0 ^{0}C. The reaction was stirred at room temperature for 1 hour, and then concentrated to afford the trifluoroacetate salt of 3-[1-(3-*tert*-butyl-1-methyl-1*H*-pyrazole-5-carbonyl)piperidin-4-yl]-*N*-methyl-*L*-alaninamide (200 mg, 100%) as a viscous gum, which was used without further purification. MS (ESI), m/z 350.3 $[M+H]^+$ (calcd for C18H32N5O2 = 350.3).

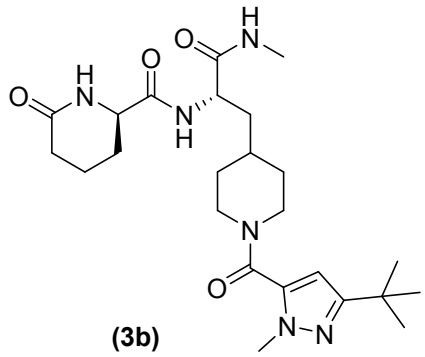
(2S)-N-[(2S)-3-[1-(3-tert-butyl-1-methyl-1H-pyrazole-5-carbonyl)piperidin-4-yl]-1-(methylamino)-1-oxopropan-2-yl]-6-oxopiperidine-2-carboxamide (3a)



(3a)

To a suspension of (2*S*)-6-oxopiperidine-2-carboxylic acid (165 mg, 1.16 mmol) and HATU (440 mg, 1.16 mmol) in DMF (4 mL) was added DIPEA (299 mg, 0.403 mL, 2.31 mmol). The resulting mixture was stirred at room temperature for 30 minutes before the trifluoroacetate salt of 3-[1-(3-*tert*-butyl-1-methyl-1*H*-pyrazole-5-carbonyl)piperidin-4-yl]-*N*-methyl-*L*-alaninamide (134 mg, 0.289 mmol) was added. The reaction was stirred at room temperature for 16 hours after which time LC-MS indicated that the starting material had been consumed. The solvent was removed *in vacuo*, and the residue purified by preparative HPLC (A : water w/0.05% ammonium hydroxide, B : acetonitrile, 20 – 40% B over 10 minutes, flow rate 25 mL/min using a Durashell 150 x 25 mm x 5 μm column) to afford after solvent removal and lyophilization (2*S*)-*N*-[(2*S*)-3-[1-(3-*tert*-butyl-1-methyl-1*H*-pyrazole-5-carbonyl)piperidin-4-yl]-1-(methylamino)-1-oxopropan-2-yl]-6-oxopiperidine-2-carboxamide **(3a)** (53 mg, 38%) as a white solid. HRMS (ESI/Q-TOF) m/z: $[M + H]^+$ calcd for C24H39N6O4 = 475.30273; Found 475.30419. MS (ESI), *m/z* 475.4 $[M+H]^+$ (calcd for C24H39N6O4 = 475.3). ^{1}H NMR (700 MHz, DMSO-d_6) δ 8.05 (d, *J* = 8.4 Hz, 1H), 7.81 (d, *J* = 2.6 Hz, 1H), 6.24 (s, 1H), 4.41 (s, 1H), 4.32 (q, *J* = 8.0 Hz, 1H), 3.93 (td, *J* = 5.9, 2.5 Hz, 1H), 3.78 (s, 3H), 3.73 (s, 2H), 2.14 (td, *J* = 6.7, 1.9 Hz, 2H), 1.87 (tt, *J* = 6.6, 3.2 Hz, 1H), 1.79 – 1.66 (m, 2H), 1.59 (tdd, J = 23.8, 11.6, 4.4 Hz, 3H), 1.24 (s, 11H), 1.16 -0.98 (m, 1H). ^{13}C NMR (175 MHz, DMSO-d_6) (several carbons not observed) δ 171.74, 171.65, 170.70, 159.08, 135.92, 101.84, 55.52, 50.10, 38.06, 27.20, 32.14, 31.65, 31.12, 30.40, 25.83, 18.46.

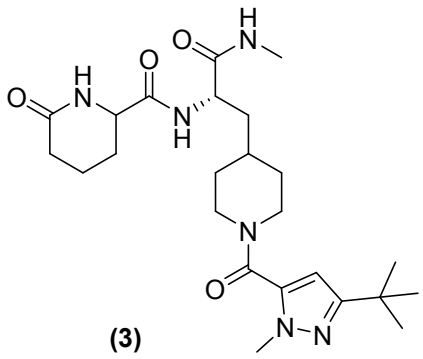
((2R)-N-[(2S)-3-[1-(3-tert-butyl-1-methyl-1H-pyrazole-5-carbonyl)piperidin-4-yl]-1-(methylamino)-1-oxopropan-2-yl]-6-oxopiperidine-2-carboxamide (3b)



(3b)

(2*R*)-*N*-[(2*S*)-3-[1-(3-*tert*-butyl-1-methyl-1*H*-pyrazole-5-carbonyl)piperidin-4-yl]-1-(methylamino)-1-oxopropan-2-yl]-6-oxopiperidine-2-carboxamide (**3b**) is accessed through the same route as (**3a**) using (2*R*)-6-oxopiperidine-2-carboxylic acid (CAS : 72002-30-3) in the final amide-bond formation step. HRMS (ESI/Q-TOF) m/z: $[M + H]^+$ calcd for C24H39N6O4 = 475.30273; Found 475.30390. ^{1}H NMR (700 MHz, DMSO-d_6) δ 8.03 (d, J = 8.5 Hz, 1H), 7.90 – 7.68 (m, 1H), 7.54 – 7.30 (m, 1H), 6.19 (s, 1H), 4.28 (m, 1H), 3.90 (m, 1H), 3.68 (s, 3H), 2.50 (s, 3H), 2.08 (t, J = 6.5 Hz, 2H), 1.88 – 1.80 (m, 1H), 1.72 – 1.60 (m, 2H), 1.60 – 1.45 (m, 3H), 1.19 (s, 9H). ^{13}C NMR (175 MHz, DMSO-d_6) (several carbons not observed) δ 171.6, 158.8, 136.1, 102.0, 54.8, 49.9, 37.3, 32.0, 31.9, 31.4, 30.7, 30.5, 18.5.

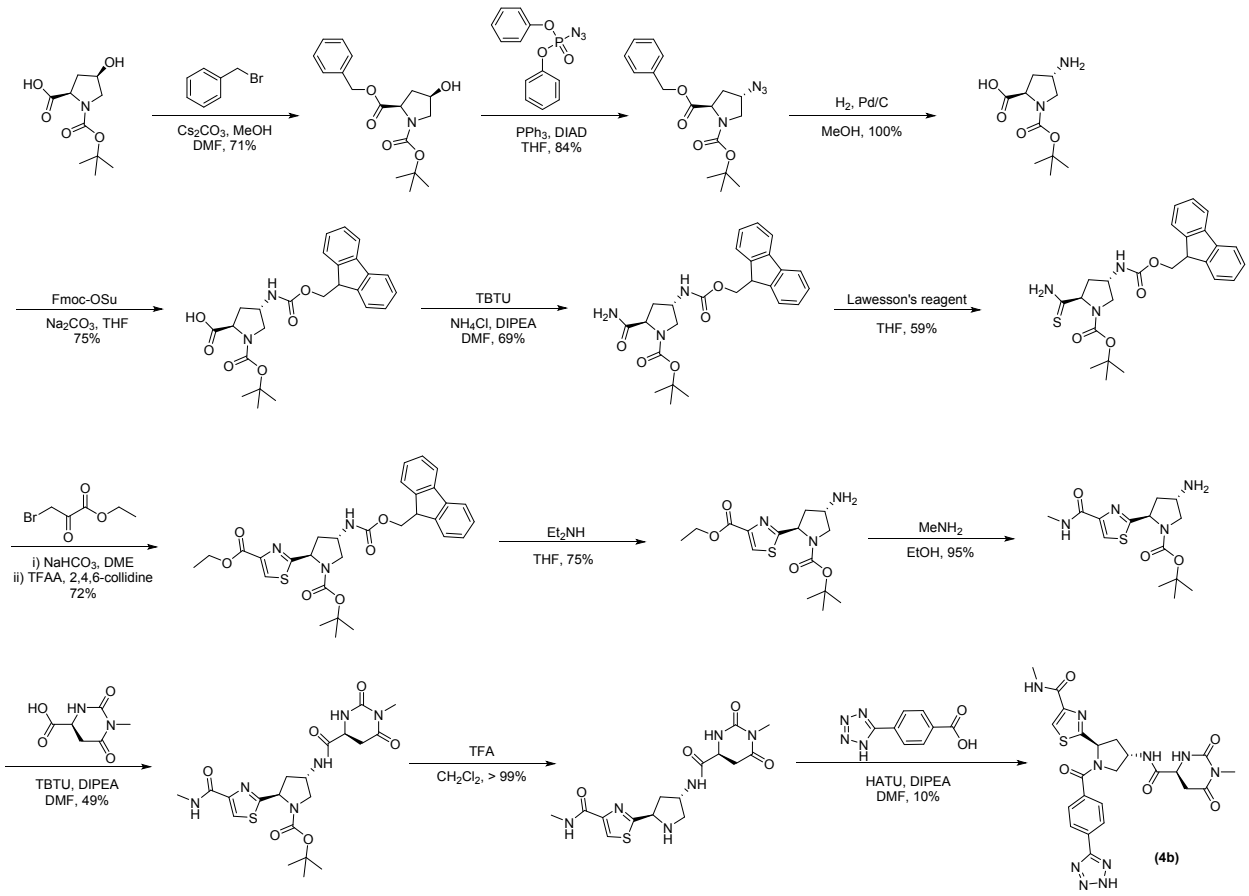
N*-[(2*S*)-3-[1-(3-*tert*-butyl-1-methyl-1*H*-pyrazole-5-carbonyl)piperidin-4-yl]-1-(methylamino)-1-oxopropan-2-yl]-6-oxopiperidine-2-carboxamide *(3)



(3)

N-[(2*S*)-3-[1-(3-*tert*-butyl-1-methyl-1*H*-pyrazole-5-carbonyl)piperidin-4-yl]-1-(methylamino)-1-oxopropan-2-yl]-6-oxopiperidine-2-carboxamide (**3**) is accessed through the same route as (**3a**) using racemic 6-oxopiperidine-2-carboxylic acid (CAS : 3770-22-7) in the final amide-bond formation step. HRMS (ESI/Q-TOF) m/z: $[M + H]^+$ calcd for C24H39N6O4 = 475.30273; Found 475.30315. ^{1}H NMR (700 MHz, DMSO-d_6) δ 8.02 (dd, J = 20.7, 8.3 Hz, 1H), 7.79 (q, J = 28.8 Hz, 1H), 7.49 (d, 1H), 6.19 (s, 1H), 4.36 (s, 1H), 4.27 (q, J = 7.6 Hz, 1H), 3.98 – 3.80 (m, 1H), 3.73 (s, 1H), 3.68 (s, 3H), 3.37 (d, J = 3.2 Hz, 1H), 2.95 (d, J = 1.9 Hz, 1H), 2.50 (d, J = 1.8 Hz, 3H), 2.17 – 1.94 (m, 2H), 1.89 – 1.78 (m, 1H), 1.73 – 1.58 (m, 2H), 1.59 – 1.42 (m, 4H), 1.19 (s, 9H). ^{13}C NMR (175 MHz, DMSO-d_6) (several carbons not observed) δ 171.9, 170.6, 159.0, 135.9, 101.8, 54.8, 54.3, 50.0, 37.8, 37.2, 32.2, 31.6, 30.6, 25.9, 18.0.

Scheme S3 - Resynthesis of (4b)



2-Benzyl 1-tert-butyl (2R,4R)-4-hydroxypyrrolidine-1,2-dicarboxylate

To an ice-bath cooled solution of (4*R*)-1-(*tert*-butoxycarbonyl)-4-hydroxy-*D*-proline (6.7 g, 28.97 mmol) in methanol (110 mL) was added a solution of aqueous cesium carbonate (4.72 g, 14.5 mmol in 72 mL of H_2O). The solution was concentrated to dryness, and the residue co-evaporated with 2-MeTHF (100 mL) to afford a white solid, which was subsequently dissolved in DMF (130 mL). To this solution at 0 ^{0}C was added benzyl bromide (4.96 g, 29.0 mmol), and the reaction stirred vigorously at room temperature for 20 hours. TLC (petroleum ether/EtOAc = 1:1) and LC-MS indicated the starting material had been consumed with a new major peak being observed corresponding to the mass of the desired product. EtOAc (150 mL) was added to the reaction mixture, which was washed with water (2 x 100 mL) followed by saturated aqueous sodium

chloride solution (2 x 50 mL). The combined organics were dried over Na_2SO_4, and concentrated to afford a residue, which was co-evaporated with THF (2 x 100 mL) to afford 2-benzyl 1-*tert*-butyl (2*R*,4*R*)-4-hydroxypyrrolidine-1,2-dicarboxylate (6.6 g, 71%) as a yellow oil, which was used directly in the next step without purification. MS (ESI), *m/z* 344.1 $[M+Na]^+$ (calcd for C17H23NNaO5 = 344.2). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.44 - 7.26 (m, 5H), 5.23 - 4.96 (m, 3H), 4.44 - 4.15 (m, 2H), 3.57 - 3.44 (m, 1H), 3.19 - 3.06 (m, 1H), 2.42 - 2.25 (m, 1H), 1.93 - 1.81 (m, 1H), 1.41 - 1.35 (m, 4H), 1.30 - 1.22 (m, 5H).

2-Benzyl 1-tert-butyl (2R,4S)-4-azidopyrrolidine-1,2-dicarboxylate

To a solution of 2-benzyl 1-*tert*-butyl (2*R*,4*R*)-4-hydroxypyrrolidine-1,2-dicarboxylate (6.6 g, 20.54 mmol) and triphenylphosphine (16.2 g, 61.6 mmol) in THF (180 mL) cooled to 5 ^{0}C was added in a dropwise manner a solution of diisopropyl azodicarboxylate (12.5g, 61.6 mmol) and diphenyl phosphoryl azide (17g, 61.6 mmol) in THF (25 mL) over 30 minutes under a nitrogen atmosphere. Upon completion of the addition, the reaction was warmed to room temperature and stirred for 40 hours. TLC (petroleum ether/EtOAc = 1:1) indicated that the starting material had been consumed, and ethanol (20 mL) was added to the reaction, which was concentrated to afford a residue, which was purified by flash chromatography (silica gel 0 – 20% EtOAc/petroleum ether) to afford 2-benzyl 1-*tert*-butyl (2*R*,4*S*)-4-azidopyrrolidine-1,2-dicarboxylate (6.0 g, 84%) as a yellow oil. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.44 - 7.25 (m, 5H), 5.24 - 5.06 (m, 2H), 4.43 - 4.20 (m, 2H), 3.59 - 3.36 (m, 2H), 2.42 - 2.26 (m, 1H), 2.24 - 2.08 (m, 1H), 1.44 - 1.34 (m, 3H), 1.31 - 1.22 (m, 6H).

(4S)-4-Amino-1-(tert-butoxycarbonyl)-D-proline

To a solution of 2-benzyl 1-*tert*-butyl (2*R*,4*S*)-4-azidopyrrolidine-1,2-dicarboxylate (6.0 g, 17.32 mmol) in methanol (120 mL) was added 10 wt% Pd/C (0.60 g, wet). The flask was re-filled and de-gassed with hydrogen (x 3), and then stirred at room temperature for 15 hours under an atmosphere of hydrogen. TLC (petroleum ether/EtOAc = 5:1) indicated that the starting material had been consumed. The reaction was filtered through celite and concentrated to afford (4*S*)-4-amino-1-(*tert*-butoxycarbonyl)-*D*-proline (3.99 g, 100%) as a slightly grey solid, which was used directly without further purification. ^{1}H NMR (400 MHz, D_2O) δ 4.31 - 4.17 (m, 1H), 4.04 - 3.93 (m, 1H), 3.87 - 3.75 (m, 1H), 3.69 - 3.50 (m, 1H), 2.54 - 2.20 (m, 2H), 1.57 - 1.30 (m, 9H).

(4S)-1-(tert-Butoxycarbonyl)-4-({[(9H-fluoren-9-yl)methoxy]carbonyl}amino)-D-proline

To a solution of (4*S*)-4-amino-1-(*tert*-butoxycarbonyl)-*D*-proline (3.60 g, 15.63 mmol) in THF (30 mL), was added a 10% aqueous solution of Na_2CO_3 (30 mL) was added. After being cooled to 0 ^{0}C using an ice-water bath, Fmoc-OSu (6.86 g, 20.3 mmol) was added. The reaction was warmed to room temperature and stirred for 2 hours. TLC (EtOAc/MeOH 7 : 3) indicated that residual Fmoc-OSu remained with a new major UV-active spot having been formed. The solvent was removed under reduced pressure with the residue partitioned between EtOAc (200 mL) and saturated aqueous ammonium chloride solution (200 mL). The pH was adjusted to ~ 6 with 1 N HCl, and the organic layer separated, and the aqueous further extracted with EtOAc (3 x 200 mL). The combined organic extracts were washed with brine (350 mL), dried over Na_2SO_4, and concentrated to give a residue, which was purified by chromatography (silica gel, 0 – 25% MeOH/EtOAc) to give (4*S*)-1-(*tert*-butoxycarbonyl)-4-({[(9*H*-fluoren-9-

yl)methoxy]carbonyl}amino)-*D*-proline (5.3 g, 75%) as a yellow gum, which was used without further purification. MS (ESI), m/z 475.2 $[M+Na]^+$ (calcd for C25H28N2NaO6 = 475.2).

tert-Butyl (2R,4S)-2-carbamoyl-4-({[(9H-fluoren-9-yl)methoxy]carbonyl}amino)pyrrolidine-1-carboxylate

To a solution of (4*S*)-1-(*tert*-butoxycarbonyl)-4-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino)-*D*-proline 2.7 g, 5.97 mmol) in DMF (30.0 mL) was added DIPEA (2.22 mL, 1.54 g, 11.9 mmol) and TBTU (2.11 g, 6.56 mmol). After being stirred for 10 minutes, ammonium chloride (1.60 g, 29.8 mmol) was added, and the reaction stirred at room temperature for 15 hours. LC-MS indicated that the starting material had been consumed with a new major peak being observed with the desired mass for the product. The solvent was removed, and the resulting residue was partitioned between EtOAc (100 mL) and water (100 mL), and the aqueous further extracted with EtOAc (2 x 100 mL). The combined organic layers were washed with brine (150 mL), dried over Na_2SO_4, and concentrated *in vacuo* to give a residue, which was purified by chromatography (silica gel, 20 – 100% EtOAc/petroleum ether) to give *tert*-butyl (2*R*,4*S*)-2-carbamoyl-4-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino)pyrrolidine-1-carboxylate (2.20 g, 69%) as a white solid, which was used without further purification. MS (ESI), *m/z* 474.2 $[M+Na]^+$ (calcd for C25H29N3NaO5 = 474.2).

tert-Butyl (2R,4S)-2-carbamothioyl-4-({[(9H-fluoren-9-yl)methoxy]carbonyl}amino) pyrrolidine-1-carboxylate

At room temperature, to a solution of *tert*-butyl (2*R*,4*S*)-2-carbamoyl-4-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino)pyrrolidine-1-carboxylate (2.10 g, 4.65 mmol) in THF (50.0 mL) was

added Lawesson's reagent (2.82 g, 6.98 mmol). The reaction was stirred at room temperature for 12 hours during which time TLC (EtOAc/petroleum ether = 1: 1) indicated that the starting material had been consumed with a new product having been formed. The reaction was concentrated to give a residue, which was partitioned between EtOAc (100 mL) and saturated aqueous $NaHCO_3$ solution (100 mL). The organic layer was separated, and the aqueous further extracted with EtOAc (3 x 100 mL). The combined organic layers were washed with brine (250 mL), dried over Na_2SO_4, and concentrated to give a residue, which was purified by chromatography (silica gel, 5 – 50% EtOAc/Petroleum ether) to afford *tert*-butyl (2*R*,4*S*)-2-carbamothioyl-4-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino)pyrrolidine-1-carboxylate (1.0 g, 59%) as a colorless gum. MS (ESI), *m/z* 490.2 $[M+Na]^+$ (calcd for C25H29N3NaO4 = 490.2). ^1H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, *J* = 7.3 Hz, 2H), 7.64 - 7.45 (m, 3H), 7.39 (t, *J* = 8.0 Hz, 2H), 7.33 (t, *J* = 8.0 Hz, 2H), 4.89 - 4.78 (m, 1H), 4.75 - 4.64 (m, 1H), 4.51 - 4.42 (m, 2H), 4.39 - 4.17 (m, 2H), 3.83 - 3.66 (m, 1H), 1.61 (s, 4H), 1.51 - 1.41 (m, 9H).

Ethyl 2-[(2R,4S)-1-(tert-butoxycarbonyl)-4-({[(9H-fluoren-9-yl)methoxy]carbonyl}amino)pyrrolidin-2-yl]-1,3-thiazole-4-carboxylate

To a suspension of *tert*-butyl (2*R*,4*S*)-2-carbamothioyl-4-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino) pyrrolidine-1-carboxylate (1.38 g, 2.95 mmol mmol) and $NaHCO_3$ (1.98 g, 23.6 mmol) in DME (30 mL) cooled in an ice-bath was added in a dropwise manner ethyl bromopyruvate (1.73 g, 8.85 mmol). Upon completion of the addition, the reaction warmed to room temperature, and stirred for 40 minutes. The reaction was cooled again to 0 ^0C, and a solution of TFAA (2.48 g, 11.8 mmol) and 2,4,6-collidine (2.86 g, 23.6 mmol) in DME (15 mL) was added. The reaction was again allowed to warm to room temperature and stirred for 2.5 hours. TLC

(EtOAc/Petroleum ether = 1: 1) and LC-MS indicated that the starting material had been consumed with a major new product formed corresponding to the mass of the desired product. The mixture was diluted with H_2O (50 mL) and extracted with EtOAc (3 x 50 mL). The organic layers were washed with 1N HCl (100 mL), H_2O (100 mL), brine (100 mL), dried over Na_2SO_4 and concentrated to afford a residue, which was purified by chromatography (silica gel, 10 – 50% EtOAc/Petroleum ether) to afford ethyl 2-[(2*R*,4*S*)-1-(*tert*-butoxycarbonyl)-4-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino) pyrrolidin-2-yl]-1,3-thiazole-4-carboxylate (1.33 g, 80%) initially as a yellow gum. Trituration with EtOAc/petroleum ether (1:20, 30 mL) followed by filtration allowed isolation of ethyl 2-[(2*R*,4*S*)-1-(*tert*-butoxycarbonyl)-4-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino) pyrrolidin-2-yl]-1,3-thiazole-4-carboxylate (1.19 g, 72%) as a white solid. MS (ESI), *m/z* 586.3 $[M+Na]^+$ (calcd for C30H33N3NaO6S = 586.2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 - 8.03 (m, 1H), 7.77 (br d, *J* = 6.8 Hz, 2H), 7.57 (br d, *J* = 7.0 Hz, 1H), 7.45 - 7.29 (m, 4H), 5.46 - 5.13 (m, 1H), 4.99 - 4.85 (m, 1H), 4.53 - 4.17 (m, 7H), 3.99 - 3.81 (m, 1H), 3.46 - 3.13 (m, 1H), 2.51 (s, 2H), 1.55 - 1.29 (m, 12H).

Ethyl 2-[(2R,4S)-4-amino-1-(tert-butoxycarbonyl)pyrrolidin-2-yl]-1,3-thiazole-4-carboxylate

To a 0 ^{0}C cooled suspension of 2-[(2*R*,4*S*)-1-(*tert*-butoxycarbonyl)-4-({[(9*H*-fluoren-9-yl)methoxy] carbonyl}amino)pyrrolidin-2-yl]-1,3-thiazole-4-carboxylate (1.19 g, 2.11 mmol) in THF (30 mL) was added diethylamine (3.26 mL, 2.32 g, 31.7 mmol). The reaction warmed to room temperature and stirred for 15 hours, after which time, TLC (CH_2Cl_2/MeOH = 10 : 1) and LC-MS indicated that no starting material remained. The reaction was concentrated to give a residue, which was purified by chromatography (silica gel, 1 – 10% MeOH/CH_2Cl_2) to give ethyl 2-[(2*R*,4*S*)-4-amino-1-(*tert*-butoxycarbonyl)pyrrolidin-2-yl]-1,3-thiazole-4-carboxylate (540 mg,

75%) as a colorless gum. MS (ESI), *m/z* 364.1 $[M+Na]^+$ (calcd for C15H23N3NaO4S = 364.1). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.56 - 8.21 (m, 1H), 5.20 - 4.97 (m, 1H), 4.37 - 4.16 (m, 2H), 3.64 - 3.42 (m, 3H), 3.21 - 3.00 (m, 1H), 2.19 - 1.65 (m, 1H), 1.49 - 1.11 (m, 14H).

tert-Butyl (2R,4S)-4-amino-2-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidine-1-carboxylate

A solution of ethyl 2-[(2*R*,4*S*)-4-amino-1-(*tert*-butoxycarbonyl)pyrrolidin-2-yl]-1,3-thiazole-4-carboxylate (71.5 mg, 0.209 mmol) in methylamine (2 mL, 30% in ethanol) was stirred at 30 ^{0}C for 16 hours. TLC (CH_2Cl_2/MeOH = 10 : 1) indicated that all of the starting material had been consumed with a new UV active spot being observed. The mixture was concentrated under vacuum to afford *tert*-butyl (2*R*,4*S*)-4-amino-2-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidine-1-carboxylate (260 mg, >99%) as a white solid, which was used without further purification.

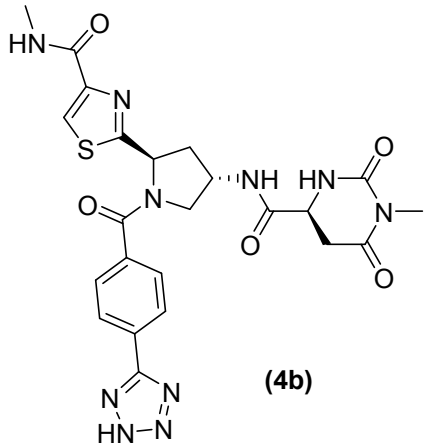
tert-Butyl (2R,4S)-2-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]-4-{[(4S)-1-methyl-2,6-dioxo-1,3-diazinane-4-carbonyl]amino}pyrrolidine-1-carboxylate

To a solution of *tert*-butyl (2*R*,4*S*)-4-amino-2-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidine-1-carboxylate (84 mg, 0.24 mmol), TBTU (99 mg, 0.31 mmol) and (4*S*)-1-methyl-2,6-dioxo-1,3-diazinane-4-carboxylic acid (53.2 mg, 0.31 mmol) in DMF (2 mL) was added DIPEA (100 mg, 0.78 mmol) at room temperature. The reaction was then stirred at room temperature for 15 hours. The reaction was concentrated under vacuum to afford a residue, which was purified by chromatography (silica gel, 0 – 10% MeOH/CH_2Cl_2) to give *tert*-butyl (2*R*,4*S*)-2-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]-4-{[(4*S*)-1-methyl-2,6-dioxo-1,3-diazinane-4-carbonyl]amino}pyrrolidine-1-carboxylate (60 mg, 49%) as a white solid, which was used without further purification. MS (ESI), *m/z* 381.0 $[M+H-Boc]^+$ (calcd for C15H21N6O4S = 381.1).

(4S)-1-Methyl-N-{(3S,5R)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide – trifluoroacetate salt

To a solution of *tert*-butyl (2*R*,4*S*)-2-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]-4-{[(4*S*)-1-methyl-2,6-dioxo-1,3-diazinane-4-carbonyl]amino}pyrrolidine-1-carboxylate (60 mg, 0.12 mmol) in CH_2Cl_2 (2 mL) was added trifluoroacetic acid (2 mL) at 0 ^{0}C. After the addition was completed, the reaction was stirred for 1 hour at room temperature. TLC (CH_2Cl_2/MeOH = 10 : 1) indicated that the starting material had been consumed. The reaction was concentrated, and the residue azeotroped with CH_2Cl_2 (3 x 10 mL) to afford (4*S*)-1-methyl-*N*-{(3*S*,5*R*)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide trifluoroacetate salt (61 mg, >99%) as a yellow gum, which was used without further purification. MS (ESI), *m/z* 403.0 $[M+Na]^+$ (calcd for C15H20N6NaO4S = 403.1).

(4S)-1-Methyl-N-{(3S,5R)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]-1-[4-(1H-tetrazol-5-yl)benzoyl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide (4b)

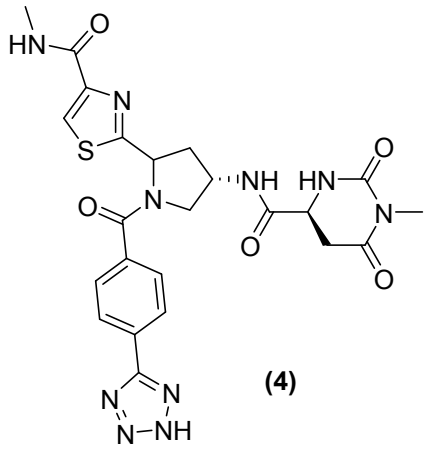


(4b)

To a solution of (4*S*)-1-methyl-*N*-{(3*S*,5*R*)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide trifluoroacetate salt (69.0 mg, 0.14 mmol), 4-(1*H*-

tetrazol-5-yl)benzoic acid (26.9 mg, 0.14 mmol) in DMF (2 mL) was added DIPEA (91.5 mg, 0.71 mmol) and HATU (53.8 mg, 0.142 mmol) at room temperature. The reaction was stirred at room temperature for 15 hours after which time LC-MS indicated that the starting material had been consumed. The solvent was removed *in vacuo*, and the residue purified by preparative HPLC (A : water w/0.225% formic acid, B : methanol, 5 – 25% B over 8 minutes, flow rate 35 mL/min using a Agela Durashell C18 150 x 25 mm x 5 μm column) to afford after solvent removal and lyophilization (4*S*)-1-methyl-*N*-{(3*S*,5*R*)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]-1-[4-(1*H*-tetrazol-5-yl)benzoyl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide **(4b)** (8 mg, 10%) as a white solid. MS (ESI), m/z 553.3 $[M+H]^+$ (calcd for C23H25N10O5S = 553.2). ^{1}H NMR (400 MHz, CD_3OD) (mixture of rotamers) δ 8.21 - 8.09 (m, 3H), 7.82 - 7.71 (m, 2H), 5.84 - 5.69 (m, 1H), 5.57 - 5.42 (m, 1H), 4.61 - 4.46 (m, 1H), 4.32 - 4.20 (m, 1H), 4.18 - 3.97 (m, 2H), 3.80 - 3.68 (m, 1H), 3.60 - 3.50 (m, 1H), 3.13 - 3.03 (m, 4H), 3.02 - 2.90 (m, 4H), 2.80 - 2.44 (m, 3H).

(4S)-1-methyl-N-{(3S)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]-1-[4-(1H-tetrazol-5-yl)benzoyl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide (4)

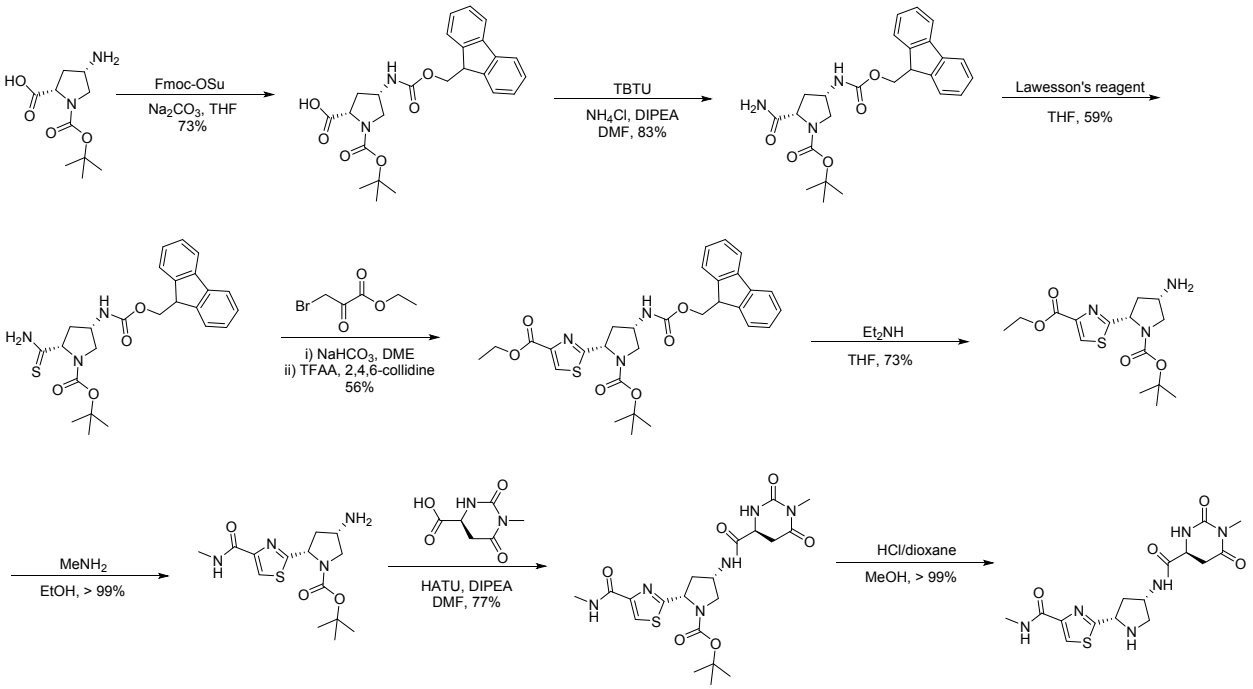


(4)

(4*S*)-1-methyl-*N*-{(3*S*)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]-1-[4-(1*H*-tetrazol-5-yl)benzoyl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide (**4**) is accessed through the same route as (**4b**) using (4*R*)-1-(*tert*-butoxycarbonyl)-4-hydroxyproline (CAS : 2139264-38-1) as the starting material. MS (ESI), m/z 553.3 $[M+H]^+$ (calcd for C23H25N10O5S = 553.2). ^1H NMR (400 MHz, CD_3OD) (mixture of rotamers) δ 8.19 - 8.01 (m, 3H), 7.70 (d, *J* = 8 Hz, 2H), 5.77 - 5.69 (m, 1H), 5.58 - 5.51 (m, 1H), 4.61 - 4.39 (m, 3H), 4.22 - 3.99 (m, 2H), 3.76 - 3.58 (m, 1H), 3.60 - 3.50 (m, 2H), 3.12 – 2.64 (m, 8H), 2.59 - 2.30 (m, 1H).

Compounds from TABLE 1 (4a, 5, 6, 7, 8)

Scheme S4 - Synthesis of (4S)-1-methyl-N-{(3S,5S)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide



(4S)-1-(tert-Butoxycarbonyl)-4-({[(9H-fluoren-9-yl)methoxy]carbonyl}amino)-L-proline

To a solution of (2*S*,4*S*)-1-Boc-4-amino-pyrrolidine-2-carboxylic acid (2.50 g, 10.9 mmol) in THF (30 mL), was added a 10% aqueous solution of Na_2CO_3 (30 mL) was added. After being cooled to

0 ^{0}C using an ice-water bath, Fmoc-OSu (4.76 g, 14.1 mmol) was added. The reaction was warmed to room temperature and stirred for 12 hours. TLC (EtOAc/MeOH 7 : 3) indicated that residual Fmoc-OSu remained with a new major UV-active spot having been formed. The solvent was removed under reduced pressure with the residue partitioned between EtOAc (100 mL) and saturated aqueous ammonium chloride solution (100 mL). The pH was adjusted to ~ 6 with 1 N HCl, and the organic layer separated, dried over Na_2SO_4, and concentrated to give a residue, which was purified by chromatography (silica gel, 0 – 100% EtOAc/petroleum ether) to give crude product as a yellow gum. Further purification was carried out by preparative HPLC (A : water w/0.225% formic acid, B : acetonitrile, 40 – 70% B over 15 minutes, flow rate 120 mL/min using a Phenomenex Synergi Max-RP 150 x 50mm x 10 μm column) to afford (4*S*)-1-(*tert*-butoxycarbonyl)-4-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino)-*L*-proline (3.58 g, 73%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.2 Hz, 2H), 7.60 – 7.58 (m, 2H), 7.40 (t, *J* = 7.2 Hz, 2H), 7.32 (t, *J* = 6.4 Hz, 2H), 5.74 (d, *J* = 6.0 Hz, 1H), 4.59 (d, *J* = 8.4 Hz, 1H), 4.36 – 4.22 (m, 4 H), 3.56 – 3.51 (m, 2 H), 2.52 – 2.49 (m, 1 H), 2.33 – 2.31 (m, 1 H), 1.44 (s, 9 H).

tert-Butyl (2S,4S)-2-carbamoyl-4-({[(9H-fluoren-9-yl)methoxy]carbonyl}amino)pyrrolidine-1-carboxylate

To a solution of (4*S*)-1-(*tert*-butoxycarbonyl)-4-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino)-*L*-proline (1.3 g, 2.87 mmol) in DMF (28.0 mL) was added DIPEA (1.02 mL, 743 mg, 5.75 mmol) and TBTU (1.11 g, 3.45 mmol). After being stirred for 10 minutes, ammonium chloride (768 mg, 14.4 mmol) was added, and the reaction stirred at room temperature for 15 hours. TLC (EtOAc/Petroleum ether = 1:1, and neat EtOAc) indicated that the starting material had been consumed with a new major spot observed. Water (25 mL) was added, and the resulting mixture

extracted with EtOAc (3 x 40 mL). The organic layer was separated, washed with water (50 mL), dried over Na_2SO_4, and concentrated *in vacuo* to give a residue, which was purified by chromatography (silica gel, 0 – 80% EtOAc/petroleum ether) to give *tert*-butyl (2*S*,4*S*)-2-carbamoyl-4-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino)pyrrolidine-1-carboxylate (1.08 g, 83%) as a white solid. MS (ESI), *m/z* 474.1 $[M+Na]^+$ (calcd for C25H29N3NaO5 = 474.2). ^1H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.2 Hz, 2H), 7.63 – 7.61 (m, 2H), 7.39 (t, *J* = 7.6 Hz, 2H), 7.32 (t, *J* = 5.6 Hz, 2H), 6.76 (br s, 1 H), 5.53 (br s, 1H), 4.48 (d, *J* = 8.0 Hz, 1H), 4.37 – 4.27 (m, 4 H), 3.57 – 3.51 (m, 2 H), 2.45 – 2.41 (m, 1 H), 2.23 – 2.21 (m, 1 H), 1.49 (s, 9 H).

tert-Butyl (2S,4S)-2-carbamothioyl-4-({[(9H-fluoren-9-yl)methoxy]carbonyl}amino) pyrrolidine-1-carboxylate

At room temperature, to a solution of *tert*-butyl (2*S*,4*S*)-2-carbamoyl-4-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino)pyrrolidine-1-carboxylate (1.64 g, 3.63 mmol) in THF (40.0 mL) was added Lawesson's reagent (1.47 g, 3.63 mmol). The reaction was stirred at room temperature for 12 hours during which time TLC (EtOAc/Petroleum ether = 1: 1, visualized with I_2) indicated that although some starting material remained, a new product was being formed. The reaction was concentrated to give a residue, which was directly purified by chromatography (silica gel, 20 – 50% EtOAc/petroleum ether) to afford *tert*-butyl (2*S*,4*S*)-2-carbamothioyl-4-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino)pyrrolidine-1-carboxylate (1.0 g, 59%) as a white solid. MS (ESI), *m/z* 490.1 $[M+Na]^+$ (calcd for C25H29N3NaO4S = 490.2). ^1H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, *J* = 7.3 Hz, 2H), 7.60 (br d, J = 6.5 Hz, 2H), 7.39 (t, *J* = 8.0 Hz, 2H), 7.33 (t, *J* = 8.0 Hz, 2H), 4.71 (br s, 2H), 4.35 (br s, 1H), 4.28 (br s, 1H), 4.26 - 4.19 (m, 1H), 3.84 - 3.64 (m, 1H), 3.49 (br d, *J* = 12.0 Hz, 1H), 2.44 (br s, 1 H), 1.84 (br s, 4H), 1.48 (s, 9H).

Ethyl 2-[(2S,4S)-1-(tert-butoxycarbonyl)-4-({[(9H-fluoren-9-yl)methoxy]carbonyl}amino) pyrrolidin-2-yl]-1,3-thiazole-4-carboxylate

To a suspension of *tert*-butyl (2*S*,4*S*)-2-carbamothioyl-4-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino) pyrrolidine-1-carboxylate (1.0 g, 2.139 mmol) and $NaHCO_3$ (1.44 g, 17.1 mmol) in DME (16 mL) cooled in an ice-bath was added in a dropwise manner ethyl bromopyruvate (1.25 g, 6.42 mmol). Upon completion of the addition, the reaction was warmed to room temperature, and stirred for 40 minutes. The reaction was cooled again to 0 ^{0}C, and a solution of TFAA (1.80 g, 8.55 mmol) and 2,4,6-collidine (2.07g, 17.1 mmol) in DME (8 mL) was added. The reaction was warmed to room temperature and stirred for 4 hours. TLC (EtOAc/petroleum ether = 1: 1, visualized with I_2) indicated that traces of starting material remained, with a major new spot being observed. The mixture was diluted with H_2O (40 mL), and extracted with EtOAc (50 mL). The organic layer was washed with 1N HCl (20 mL), H_2O (20 mL), brine (15 mL), dried over Na_2SO_4 and concentrated to afford a residue, which was purified by chromatography (silica gel, 0 – 33% EtOAc/petroleum ether) to afford a yellow gum. Trituration with TBME (20 mL) and petroleum ether (8 mL) gave ethyl 2-[(2*S*,4*S*)-1-(*tert*-butoxycarbonyl)-4-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino) pyrrolidin-2-yl]-1,3-thiazole-4-carboxylate (0.68 g, 56%) as a white solid. MS (ESI), *m/z* 586.1 $[M+Na]^+$ (calcd for C30H33N3NaO6S = 586.2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (br s, 1H), 7.75 (d, *J* = 7.5 Hz, 2H), 7.62 (br d, *J* = 8.5 Hz, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.26 (s, 2H), 7.32 - 7.25 (m, 1H), 5.30 (br s, 1H), 4.53 (br s, 1H), 4.42 - 4.29 (m, 4H), 4.25 (br d, *J* = 7.0 Hz, 1H), 3.79 – 3.75 (m, 1H), 3.54 (br d, *J* = 11.8 Hz, 1H), 2.61 (br s, 2H), 1.45 (br s, 9H), 1.31 (t, J = 8 Hz, 3H).

Ethyl 2-[(2S,4S)-4-amino-1-(tert-butoxycarbonyl)pyrrolidin-2-yl]-1,3-thiazole-4-carboxylate

To a 0 ^{0}C cooled suspension of 2-[(2*S*,4*S*)-1-(*tert*-butoxycarbonyl)-4-({[(9*H*-fluoren-9-yl)methoxy] carbonyl}amino)pyrrolidin-2-yl]-1,3-thiazole-4-carboxylate (680 mg, 1.21 mmol) in THF (12 mL) was added diethylamine (1.86 mL, 1.32 g, 18.1 mmol). The reaction was warmed to room temperature and stirred for 7 hours, after which time, TLC (CH_2Cl_2/MeOH = 10:1) indicated that no starting material remained. The reaction was concentrated to give a residue, which was purified by chromatography (silica gel, 0 – 10% MeOH/CH_2Cl_2) to give ethyl 2-[(2*S*,4*S*)-4-amino-1-(*tert*-butoxycarbonyl)pyrrolidin-2-yl]-1,3-thiazole-4-carboxylate (300 mg, 73%) as a white solid. MS (ESI), *m/z* 342.1 $[M+H]^+$ (calcd for C15H24N3O4S = 342.2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 (s, 1H), 5.35 - 5.06 (m, 1H), 4.41 (q, *J* = 6.9 Hz, 2H), 4.01 - 3.57 (m, 2H), 3.38 - 3.16 (m, 1H), 2.77 - 2.55 (m, 1H), 2.37 - 1.95 (m, 1H), 1.75 - 1.06 (m, 14H).

tert-Butyl (2S,4S)-4-amino-2-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidine-1-carboxylate

A solution of ethyl 2-[(2*S*,4*S*)-4-amino-1-(*tert*-butoxycarbonyl)pyrrolidin-2-yl]-1,3-thiazole-4-carboxylate (257 mg, 0.753 mmol) in methylamine (8 mL, 30% in ethanol) was stirred at 30 ^{0}C for 16 hours. TLC (CH_2Cl_2/MeOH = 10:1) indicated that all the starting material had been consumed with a new UV active spot being observed. The mixture was concentrated under vacuum to afford *tert*-butyl (2*S*,4*S*)-4-amino-2-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidine-1-carboxylate (260 mg, >99%) as a colorless gum, which was used without further purification. MS (ESI), *m/z* 326.9 $[M+H]^+$ (calcd for C14H23N4O3S = 327.2).

tert-Butyl (2S,4S)-2-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]-4-{[(4S)-1-methyl-2,6-dioxo-1,3-diazinane-4-carbonyl]amino}pyrrolidine-1-carboxylate

To a solution of *tert*-butyl (2*S*,4*S*)-4-amino-2-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidine-1-carboxylate (195 mg, 0.60 mmol) and (4*S*)-1-methyl-2,6-dioxo-1,3-diazinane-4-carboxylic acid (123 mg, 0.72 mmol) in DMF (4 mL) was added DIPEA (232 mg, 1.79 mmol) followed by HATU (273 mg, 0.72 mmol) at room temperature. The reaction was then stirred at room temperature for 15 hours. TLC (CH_2Cl_2/MeOH = 10:1) indicated that the starting material had been consumed with a new UV-active spot being observed. The reaction was concentrated under vacuum to afford a residue, which was purified by chromatography (silica gel, 0 – 5% MeOH/CH_2Cl_2) to give *tert*-butyl (2*S*,4*S*)-2-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]-4-{[(4*S*)-1-methyl-2,6-dioxo-1,3-diazinane-4-carbonyl]amino}pyrrolidine-1-carboxylate (220 mg, 77%) as a white solid, which was used without further purification. MS (ESI), *m/z* 481.1 $[M+H]^+$ (calcd for C20H29N6O6S = 481.2).

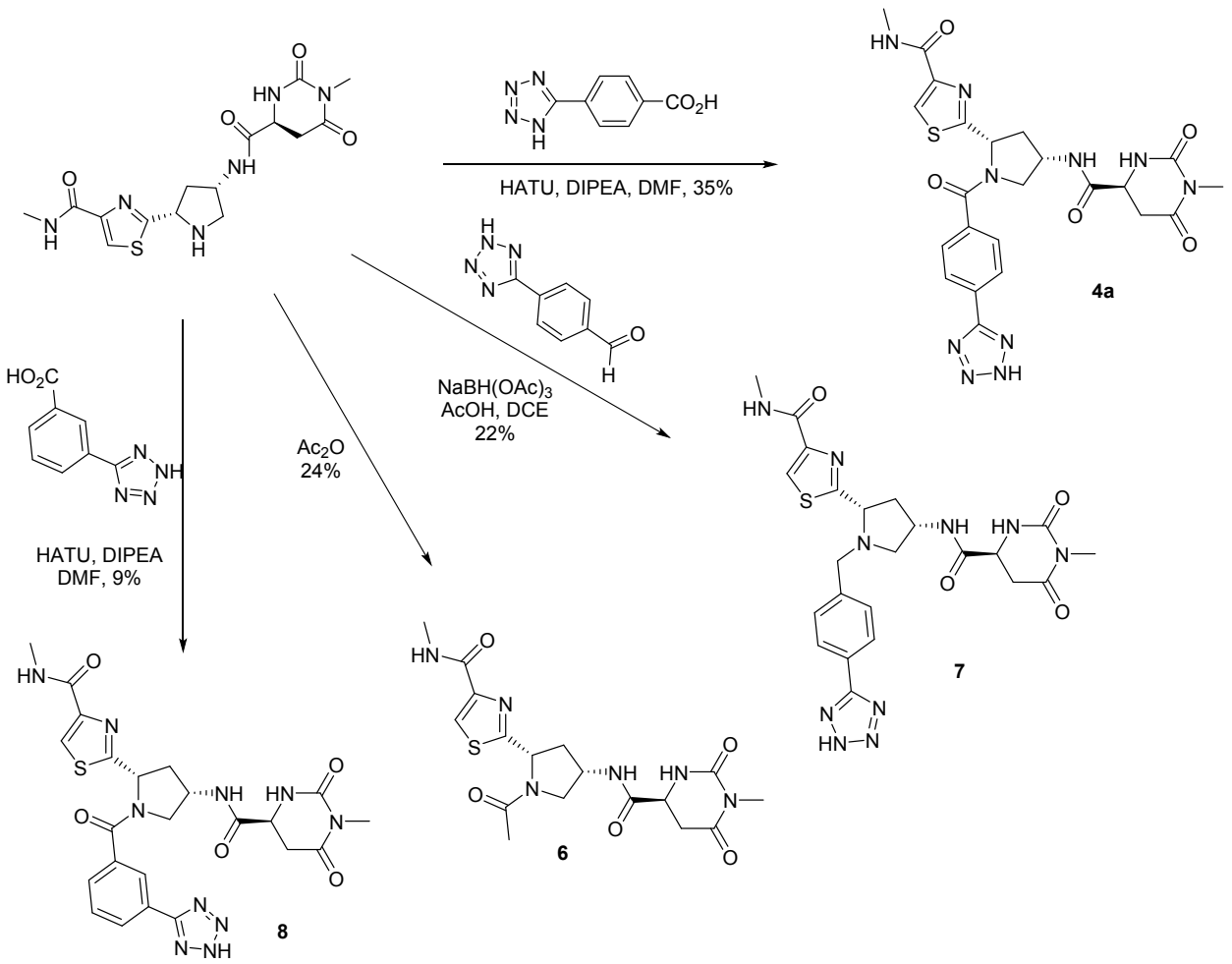
(4S)-1-Methyl-N-{(3S,5S)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide – hydrochloride salt

To a solution of *tert*-butyl (2*S*,4*S*)-2-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]-4-{[(4*S*)-1-methyl-2,6-dioxo-1,3-diazinane-4-carbonyl]amino}pyrrolidine-1-carboxylate (210 mg, 0.437 mmol) in methanol (3 mL) was added 4M HCl in dioxane (8 mL) at 0 ^{0}C. After the addition was completed, the reaction was warmed to room temperature and stirred for 15 hours. LC-MS indicated that the starting material had been consumed. The solvent was removed *in vacuo*, with the residue being re-dissolved in water (3 mL). Lyophilization over 24 hours afforded (4*S*)-1-methyl-*N*-{(3*S*,5*S*)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide hydrochloride salt (180 mg, 99%) as a colorless solid, which was used without further purification. MS (ESI), *m/z* 403.1 $[M+Na]^+$ (calcd for C15H20N6NaO4S = 403.1).

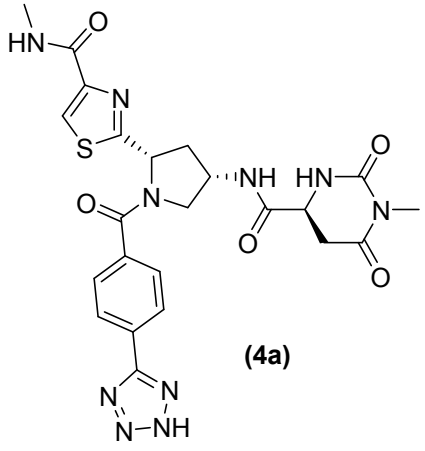
Alternative Deprotection to afford (4S)-1-methyl-N-{(3S,5S)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide – trifluoroacetate salt

To a solution of *tert*-butyl (2*S*,4*S*)-2-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]-4-{[(4*S*)-1-methyl-2,6-dioxo-1,3-diazinane-4-carbonyl]amino}pyrrolidine-1-carboxylate (100 mg, 0.208 mmol) in CH_2Cl_2 (2 mL) was added trifluoroacetic acid (2 mL) at 15 ^{0}C. After the addition was completed, the reaction was stirred for 3 hours at room temperature. LC-MS indicated that the starting material had been consumed. The reaction was concentrated, and the residue azeotroped with CH_2Cl_2 (3 x 10 mL) to afford (4*S*)-1-methyl-*N*-{(3*S*,5*S*)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide trifluoroacetate salt (100 mg, 97%) as a yellow gum, which was used without further purification. MS (ESI), *m/z* 403.0 $[M+Na]^+$ (calcd for C15H20N6NaO4S = 403.1).

Scheme S5 - Syntheses of Compounds (4a), (6), (7), and (8)

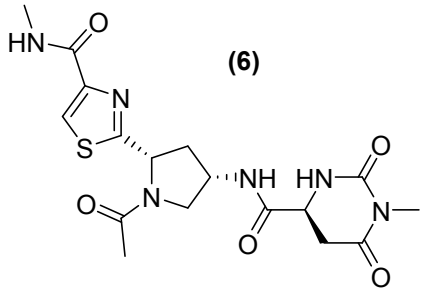


(4S)-1-methyl-N-{(3S,5S)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]-1-[4-(1H-tetrazol-5-yl)benzoyl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide (4a)



To a solution of (4*S*)-1-methyl-*N*-{(3*S*,5*S*)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide trifluoroacetate salt (100.0 mg, 0.202 mmol), 4-(1H-tetrazol-5-yl)benzoic acid (150.0 mg, 0.789 mmol) in DMF (5.0 mL) was added DIPEA (544.0 mg, 4.21 mmol) and HATU (300.0 mg, 0.789 mmol) at room temperature. The reaction was stirred at room temperature for 15 hours after which time LC-MS indicated that the starting material had been consumed. The solvent was removed *in vacuo*, and the residue purified by preparative HPLC (A : water w/0.225% formic acid, B : methanol, 20 – 40% B over 8 minutes, flow rate 35 mL/min using a Agela Durashell C18 150 x 25 mm x 5 μm column) to afford after solvent removal and lyophilization (4*S*)-1-methyl-*N*-{(3*S*,5*S*)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]-1-[4-(1*H*-tetrazol-5-yl)benzoyl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide **(4a)** (26 mg, 23%) as a white solid. HRMS (ESI/Q-TOF) m/z: $[M + H]^+$ calcd for C23H25N10O5S = 553.1652; Found 553.1734. MS (ESI), m/z 553.1 $[M+H]^+$ (calcd for C23H25N10O5S = 553.2). ^{1}H NMR (400 MHz, DMSO-d_6) (mixture of rotamers) δ 8.46 - 8.29 (m, 2H), 8.23 - 8.11 (m, 3H), 7.88 - 7.77 (m, 3H), 5.57 (t, J = 8.1 Hz, 1H), 4.36 - 4.25 (m, 1H), 3.93 - 3.86 (m, 1H), 3.83 - 3.73 (m, 1H), 3.61 - 3.44 (m, 1H), 2.98 - 2.88 (m, 5H), 2.86 - 2.73 (m, 4H), 2.63 - 2.54 (m, 2H), 2.25 - 2.13 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.26, 170.99, 169.03, 168.75, 161.39, 154.23, 149.99, 138.39, 128.90, 127.55, 123.80, 57.50, 54.41, 48.81, 37.32, 34.09, 26.70, 26.30.

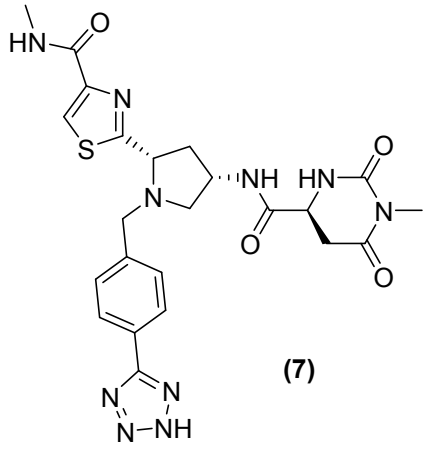
(4S)-N-{(3S,5S)-1-acetyl-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidin-3-yl}-1-methyl-2,6-dioxo-1,3-diazinane-4-carboxamide (6)



(6)

A solution of the hydrochloride salt of (4*S*)-1-methyl-*N*-{(3*S*,5*S*)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide (50 mg, 0.12 mmol) in acetic anhydride (2 mL) was stirred at 50 ^{0}C for 5 hours. LC-MS indicated that the starting material had been consumed with the main new peak showing the mass of the desired product. The solvent was removed with the residue being purified by preparative TLC (silica gel, CH_2Cl_2/MeOH = 10:1). The major band was isolated with the silica being washed with MeOH. Filtration followed by removal of the solvent afforded a residue, which was taken up in water, and lyophilized to afford (4*S*)-*N*-{(3*S*,5*S*)-1-acetyl-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidin-3-yl}-1-methyl-2,6-dioxo-1,3-diazinane-4-carboxamide **(6)** (12 mg, 24% yield) as a white solid. HRMS (ESI/Q-TOF) m/z: $[M + H]^+$ calcd for C17H23N6O4S = 423.13724; Found 423.14609. MS (ESI), m/z 445.0 $[M+Na]^+$ (calcd for C17H22N6NaO5S = 445.1). ^{1}H NMR (600 MHz, DMSO-d_6) (mixture of rotamers) δ 8.39 - 8.23 (m, 1H), 8.18 (s, 1H), 7.78 (d, J = 3.1 Hz, 1H), 5.47 - 5.21 (m, 1H), 4.40 - 4.30 (m, 1H), 4.43 - 4.17 (m, 1H), 4.07 - 3.74 (m, 1H), 3.49 - 3.24 (m, 1H, overlapping with water), 2.93 (s, 2H), 2.82 - 2.73 (m, 2H), 2.68 - 2.54 (m, 1H), 2.20 - 2.05 (m, 1H), 2.03 - 1.99 (m, 1H), 1.84 - 1.79 (m, 1H), 2.04 - 1.79 (m, 1H).^{13}C NMR (176 MHz, DMSO-d_6) δ 173.10, 170.30, 170.18, 169.16, 168.25, 160.95, 153.73, 153.70, 148.99, 123.70, 123.24, 58.25, 56.60, 52.41, 48.46, 48.37, 47.40, 36.84, 33.63, 33.59, 26.18, 25.71, 22.41, 21.88.

(4S)-1-methyl-N-[(3S,5S)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]-1-{[4-(1H-tetrazol-5-yl)phenyl]methyl}pyrrolidin-3-yl]-2,6-dioxo-1,3-diazinane-4-carboxamide (7)

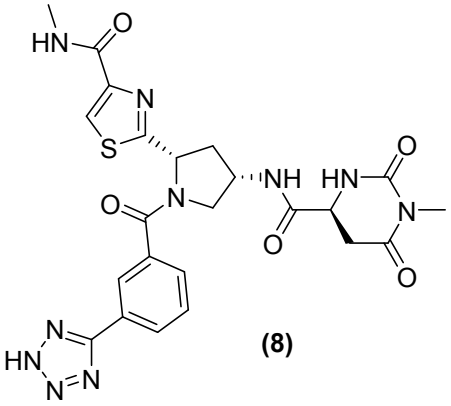


(7)

To a solution of the hydrochloride salt of (4*S*)-1-methyl-*N*-{(3*S*,5*S*)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide (80 mg, 0.19 mmol) and 4-(1*H*-tetrazol-5-yl)benzaldehyde (67 mg, 0.384 mmol) in anhydrous 1,2-dichloroethane (3 mL) was added triethylamine (39 mg, 0.384 mmol), acetic acid (23 mg, 0.384 mmol) and sodium triacetoxyborohydride (122 mg, 0.576 mmol) at 0 ^{0}C. The reaction mixture was warmed to room temperature, and stirred for 16 hours after which time, LC-MS indicated that the starting material had been consumed with a new major peak displaying the desired mass for the product. The solvent was removed, and the residue purified by preparative HPLC (A : water w/0.05% ammonium hydroxide, B : aectonitrile, 0 – 10% B over 10 minutes, flow rate 25 mL/min using a Xbridge C18 150 x 30 mm x 10 μm column) to afford after solvent removal and lyophilization (4*S*)-1-methyl-*N*-[(3*S*,5*S*)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]-1-{[4-(1H-tetrazol-5-yl)phenyl]methyl}pyrrolidin-3-yl]-2,6-dioxo-1,3-diazinane-4-carboxamide **(7)** (23 mg, 22%) as a white solid. HRMS (ESI/Q-TOF) m/z: $[M + H]^+$ calcd for C23H27N10O4S = 539.18592; Found 539.19447. MS (ESI), m/z 539.1 $[M+H]^+$ (calcd for C23H27N10O4S = 539.2). ^{1}H NMR (600 MHz, DMSO-d_6) (mixture of rotamers) δ 8.34 - 8.12 (m, 3H), 8.01 (d, J = 8.1 Hz, 2H), 7.81 - 7.70

(m, 1H), 7.53 (d, *J* = 8.1 Hz, 1H), 4.33 - 4.08 (m, 3H), 4.00 - 3.90 (m, 2H), 3.52 - 3.40 (m, 3H), 3.18 - 3.05 (m, 1H), 2.98 - 2.85 (m, 4H), 2.81 - 2.65 (m, 5H), 2.59 - 2.54 (m, 1H), 1.83 - 1.68 (m, 1H).^{13}C NMR (176 MHz, DMSO) δ 176.11, 170.07, 168.29, 161.00, 156.11, 153.79, 149.95, 140.85, 130.26, 130.02, 129.00, 126.73, 126.32, 124.44, 123.64, 64.55, 58.21, 57.22, 48.06, 47.83, 45.71, 40.73, 26.15, 25.73.

(4S)-1-Methyl-N-{(3S,5S)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]-1-[3-(1H-tetrazol-5-yl)benzoyl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide (8)

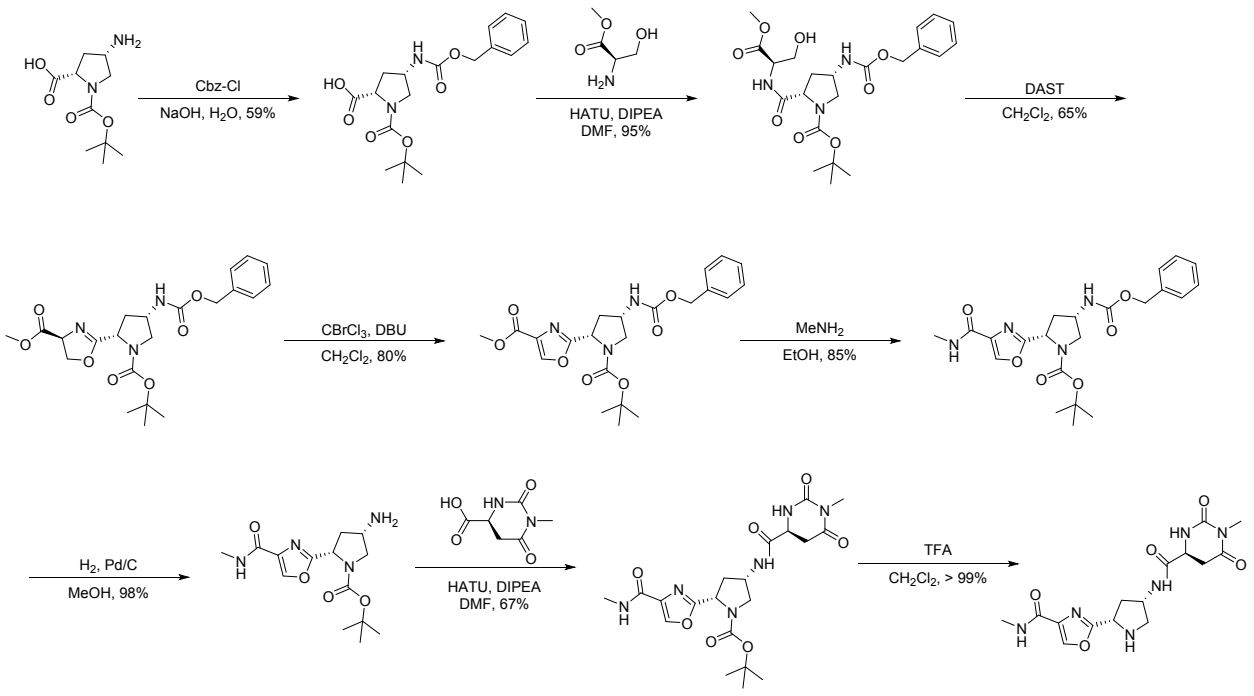


(8)

To a solution of the hydrochloride salt of (4*S*)-1-methyl-*N*-{(3*S*,5*S*)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide (60 mg, 0.14 mmol) and 3-(1*H*-tetrazol-5-yl)benzoic acid (27.4 mg, 0.144 mmol), HATU (54.7 mg, 0.144 mmol) in DMF (2 mL) was added DIPEA (93 mg, 0.72 mmol). After the addition, the reaction was stirred at 30 ^{0}C for 15 hours. LC-MS indicated that the starting material had been consumed with a new peak corresponding to the mass of the desired product being observed. The solvent was removed, and the residue was subjected to preparative HPLC (A : water w/0.225% formic acid, B : acetonitrile, 10 – 30% B over 10 minutes, flow rate 25 mL/min using a Agela Durashell C18 150 x 25 mm x 5 μm column) to afford after solvent removal and lyophilization 15 mg of the desired product

contaminated with PF_6 residues. This was subjected to a second purification by preparative HPLC (A : water w/0.05% ammonium hydroxide, B : acetonitrile, 5 – 30% B over 10 minutes, flow rate 25 mL/min using a DuraShell C18 150 x 25 mm x 5 μm column) to afford after solvent removal and lyophilization (4*S*)-1-methyl-*N*-{(3*S*,5S)-5-[4-(methylcarbamoyl)-1,3-thiazol-2-yl]-1-[3-(1H-tetrazol-5-yl)benzoyl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide **(8)** (7 mg, 9%) as a colorless solid. HRMS (ESI/Q-TOF) m/z: $[M + H]^+$ calcd for C27H25N10O5S = 553.1652; Found 553.1728. MS (ESI), m/z 553.1 $[M+H]^+$ (calcd for C27H25N10O5S = 553.2). ^{1}H NMR (400 MHz, DMSO-d_6) (mixture of rotamers) δ 8.46 - 8.28 (m, 2H), 8.26 - 8.14 (m, 3H), 7.84 - 7.70 (m, 3H), 5.58 (br t, *J* = 8.1 Hz, 1H), 4.39 - 4.26 (m, 1H), 3.88 (td, *J* = 3.6, 7.0 Hz, 1H), 3.84 - 3.74 (m, 1H), 3.58 (dd, *J* = 8.9, 10.2 Hz, 2H), 2.96 - 2.86 (m, 4H), 2.83 - 2.72 (m, 4H), 2.58 (br dd, *J* = 3.1, 16.6 Hz, 1H), 2.27 - 2.14 (m, 1H).^{13}C NMR (101 MHz, DMSO) δ =173.29, 170.92, 169.09, 168.75, 161.39, 156.02, 154.18, 150.00, 137.18, 130.13, 129.97, 129.34, 126.12, 125.80, 123.81, 57.56, 54.38, 48.85, 48.78, 37.43, 34.01, 26.70, 26.29.

Scheme S6 - Synthesis of (4S)-1-methyl-N-{(3S,5S)-5-[4-(methylcarbamoyl)-1,3-oxazol-2-yl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide



(4S)-4-{[(Benzyloxy)carbonyl]amino}-1-(tert-butoxycarbonyl)-L-proline

To a solution of (2*S*,4*S*)-1-Boc-4-amino-pyrrolidine-2-carboxylic acid (1.5 g, 6.5 mmol) in water (32.6 mL) was added 2 N NaOH (3.26 mL, 6.51 mmol) The mixture was cooled to 0 ^{0}C, and Cbz-Cl (1.22 g, 0.98 mL, 7.17 mmol) was added in a dropwise manner. A further aliquot of 2 N NaOH (3.26 mL, 6.51 mmol) was then added to the reaction mixture, and the resulting solution stirred at 0 ^{0}C for 1 hour, before being warmed to room temperature and stirred for 3 hours. Further portions of both 2 N NaOH (6.51 mL, 13.0 mmol) and Cbz-Cl (1.22 g 0.98 mL, 7.17 mmol) were added at 0 ^{0}C, and the reaction mixture stirred at room temperature for 16 hours. LC-MS indicated that the starting material was consumed, and the reaction was then washed with TBME (100 mL) to remove excess Cbz-Cl. The aqueous layer was acidified to pH ~ 3 with solid citric acid and extracted with EtOAc (3 x 80 mL). The organic layers were washed with water (100 mL), and brine (100 mL), dried over $MgSO_4$, and concentrated to afford (4*S*)-4-{[(benzyloxy)carbonyl]amino}-1-(t*ert*-

butoxycarbonyl)-*L*-proline (1.4 g, 59%) as a white solid, which was used without further purification. MS (ESI), *m/z* 387.0 $[M+Na]^+$ (calcd for C18H24N2NaO6 = 387.2).

Methyl (4S)-4-{[(benzyloxy)carbonyl]amino}-1-(tert-butoxycarbonyl)-L-prolyl-D-serinate

To a solution of (4*S*)-4-{[(benzyloxy)carbonyl]amino}-1-(*tert*-butoxycarbonyl)-*L*-proline (1400 mg, 3.842 mmol), *L*-serine methyl ester (658 mg, 4.23 mmol) and HATU (1.61 g, 4.23 mmol) in DMF (20 mL) was added DIPEA (1.24 g, 9.61 mmol). After the addition was complete, the reaction was stirred at room temperature for 16 hours. Water (50 mL) was added to the reaction, which was then extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with saturated $NaHCO_3$ (50 mL), 1N citric acid (50 mL), and brine (50 mL), then dried over Na_2SO_4. Removal of the solvent under vacuum afforded methyl (4*S*)-4-{[(benzyloxy)carbonyl]amino}-1-(*tert*-butoxycarbonyl)-*L*-prolyl-*D*-serinate (1.7 g, 95%) as a yellow gum, which was used without further purification. MS (ESI), *m/z* 488.1 $[M+Na]^+$ (calcd for C22H31N3NaO8 = 488.2).

Methyl (4S)-2-[(2S,4S)-4-{[(benzyloxy)carbonyl]amino}-1-(tert-butoxycarbonyl)pyrrolidin-2-yl]-4,5-dihydro-1,3-oxazole-4-carboxylate

DAST (2.66 g, 16.5 mmol) was added in a dropwise manner to a solution of methyl (4*S*)-4-{[(benzyloxy)carbonyl]amino}-1-(*tert*-butoxycarbonyl)-*L*-prolyl-*D*-serinate (1.92 g, 4.13 mmol) in CH_2Cl_2 (30 mL) at -70 ^{0}C. After the addition was complete, the reaction was stirred at -10 ^{0}C for 1.5 hours. TLC (CH_2Cl_2/MeOH = 20 : 1) indicated that the starting material had been consumed, and the reaction was quenched with saturated aqueous $NaHCO_3$ solution (20 mL). The mixture was extracted with CH_2Cl_2 (3 x 20 mL), and the combined organic layers washed with brine, dried over Na_2SO_4, and the solvent removed under vacuum to afford a residue, which was

purified by chromatography (silica gel, 0 – 100% EtOAc/petroleum ether) to afford methyl (4*S*)-2-[(2*S*,4*S*)-4-{[(benzyloxy)carbonyl]amino}-1-(tert-butoxycarbonyl)pyrrolidin-2-yl]-4,5-dihydro-1,3-oxazole-4-carboxylate (1.2 g, 65%) as a yellow solid, which was used without further purification. MS (ESI), *m/z* 448.1 $[M+H]^+$ (note under acid-mediated LC-MS conditions, the compound undergoes hydrolysis thus displaying two peaks) (calcd for C22H30N3O7 = 448.2).

Methyl 2-[(2S,4S)-4-{[(benzyloxy)carbonyl]amino}-1-(tert-butoxycarbonyl)pyrrolidin-2-yl]-1,3-oxazole-4-carboxylate

To a solution of methyl (4*S*)-2-[(2*S*,4*S*)-4-{[(benzyloxy)carbonyl]amino}-1-(*tert*-butoxycarbonyl)pyrrolidin-2-yl]-4,5-dihydro-1,3-oxazole-4-carboxylate (1.20 g, 2.68 mmol) in CH_2Cl_2 (30 mL) at – 30 ^{0}C was added DBU (816 mg, 5.36 mmol), and the reaction stirred for 30 minutes. To this solution was added bromotrichloromethane (638 mg, 3.22 mmol) at – 20 ^{0}C with the reaction being stirred at this temperature for 10 minutes before being warmed to room temperature and stirred for 16 hours. TLC (EtOAc/petroleum ether = 1:1) indicated that the starting material had been consumed, and the reaction was quenched with water (30 mL). The resulting mixture was extracted with CH_2Cl_2 (3 x 25 mL), and the combined organic layers washed with brine (50 mL), dried over Na_2SO_4 and the solvent removed under vacuum to afford a residue, which was purified by chromatography (silica gel, 0 – 55% EtOAc/petroleum ether) to give methyl 2-[(2*S*,4*S*)-4-{[(benzyloxy)carbonyl]amino}-1-(*tert*-butoxycarbonyl)pyrrolidin-2-yl]-1,3-oxazole-4-carboxylate (950 mg, NMR indicates the presence of ~ 0.15 equiv. of CH_2Cl_2, 80%) as a white solid. MS (ESI), *m/z* 468.0 $[M+Na]^+$ (calcd for C22H27N3NaO7 = 468.2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (s, 1 H), 7.37 – 7.32 (m, 5 H), 5.13 – 4.96 (m, 3 H), 4.49 – 4.47 (m, 1 H),

3.82 – 3.79 (m, 4 H), 3.57 – 3.49 (m, 1 H), 2.70 – 2.61 (m, 1 H), 2.20 – 2.17 (m, 1 H), 1.30 – 1.26 (m, 9 H).

tert-Butyl (2S,4S)-4-{[(benzyloxy)carbonyl]amino}-2-[4-(methylcarbamoyl)-1,3-oxazol-2-yl]pyrrolidine-1-carboxylate

A solution of methyl 2-[(2*S*,4*S*)-4-{[(benzyloxy)carbonyl]amino}-1-(*tert*-butoxycarbonyl)pyrrolidin-2-yl]-1,3-oxazole-4-carboxylate (450 mg, 1.01 mmol) in $MeNH_2$ (10 mL, 30% in ethanol) was stirred at 20 ^{0}C for 16 hours. LC-MS indicated that the starting material had been consumed. The mixture was concentrated under vacuum to afford *tert*-butyl (2*S*,4*S*)-4-{[(benzyloxy)carbonyl]amino}-2-[4-(methylcarbamoyl)-1,3-oxazol-2-yl]pyrrolidine-1-carboxylate (380 mg, 85%) as a white solid, which was used in the next step without any further purification. MS (ESI), *m/z* 467.1 $[M+Na]^+$ (calcd for C22H28N4NaO6 = 467.2).

tert-Butyl (2S,4S)-4-amino-2-[4-(methylcarbamoyl)-1,3-oxazol-2-yl]pyrrolidine-1-carboxylate

A solution of *tert*-butyl (2*S*,4*S*)-4-{[(benzyloxy)carbonyl]amino}-2-[4-(methylcarbamoyl)-1,3-oxazol-2-yl]pyrrolidine-1-carboxylate (330 mg, 0.742 mmol) and wet Pd/C (60 mg, 10% wt) in methanol (10 mL) was hydrogenated under balloon pressure (15 psi) at room temperature for 2 hours. TLC (EtOAc/petroleum ether = 1:1) indicated that the starting material had been consumed, and the reaction mixture was filtered through a pad of celite. Evaporation of the solvent under vacuum gave a residue, which upon being dried under high vacuum afforded *tert*-butyl (2*S*,4*S*)-4-amino-2-[4-(methylcarbamoyl)-1,3-oxazol-2-yl]pyrrolidine-1-carboxylate (225 mg, 98%) as a yellow solid, which was used without further purification. MS (ESI), *m/z* 310.9 $[M+H]^+$ (calcd for C14H23N4O4 = 311.2). ^{1}H NMR (400 MHz, CD_3OD) δ 8.33 – 8.28 (m, 1 H), 4.95, (t, J = 6.8

Hz, 1 H), 3.83 – 3.79 (m, 1 H), 3.60 (quin., J = 6.4 Hz, 1 H), 2.72 (s, 3 H), 2.68 – 2.65 (m, 1 H), 1.98 – 1.94 (m, 1 H), 1.45 – 1.27 (m, 9 H).

tert-Butyl (2S,4S)-2-[4-(methylcarbamoyl)-1,3-oxazol-2-yl]-4-{[(4S)-1-methyl-2,6-dioxo-1,3-diazinane-4-carbonyl]amino}pyrrolidine-1-carboxylate

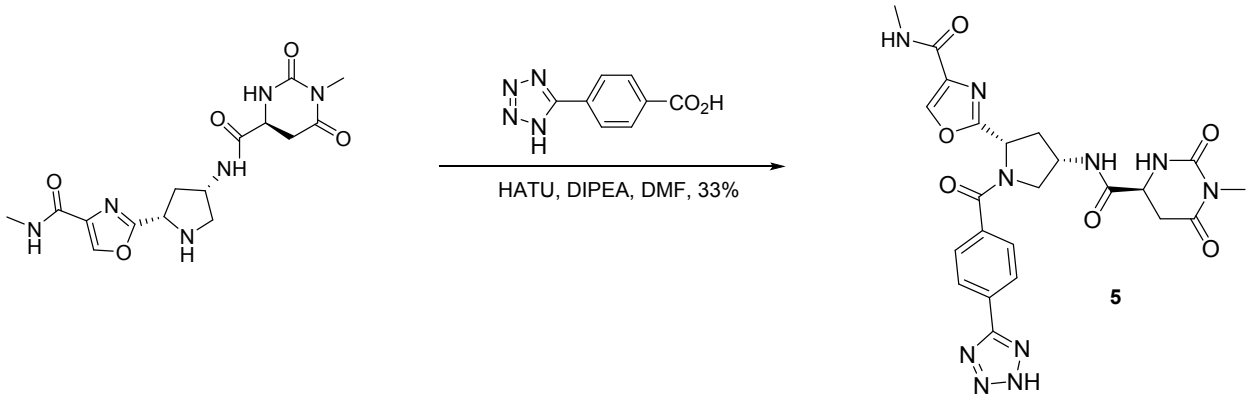
To a solution of *tert*-butyl (2*S*,4*S*)-4-amino-2-[4-(methylcarbamoyl)-1,3-oxazol-2-yl]pyrrolidine-1-carboxylate (70 mg, 0.23 mmol) and (4*S*)-1-methyl-2,6-dioxo-1,3-diazinane-4-carboxylic acid (46.6 mg, 0.271 mmol), HATU (103 mg, 0.271 mmol) in DMF (2 mL) was added DIPEA (87.5 mg, 0.677 mmol). The reaction mixture was stirred at 30 ^{0}C for 15 hours after which time, LC-MS indicated the starting material had been consumed with a new major peak displaying the desired product mass being observed. The solvent was removed, and the residue purified by chromatography (silica gel, 0-25% MeOH/CH_2Cl_2) to afford *tert*-butyl (2*S*,4*S*)-2-[4-(methylcarbamoyl)-1,3-oxazol-2-yl]-4-{[(4*S*)-1-methyl-2,6-dioxo-1,3-diazinane-4-carbonyl]amino}pyrrolidine-1-carboxylate (70 mg, 67%) as a white solid, which was used in the next step without further purification. MS (ESI), m/z 465.0 $[M+H]^+$ (calcd for C20H29N6O7 = 465.2). Note, certain LC-MS conditions detected a ~ 20% unidentified impurity in this material.

(4S)-1-Methyl-N-{(3S,5S)-5-[4-(methylcarbamoyl)-1,3-oxazol-2-yl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide (trifluoroacetate salt)

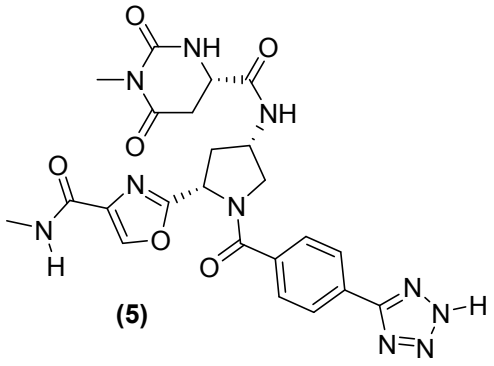
To a solution of *tert*-butyl (2*S*,4*S*)-2-[4-(methylcarbamoyl)-1,3-oxazol-2-yl]-4-{[(4*S*)-1-methyl-2,6-dioxo-1,3-diazinane-4-carbonyl]amino}pyrrolidine-1-carboxylate (70 mg, 0.15 mmol) in CH_2Cl_2 (2 mL) cooled in an ice-bath was added trifluoroacetic acid (1 mL). After the addition was completed, the reaction was warmed to room temperature, and stirred for 1 hour. LC-MS indicated

the reaction was complete, and the solvent was removed under vacuum with the residue being azeotroped with CH_2Cl_2 (3 x 5 mL) to afford the trifluoroacetate salt of (4*S*)-1-methyl-*N*-{(3*S*,5*S*)-5-[4-(methylcarbamoyl)-1,3-oxazol-2-yl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide (80 mg crude, >99%) as a viscous gum, which was used in the next step without further purification.

Scheme S7 - Synthesis of (4S)-1-methyl-N-{(3S,5S)-5-[4-(methylcarbamoyl)-1,3-oxazol-2-yl]-1-[4-(1H-tetrazol-5-yl)benzoyl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide (5)



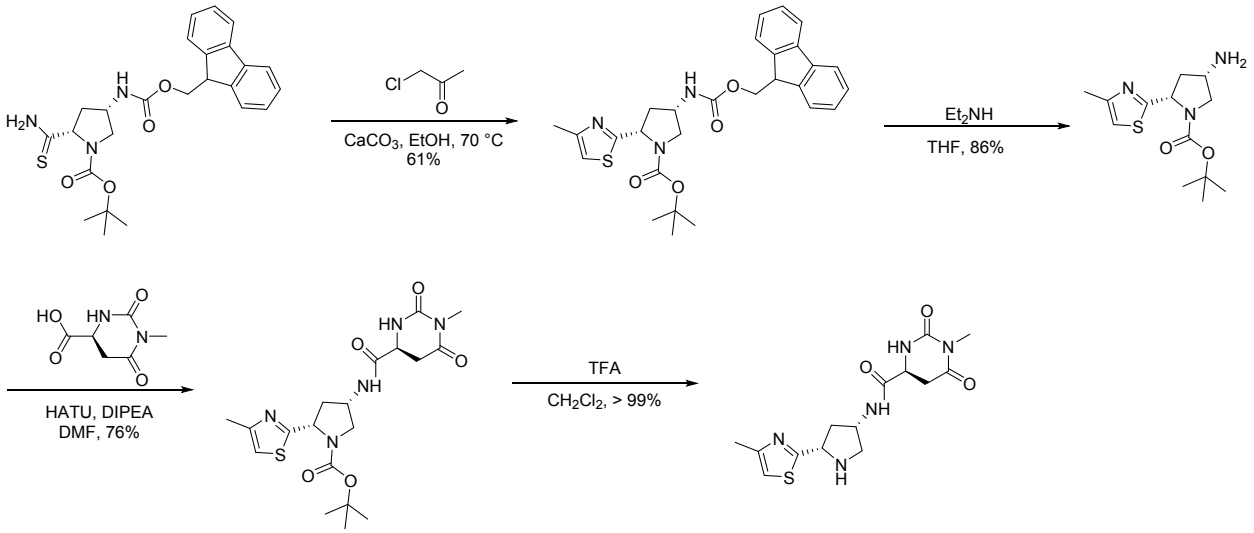
(4S)-1-methyl-N-{(3S,5S)-5-[4-(methylcarbamoyl)-1,3-oxazol-2-yl]-1-[4-(1H-tetrazol-5-yl)benzoyl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide (5)



To a solution of the trifluoroacetate salt of (4*S*)-1-Methyl-*N*-{(3*S*,5*S*)-5-[4-(methylcarbamoyl)-1,3-oxazol-2-yl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide (80 mg, 0.15 mmol) and

4-(1*H*-tetrazol-5-yl)benzoic acid (31 mg, 0.162 mmol), HATU (62 mg, 0.162 mmol) in DMF (2 mL) was added DIPEA (95.1 mg, 0.736 mmol). After the addition was completed, the reaction was stirred at room temperature for 15 hours. LC-MS indicated the starting material had been consumed with a new major peak with the desired product mass being observed. The solvent was removed, and the residue was subjected to preparative HPLC (A : water w/0.05% ammonium hydroxide, B : methanol, 5 – 25% B over 8 minutes, flow rate 35 mL/min using a Agela Durashell C18 150 x 25 mm x 5 μm column) to afford after solvent removal and lyophilization 36 mg of the desired product, which was shown to contain impurities by NMR. This was subjected to a second purification by preparative HPLC (A : water w/0.05% ammonium hydroxide, B : acetonitrile, 5 – 25% B over 10 minutes, flow rate 25 mL/min using a DuraShell C18 150 x 25 mm x 5 μm column) to afford after solvent removal and lyophilization (4*S*)-1-methyl-*N*-{(3*S*,5*S*)-5-[4-(methylcarbamoyl)-1,3-oxazol-2-yl]-1-[4-(1*H*-tetrazol-5-yl)benzoyl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide **(5)** (26 mg, 33%) as a white solid. HRMS (ESI/Q-TOF) m/z: $[M + H]^+$ calcd for C23H25N10O6 = 536.18803; Found 536.18948. MS (ESI), m/z 537.0 $[M+H]^+$ (calcd for C23H25N10O6 = 537.2). HRMS (6200 series TOF/6500 series). Found m/z = 536.18948 $[M+H]^+$. Target m/z = 536.18803. ^{1}H NMR (600 MHz, DMSO-d_6) (mixture of rotamers) δ 8.61 - 8.30 (m, 2H), 8.23 - 8.02 (m, 3H), 7.79 (d, J = 3.1 Hz, 1H), 7.63 (br d, J = 6.4 Hz, 2H), 7.31 - 6.95 (m, 2H), 5.31 (br d, J = 7.2 Hz, 1H), 4.42 - 4.20 (m, 1H), 4.00 - 3.77 (m, 2H), 2.92 (s, 3H), 2.79 - 2.59 (m, 5H), 2.16 - 1.94 (m, 1H).^{13}C NMR (176 MHz, DMSO) δ 170.41, 168.30, 163.56, 160.30, 157.91, 153.69, 141.11, 136.09, 135.41, 130.85, 127.90, 126.03, 53.51, 53.03, 48.31, 48.12, 35.28, 33.51, 26.18, 25.43.

Scheme S8 - Synthesis of (4*S*)-1-methyl-*N*-[(3*S*,5*S*)-5-(4-methyl-1,3-thiazol-2-yl)pyrrolidin-3-yl]-2,6-dioxo-1,3-diazinane-4-carboxamide



***tert*-Butyl (2S,4S)-4-({[(9H-fluoren-9-yl)methoxy]carbonyl}amino)-2-(4-methyl-1,3-thiazol-2-yl)pyrrolidine-1-carboxylate**

A mixture of *tert*-butyl (2*S*,4*S*)-2-carbamothioyl-4-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino)pyrrolidine-1-carboxylate (20.7 g, 46.4 mmol), 1-chloropropan-2-one (8.19 g, 88.5 mmol) and calcium carbonate (13.3 g, 133 mmol) in anhydrous EtOH (210 mL) was heated at 70 ^{0}C for 15 hours. TLC (petroleum ether/EtOAc = 1:1) and LC-MS indicated that the reaction was > 90% complete. The reaction was allowed to cool, filtered, and the solvent removed *in vacuo* to afford a residue, which was purified by chromatography (silica gel, EtOAc/petroleum ether 0 – 60%) to afford *tert*-butyl (2*S*,4*S*)-4-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino)-2-(4-methyl-1,3-thiazol-2-yl)pyrrolidine-1-carboxylate (13.68 g, 61%) as a light yellow solid. MS (ESI), *m/z* 506.0 $[M+H]^+$ (calcd for C28H32N3O4S = 506.2). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.94 - 7.84 (m, 2H), 7.72 - 7.59 (m, 3H), 7.46 - 7.26 (m, 4H), 7.20 - 7.09 (m, 1H), 5.02 (br s, 1H), 4.39 - 4.26 (m,

2H), 4.23 - 3.99 (m, 2H), 3.87 - 3.68 (m, 1H), 3.23 - 3.08 (m, 1H), 2.28 (s, 3H), 1.49 - 1.14 (m, 9H).

***tert*-Butyl (2*S*,4*S*)-4-amino-2-(4-methyl-1,3-thiazol-2-yl)pyrrolidine-1-carboxylate**

To a solution of *tert*-butyl (2*S*,4*S*)-4-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino)-2-(4-methyl-1,3-thiazol-2-yl)pyrrolidine-1-carboxylate (13.68 g, 27.1 mmol) in THF (270 mL) cooled in an ice-bath was added diethylamine (42.0 mL, 406 mmol). The reaction mixture was warmed to room temperature and stirred for 15 hours after which time TLC (petroleum ether/EtOAc = 1:1 and CH_2Cl_2/MeOH = 10:1) showed the reaction to be complete. The volatiles were evaporated, and the residue subjected to chromatography (silica gel, EtOAc/petroleum ether 0 – 100% then MeOH/CH_2Cl_2 0 – 20%) to afford *tert*-butyl (2*S*,4*S*)-4-amino-2-(4-methyl-1,3-thiazol-2-yl)pyrrolidine-1-carboxylate (6.62 g, 86%) as a yellow oil. MS (ESI), *m/z* 283.9 $[M+H]^+$ (calcd for C13H22N3O2S = 284.1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.75 (s, 1H), 5.25 - 4.95 (m, 1H), 3.99 - 3.72 (m, 1H), 3.68 - 3.53 (m, 1H), 3.43 (s, 1H), 3.32 - 3.17 (m, 1H), 2.55 - 2.55 (m, 1H), 2.38 (d, *J* = 0.8 Hz, 3H), 2.15 - 1.87 (m, 4H), 1.57 - 1.18 (m, 9H).

***tert*-Butyl (2*S*,4*S*)-4-{[(4*S*)-1-methyl-2,6-dioxo-1,3-diazinane-4-carbonyl]amino}-2-(4-methyl-1,3-thiazol-2-yl)pyrrolidine-1-carboxylate**

To a stirred solution of *tert*-butyl (2*S*,4*S*)-4-amino-2-(4-methyl-1,3-thiazol-2-yl)pyrrolidine-1-carboxylate (2.84 g, 10.02 mmol) and (4*S*)-1-methyl-2,6-dioxo-1,3-diazinane-4-carboxylic acid (1.81 mg, 10.5 mmol) in dry DMF (30 mL) was added HATU (5.06 g, 13.3 mmol) followed by DIPEA (5.43 g, 7.31 mL, 42.0 mmol) at 0 ^{0}C under a N_2 atmosphere. The reaction was stirred for 3 hours, after which time LC-MS indicated the reaction was complete. The mixture was poured into ice-water (50 mL) and extracted with EtOAc (3 x 50 mL) followed by CH_2Cl_2 (3 x 50 mL).

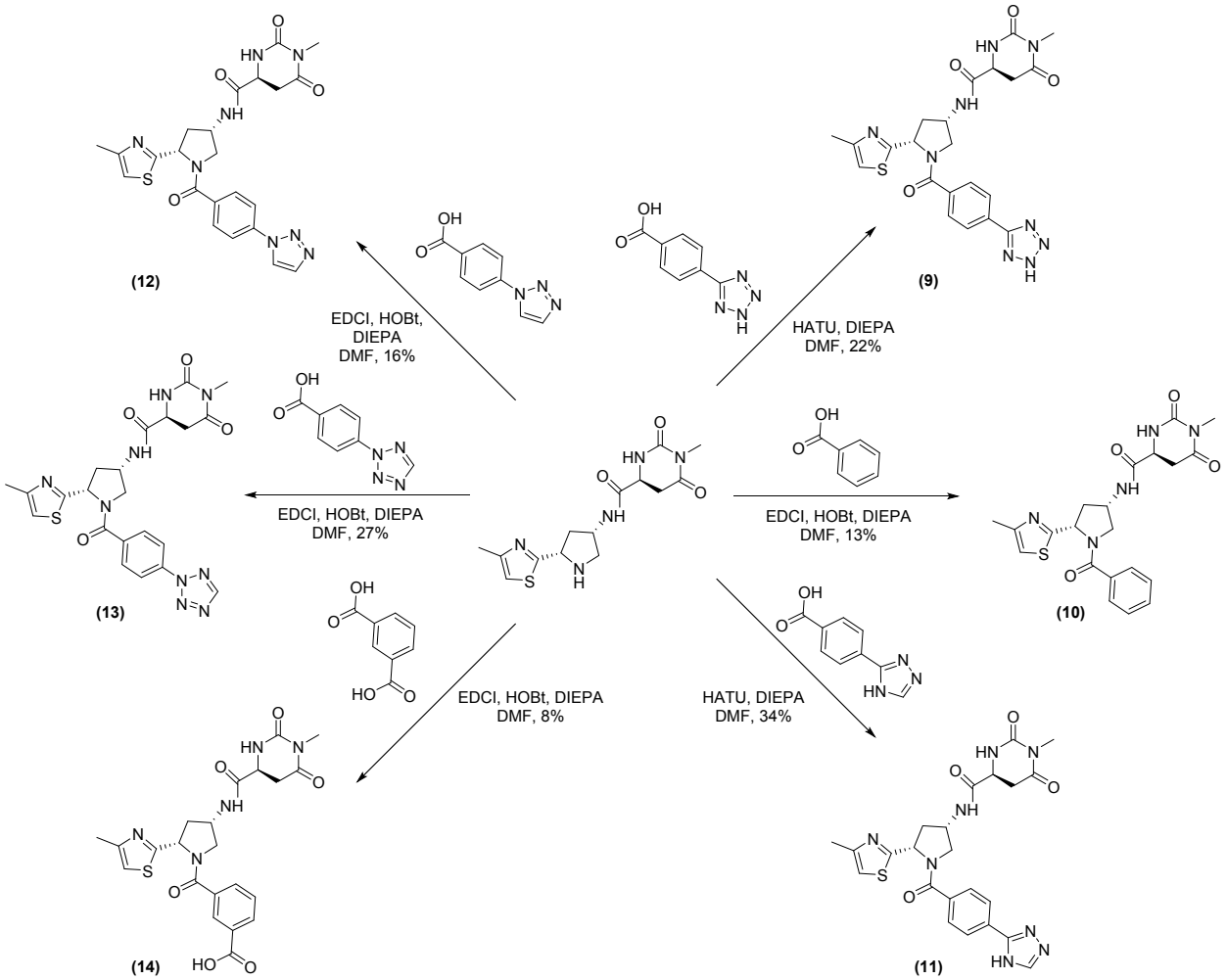
The combined extracts were dried over Na_2SO_4, and the solvents removed *in vacuo* to afford the crude product as a black gum. The residue was purified by preparative HPLC (A : water w/0.05% ammonium hydroxide, B : acetonitrile, 20 – 40% B over 15 minutes, flow rate 110 mL/min using a Phenomenex Gemini C18 250 x 50 mm x 10 µm column) to afford after solvent removal and lyophilization *tert*-butyl (2*S*,4*S*)-4-{[(4*S*)-1-methyl-2,6-dioxo-1,3-diazinane-4-carbonyl]amino}-2-(4-methyl-1,3-thiazol-2-yl)pyrrolidine-1-carboxylate (3.32 g, 76%) as a white solid. MS (ESI), *m/z* 438.1 $[M+H]^+$ (calcd for C19H28N5O5S = 438.2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.64 - 9.34 (m, 1H), 6.88 (br d, *J* = 3.8 Hz, 1H), 6.16 (br d, *J* = 12.5 Hz, 1H), 5.28 - 5.08 (m, 1H), 4.79 - 4.63 (m, 1H), 4.19 - 4.06 (m, 1H), 3.79 - 3.62 (m, 1H), 3.57 - 3.38 (m, 1H), 3.17 (s, 3H), 3.08 - 2.96 (m, 1H), 2.87 - 2.76 (m, 1H), 2.68 - 2.52 (m, 1H), 2.48 (s, 3H), 2.34 - 2.16 (m, 1H), 1.89 (br s, 1H), 1.42 (br d, *J* = 6.0 Hz, 9H).

(4*S*)-1-Methyl-*N*-[(3*S*,5*S*)-5-(4-methyl-1,3-thiazol-2-yl)pyrrolidin-3-yl]-2,6-dioxo-1,3-diazinane-4-carboxamide

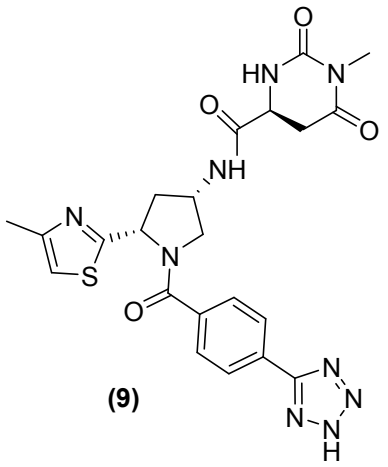
To a solution of *tert*-butyl (2*S*,4*S*)-4-{[(4*S*)-1-methyl-2,6-dioxo-1,3-diazinane-4-carbonyl]amino}-2-(4-methyl-1,3-thiazol-2-yl)pyrrolidine-1-carboxylate (3.32 g, 7.59 mmol) in CH_2Cl_2 (30 mL) at 0 ^{0}C was added trifluoroacetic acid (15 mL) in a dropwise manner. Upon completion of the addition, the reaction was warmed to room temperature, and stirred for 3 hours after which time LC-MS indicated that the starting material had been consumed. The solvent was removed *in vacuo*, and the residue dissolved in TBME (30 mL) and stirred for 30 minutes. The mixture was concentrated, and the residue partitioned between water (20 mL) and EtOAc (10 mL). Lyophilization of the aqueous afforded 3.29 g of crude product, which was further purified by SFC (Daicel ChiralPak AD 250mm x 50mm x 10µm eluting with 40% EtOH with 0.1% ammonium

hydroxide/CO_2 at a flow rate of 200 mL/min) to afford (4*S*)-1-methyl-*N*-[(3*S*,5*S*)-5-(4-methyl-1,3-thiazol-2-yl)pyrrolidin-3-yl]-2,6-dioxo-1,3-diazinane-4-carboxamide (2.55 g, 99%) as a brown solid. MS (ESI), *m/z* 337.9 $[M+H]^+$ (calcd for C14H20N5O3S = 338.1). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.53 (br d, *J* = 6.5 Hz, 1H), 7.89 (d, *J* = 3.3 Hz, 1H), 7.37 (s, 1H), 7.21 (br d, *J* = 18.1 Hz, 1H), 4.91 (dd, *J* = 7.3, 9.8 Hz, 1H), 4.40 (sxt, *J* = 7.4 Hz, 1H), 3.95 (td, *J* = 3.6, 7.1 Hz, 1H), 3.46 (br dd, *J* = 7.8, 11.5 Hz, 2H), 3.02 - 2.98 (m, 1H), 2.95 (s, 3H), 2.74 (td, *J* = 7.3, 12.9 Hz, 1H), 2.64 (dd, *J* = 3.1, 16.4 Hz, 1H), 2.39 (s, 3H), 2.11 - 2.01 (m, 1H).

Scheme S9 - Syntheses of Compounds from TABLE 2 (9), (10), (11), (12), (13), and (14).



(4*S*)-1-methyl-*N*-{(3*S*,5*S*)-5-(4-methyl-1,3-thiazol-2-yl)-1-[4-(2*H*-tetrazol-5-yl)benzoyl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide (9)

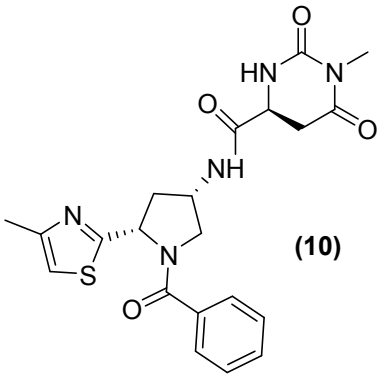


(9)

To a solution of 4-(2*H*-tetrazol-5-yl)benzoic acid (40.6 mg, 0.21 mmol) and HATU (81 mg, 0.213 mmol) in DMF (2 mL) was added DIPEA (115 mg, 155 µL, 0.89 mmol) followed by a solution of (4*S*)-1-methyl-*N*-[(3*S*,5*S*)-5-(4-methyl-1,3-thiazol-2-yl)pyrrolidin-3-yl]-2,6-dioxo-1,3-diazinane-4-carboxamide (60 mg, 0.18 mmol) in DMF (1 mL). The reaction was stirred at room temperature for 16 hours after which time, LC-MS indicated that the starting material had been consumed. The solvent was removed, and the residue purified by SFC (ZymorSPHER Diol-Monol AD 150mm x 21.1 mm x 5µm eluting with 16 - 40% MeOH/CO_2 over 5 minutes at a flow rate of 80 mL/min) to afford (4*S*)-1-methyl-*N*-{(3*S*,5*S*)-5-(4-methyl-1,3-thiazol-2-yl)-1-[4-(2*H*-tetrazol-5-yl)benzoyl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide **(9)** (19.5 mg, 22%) as a white solid. MS (ESI), *m/z* 510.1 $[M+H]^+$ (calcd for C22H24N9O4S = 510.2). ^{1}H NMR (400 MHz, DMSO-d_6) (mixture of rotamers) δ 8.40 (d, *J* = 7.0 Hz, 1H), 8.25 - 7.99 (m, 2H), 7.78 (br d, *J* = 1.0 Hz, 1H), 7.66 - 7.41 (m, 2H), 7.26 - 7.12 (m, 2H), 7.12 - 6.87 (m, 2H), 5.80 - 5.33 (m, 1H), 4.36 - 4.07 (m, 1H), 3.97 - 3.81 (m, 1H), 3.59 - 3.43 (m, 1H), 3.00 - 2.78 (m, 3H), 2.76 - 2.63 (m,1H), 2.58 (br d, *J* = 3.2 Hz, 1H), 2.40 - 2.26 (m, 3H), 2.22 - 2.07 (m, 1H). ^{13}C NMR (101 MHz,

DMSO-d_6) δ = 171.83, 170.84, 169.42, 168.75, 160.98, 154.19, 152.59, 151.91, 135.76, 126.53, 114.45, 57.19, 54.51, 48.92, 48.72, 37.27, 34.04, 26.71, 17.31.

(4*S*)-*N*-[(3*S*,5*S*)-1-benzoyl-5-(4-methyl-1,3-thiazol-2-yl)pyrrolidin-3-yl]-1-methyl-2,6-dioxo-1,3-diazinane-4-carboxamide (10)

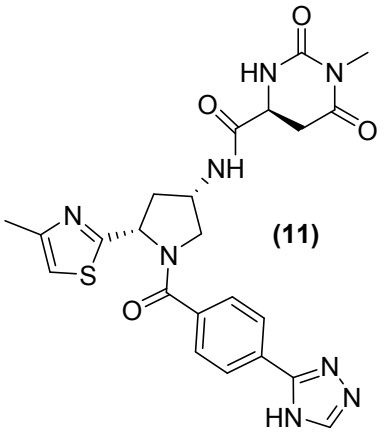


(10)

To a solution of (4*S*)-1-methyl-*N*-[(3*S*,5*S*)-5-(4-methyl-1,3-thiazol-2-yl)pyrrolidin-3-yl]-2,6-dioxo-1,3-diazinane-4-carboxamide (80 mg, 0.24 mmol) and benzoic acid (38 mg, 0.31 mmol) in DMF (2 mL) was added DIPEA (153 mg, 207 µL, 1.19 mmol), HOBt (42 mg, 0.31 mmol) and EDCI (59 mg, 0.31 mmol) at – 20 ^{0}C. The reaction was warmed to room temperature, and then stirred for 16 hours after which time, LC-MS indicated that the starting material had been consumed. The solvent was removed, and the residue purified twice by preparative HPLC (A : water w/0.05% ammonium hydroxide, B : acetonitrile, 8 – 48% B over 10 minutes, flow rate 25 mL/min using a Xtimate C18 150 x 25 mm x 5 µm column) to afford after solvent removal and lyophilization (4*S*)-*N*-[(3*S*,5*S*)-1-benzoyl-5-(4-methyl-1,3-thiazol-2-yl)pyrrolidin-3-yl]-1-methyl-2,6-dioxo-1,3-diazinane-4-carboxamide **(10)** (13 mg, 13%) as a white solid. MS (ESI), *m/z* 442.1 $[M+H]^+$ (calcd for C21H25N5O4S = 442.2). ^{1}H NMR (400 MHz, DMSO-d_6) (mixture of

rotamers) δ 8.41 (br d, *J* = 6.5 Hz, 1H), 7.80 (br d, *J* = 2.8 Hz, 1H), 7.52 (br d, *J* = 8.3 Hz, 5H), 7.18 (s, 1H), 5.49 (br t, *J* = 8.0 Hz, 1H), 4.31 - 4.16 (m, 1H), 3.89 (td, *J* = 3.5, 6.8 Hz, 1H), 3.80 - 3.64 (m, 1H), 3.44 (br t, *J* = 9.7 Hz, 1H), 2.97 (br s, 1H), 2.95 - 2.87 (m, 4H), 2.82 - 2.65 (m, 1H), 2.64 - 2.56 (m, 1H), 2.42 - 2.30 (m, 3H), 2.25 - 2.13 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ = 171.82, 170.90, 169.67, 168.76, 154.20, 151.91, 136.27, 131.08, 128.90, 127.83, 114.44, 57.07, 54.55, 48.88, 48.68, 37.15, 34.05, 26.70, 17.30.

(4*S*)-1-Methyl-*N*-{(3*S*,5*S*)-5-(4-methyl-1,3-thiazol-2-yl)-1-[4-(4*H*-1,2,4-triazol-3-yl)benzoyl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide (11)

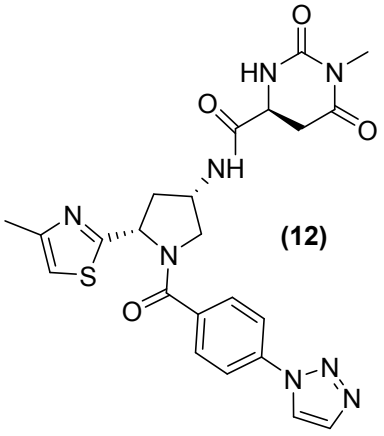


(11)

To a solution of (4*S*)-1-methyl-*N*-[(3*S*,5*S*)-5-(4-methyl-1,3-thiazol-2-yl)pyrrolidin-3-yl]-2,6-dioxo-1,3-diazinane-4-carboxamide (80 mg, 0.24 mmol) and 4-(4*H*-1,2,4-triazol-3-yl)benzoic acid (54 mg, 0.29 mmol) in DMF (2 mL) was added DIPEA (184 mg, 248 µL, 1.42 mmol) and HATU (108 mg, 0.29 mmol) at – 20 ^{0}C. The reaction was warmed to room temperature, and then stirred for 16 hours after which time, LC-MS indicated that the starting material had been consumed. The solvent was removed, and the residue purified by preparative HPLC (A : water w/0.05% ammonium hydroxide, B : acetonitrile, 11 – 31% B over 10 minutes, flow rate 25 mL/min using a DuraShell C18 150 x 25 mm x 5 µm column) to afford after solvent removal and

lyophilization (4*S*)-1-methyl-*N*-{(3*S*,5*S*)-5-(4-methyl-1,3-thiazol-2-yl)-1-[4-(4*H*-1,2,4-triazol-3-yl)benzoyl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide **(11)** (41 mg, 34%) as a white solid. MS (ESI), *m/z* 508.8 $[M+H]^+$ (calcd for C23H25N8O4S = 509.2). ^{1}H NMR (400 MHz, DMSO-d_6) (mixture of rotamers) δ 8.41 (s, 1H), 8.19 (br d, *J* = 5.5 Hz, 1H), 8.09 (br d, *J* = 8.0 Hz, 2H), 7.58 (br d, *J* = 7.0 Hz, 2H), 7.12 (s, 1H), 5.52 (br t, *J* = 7.7 Hz, 1H), 4.32 (br d, *J* = 7.3 Hz, 1H), 3.97 - 3.82 (m, 2H), 3.51 (dd, *J* = 8.4, 10.7 Hz, 1H), 2.95 (s, 3H), 2.93 - 2.91 (m, 1H), 2.67 (br d, *J* = 5.0 Hz, 1H), 2.63 (br d, *J* = 5.0 Hz, 1H), 2.37 (s, 3H), 2.27 - 2.17 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ = 171.72, 170.88, 169.27, 168.75, 154.20, 151.92, 146.92, 136.62, 132.79, 128.52, 127.57, 126.26, 114.48, 57.16, 54.50, 48.90, 48.70, 37.19, 34.06, 26.70, 17.31.

(4*S*)-1-methyl-*N*-{(3*S*,5*S*)-5-(4-methyl-1,3-thiazol-2-yl)-1-[4-(1*H*-1,2,3-triazol-1-yl)benzoyl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide (12)

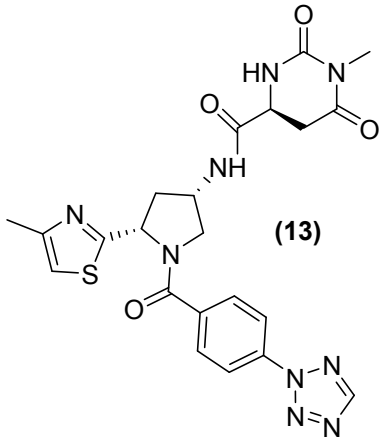


(12)

To a solution of (4*S*)-1-methyl-*N*-[(3*S*,5*S*)-5-(4-methyl-1,3-thiazol-2-yl)pyrrolidin-3-yl]-2,6-dioxo-1,3-diazinane-4-carboxamide (80 mg, 0.24 mmol) and 4-(1*H*-1,2,3-triazol-1-yl)benzoic acid (54 mg, 0.29 mmol) in DMF (2 mL) was added DIPEA (153 mg, 207 µL, 1.19 mmol), HOBt (42 mg, 0.31 mmol) and EDCI (59 mg, 0.31 mmol) at – 20 ^{0}C. The reaction was warmed to room temperature, and then stirred for 16 hours after which time, LC-MS indicated that the starting

material had been consumed. The solvent was removed, and the residue purified twice by preparative HPLC (initially A : water w/0.05% ammonium hydroxide, B : acetonitrile, 5 – 45% B over 10 minutes, flow rate 25 mL/min using a Agela DuraShell 250 x 50 mm x 5 μm column followed by A : water w/0.225% formic acid, B : acetonitrile, 6 – 46% B over 10 minutes, flow rate 25 mL/min using a Xtimate C18 150 x 25 mm x 5 μm column) to afford after solvent removal and lyophilization (4*S*)-1-methyl-*N*-{(3*S*,5*S*)-5-(4-methyl-1,3-thiazol-2-yl)-1-[4-(1*H*-1,2,3-triazol-1-yl)benzoyl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide **(12)** (19 mg, 16%) as a white solid. MS (ESI), *m/z* 509.3 $[M+H]^+$ (calcd for C23H25N8O4S = 509.2). ^{1}H NMR (400 MHz, CD_3OD) (mixture of rotamers) δ 8.65 (s, 1H), 8.09 - 7.98 (m, 2H), 7.95 (s, 1H), 7.83 (br d, *J* = 8.2 Hz, 2H), 7.11 (s, 1H), 5.64 (br t, *J* = 7.9 Hz, 1H), 4.50 - 4.36 (m, 1H), 4.07 (dd, *J* = 5.2, 6.4 Hz, 1H), 4.02 - 3.92 (m, 1H), 3.76 - 3.65 (m, 1H), 3.08 (s, 3H), 3.01 (br dd, *J* = 6.7, 16.6 Hz, 1H), 2.92 - 2.71 (m, 2H), 2.46 (s, 3H), 2.33 - 2.20 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ = 171.52, 170.92, 168.76, 168.62, 154.20, 151.92, 138.41, 136.09, 135.10, 129.86, 123.87, 120.48, 114.55, 57.22, 54.51, 48.88, 48.69, 37.14, 34.07, 26.71, 17.31.

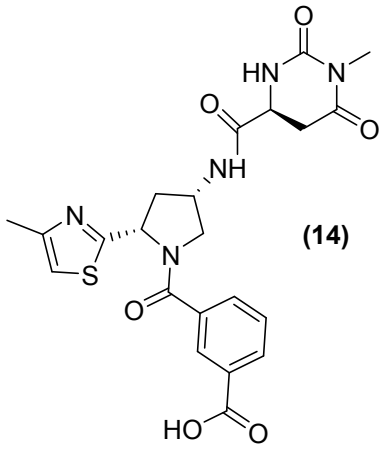
(4*S*)-1-methyl-*N*-{(3*S*,5*S*)-5-(4-methyl-1,3-thiazol-2-yl)-1-[4-(2*H*-tetrazol-2-yl)benzoyl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide (13)



(13)

To a solution of (4*S*)-1-methyl-*N*-[(3*S*,5*S*)-5-(4-methyl-1,3-thiazol-2-yl)pyrrolidin-3-yl]-2,6-dioxo-1,3-diazinane-4-carboxamide (80 mg, 0.24 mmol) and 4-(2*H*-tetrazol-2-yl)benzoic acid (54 mg, 0.29 mmol) in DMF (2 mL) was added DIPEA (184 mg, 242 μL, 1.42 mmol), HOBt (42 mg, 0.31 mmol) and EDCI (59 mg, 0.31 mmol) at – 20 ^{0}C. The reaction was warmed to room temperature, and then stirred for 16 hours after which time, LC-MS indicated that the starting material had been consumed. The solvent was removed, and the residue purified by preparative HPLC (initially A : water w/0.05% ammonium hydroxide, B : acetonitrile, 0 – 40% B over 10 minutes, flow rate 25 mL/min using a DuraShell 150 x 25 mm x 5 μm column) to afford after solvent removal and lyophilization (4*S*)-1-methyl-*N*-{(3*S*,5*S*)-5-(4-methyl-1,3-thiazol-2-yl)-1-[4-(2*H*-tetrazol-2-yl)benzoyl]pyrrolidin-3-yl}-2,6-dioxo-1,3-diazinane-4-carboxamide **(13)** (32 mg, 27%) as a white solid. MS (ESI), *m/z* 509.9 $[M+H]^+$ (calcd for C22H24N9O4S = 510.2). ^{1}H NMR (400 MHz, DMSO-d_6) (mixture of rotamers) δ 10.18 (s, 1H), 8.48 (br d, *J* = 6.8 Hz, 1H), 8.04 (br d, *J* = 8.3 Hz, 2H), 7.88 - 7.70 (m, 3H), 7.21 (s, 1H), 5.51 (t, *J* = 8.3 Hz, 1H), 4.25 (br s, 1H), 3.96 - 3.86 (m, 1H), 3.76 (br dd, *J* = 7.3, 9.8 Hz, 1H), 3.54 - 3.47 (m, 1H), 3.01 - 2.85 (m, 4H), 2.81 - 2.69 (m, 1H), 2.63 - 2.55 (m, 1H), 2.40 - 2.33 (m, 3H), 2.29 - 2.17 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ = 171.41, 170.97, 168.75, 168.43, 154.21, 151.91, 142.91, 137.19, 135.54, 129.71, 121.60, 114.60, 57.21, 54.50, 48.85, 48.67, 37.09, 34.06, 26.70, 17.31.

3-[(2*S*,4*S*)-4-{[(4*S*)-1-methyl-2,6-dioxo-1,3-diazinane-4-carbonyl]amino}-2-(4-methyl-1,3-thiazol-2-yl)pyrrolidine-1-carbonyl]benzoic acid (14)



(14)

To a solution of (4*S*)-1-methyl-*N*-[(3*S*,5*S*)-5-(4-methyl-1,3-thiazol-2-yl)pyrrolidin-3-yl]-2,6-dioxo-1,3-diazinane-4-carboxamide (80 mg, 0.24 mmol) and benzene-1,4-dicarboxylic acid (47 mg, 0.29 mmol) in DMF (2 mL) was added DIPEA (184 mg, 242 µL, 1.42 mmol), HOBt (42 mg, 0.31 mmol) and EDCI (59 mg, 0.31 mmol) at – 20 ^{0}C. The reaction was warmed to room temperature, and then stirred for 16 hours after which time, LC-MS indicated that the starting material had been consumed. The solvent was removed, and the residue purified by preparative HPLC (initially A : water w/0.225% formic acid, B : acetonitrile, 4 – 44% B over 10 minutes, flow rate 25 mL/min using a Xtimate C18 150 x 25 mm x 5 µm column) to afford after solvent removal and lyophilization 3-[(2*S*,4*S*)-4-{[(4*S*)-1-methyl-2,6-dioxo-1,3-diazinane-4-carbonyl]amino}-2-(4-methyl-1,3-thiazol-2-yl)pyrrolidine-1-carbonyl]benzoic acid **(14)** (9 mg, 8%) as a white solid. HRMS (ESI/Q-TOF) m/z: $[M + H]^+$ calcd for C24H24N5O6S = 486.14418; Found 486.14474. MS (ESI), *m/z* 486.1 $[M+H]^+$ (calcd for C22H24N5O6S = 486.1). ^{1}H NMR (700 MHz, DMSO-d_6) δ 8.37 (d, *J* = 6.7 Hz, 1H), 7.98 (d, *J* = 7.8 Hz, 2H), 7.58 (br d, *J* = 7.8 Hz, 2H), 7.12 (s, 1H), 5.43 (t, *J* = 8.2 Hz, 1H), 4.30 – 4.11 (m, 1H), 3.91 – 3.79 (m, 1H), 3.68 – 3.57 (m, 1H), 3.39 (t, *J* = 9.6 Hz, 1H), 2.88 (d, *J* = 7.0 Hz, 1H), 2.86 (s, 3H), 3.31 (s, 3H), 2.19 – 2.04 (m, 1H). ^{13}C NMR

(175 MHz, DMSO-d_6) δ = 170.83, 169.94, 168.48, 166.26, 153.35, 151.60, 139.21, 132.19, 128.98, 126.89, 113.46, 56.33, 53.51, 48.01, 47.75, 36.70, 33.40, 26.05, 16.75.