Targeting the von Hippel-Lindau E3 Ubiquitin Ligase using Small Molecules to Disrupt the VHL/HIF-1α Interaction

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Supporting Information

1. Computational Protocols

De-novo design

The crystal structure of the HIF-1α peptide in complex with Elongin B/Elongin C/VHL was used as a starting point for all computations.[1] The structures of Elongin B and C chains, distant from the VHL/HIF-1α binding site, were discarded and the HIF-1α peptide was removed. The protein preparation wizard of the software Maestro was used to add missing hydrogen atoms, choose the protonation state of protein side chains, and minimize the energy of the protein.[2] A structural model of the hydroxyproline moiety of the HIF-1α peptide bound to VHL (including a conserved water molecule) was used to generate several hydroxyproline analogs using the *de novo* design program BOMB.[3] Using the software VMD, plausible designs were visualized,[4] and considered for experimental testing according to their predicted interactions and ease of synthesis. Figure S1 depicts computed interactions for **4**.

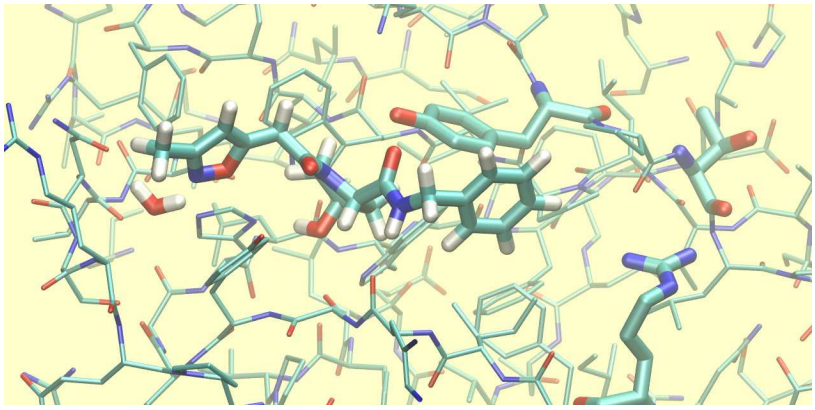


Figure S1. BOMB computed interactions for **4** in complex with VHL. The ligand, structural water and receptor residues Tyr98, Thr100 and Arg107 are shown as thick sticks.

2. Biochemical Methods

Cloning, expression and purification of the $V_{54-213}BC$ complex

vhl$_{54-213}$ was amplified from a pET28a plasmid containing *vhl*$_{1-213}$ (gift from Dr. Judith Frydman, Stanford), and inserted in the pHAT4 and pET28a vectors to yield a six Histidine-tag at the N-terminus, giving rise to the pIVM_02 and pIVM_03 plasmids. The pCDF_1 plasmid, a pDUET_1 plasmid, encodes for both *EloB*$_{1-120}$ and *EloC*$_{17-112}$, was a gift from Dr. Alex Bullock (SGC Oxford).

The $_{H6}V_{54-213}BC$ complex was expressed from BL21(DE3)(pIVM_02+pCDF_1) or BL21(DE3)(pIVM_03+pCDF_1). A preculture from one colony of BL21(DE3)(pIVM_02+pCDF_1) or BL21(DE3)(pIVM_03+pCDF_1) was grown overnight at 37°C in LB supplemented with 100 μg/ml ampicilin or 50 μg/ml kanamycin and 50 μg/ml streptomycin. This preculture was diluted 100 times to inoculate LB or TB cultures supplemented with 100 μg/ml ampicilin or 50 μg/ml kanamycin and 50 μg/ml streptomycin, for BL21(DE3)(pIVM_02+pCDF_1) and BL21(DE3)(pIVM_03+pCDF_1), respectively, and grown at 37°C until the OD_{600} reached 0.7. After lowering the temperature to 18°C or 25°C, for BL21(DE3)(pIVM_02+pCDF_1) and BL21(DE3)(pIVM_03+pCDF_1), respectively, protein expression was induced with 0.5 mM IPTG, and the cultures were further incubated overnight. Cells were harvested by centrifugation for 15 minutes at 5000 rpm, using a Beckman JLA8.1000 centrifuge, at 4°C. The cell pellet was resuspended in 20 mM Tris pH 8.0, 500 mM NaCl, 10 mM imidazole, supplemented with Complete, EDTA free, protease inhibitor mix (as specified by the supplier, Roche), and lysed by French press. After 30 min incubation at RT with 10 μg/ml DNaseI and 10 mM $MgCl_2$, the cell debris was removed by centrifugation for 40 minutes at 20000 rpm, using a Beckman JA25.50 centrifuge, at 4°C. The cell lysate was subsequently loaded on a NiNTA column equilibrated in 20 mM Tris pH 8.0, 500 mM NaCl, 10 mM imidazole. The $_{H6}V_{54-213}BC$ complex was eluted using a linear gradient to 500 mM imidazole in the same buffer. To remove the His_6 tag the pooled elution fractions were incubated O/N at 4°C with 0.5U of thrombin per mg of protein. The thrombin was removed using a benzamidine sepharose column, while $V_{54-213}BC$ and $_{H6}V_{54-213}BC$ were separated using a second Ni sepharose. After adding 1 mM DTT and lowering the NaCl concentration, the flow through containing the $V_{54-213}BC$ complex was loaded on a MonoQ anion exchange column, equilibrated in 20 mM Bis-Tris pH 7.0, 1 mM DTT. $V_{54-213}BC$ was eluted using a linear gradient to 1 M NaCl in the same buffer and loaded on a Superdex75 16/60 Prep Grade column, equilibrated in 20 mM Bis-Tris pH 7.0, 150 mM NaCl, 1mM DTT. The $V_{54-213}BC$ complexes from BL21(DE3)(pIVM_02+pCDF_1) and BL21(DE3)(pIVM_03+pCDF_1) will hereafter be called $V_{54}BC$_pHAT4 and $V_{54}BC$_pET28a, respectively.

NMR spectroscopy

^{1}H NMR spectroscopic experiments were performed at 278 K on a 700 MHz Bruker NMR spectrometer equipped with a 5 mm triple TXI cryoprobe with *z* gradients. The resulting spectra were analyzed with TopSpin. Each compound was run as 3 samples according to the following compositions:

a) *Control sample* = 1mM compound, 2% (v/v) d_6-DMSO, 20µM TSP, 50mM $NaPO_4$ pH 7.0, 10% (v/v) D_2O.
b) *+ protein sample* = control sample + 20µM $_{H6}V_{54\text{-}213}$BC complex.
c) *+ protein + displacer sample* = control sample + 20µM $_{H6}V_{54\text{-}213}$BC complex + 500µM HIF-1α 10-mer peptide (DEALA-Hyp-YIPD).

All samples were made up to 200µl in 3mm capillaries. (Trimethylsilyl)-propionic acid-d_4 (TSP; 20µM) was present in all samples for calibration purposes.

*Water-l*igand *o*bserved by *g*radient *s*pectroscop*y* (WaterLOGSY)[5] experiments employed a 20 ms selective Gaussian 180° pulse at the water frequency and an NOE mixing time of 1 s. Water suppression was achieved by using a double-gradient echo excitation sculpting sequence including a 5 ms selective pulse with gradients.[6]

Crystallization of the $V_{54\text{-}213}$BC complex

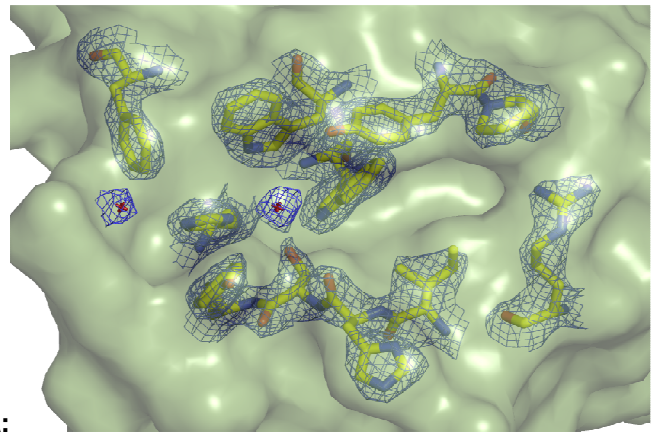
The purified $V_{54\text{-}213}$BC complexes were concentrated to 10 mg/ml using 10kDa cut-off Vivaspin concentrators. Initial crystals of the $V_{54\text{-}213}$BC_pHAT4 complex were grown using hanging drop vapour diffusion, at 4°C, in 0.1 mM Na cacodylate pH 5.7, 15% PEG8000, 0.2 M Mg acetate, 5 mM DTT. $V_{54\text{-}213}$BC_pHAT4, and $V_{54\text{-}213}$BC_pET28a crystals were also grown in these conditions at 12°C and 18°C, using seeds from the crystals grown at 4°C. For both $V_{54\text{-}213}$BC_pHAT4 and $V_{54\text{-}213}$BC_pET28a these initial conditions were also optimized by changing the buffer to 0.1 M Na citrate pH 5.8 and increasing the DTT concentration to 50 mM. Crystals were cryo-protected using 20% v/v glycerol and stored in liquid nitrogen.

In order to collect data for the **15** bound V_{54}BC complex, V_{54}BC_pHAT4 crystals were soaked overnight in a 1 mM **15** solution in 0.1 mM Na citrate pH 5.8, 15% PEG8000, 0.2 M Mg acetate, 50 mM DTT.

Data collection and crystal structure solution

X-ray data of the V_{54}BC_pHAT4 and V_{54}BC_pET28a crystals were collected at 100 K at the Diamond (beamline IO3) and ESRF (beamline ID14-4) synchrotron facilities and processed using XDS. The structure of the apo V_{54}BC_pET28a complex was solved by molecular replacement using Phaser[7] from the CCP4i suite[8] and the pdb deposition 1VCB[9] as a search model. The structure was completed in Coot[10] and refined using Refmac5[11] from the CCP4i suite. The structure of the **15** bound V_{54}BC_pHAT4 complex was solved by molecular replacement using Phaser from the CCP4i suite using the refined apo V_{54}BC_pET28a structure as a search model. The structure was completed in Coot[10] and refined using Refmac5[11] from the CCP4i suite. Data collection statistics and refinement parameters are summarized in Table 1.

A:



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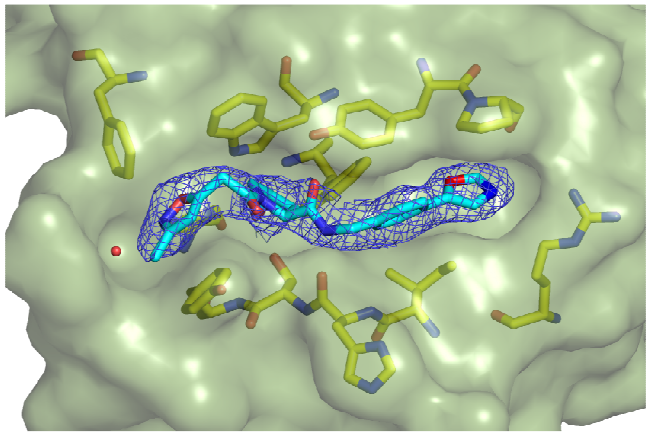

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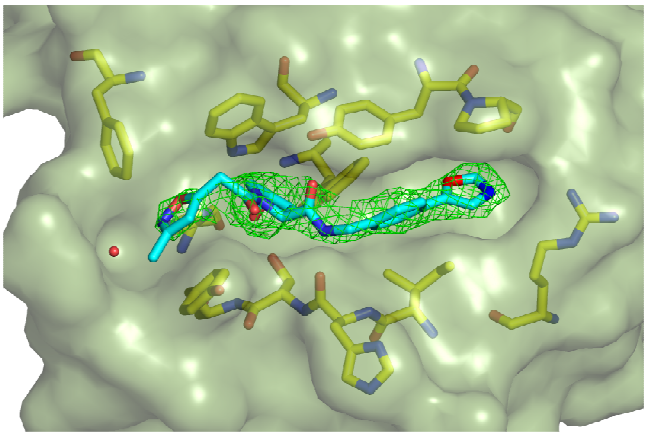


Figure S2. Crystal structures of V_{54}BC apo (A) and in complex with **15** (B and C). Electron density ($2F_o–F_c$) superimposed around Hyp binding site residues (sticks, yellow carbons) and conserved water molecules (red dots), and **15** (sticks, cyan carbons) are shown in blue and are contoured at 1.2σ. An unbiased omit map ($F_o–F_c$) is also shown in green contoured at 2.5σ around the modeled inhibitor (panel C). The protein surface is shown in green at 50% transparency.

Table 1. Crystallographic data collection statistics and refinement parameters

Dataset	**V_{54}BC_ apo**	**V_{54}BC_15**
Data collection		
Synchrotron	Diamond	ESRF
Beamline	IO3	ID14-4
Wavelenght (λ)	0.9763	0.97625
Processing		
Space group	$P4_122$	$P4_122$
Cell parameters (Å)	93.08 93.08 364.58	93.74 93.74 363.18

($\alpha=\beta=\gamma=90^o$)		
Resolution, Å (outer shell) [(c)]	50.0-2.8 (2.96-2.80)	50.0-2.9 (3.06-2.90)
Total reflections	380657(44909)	166014(14453)
Unique reflections	75468(12079)	34397(4807)
Completeness	98.2(92.9)	93.9(91.9)
Multiplicity	5.0(3.7)	4.8(3.0)
Rmerge (%)	10.8 (45.8)	11.9(51.1)
$\langle I/\sigma(I)\rangle$	12.30 (2.87)	9.6(2.5)
Mosaicity, (°)	0.238	0.298
Refinement		
Resolution range (Å)	46.54-2.80	41.9-2.9
Reflections	38610	32437
Percentage observed	100	91.83
R_{cryst} (%)[(a)]	22.26	23.1
R_{free} (%)[(b)]	31.93	34.69
RMS		
Bonds (Å)	0.0219	0.024
Angles (°)	2.2592	2.5

Ramachandran Plot		
Most favored (%)	86.1	79.5
Additionally allowed (%)	10.4	14.6
Disallowed (%)	3.5	5.9
PDB code	3zrf	3zrc

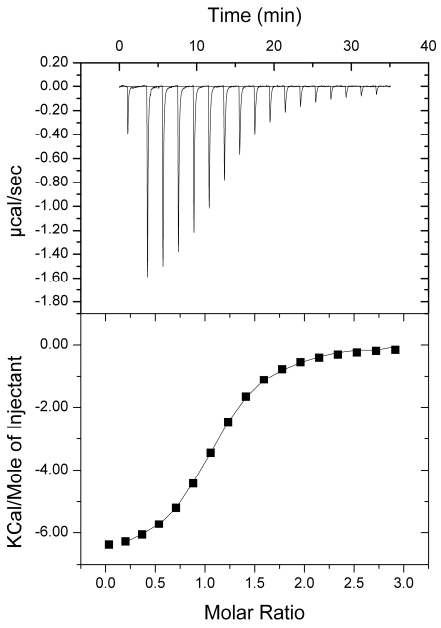
[a] $R_{cryst} = \Sigma||F_{obs}|-|F_{calc}||/\Sigma|F_{obs}|$, F_{obs} and F_{calc} are observed and calculated structure factor amplitudes

[b] R_{free} as for R_{cryst} using a random subset of the data excluded from the refinement

(c) Data in brackets are for the highest resolution shell

Isothermal titration calorimetry (ITC)

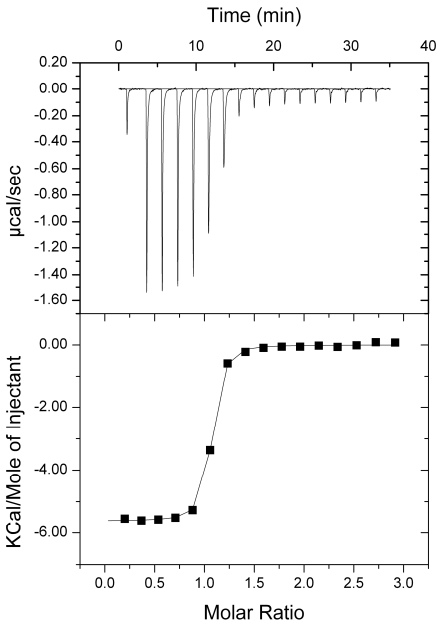
ITC experiments were performed using an ITC200 instrument from Microcal Inc. (GE Healthcare) at 25 ˚C. HIF-1α peptide DEALA-Hyp-YIPD and **15** were dissolved in 20 mM Bis-Tris pH 7.0, 150 mM NaCl, 1 mM DTT at 1 mM concentration, and titrated into 100 μM V_{54}BC_pET28a, equilibrated in the same buffer. The titration comprised 16 × 2.4 μL injections of ligand solution of 5 s duration at 2 min intervals. An initial injection of ligand (0.5 μL) was made and discarded during data analysis. The data were fitted to a single binding site model using the Microcal LLC ITC200 Origin software provided by the manufacturer. ITC traces together with the results of the data fitting are shown below.



K_d = 5.4 ± 0.2 μM

ΔH = –6.84 ± 0.05 kcal/mol

–TΔS = –0.3 ± 0.1 kcal/mol



K_d = 180 ± 20 nM

ΔH = –5.61 ± 0.02 kcal/mol

–TΔS = –3.6 ± 0.1 kcal/mol

Figure S3. ITC titrations of **15** (left) and DEALA-Hyp-YIPD (right) into V_{54}CB complex.

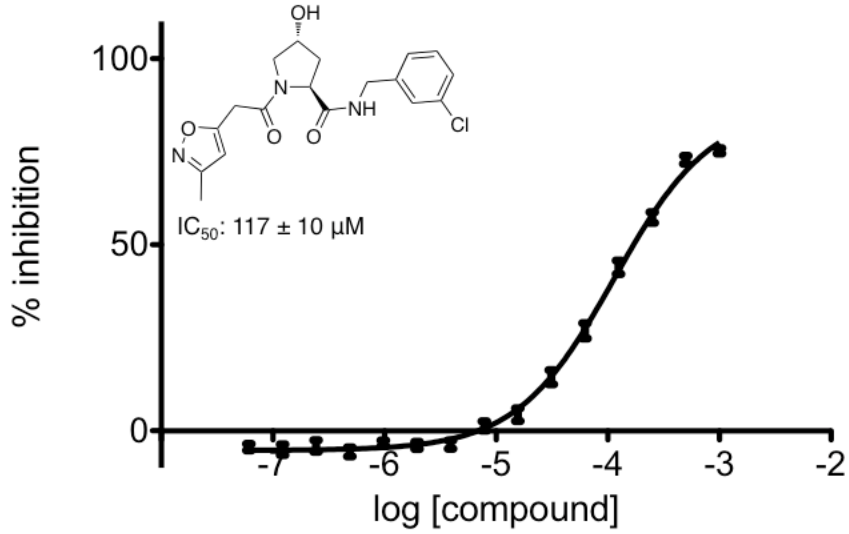
Protein expression and purification of $V_{1-213}BC$

E. coli (BL21) were cotransformed with 6xHis tagged VHL (pET28a) and Elongins B and C (pCDF). The cells were then grown in 2 liters of 2xYT media with 50 μg/mL kanamycin and 50 μg/mL streptomycin at 37°C. The broth was cooled to 24°C and the bacteria induced with 0.5mM IPTG at 24°C for 19 hours and then pelleted at 4°C. The pellet was resuspended in 30 mL lysis buffer (50 mM Tris, 300 mM NaCl, 10 mM imidazole, pH 8.0 with 30 μL of 10% NP40 and 1x Roche EDTA free protease inhibitors added) and lysed with a sonicating probe (Branson Sonifier 450) for 4x 60 s with a 50% duty cycle. The protein was then purified using HisPur Cobalt resin (Thermo Scientific) and exchanged into VHL buffer (50 mM Tris, 200 mM NaCl, 2 mM DTT, pH 7.5) using PD-10 columns (GE Healthcare). The concentration of protein was measured by Bradford assay and the protein snap frozen and stored at -80°C.

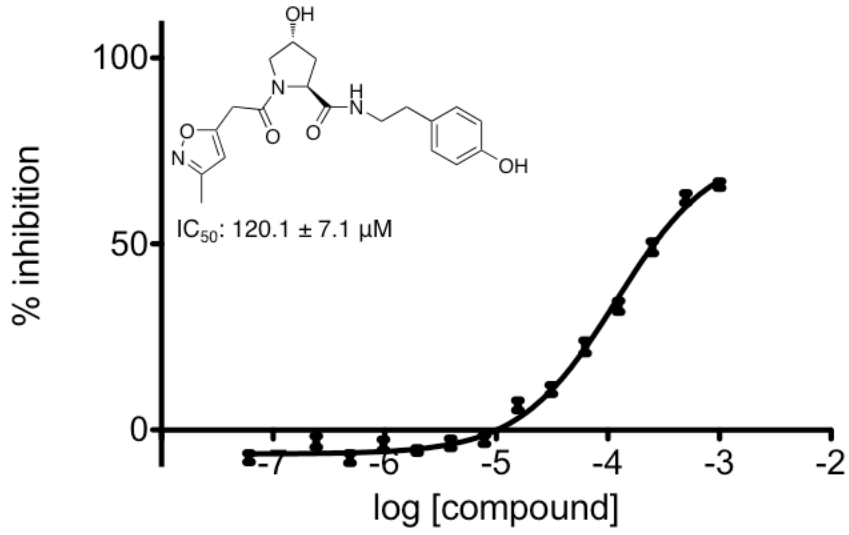
Fluorescence Polarization Assay

VHL ligands were dissolved in DMSO (100 mM), and then diluted 10 fold with VHL buffer. The compounds were then diluted 2 fold with 10% DMSO in buffer (20 μL into μL of 10% DMSO in buffer) 14 times. Aqueous DEALA-Hyp-YIPD was used a positive control. 278 μM FAM-DEALAHyp-YIPD (DMSO) was diluted 1000 fold into VHL buffer. For polarization displacement assays, 9 μL of 1 μM $V_{1-213}CB$ (450 nM final), 2 μL of VHL Ligands (VL) compounds in 10% DMSO (1% DMSO final), and 9 μL of 278 nM FAM-DEALAHyp-YIPD were added to a 384 well plate (Corning 3575). The plate was then shaken for 1 minute, and centrifuged for 1 minute, before reading fluorescence polarization on a Perkin Elmer Envision 2101 Multilabel reader (excitation 486 nM, emission 535 nM). Wells containing $V_{1-213}CB$, DMSO vehicle, FAM-DEALAHyp-YIPD served as maximum polarization (or minimum displacement) Wells containing buffer in place of $V_{1-213}CB$, DMSO vehicle, FAM-DEALAHyp-YIPD served as minimum polarization (or maximum displacement). The percent inhibition was determined by normalizing to maximum and minimum polarization, and graphed against the log [VL]. IC_{50} values were then determined using Prism 5 for each replicate (n=9), which were then averaged to determine the average IC_{50} and the standard error of the mean (SEM).

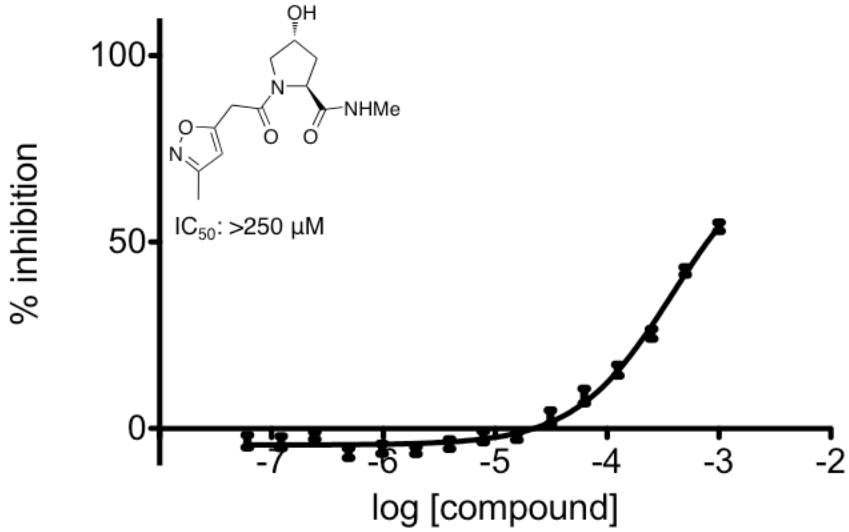
1


OH
N
O
N
O
O
NH
Cl
IC50: 117 ± 10 µM
100
50
0
% inhibition
-7
-6
-5
-4
-3
-2
log [compound]

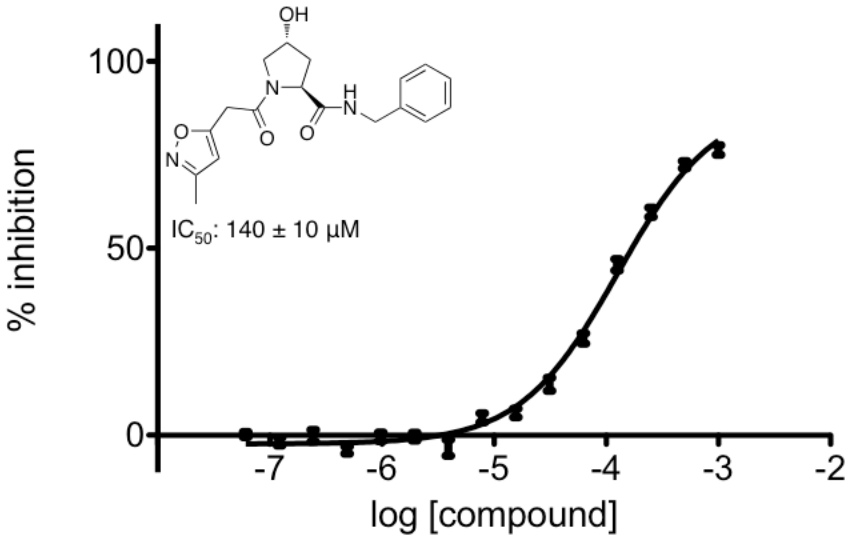
2


OH
N
O
N
O
O
H
N
OH
IC50: 120.1 ± 7.1 µM
100
50
0
% inhibition
-7
-6
-5
-4
-3
-2
log [compound]

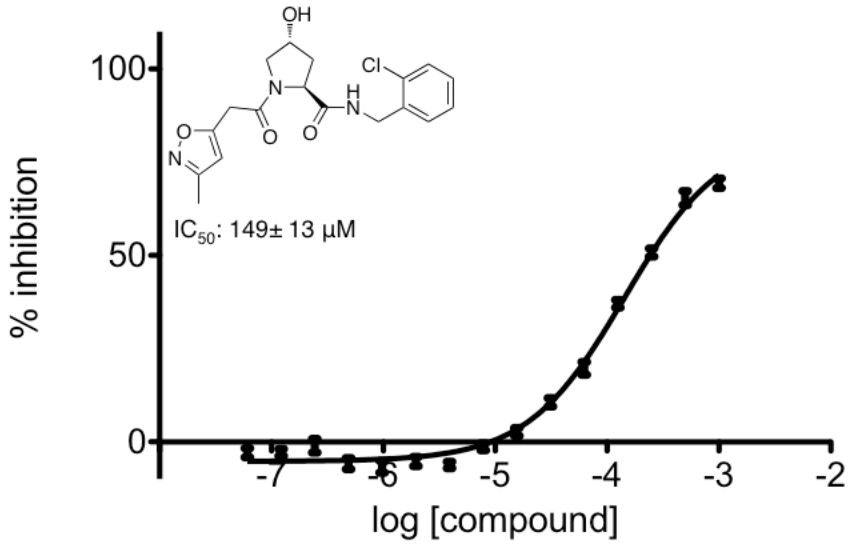
3


OH
N
O
N
O
O
NHMe
IC50: >250 µM
100
50
0
% inhibition
-7
-6
-5
-4
-3
-2
log [compound]

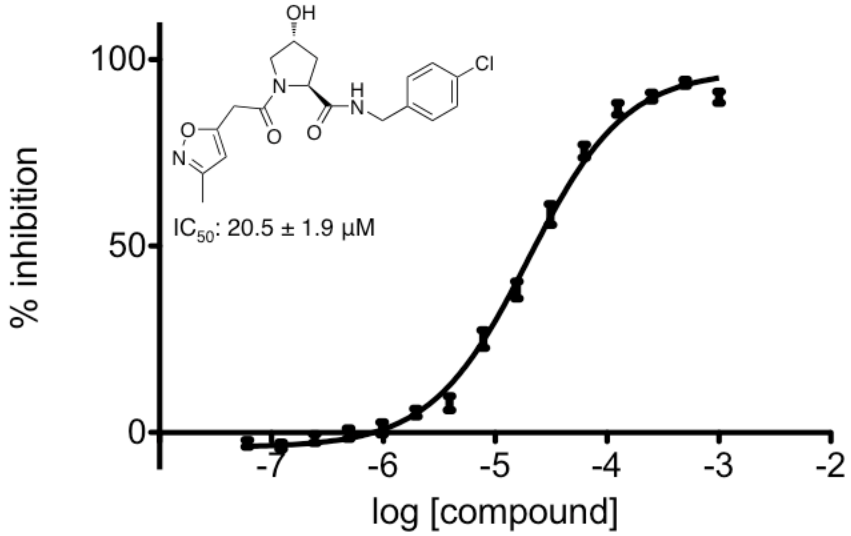
4


OH
N
O
N
O
O
H
N
IC50: 140 ± 10 µM
100
50
0
% inhibition
-7
-6
-5
-4
-3
-2
log [compound]

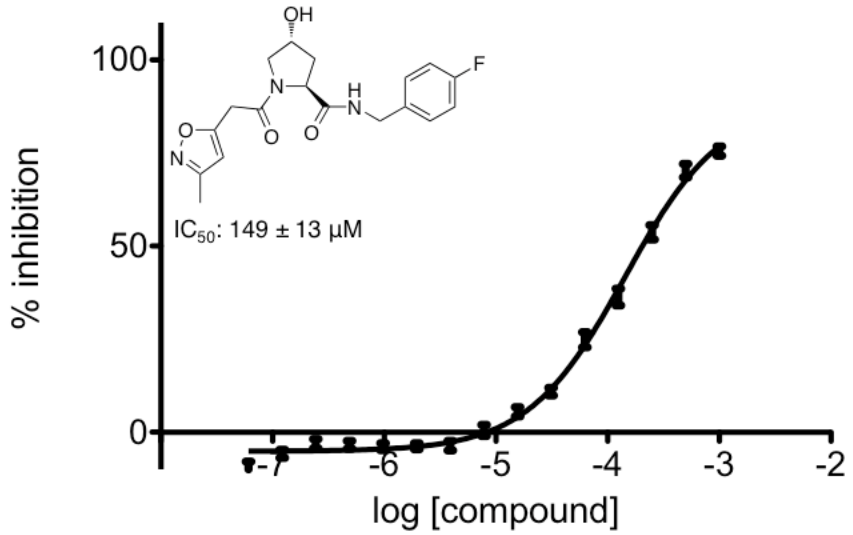
5


OH
Cl
N
O
H
N
O
O
N
IC50: 149± 13 µM
100
50
0
% inhibition
-7
-6
-5
-4
-3
-2
log [compound]

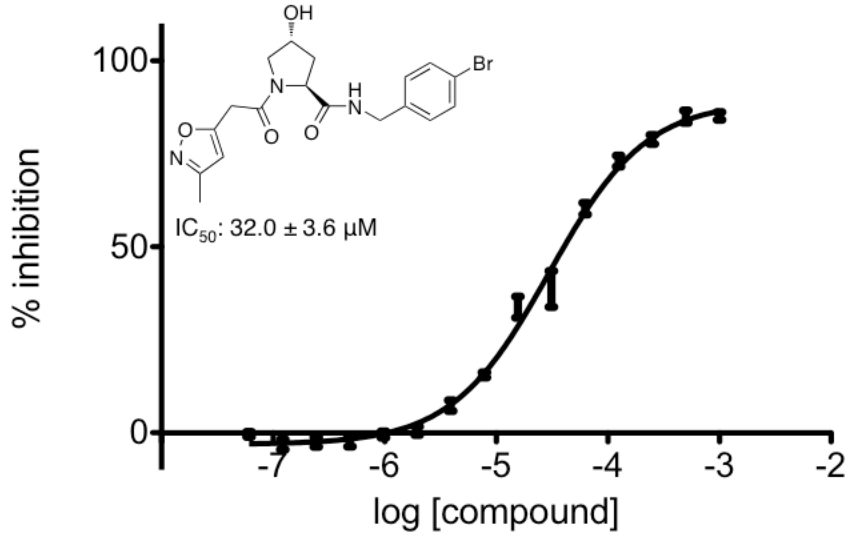
6


OH
Cl
N
O
H
N
O
O
N
IC50: 20.5 ± 1.9 µM
100
50
0
% inhibition
-7
-6
-5
-4
-3
-2
log [compound]

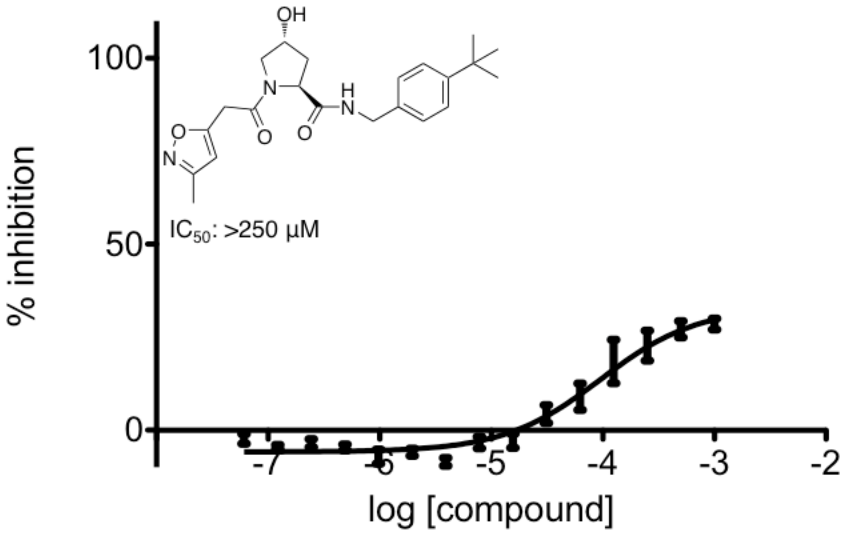
7


OH
F
H
N
O
N
O
O
IC50: 149 ± 13 µM
100
50
0
% inhibition
-7
-6
-5
-4
-3
-2
log [compound]

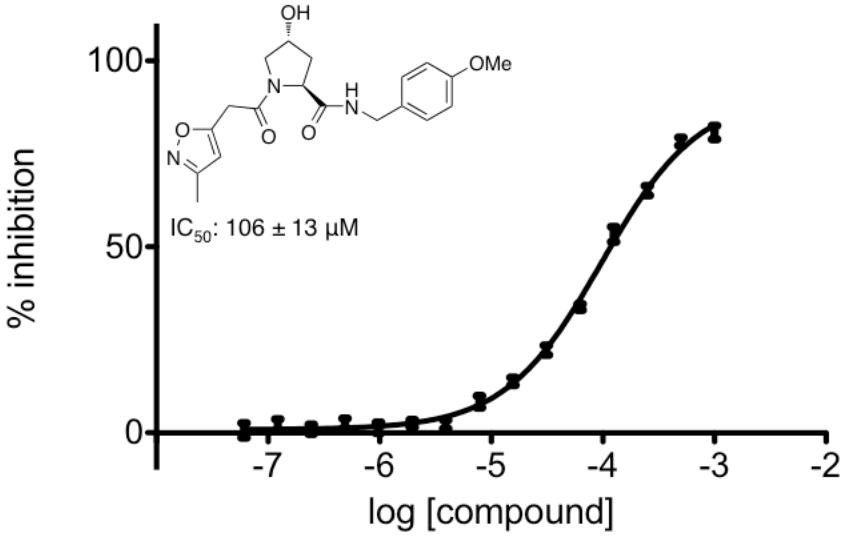
8


OH
Br
H
N
O
N
O
O
IC50: 32.0 ± 3.6 µM
100
50
0
% inhibition
-7
-6
-5
-4
-3
-2
log [compound]

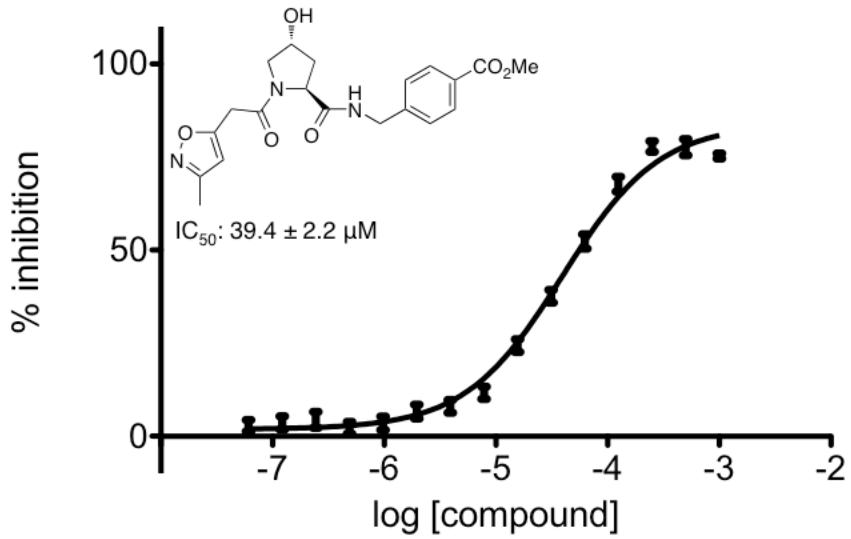
9


OH
N
O
N
O
O
H
N
IC50: >250 µM
100
50
0
% inhibition
-7
-6
-5
-4
-3
-2
log [compound]

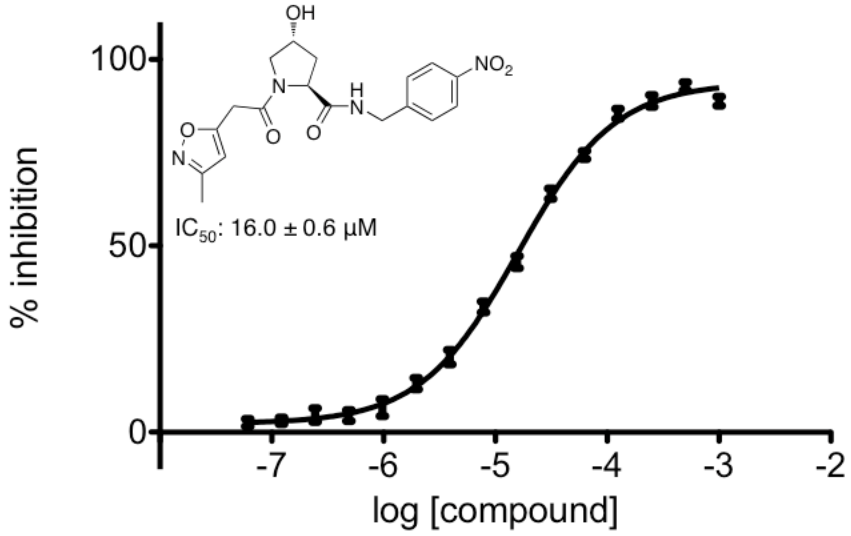
10


OH
N
O
N
O
O
H
N
OMe
IC50: 106 ± 13 µM
100
50
0
% inhibition
-7
-6
-5
-4
-3
-2
log [compound]

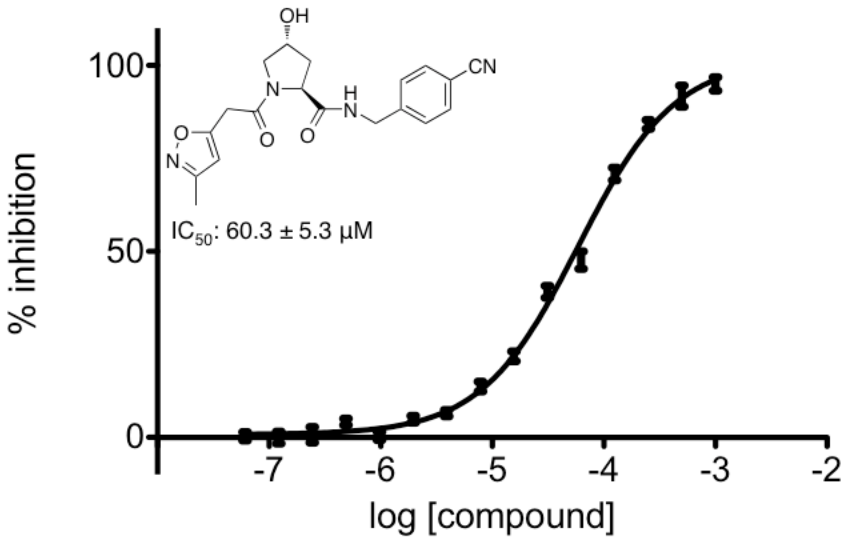
11


OH
CO2Me
IC50: 39.4 ± 2.2 µM
% inhibition
100
50
0
-7
-6
-5
-4
-3
-2
log [compound]

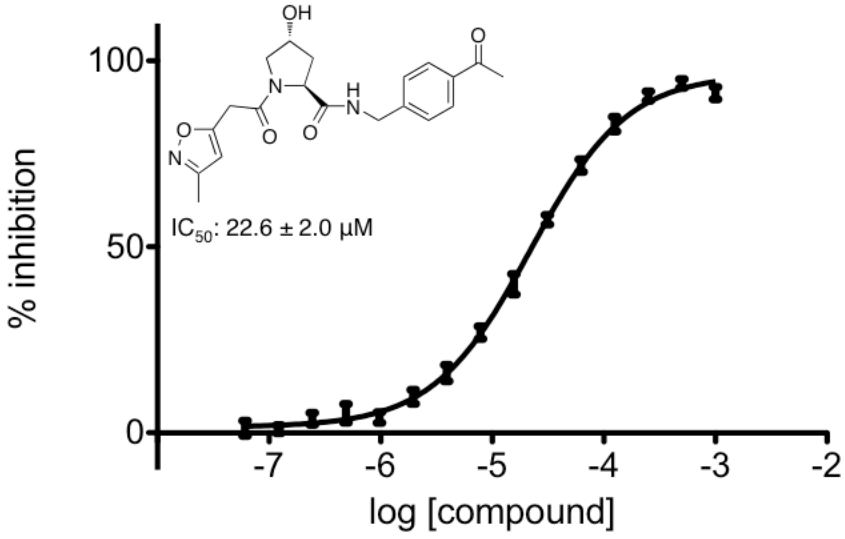
12


OH
NO2
IC50: 16.0 ± 0.6 µM
% inhibition
100
50
0
-7
-6
-5
-4
-3
-2
log [compound]

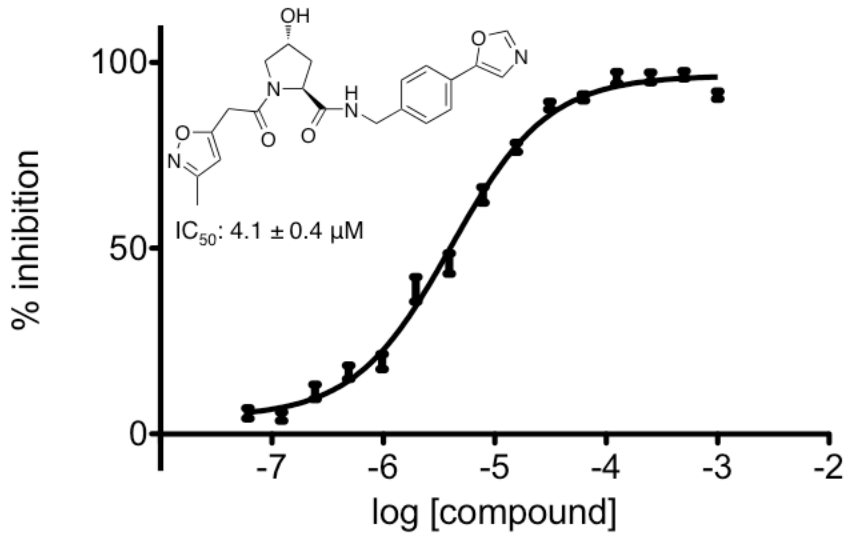
13


OH
CN
H
N
O
N
O
O
IC50: 60.3 ± 5.3 µM
100
50
0
% inhibition
-7
-6
-5
-4
-3
-2
log [compound]

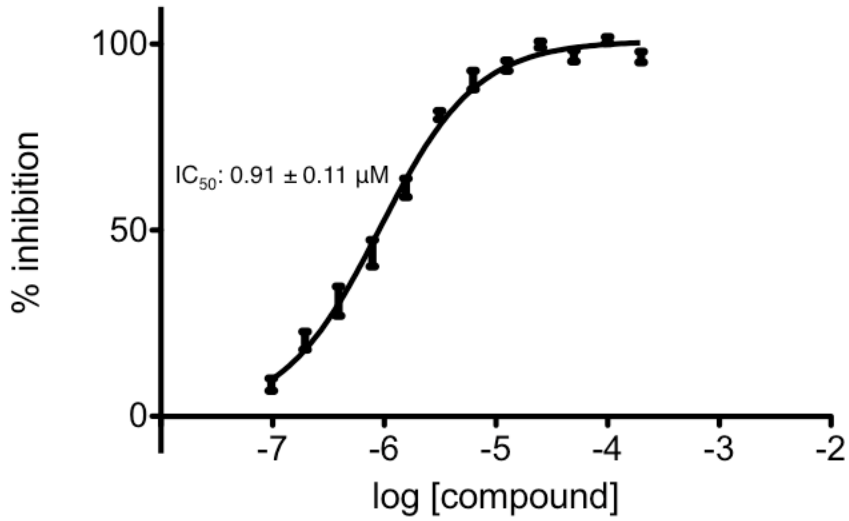
14


OH
O
H
N
O
N
O
O
IC50: 22.6 ± 2.0 µM
100
50
0
% inhibition
-7
-6
-5
-4
-3
-2
log [compound]

15

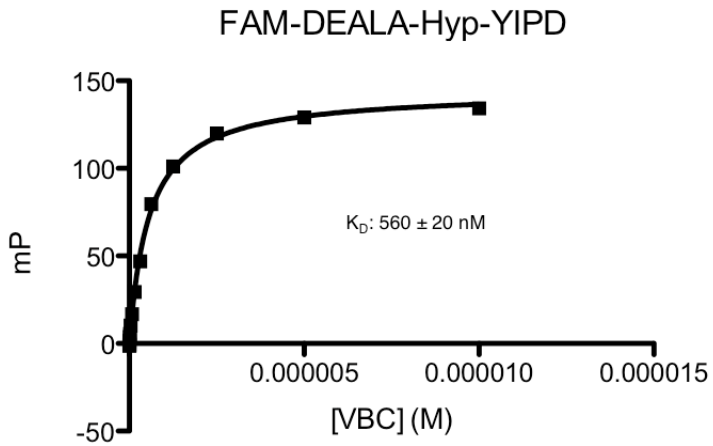


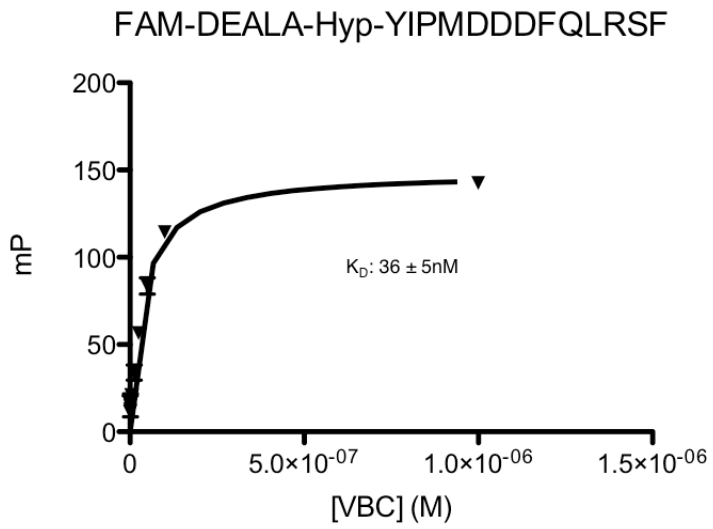
DEALA-Hyp-YIPD



K_d determination of Fluorescent peptides

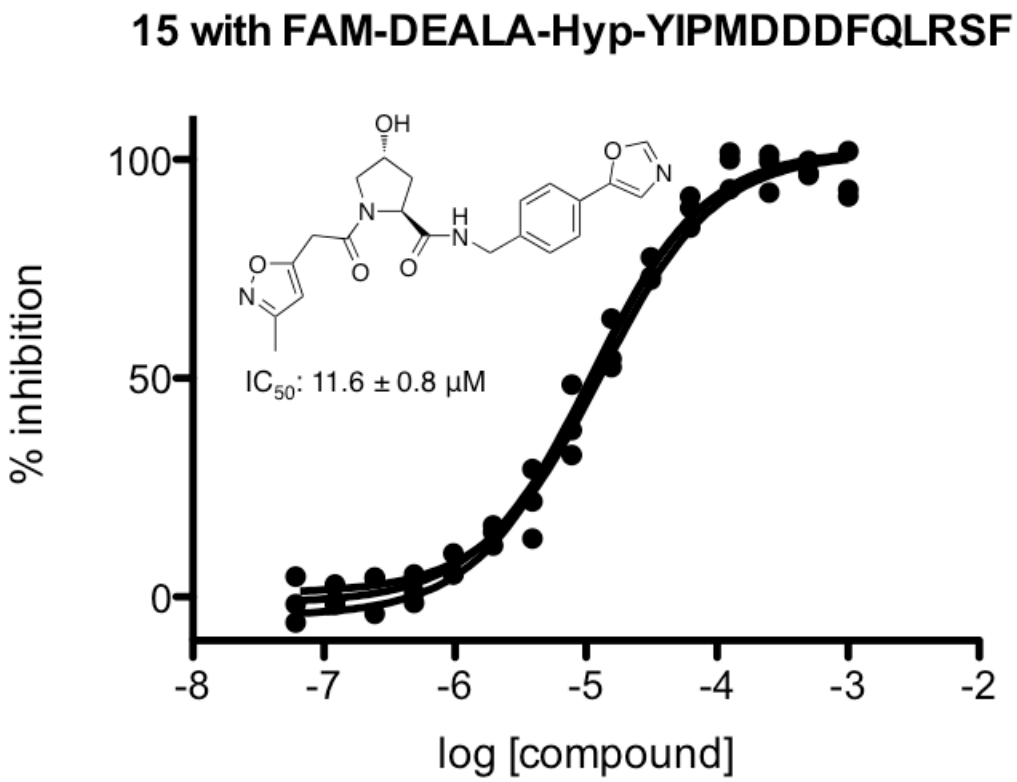
The K_ds of FAM-DEALA-Hyp-YIPD and FAM-DEALA-Hyp-YIPMDDDFQLRSF were determined by fluorescence polarization. 278 μM FAM-DEALAHyp-YIPD (DMSO) was diluted 1000 fold into VHL buffer and titrated against 2 fold serial dilutions were made of $V_{1\text{-}213}$CB, starting from 22 μM. 5 μM FAM-DEALAHyp-YIPMDDDFQLRSF was diluted 450 fold into VHL buffer and titrated against 2.2 μM $V_{1\text{-}213}$CB, and 2 fold serial dilutions were made of $V_{1\text{-}213}$CB, starting from 220 nM. 9 μL of $V_{1\text{-}213}$CB, 2 μL of 10% DMSO (1% DMSO final), and 9 μL of 278 nM FAM-DEALAHyp-YIPD or 11.1 nM FAM-DEALA-Hyp-YIPMDDDFQLRSF were added to a 384 well plate (Corning 3575). The plate was then shaken for 1 minute, and centrifuged for 1 minute, before reading fluorescence polarization on a Perkin Elmer Envision 2101 Multilabel reader (excitation 486 nM, emission 535 nM). K_ds were then determined using Prism 5 (n=3).





Displacement of FAM-DEALA-Hyp-YIPMDDDFQLRSF by 15

To show that **15** is capable of displacing a longer peptide derived from HIF-1α that binds to the secondary binding site as well as the primary binding site,[1] the FP displacement assay was also performed with FAM-DEALA-Hyp-YIPMDDDFQLRSF. **15** was dissolved in DMSO (100 mM), and then diluted 10 fold with VHL buffer. It was then diluted 2 fold with 10% DMSO in buffer (20 μL into μL of 10% DMSO in buffer) 14 times. 5 μM FAM-DEALAHyp-YIPD (DMSO) was diluted 450 fold into VHL buffer. For polarization displacement assays, 9 μL of 222 nM V_{1-213}CB (100 nM final), 2 μL of **15** in 10% DMSO (1% DMSO final), and 9 μL of 11.1 nM FAM-DEALAHyp-YIPD (5 nM final) were added to a 384 well plate (Corning 3575). The plate was then shaken for 1 minute, and centrifuged for 1 minute, before reading fluorescence polarization on a Perkin Elmer Envision 2101 Multilabel reader (excitation 486 nM, emission 535 nM). Wells containing V_{1-213}CB, DMSO vehicle, FAM-DEALAHyp-YIPD served as maximum polarization (or minimum displacement) Wells containing buffer in place of V_{1-213}CB, DMSO vehicle, FAM-DEALAHyp-YIPD served as minimum polarization (or maximum displacement). The percent inhibition was determined by normalizing to maximum and minimum polarization, and graphed against the log [VL]. IC_{50} values were then determined using Prism 5 for each replicate (n=3), which were then averaged to determine the average IC_{50} and the standard error of the mean (SEM).



3. Synthetic Methods

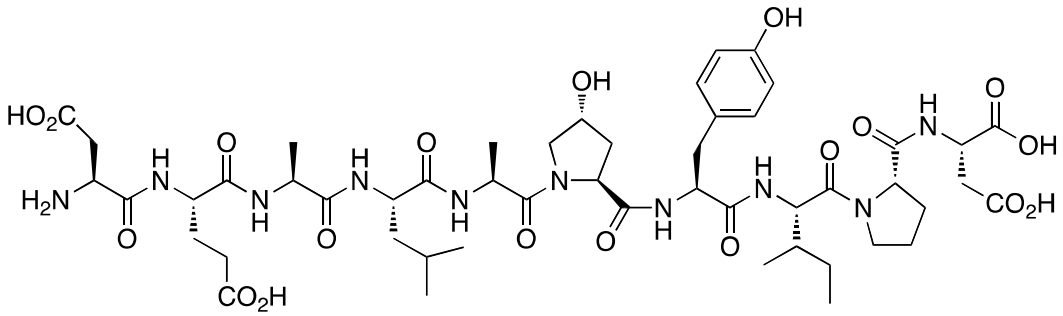
General Chemistry

All reactions were performed in oven-dried or flame-dried glassware fitted with rubber septa under a positive pressure of nitrogen, unless otherwise noted. Air-and moisture-sensitive liquids were transferred via syringe or cannula. THF was distilled from sodium/benzophenone. Dichloromethane was distilled from calcium hydride. Analytical thin layer chromatography (TLC) was performed using glass plates precoated with silica gel (0.25 mm). TLC plates were visualized by exposure to UV light (UV) or $KMnO_4$. Flash column chromatography was performed using silica gel 60 (230-400 mesh, Merck) with the indicated solvents.

^1H and ^{13}C spectra were recorded on Bruker Avance DPX-500 or Bruker Avance DPX- 400 NMR spectrometers. ^1H NMR spectra are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), integration, and coupling constant (J) in Hertz (Hz). ^1H NMR chemical shifts are reported relative to $CDCl_3$ (7.26 ppm), d_6-DMSO (2.50 ppm) and d_4-MeOD (3.31 ppm). ^{13}C NMR was recorded relative to the central line of $CDCl_3$ (77.16 ppm), d_6-DMSO (39.52 ppm) and d_4-MeOD (49.00 ppm). In most cases, only peaks of the major rotamer are reported. Mass spectra were obtained using a Perkin-Elmer API 150 EX spectrometer. MALDI-TOF analyses of purified samples were performed in a Voyager-DE-PRO 6268 (Applied Biosystems) using cyano-4-hydroxycinnamic acid matrices. Unless otherwise noted, HPLC was performed using a Dynamax SD200 solvent delivery system connected to a Dynamax UV-1 Absorbance Detector with a YMC-Pack ODS-AM preparative column (250 x 20 mm, 5 µm particle size, 12 nm pore size). A linear gradient of MeCN in H_2O from 20% to 100% MeCN, with constant 0.1% TFA was run over 40 minutes.

Peptidic Reagents

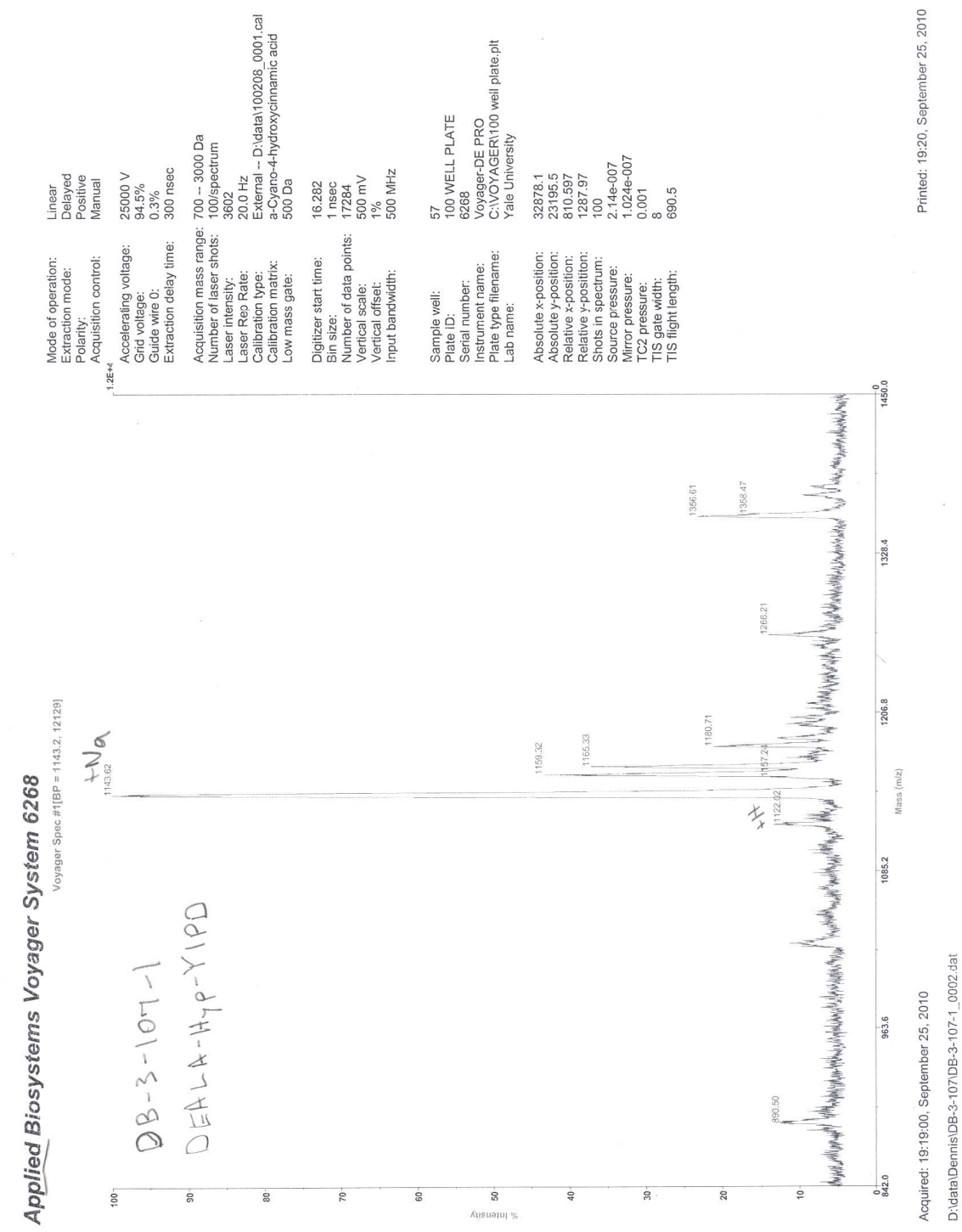
DEALA-Hyp-YIPD



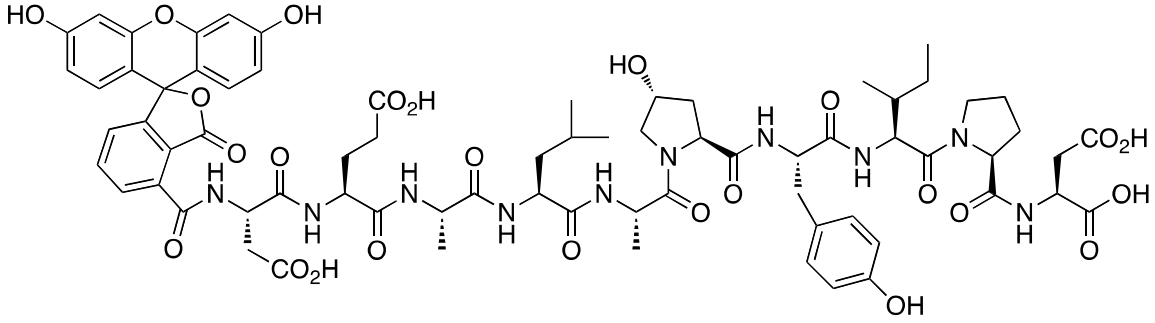
Exact Mass: 1118.51
Molecular Weight: 1119.18

DEALA-Hyp-YIPD was ordered from the W.M. Keck Foundation's Small Scale Peptide Synthesis service. It was analyzed by MALDI and used without further purification.

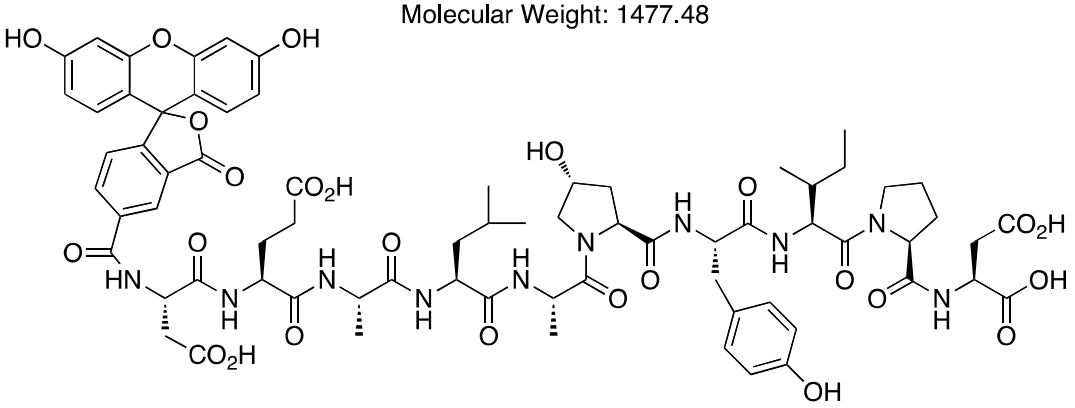
MALDI:


Applied Biosystems Voyager System 6268
Voyager Spec #1[BP = 1143.2, 12129]
DB-3-107-1
DEALA-Hyp-YIPD
+Na
+H
1143.62
1159.32
1165.33
1180.71
1122.02
1157.24
1266.21
1356.61
1358.47
890.50
% Intensity
Mass (m/z)
Mode of operation: Linear
Extraction mode: Delayed
Polarity: Positive
Acquisition control: Manual
Accelerating voltage: 25000 V
Grid voltage: 94.5%
Guide wire 0: 0.3%
Extraction delay time: 300 nsec
Acquisition mass range: 700 -- 3000 Da
Number of laser shots: 100/spectrum
Laser intensity: 3602
Laser Rep Rate: 20.0 Hz
Calibration type: External -- D:\data\100208_0001.cal
Calibration matrix: a-Cyano-4-hydroxycinnamic acid
Low mass gate: 500 Da
Digitizer start time: 16.282
Bin size: 1 nsec
Number of data points: 17284
Vertical scale: 500 mV
Vertical offset: 1%
Input bandwidth: 500 MHz
Sample well: 57
Plate ID: 100 WELL PLATE
Serial number: 6268
Instrument name: Voyager-DE PRO
Plate type filename: C:\VOYAGER\100 well plate.plt
Lab name: Yale University
Absolute x-position: 32878.1
Absolute y-position: 23195.5
Relative x-position: 810.597
Relative y-position: 1287.97
Shots in spectrum: 100
Source pressure: 2.14e-007
Mirror pressure: 1.024e-007
TC2 pressure: 0.001
TIS gate width: 8
TIS flight length: 690.5
Acquired: 19:19:00, September 25, 2010
D:\data\Dennis\DB-3-107\DB-3-107-1_0002.dat
Printed: 19:20, September 25, 2010

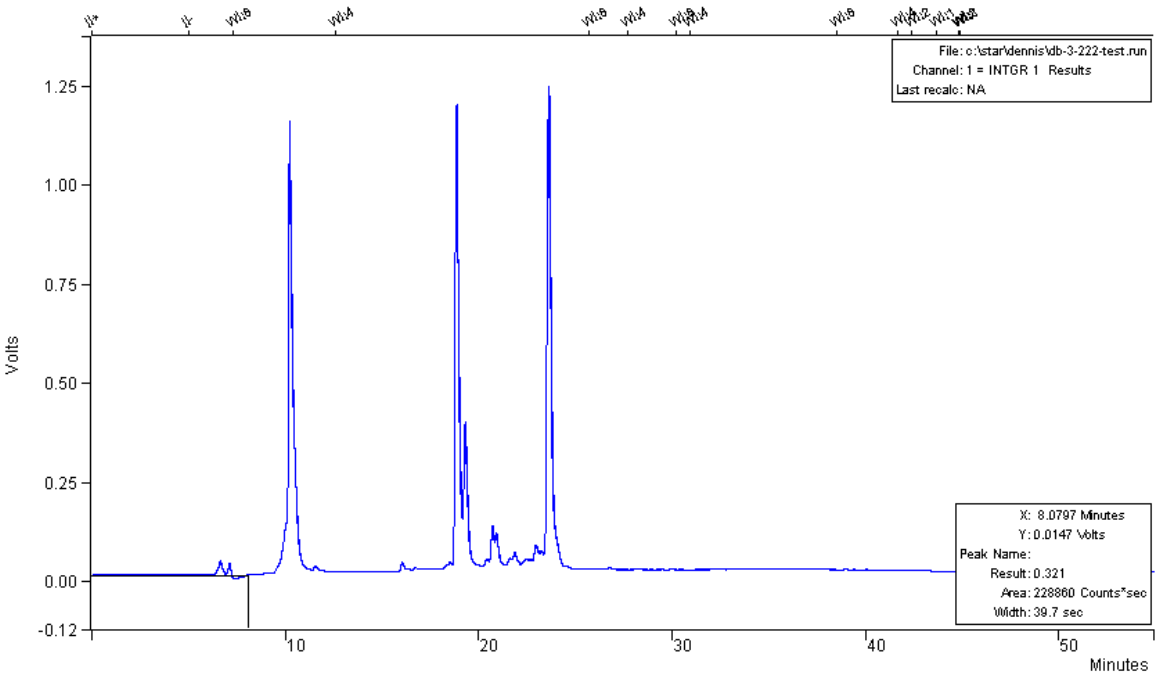
5,6-FAM-DEALA-Hyp-YIPD



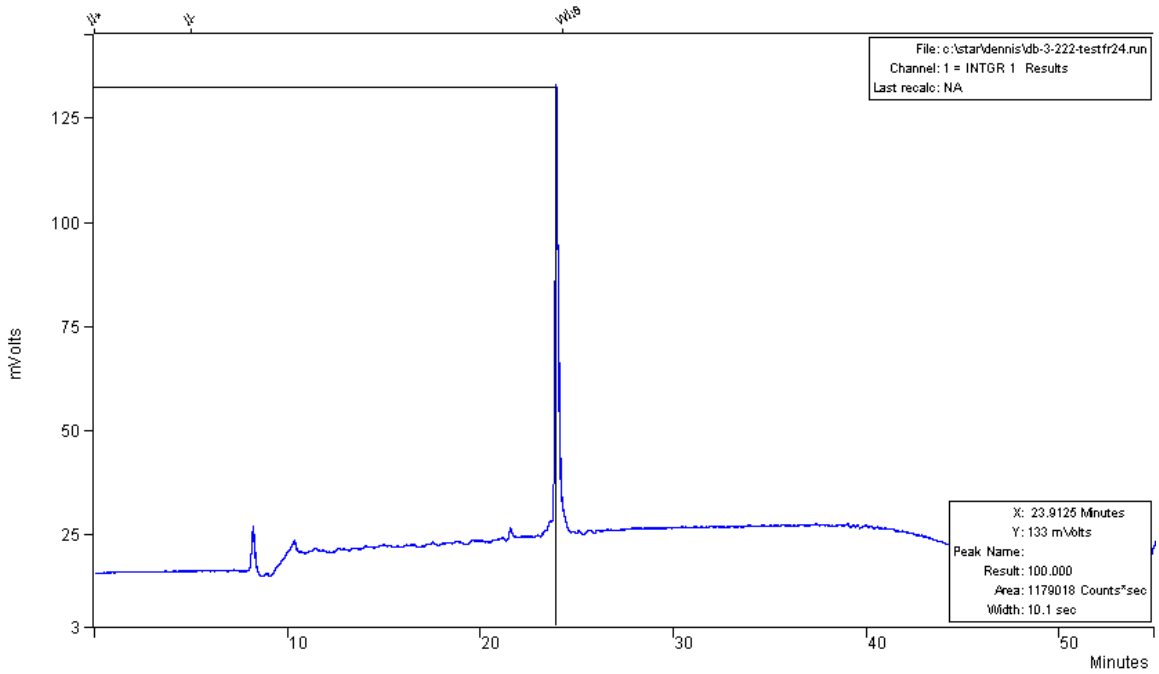
Molecular Weight: 1477.48



5,6-FAM-DEALA-Hyp-YIPD was ordered from the W.M. Keck Foundation's Small Scale Peptide Synthesis service as a mixture of the 5 and 6 FAM isomers. It was purified by HPLC, and analyzed by HPLC and MALDI.

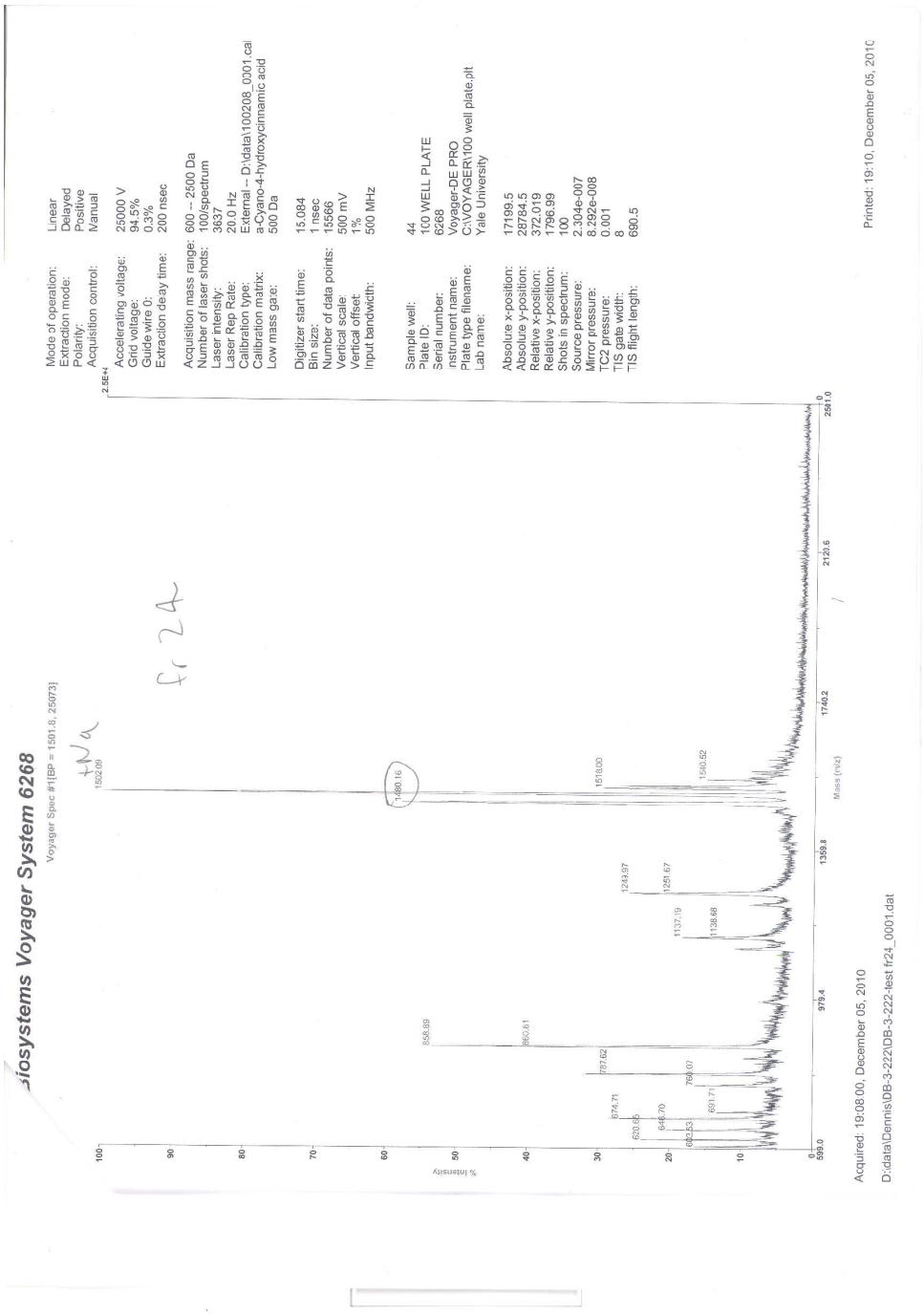


HPLC trace of crude fluorescent peptide.



HPLC trace of purified fluorescent peptide.

MALDI:


Biosystems Voyager System 6268
Voyager Spec #1[BP = 1501.8, 25073]
Mode of operation: Linear
Extraction mode: Delayed
Polarity: Positive
Acquisition control: Manual
Accelerating voltage: 25000 V
Grid voltage: 94.5%
Guide wire 0: 0.3%
Extraction delay time: 200 nsec
Acquisition mass range: 600 -- 2500 Da
Number of laser shots: 100/spectrum
Laser intensity: 3637
Laser Rep Rate: 20.0 Hz
Calibration type: External -- D:\data\100208_0301.cal
Calibration matrix: a-Cyano-4-hydroxycinnamic acid
Low mass gate: 500 Da
Digitizer start time: 15.084
Bin size: 1 nsec
Number of data points: 15566
Vertical scale: 500 mV
Vertical offset: 1%
Input bandwidth: 500 MHz
Sample well: 44
Plate ID: 100 WELL PLATE
Serial number: 6268
Instrument name: Voyager-DE PRO
Plate type filename: C:\VOYAGER\100 well plate.plt
Lab name: Yale University
Absolute x-position: 17199.5
Absolute y-position: 28784.5
Relative x-position: 372.019
Relative y-position: 1796.99
Shots in spectrum: 100
Source pressure: 2.304e-007
Mirror pressure: 8.292e-008
TOF2 pressure: 0.001
TIS gate width: 8
TIS flight length: 690.5
Fr 24
1502.09
1480.16
1518.00
1540.52
858.89
860.61
793.62
769.07
674.71
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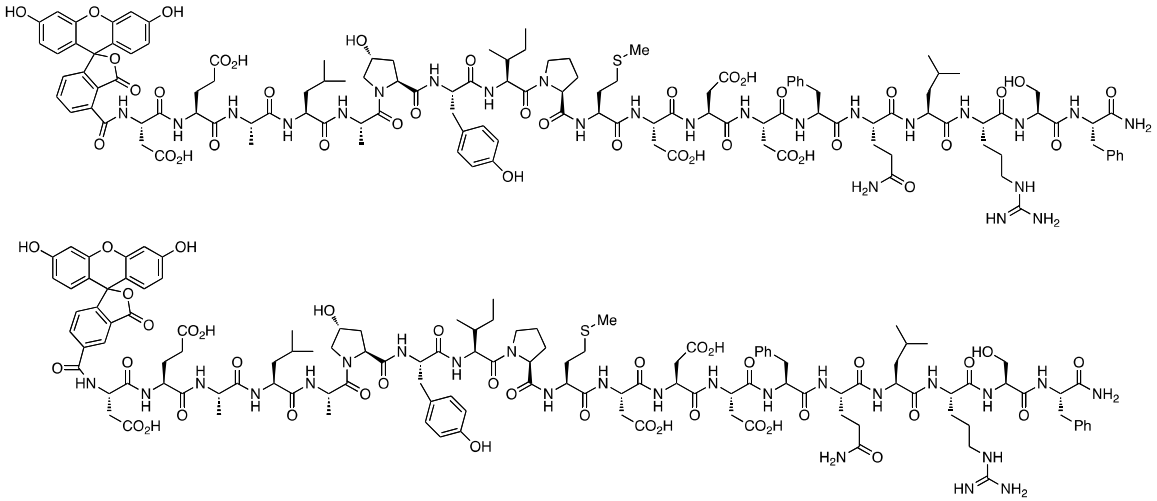
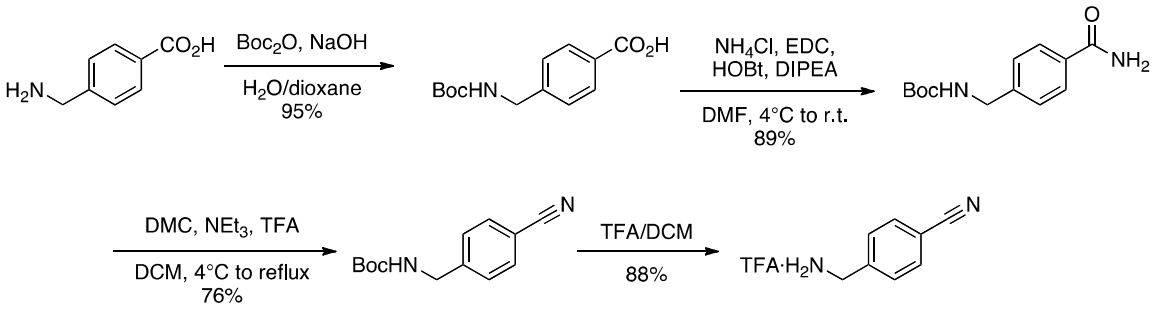
646.70
682.53
691.71
1243.97
1251.67
1137.19
1138.68
% Intensity
Mass (m/z)
Acquired: 19:08:00, December 05, 2010
D:\data\Dennis\DB-3-222\DB-3-222-test fr24_0001.dat
Printed: 19:10, December 05, 2010

5,6-FAM-DEALA-Hyp-YIPMDDDFQLRSF-NH_2

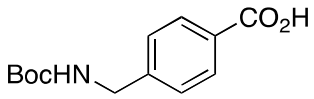


5,6-FAM-DEALA-Hyp-YIPMDDDFQLRSF-NH_2 was synthesized on Rink amide resin, using standard solid phase peptide synthesis techniques. The peptide was cleaved from the resin using 82.5/5/5/5/2.5 TFA/thioanisole/water/phenol/EDT (1,2-Ethandithiol), and purified by HPLC. **MS** (MALDI) 2617.167 (M+H).

Synthesis and Characterization of VHL Ligands

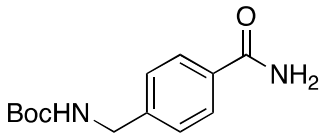


4-(((tert-butoxycarbonyl)amino)methyl)benzoic acid (Boc-Amb)



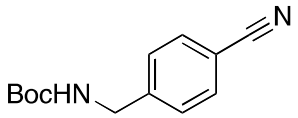
4-(Aminomethyl)benzoic acid (Amb-OH) (5.01 g, 33.1 mmol, 1 eq) was dissolved in dioxane (80 mL) and water (40 mL) at room temperature. 1M NaOH (aq) (40 mL, 40 mmol, 1.2 eq) was added and the solution was cooled to 4 °C in an ice bath. Di-*tert*-butyl dicarbonate (7.97 g, 36.5 mmol, 1.1 eq) was added and the solution was allowed to warm slowly to room temperature. After 15 hours, the dioxane was removed under reduced pressure. The remaining aqueous solution was acidified to pH 2 with 10% potassium bisulfate (aq) and extracted with EtOAc. The organic layer was dried over sodium sulfate, filtered and condensed to give Boc-Amb-OH as a white solid that gave a ^{1}H NMR spectrum that matched the literature.[12]

***tert*-butyl 4-carbamoylbenzylcarbamate (Boc-Amb-NH_2)**



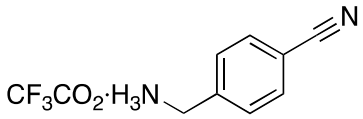
Boc-Amb-OH (3.0 g, 11.0 mmol, 1 eq) was dissolved in MeCN (60 mL) and DMF (15 mL) at room temperature. The solution was cooled to 4 °C. Ammonium chloride (0.96 g, 17.9 mmol, 1.5 eq), EDC (3.66 g, 19.1 mmol, 1.6 eq), HOBt (2.74 g, 20.3 mmol, 1.7 eq) and DIPEA (9.3 mL, 53.7 mmol, 4.5 eq) were then added. The N_2 line was removed and the solution was allowed to warm to room temperature slowly under a septum for 22 hours after which most of the MeCN was removed under reduced pressure. The resulting solution was diluted with EtOAc, washed with saturated ammonium chloride (aq), saturated sodium bicarbonate, water and brine. The organic layer was dried over sodium sulfate, filtered and condensed to give Boc-Amb-NH_2 as a white solid with spectra matching the literature data.[13]

tert-butyl 4-cyanobenzylcarbamate

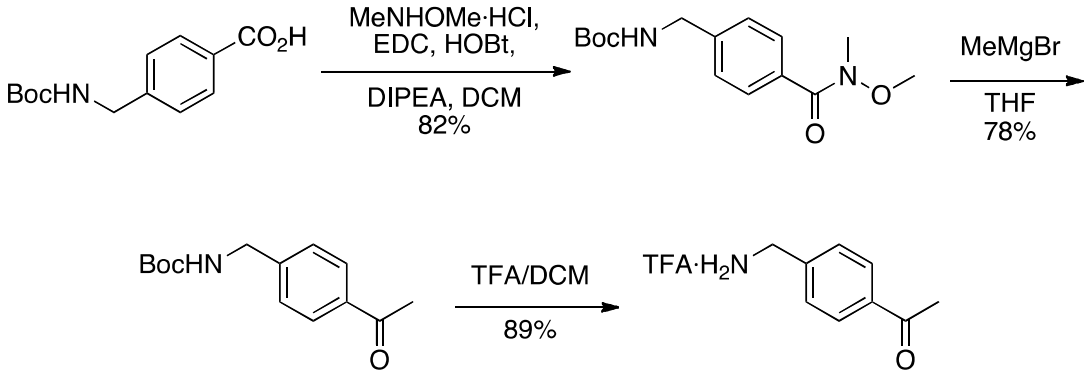


Boc-Amb-NH_2 (2.57 g, 10.3 mmol, 1 eq) was dissolved in DCM (137 mL) at room temperature. The solution was cooled to 4 °C and TFA (0.8 mL, 10.3 mmol, 1 eq) was added. In quick succession, 2-chloro-1,3-dimethylimidazolinium chloride (DMC)[14] (2.87 g, 17.0 mmol, 1.65 eq) and triethylamine (5.03 mL, 36.1 mmol, 3.5 eq) were added. The solution was then warmed to room temperature, fitted with a condenser and placed in an oil bath at 50 °C for 21 hours. The solution was then cooled to room temperature, diluted with DCM and washed with water. The organic layer was dried over sodium sulfate, filtered and condensed. Purification by column chromatography (20 to 50% EtOAc/hexanes) gave a white solid (1.82 g, 7.84 mmol, 76%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.52 (d, *J* = 7.9 Hz, 2H), 7.32 (d, *J* = 7.9 Hz, 2H), 5.33 (s, 1H), 4.28 (d, *J* = 5.9 Hz, 2H), 1.38 (s, 9H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 156.05, 144.92, 132.30, 127.75, 118.84, 110.82, 79.83, 44.07, 28.35. **MS** (ESI) 233.1 (M+H), 176.1 (M-tBu).

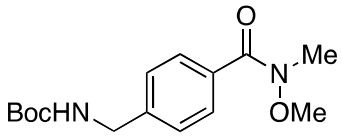
4-(aminomethyl)benzonitrile trifluoroacetate salt



tert-butyl 4-cyanobenzylcarbamate (57.7 mg, 0.248 mmol, 1 eq) was dissolved in DCM (4 mL) at room temperature. TFA (1 mL) was added and the solution was stirred for 15 hours, after which the disappearance of starting material was confirmed by TLC (50% EtOAc/hexanes). The mixture was concentrated under reduced pressure to give the trifluoroacetate salt of 4-(aminomethyl)benzonitrile (53.7 mg, 0.218 mmoll, 88%) as a cream colored solid, which was used without further purification. **^{1}H NMR** (500 MHz, MeOD) δ 7.89 – 7.74 (m, 2H), 7.65 (d, *J* = 8.4 Hz, 2H), 4.22 (s, 2H). **^{13}C NMR** (126 MHz, MeOD) δ 139.79, 133.93, 130.92, 119.15, 114.00, 43.70. **MS** (ESI) 132.9 (M – $CF_3CO_2^-$).

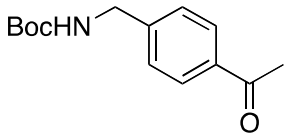


tert-butyl 4-(methoxy(methyl)carbamoyl)benzylcarbamate (Boc-Amb-N(OMe)Me)



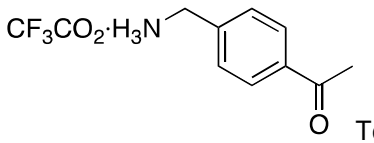
Boc-Amb-OH (2.55 g, 10.16 mmol, 1 eq) was dissolved in DCM (68 mL) and cooled to 4 °C in an ice bath. EDC (2.34 g, 12.2 mmol, 1.2 eq), HOBt (1.65 g, 12.2 mmol, 1.2 eq) and DIPEA (6.2 mL, 35.6 mmol, 3.5 eq) were added. The solution was stirred for 30 minutes and then N,O-Dimethylhydroxylamine hydrochloride (1.09 g, 11.2 mmol, 1.1 eq) was added. The solution warmed slowly to room temperature and after 21 hours was poured into brine, with a small amount of chloroform to break the resulting emulsion. After separation, the aqueous layer was extracted twice with EtOAc. The combined organic layer was dried over sodium sulfate, filtered and condensed. Purification by column chromatography (40 to 75% EtOAc/hexanes) gave a colorless oil (2.45 g, 8.33 mmol, 82%). **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.65 (d, *J* = 8.2 Hz, 2H), 7.31 (d, *J* = 8.1 Hz, 2H), 4.88 (s, 1H), 4.36 (d, *J* = 5.1 Hz, 2H), 3.55 (s, 3H), 3.35 (d, *J* = 4.7 Hz, 3H), 1.47 (s, 9H). **^{13}C NMR** (126 MHz, CDCl3) δ 169.77, 156.04, 141.79, 133.21, 128.76, 127.03, 79.87, 61.20, 44.50, 33.88, 28.55. **MS** (ESI) 295.2 (M+H).

tert-butyl 4-acetylbenzylcarbamate



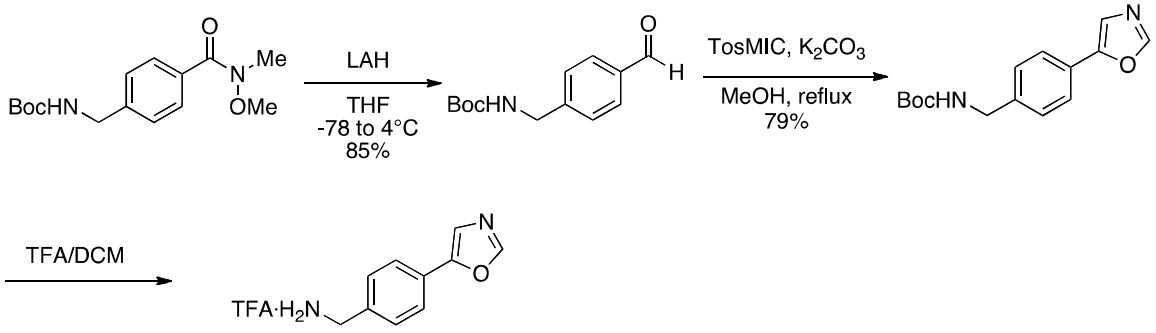
To a stirred solution of Boc-Amb-N(OMe)Me (441 mg, 1.50 mmol) in THF (4.5 mL) at -78 °C was added dropwise a solution of methylmagnesium bromide (3 M in Et_2O, 1.25 mL, 3.75 mmol). The reaction mixture was stirred at -78 °C for 1.0 h, at rt for 14 h, and cooled to -20 °C. The resulting mixture was quenched with saturated aqueous NH_4Cl solution, and extracted twice with ethyl acetate. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. The residue was purified by flash chromatography on silica gel to afford ketone **3** (291 mg, 78%). **^{1}H NMR** (500 MHz, $CDCl_3$) d 7.91 (d, *J* = 8.2 Hz, 2H), 7.36 (d, *J* = 8.2 Hz, 2H), 4.98 (brs, 1H), 4.37 (d, *J* = 5.8 Hz, 2H), 2.58 (s, 3H), 1.46 (s, 3H). **^{13}C NMR** (125 MHz, $CDCl_3$) d 197.7, 155.8, 144.5, 136.2, 128.7, 127.3, 79.8, 44.3, 28.3, 26.6.

1-(4-(aminomethyl)phenyl)ethanone trifluoroacetate salt

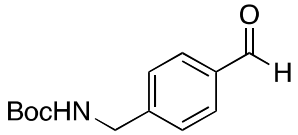


To a solution of ketone **3** (250 mg, 1.0 mmol) in DCM (3.0 mL) at 0 °C were added TFA (0.5 mL). The bath was removed and the reaction mixture was stirred at rt for 12 h. TFA and solvent were removed in vacuo. The residue was purified by flash chromatography on silica gel to afford amine **4** as TFA salt (235 mg, 89%) as a white solid. ^{1}H **NMR** (500 MHz,

MeOD) d 8.05 (d, *J* = 8.3 Hz, 2H), 7.58 (d, *J* = 8.2 Hz, 2H), 4.19 (s, 2H), 2.61 (s, 3H). **^{13}C NMR** (125 MHz, MeOD) d 199.7, 139.5, 138.8, 130.2, 130.1, 43.8, 26.7.

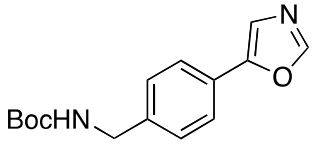


***tert*-butyl 4-formylbenzylcarbamate (Boc-Amb-H)**



Boc-Amb-N(OMe)Me (2.45 g, 8.33 mmol, 1 eq) was dissolved in THF (83 mL) and cooled to -78 °C in a dry ice/acetone bath. Lithium aluminum hydride (0.41 g, 10.83 mmol, 1.3 eq) was added in 2 portions over 5 minutes. After 50 minutes, the suspension was warmed to 4 °C in an ice bath. After 3.5 hours, the reaction was deemed complete by TLC (mini workup in 10% potassium bisulfate and EtOAc, 50% EtOAc/hexanes) and the reaction was quenched by the slow addition of 10% potassium bisulfate at 4 °C. The mixture was warmed to room temperature, and stirred for 30 minutes. Most of the THF was removed under reduced pressure and mixture was diluted with water and extracted thrice with EtOAc. The combined organic layer was washed once with brine, dried over sodium sulfate, filtered and condensed. Purification by column chromatography (40 to 50% EtOAc/hexanes) gave Boc-Amb-H as a white solid (1.66 g, 7.1 mmol, 85%). **^{1}H NMR** (500 MHz, $CDCl_3$) δ 9.96 (s, 1H), 7.81 (d, *J* = 8.2 Hz, 2H), 7.41 (d, *J* = 8.0 Hz, 2H), 5.12 (s, 1H), 4.37 (d, *J* = 5.6 Hz, 2H), 1.44 (s, 9H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 191.94, 156.03, 146.30, 135.62, 130.14, 127.78, 79.92, 44.44, 28.46. **MS** (ESI) 235.9 (M+H), 180.2 (M-tBu).

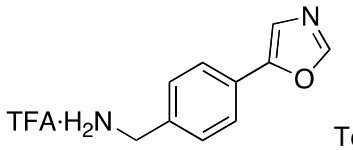
tert-butyl 4-(oxazol-5-yl)benzylcarbamate



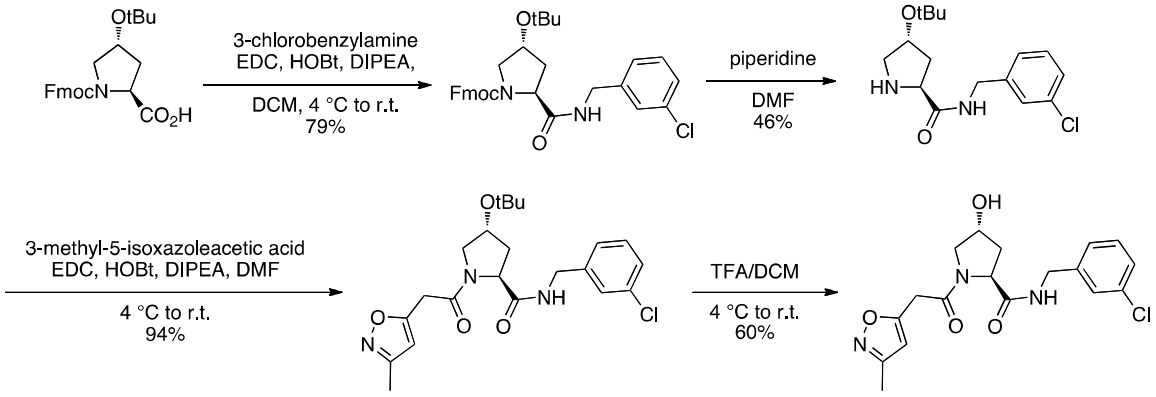
Potassium carbonate (0.13 g, 0.94 mmol, 1.2 eq) and toluenesulfonylmethyl isocyanide (0.184 g, 0.94 mmol, 1.2 eq) were added to MeOH (7.8 mL) at room temperature. The round bottom was fitted with a reflux condenser and heated to 45 °C. After 15 minutes, Boc-Amb-H (0.1835 g, 0.78 mmol, 1 eq) was added and the mixture was heated to 75 °C for 3 hours and then cooled to room temperature. The MeOH was removed under reduced pressure and the crude material was

resuspended in EtOAc and 1:2 mixture of saturated sodium carbonate to water and separated. The aqueous layer was then extracted once with EtOAc. The combined organic layer was dried over sodium sulfate, filtered and condensed. Purification by column chromatography (20 to 35% EtOAc/hexanes) gave a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91 (s, 1H), 7.62 (d, *J* = 8.3 Hz, 2H), 7.35 (ob d, 2H), 7.34 (ob s, 1), 4.88 (s, 1H), 1.47 (s, 9H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 156.02, 151.40, 150.47, 139.78, 128.01, 126.84, 124.67, 121.47, 79.68, 44.38, 28.47. **MS** (ESI) 275.5 (M+H).

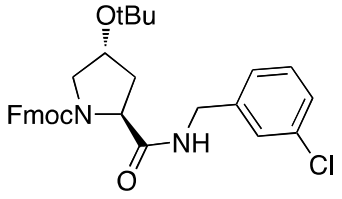
(4-(oxazol-5-yl)phenyl)methanamine trifluoroacetate salt



To a solution of tert-butyl 4-(oxazol-5-yl)benzylcarbamate (1.09 g) in DCM (40 mL), TFA (4 mL) was added at room temperature. The solution was stirred for 16 hours and concentrated under reduced pressure to yield the trifluoroacetate salt of (4-(oxazol-5-yl)phenyl)methanamine (1.984 g) as a cream colored solid, which was used without further purification. **^{1}H NMR** (400 MHz, MeOD) δ 8.29 (s, 1H), 7.83 (d, *J* = 8.4 Hz, 2H), 7.60 (s, 1H), 7.56 (d, *J* = 8.5 Hz, 2H), 4.16 (s, 2H). **MS** (ESI) 175.3 (M- $CF_3CO_2^-$).

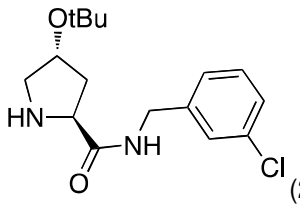


(2*S*,4*R*)-(9*H*-fluoren-9-yl)methyl 4-(*tert*-butoxy)-2-((3-chlorobenzyl)carbamoyl)pyrrolidine-1-carboxylate



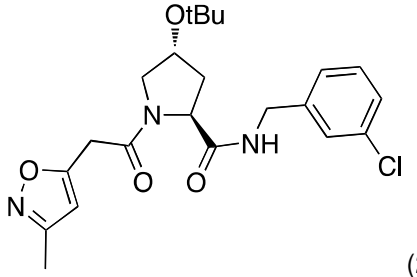
Fmoc-Hyp(OtBu)-OH (0.50 g, 1.22 mmol, 1 eq) was dissolved in DCM (6.1 mL) and cooled to 4 °C in an ice bath. EDC (0.282 g, 1.47 mmol, 1.2 eq), HOBt (0.215 g, 1.59 mmol, 1.3 eq), DIPEA (0.25 mL, 1.47 mmol, 1.2 eq) and 3-chlorobenzylamine (0.17 mL, 1.34 mmol, 1.1 eq) were added and the solution was allowed to warm to room temperature slowly. After 18 hours, the DCM was removed under reduced pressure and the crude material was resuspended in EtOAc and washed with 1M HCl (aq), saturated sodium bicarbonate, water and brine. The organic layer was dried over sodium sulfate, filtered and condensed. Purification by column chromatography (40 to 50% EtOAc/hexanes) gave a white solid (0.51 g, 0.96 mmol, 79%). **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.1 Hz, 2H), 7.58 (d, *J* = 7.0 Hz, 2H), 7.40 (t, *J* = 7.4 Hz, 2H), 7.30 (dd, *J* = 9.0, 5.7 Hz, 2H), 7.25 – 7.11 (m, 4H), 4.50 – 4.10 (m, 7H), 3.66 – 3.54 (m, 1H), 3.30 (dd, *J* = 10.6, 6.2 Hz, 1H), 2.52 (s, 1H), 2.02 – 1.89 (m, 1H), 1.25 – 1.14 (m, 9H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 171.45, 156.54, 152.44, 143.91, 141.51, 134.65, 131.02, 130.03, 127.94, 127.55, 127.25, 125.61, 125.20, 120.17, 100.14, 74.28, 69.81, 68.10, 59.24, 53.21, 47.33, 42.97, 35.97, 28.45. **MS** (ESI) 533.7 (M+H).

(2S,4R)-4-(tert-butoxy)-N-(3-chlorobenzyl)pyrrolidine-2-carboxamide



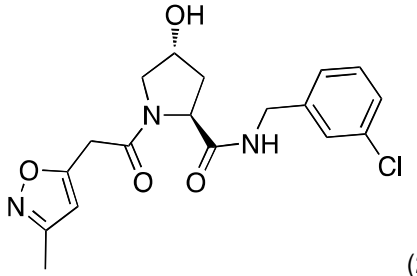
(2*S*,4*R*)-(9*H*-fluoren-9-yl)methyl 4-(*tert*-butoxy)-2-((3-chlorobenzyl)carbamoyl)pyrrolidine-1-carboxylate (0.148 g, 0.278 mmol, 1 eq) was dissolved in DCM (5.6 mL) and cooled to 4 °C in an ice bath. Piperidine (0.275 mL, 2.78 mmol, 10 eq) was added and the solution was allowed to warm slowly to room temperature. After 15 hours, the solution was concentrated under reduced pressure and purified by column chromatograph (0 to 40% EtOAc/hexanes then DCM to 10% MeOH/DCM) to give a white foamy solid (39.7 mg, 0.128 mmol, 46%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.01 (s, 1H), 7.24 (q, *J* = 4.7 Hz, 3H), 7.18 – 7.10 (m, 1H), 4.40 (d, *J* = 6.2 Hz, 2H), 4.19 – 4.07 (m, 1H), 3.97 (dd, *J* = 9.0, 7.0 Hz, 1H), 2.93 – 2.75 (m, 2H), 2.18 (dddd, *J* = 13.6, 9.1, 3.0, 1.5 Hz, 1H), 2.08 – 2.01 (m, 1H), 1.17 (s, 9H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 175.07, 140.91, 134.59, 130.03, 127.73, 127.61, 125.82, 73.83, 72.42, 60.05, 55.08, 42.40, 39.38, 28.54. **MS** (ESI) 310.7 (M+H).

(2*S*,4*R*)-4-(*tert*-butoxy)-*N*-(3-chlorobenzyl)-1-(2-(3-methylisoxazol-5-yl)acetyl)pyrrolidine-2-carboxamide



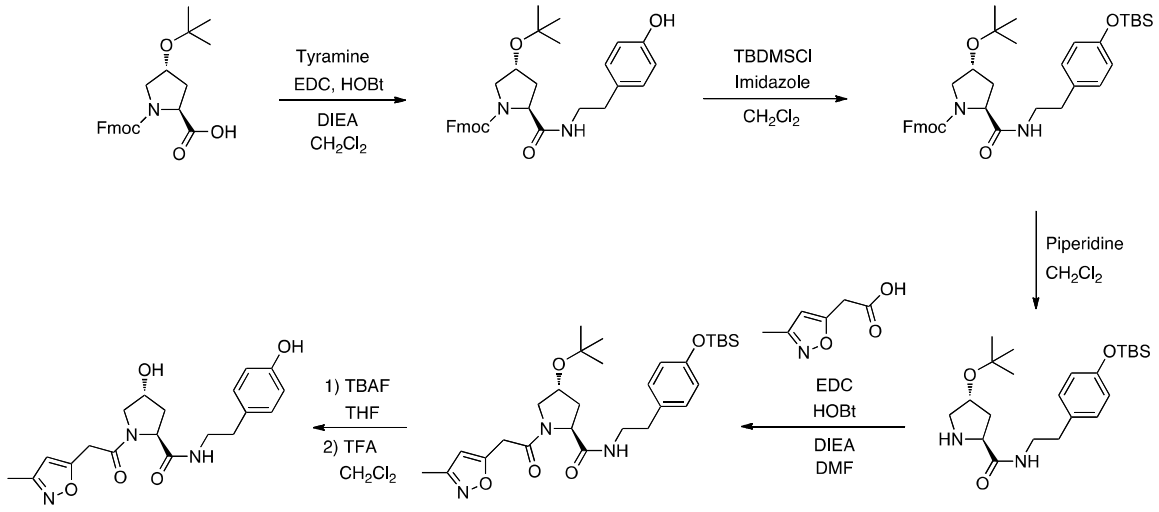
(2S,4R)-4-(tert-butoxy)-N-(3-chlorobenzyl)pyrrolidine-2-carboxamide (29.5 mg, 0.095 mmol, 1 eq) was dissolved in DMF (0.5 mL) and cooled to 4 °C in an ice bath. 3-Methyl-5-isoxazoleacetic acid (17 mg, 0.123 mmol, 1.3 eq), EDC (25 mg, 0.133 mmol, 1.4 eq), HOBT (19 mg, 0.143 mmol, 1.5 eq) and DIPEA (0.2 mL) were added and the solution was allowed to warm slowly to room temperature. After 14 hours, the solution was diluted with water, and extracted once with EtOAc. The aqueous layer was then diluted with brine, and extracted twice with EtOAc. The combined organic layer was washed with brine, and back extracted once with EtOAc. The combined organic layer was dried over sodium sulfate, filtered and condensed. Purification by column chromatography (0 to 5% MeOH/DCM) gave a colorless oil (38.7 mg, 0.089mmol, 94%). **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.33 (t, *J* = 5.6 Hz, 1H), 7.25 – 7.17 (m, 3H), 7.09 (dt, *J* = 6.8, 1.8 Hz, 1H), 6.06 (d, *J* = 11.5 Hz, 1H), 4.67 (dd, *J* = 8.4, 2.6 Hz, 1H), 4.58 – 4.47 (m, 1H), 4.43 – 4.29 (m, 2H), 3.77 (s, 3H), 3.32 (dt, *J* = 13.0, 6.5 Hz, 1H), 2.56 – 2.46 (m, 1H), 2.26 (s, 3H), 1.94 – 1.84 (m, 1H), 1.19 (d, *J* = 6.3 Hz, 9H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 170.69, 167.34, 165.24, 160.24, 140.37, 134.51, 130.03, 127.56, 127.51, 125.61, 104.17, 74.45, 69.98, 59.04, 54.12, 42.95, 35.52, 33.40, 28.36, 11.56. **MS** (ESI) 433.8 (M+H).

(2*S*,4*R*)-*N*-(3-chlorobenzyl)-4-hydroxy-1-(2-(3-methylisoxazol-5-yl)acetyl)pyrrolidine-2-carboxamide (1)

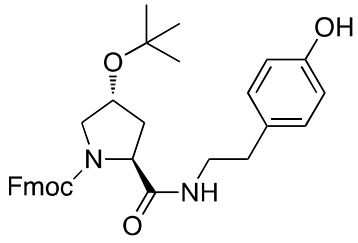


(2*S*,4*R*)-4-(*tert*-butoxy)-*N*-(3-chlorobenzyl)-1-(2-(3-methylisoxazol-5-yl)acetyl)pyrrolidine-2-carboxamide (38.7 mg, 0.089 mmol, 1 eq) was dissolved in DCM (0.9 mL) and cooled to 4 °C in an ice bath. TFA (0.069 mL, 0.89 mmol, 10 eq) was added and the solution was allowed to warm slowly to room temperature. After 16 hours, the solution was condensed and purified by column chromatography (0 to 7.5% MeOH/DCM) to give **1** as a white solid (20.5 mg, 0.054 mmol, 60%). **^{1}H NMR** (500MHz, MeOD):δ8.684 (1H, s); 7.33-

7.23 (4H, m); 6.24 (1H, s); 4.56-4.53 (1H, t, J= 8 Hz); 4.51-4.50 (1H, m); 4.39-4.37 (2H, m); 3.96-3.92 (2H, m); 3.81-3.3.78 (1H, dd, J= 9 Hz, 4 Hz); 3.64-3.62 (1H, m); 2.28-2.24 (4H, m); 2.09-2.04 (1H, m).**^{13}C NMR (**125MHz, MeOD):δ174.56, 168.67, 167.68, 161.58, 142.25, 135.35, 131.04, 128.43, 128.19, 126.76, 105.37, 70.86, 60.78, 56.96, 43.60, 39.33, 33.90, 11.21. **MS** (ESI) 378.2 (M+H).



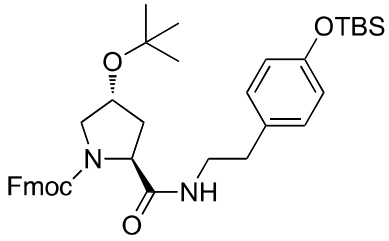
(2S,4R)-(9H-fluoren-9-yl)methyl 4-(tert-butoxy)-2-((4-hydroxyphenethyl)carbamoyl)pyrrolidine-1-carboxylate



To a solution of Fmoc-O-*tert*-butyl-l-4-hydroxyproline (Fmoc-Hyp(*t*-Bu)-OH, Chem-Impex International, 410 mg, 1.0 mmol) in DCM (10 mL) at room temperature were added tyramine (151 mg, 1.1 mmol), DIPEA (0.58 mL, 3.3 mmol), and HOBt (162 mg, 1.2 mmol). The mixture was cooled to 0 °C, and then EDC (230 mg, 1.2 mmol) was added to the mixture at 0 °C. The reaction mixture was allowed to warm to rt, stirred at rt for 16 h, and cooled to 0 °C. The resulting mixture was quenched with H_2O (20 mL) and extracted twice with ethyl acetate. The combined extracts were washed with brine, dried over anhydrous Na_2SO_4, filtered, and evaporated. The concentrate was purified by column chromatography (eluting with 100% DCM initially, grading to 5% CH_3OH in DCM) on silica gel to afford the coupled product (470 mg, 89%). **^{1}H NMR** (500 MHz, $CDCl_3$) d 7.74 (d, *J* = 7.3 Hz, 2H), 7.56 (dd, *J* = 6.4, 6.4 Hz, 3/2H), 7.49-7.44 (m, 1/2H), 7.39-7.36 (m, 2H), 7.30-7.28 (m, 2H), 6.95 (d, *J* = 8.1 Hz, 3/2H), 6.87 (d, *J* = 7.0 Hz, 1/2H), 6.68-6.64 (m, 3H), 6.07 & 5.95 (due to the rotamers, both s, 1H), 4.40-4.30 (m, 2H), 4.28-4.18 (m, 2H), 3.58 (dd, *J* = 10.4, 6.4 Hz, 1H), 3.42-3.38 (m, 2H), 3.26 (dd, *J* = 10.4, 6.4

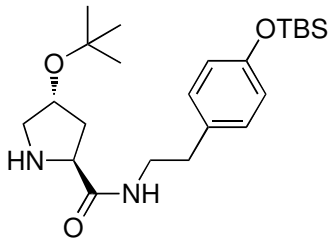
Hz, 2H), 2.71-2.63 (m, 3/2H), 2.56-2.53 (m, 1/2H), 2.40-2.37 (m, 1H), 1.91-1.85 (m, 1H), 1.21 & 1.15 (due to the rotamers, both s, 9H). **^{13}C NMR** (asterisk denotes the signals of the minor rotamer, 125 MHz, $CDCl_3$) d 171.4, 156.1, 154.6, 143.8, *143.7, 141.3, *141.2, *130.3, 129.7, 127.8, 127.1, 127.0, 125.1, 125.0, 120.0, *115.5, 115.3, 74.1, 69.5, *68.6, 67.9, *67.3, 59.1, *53.4, 53.1, 47.0, 40.9, *40.6, 36.0, 34.6, 28.2. **MS** (ESI) [M+H] 529.5.

(2S,4R)-(9H-fluoren-9-yl)methyl 4-(tert-butoxy)-2-((4-((tert-butyldimethylsilyl)oxy)phenethyl)carbamoyl)pyrrolidine-1-carboxylate



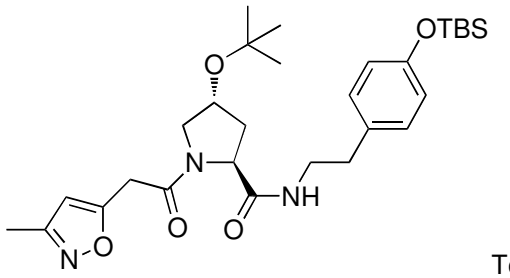
To a solution of (2S,4R)-(9H-fluoren-9-yl)methyl 4-(tert-butoxy)-2-((4-((tert-butyldimethylsilyl)oxy)phenethyl)carbamoyl)pyrrolidine-1-carboxylate (400 mg, 0.757 mmol) in DCM (7.5 mL) at 0 ^{o}C were added imidazole (62 mg, 0.908 mmol) and TBDMSCl (126 mg, 0.833 mmol). The reaction mixture was allowed to rt, stirred at rt for 18 h, and cooled to 0 ^{o}C. The reaction mixture was allowed to warm to rt, stirred at rt for 16 h, and cooled to 0 ^{o}C. The resulting mixture was quenched with H_2O (10 mL) and extracted twice with ethyl acetate. The combined extracts were washed with brine, dried over anhydrous Na_2SO_4, filtered, and evaporated. The concentrate was chromatographed (eluting with 10% ethyl acetate in hexane initially, grading to 30% ethyl acetate in hexane) on silica gel to provide the TBS ether (469 mg, 96%). **^{1}H NMR** (400 MHz, $CDCl_3$) d 7.76 (d, *J* = 7.0 Hz, 2H), 7.58 (d, *J* = 7.0 Hz, 2H), 7.40 (dd, *J* = 7.4, 7.4 Hz, 2H), 7.02 & 6.93 (due to the rotamers, both d, *J* = 7.5 Hz, 2H), 6.73 (d, *J* = 8.4 Hz, 2H), 4.45-4.13 (m, 5H), 3.60-3.25 (m, 4H), 2.72 & 2.60 (due to the rotamers, both s, 2H), 2.45-2.43 (m, 1H), 1.93-1.88 (m, 1H), 1.21 (s, 9H), 0.95 (s, 9H), 0.15 (s, 6H). **^{13}C NMR** (asterisk denotes the signals of the minor rotamer, 100 MHz, $CDCl_3$) d 171.2, 156.1, 154.2, 143.9, *143.8, 141.3, *131.5, 129.6, 127.8, 127.1, 125.1, 120.0, 77.2, 74.1, 69.6, 67.8, *59.5, 59.1, *53.9, 53.0, 47.2, 40.9, 36.0, 34.9, *34.6, 28.3, 25.7, 18.2, -4.4. **MS** (ESI) [M+H] 643.6.

(2S,4R)-4-(tert-butoxy)-N-(4-((tert-butyldimethylsilyl)oxy)phenethyl)pyrrolidine-2-carboxamide



To a solution of (2S,4R)-(9H-fluoren-9-yl)methyl 4-(tert-butoxy)-2-((4-((tert-butyldimethylsilyl)oxy)phenethyl)carbamoyl)pyrrolidine-1-carboxylate (193 mg, 0.3 mmol) in DCM (6 mL) at 0 °C was added piperidine (0.18 mL, 3.0 mmol). The reaction mixture was allowed to warm to rt, stirred at rt for 18 h, and evaporated. The residue was purified by column chromatography (eluting with 100% DCM initially, grading to 3% CH_3OH in DCM) on silica gel to furnish the free amine (110 mg, 87%). **^{1}H NMR** (400 MHz, MeOD) d 7.08 (d, *J* = 6.7 Hz, 1H), 7.07 (d, *J* = 8.4 Hz, 1H), 6.76 (d, *J* = 8.3 Hz, 1H), 6.75 (d, *J* = 8.4 Hz, 1H), 4.19-4.14 (m, 1/2H), 4.13-4.08 (m, 1/2H), 3.70 (t, *J* = 8.0 Hz, 1H), 3.49-3.33 (m, 2H), 3.14 & 2.98 (due to the rotamers, both dd, *J* = 11.4, 5.1 Hz, 1H), 2.76-2.68 (m, 2H), 2.02-1.92 (m, 1H), 1.88-1.72 (m, 1H), 1.16 & 1.15 (due to the rotamers, both s, 9H), 0.98 (s, 9H), 0.17 (s, 6H). **^{13}C NMR** (100 MHz, MeOD) d 176.7, 155.4, 133.2, 130.9, 121.1, 121.0, 86.5, 74.9, 74.8, 73.5, 71.1, 63.1, 60.8, 58.2, 55.6, 41.5, 41.3, 40.9, 40.4, 35.6, 35.4, 28.7, 26.1, 19.0, -4.3. **MS** (ESI) [M+H] 421.2.

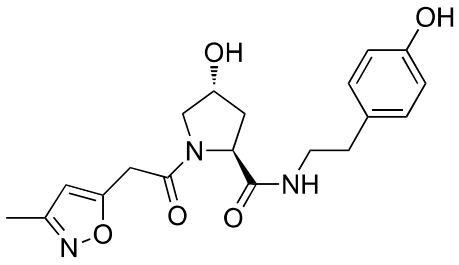
(2S,4R)-4-(tert-butoxy)-N-(4-((tert-butyldimethylsilyl)oxy)phenethyl)-1-(2-(3-methylisoxazol-5-yl)acetyl)pyrrolidine-2-carboxamide



To a solution of (2S,4R)-4-(tert-butoxy)-N-(4-((tert-butyldimethylsilyl)oxy)phenethyl)pyrrolidine-2-carboxamide (50 mg, 0.120 mmol) in DMF (3 mL) at room temperature were added 3-methyl-5-isoxazole acetic acid (Sigma-Aldrich Co., 19 mg, 0.132 mmol), DIPEA (0.070 mL, 0.396 mmol), and HOBt (19 mg, 0.144 mmol). The mixture was cooled to 0 °C and EDC (28 mg, 0.144 mmol) was added to the mixture. The reaction mixture was allowed to warm to rt, further stirred at rt for 15 h, and cooled to 0 °C. The resulting mixture was quenched with H_2O (10 mL) and extracted three times with ethyl acetate. The combined extracts were washed with brine, dried over anhydrous Na_2SO_4, filtered, and evaporated. The concentrate

was purified by column chromatography (eluting with 100% DCM initially, grading to 5% CH_3OH in DCM) on silica gel to afford the coupled product (54 mg, 83%). **^{1}H NMR** (400 MHz, $CDCl_3$) d 7.01 (d, *J* = 8.4 Hz, 2H), 6.80 (t, *J* = 5.4 Hz, 1H), 6.73 (d, *J* = 8.4 Hz, 2H), 4.54 (dd, *J* = 8.4, 2.4 Hz, 1H), 4.49-4.42 (m, 1H), 3.71 (d, *J* = 3.7 Hz, 2H), 3.65 (dd, *J* = 9.9, 7.0 Hz, 1H), 3.41 (dt, *J* = 6.2, 6.2 Hz, 2H), 3.26 (dd, *J* = 9.9, 7.4 Hz, 1H), 2.76-2.63 (m, 2H), 2.44 (ddd, *J* = 12.5, 6.3, 2.4 Hz, 1H), 2.26 (s, 3H), 1.81 (dt, *J* = 12.5, 8.3 Hz, 1H), 1.18 (s, 9H), 0.95 (s, 9H), 0.16 (s, 6H). **^{13}C NMR** (100 MHz, $CDCl_3$) d 170.4, 166.8, 165.2, 162.4, 160.0, 154.1, 131.4, 129.6, 119.9, 104.0, 74.2, 69.8, 58.8, 53.8, 40.8, 36.4, 35.4, 34.7, 33.2, 31.3, 28.2, 25.6, 18.1, 11.4, -4.5. **MS** (ESI) [M+H] 544.5.

(2S,4R)-4-hydroxy-N-(4-hydroxyphenethyl)-1-(2-(3-methylisoxazol-5-yl)acetyl)pyrrolidine-2-carboxamide (2)



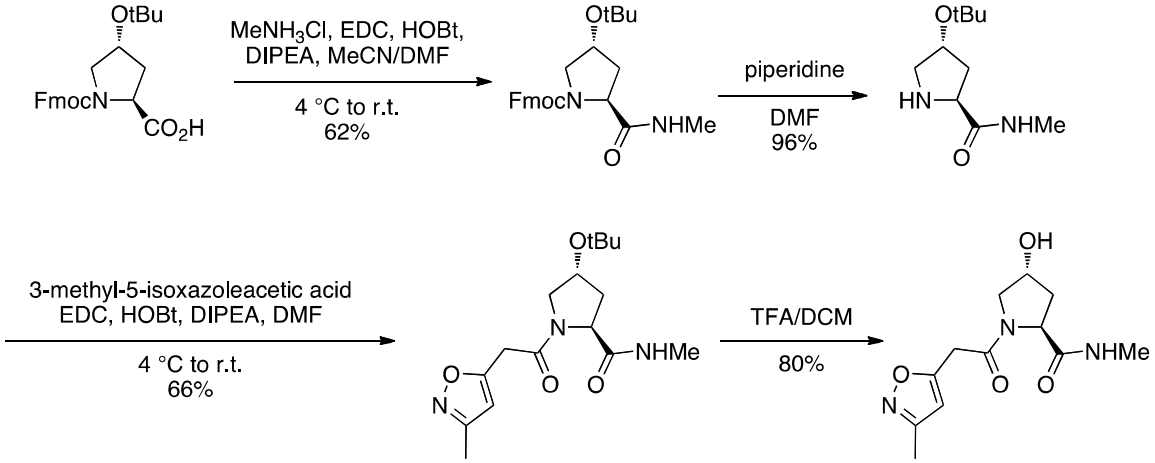
To a stirred solution of coupled product (20 mg, 0.0368 mmol) in THF (1.5 mL) at 0 °C was added TBAF (1.0 M in THF, 92 µL, 0.092 mmol). The reaction mixture was stirred at rt for 15 h and quenched at 0 °C with saturated NH_4Cl aqueous solution. The mixture was extracted twice with ethyl acetate and the combined extracts were washed with brine, dried over Na_2SO_4, filtered, and concentrated.

The crude precursor of **2** was used without further purification for the next step, but a small amount of crude sample was chromatographed on silica gel to perform ^{1}H NMR experiment.

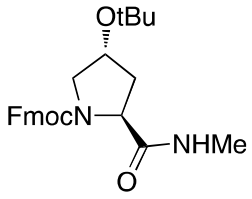
^{1}H NMR (500 MHz, $CDCl_3$) d 6.97 (d, *J* = 7.6 Hz, 2H), 6.80 (t, *J* = 8.4 Hz, 1H), 6.71 (d, *J* = 7.6 Hz, 2H), 6.07 (s, 1H), 4.53 (dd, *J* = 7.3, 3.6 Hz, 1H), 4.49-4.43 (m, 1H), 3.72 & 3.71 (due to the rotamers, both s, 2H), 3.71-3.68 (m, 1H), 3.43-3.37 (m, 2H), 3.27 (dd, *J* = 13.1, 8.3 Hz, 1H), 2.74-2.61 (m, 2H), 2.38 (ddd, *J* = 12.5, 6.1, 2.3 Hz, 1H), 2.25 (s, 3H), 1.87 (dt, *J* = 12.9, 8.2 Hz, 1H), 1.17 (s, 9H).

To a solution of the crude precursor of **2** in DCM (1.5 mL) at 0 °C was added TFA (0.3 mL). The bath was removed and the reaction mixture was stirred at rt for 14 h and concentrated. The residue was chromatographed (eluting with 2% CH_3OH in DCM initially, grading to 8% CH_3OH in

DCM) on silica gel to give **2** (12.5 mg, 92%, 2 steps yield). **^{1}H NMR** (500 MHz, MeOD) d 8.33 & 8.13 (due to the rotamers, both s, 1H), 7.02 (d, *J* = 8.3 Hz, 2H), 6.69 & 6.65 (due to the rotamers, both d, *J* = 8.3 Hz, 2H), 6.22 & 6.10 (due to the rotamers, both s, 1H), 4.43 (d, *J* = 7.7 Hz, 2H), 3.89 (d, *J* = 4.7 Hz, 2H), 3.74 (dd, *J* = 11.0, 4.3 Hz, 1H), 3.57 (d, *J* = 11.0 Hz, 1H), 3.42-3.36 (m, 2H), 2.73-2.63 (m, 2H), 2.25 (s, 3H), 2.16-2.12 (m, 1H), 1.96-1.91 (m, 1H). **^{13}C NMR** (asterisk denotes the signals of the minor rotamer, 125 MHz, MeOD) d 174.2, 174.1, *173.9, *173.8, *169.0, 168.6, 167.6, *167.4, 161.6, *161.5, *157.0, 156.9, *131.2, 130.8, *116.2, 116.1, *105.6, 105.4, 70.7, *69.2, *61.0, *60.9, 60.7, 60.6, 56.9, *56.2, 42.4, 42.3, *42.0, *41.9, 41.5, 39.3, 35.5, *35.3, 33.9, *32.9, 11.2. **MS** (ESI) [M+H] 374.1, [2M+Na] 769.6.



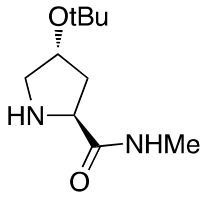
(2*S*,4*R*)-(9*H*-fluoren-9-yl)methyl 4-(*tert*-butoxy)-2-(methylcarbamoyl)pyrrolidine-1-carboxylate (Fmoc-Hyp(OtBu)-NHMe)



Fmoc-Hyp(OtBu)-OH (0.30 g, 0.73 mmol, 1 eq) was dissolved in MeCN (5 mL) and DMF (1.5 mL) and cooled to 4 °C in an ice bath. Methylamine hydrochloride (0.10 g, 1.46 mmol, 2 eq), EDC (0.29 g, 1.53 mmol, 2.1 eq), HOBt (0.22 g, 1.61 mmol, 2.2 eq) and DIPEA (0.52 mL, 2.99 mmol, 4.1 eq) were added. The N_2 line was removed and the solution was allowed to warm to room temperature slowly under a septum for 12 hours after which most of the MeCN was removed under reduced pressure. The mixture was then diluted with EtOAc and washed with 1 M HCl (aq), saturated sodium bicarbonate, water and brine. The organic layer was dried over sodium sulfate, filtered and condensed. Purification by column chromatography (50 to 100% EtOAc/hexanes) gave Fmoc-Hyp(OtBu)-NHMe as a white foamy solid (0.19 g, 0.45 mmol, 62%). **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.58 (t, *J* = 7.7 Hz, 2H), 7.40 (t, *J* = 7.2 Hz,

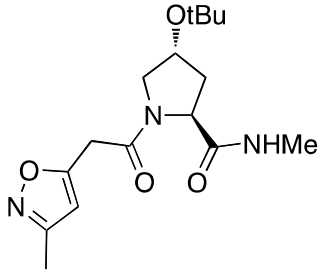
2H), 7.31 (t, *J* = 7.0 Hz, 2H), 6.70 (s, 1H), 4.44 – 4.24 (m, 5H), 3.65 – 3.53 (m, 1H), 3.27 (dd, *J* = 10.0, 6.4 Hz, 1H), 2.77 (d, *J* = 4.0 Hz, 3H), 2.59 (s, 1H), 2.46 (s, 1H), 1.21 (s, 9H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 172.00, 156.25, 143.80, 141.42, 127.87, 127.17, 125.13, 120.12, 74.17, 69.72, 67.85, 59.10, 53.16, 47.28, 36.10, 28.38, 26.37. **MS** (ESI) 423.2 (M+H).

(2S,4R)-4-(tert-butoxy)-N-methylpyrrolidine-2-carboxamide (HN-Hyp(OtBu)-NHMe)



Fmoc-Hyp(OtBu)-NHMe (89 mg, 0.21 mmol, 1 eq) was dissolved in DMF (2.1 mL) in a capped vial. Piperidine (0.21 mL, 2.1 mmol, 10 eq) was added. The solution was stirred for 18 hours and condensed. Purification by column chromatography (0 to 10 % MeOH/DCM) gave HN-Hyp(OtBu)-NHMe as a colorless oil (40.2 mg, 0.201 mmol, 96%). **^{1}H NMR** (500 MHz, MeOD) δ 4.25 (dt, *J* = 8.9, 3.1 Hz, 1H), 3.76 (t, *J* = 8.1 Hz, 1H), 3.31 (dt, *J* = 3.2, 1.6 Hz, 1H), 3.08 (dd, *J* = 11.5, 5.1 Hz, 1H), 2.78 – 2.72 (m, 4H), 2.05 – 1.96 (m, 1H), 1.92 – 1.81 (m, 1H), 1.19 (d, *J* = 5.0 Hz, 9H). **^{13}C NMR** (126 MHz, MeOD) δ 177.44, 74.87, 73.59, 60.86, 55.71, 40.79, 28.67, 26.11. **MS** (ESI) 201.2 (M+H).

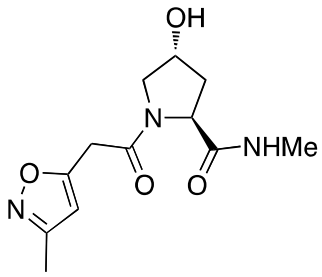
(2S,4R)-4-(tert-butoxy)-N-methyl-1-(2-(3-methylisoxazol-5-yl)acetyl)pyrrolidine-2-carboxamide



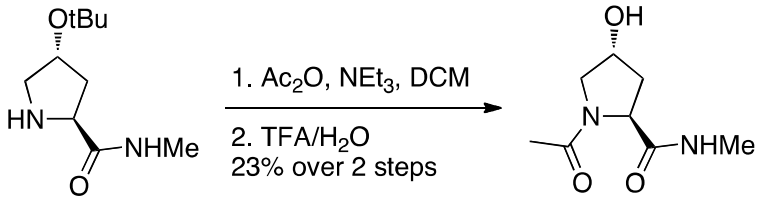
HN-Hyp(OtBu)-NHMe (40 mg, 0.20 mmol, 1 eq) was dissolved in DMF (2 mL) and cooled to 4 °C in an ice bath. 3-Methyl-5-isoxazoleacetic acid (37 mg, 0.26 mmol, 1.3 eq), HOBt (41 mg, 0.30 mmol, 1.5 eq), EDC (54 mg, 0.28 mmol, 1.4 eq) and DIPEA (0.14 mL, 0.8 mmol, 4 eq) were added and solution was allowed to warm slowly to room temperature. After 15 hours, the solution was diluted with water and extracted thrice with EtOAc. The combined organic layers were washed once with brine, and the combined aqueous layers were back extracted once with EtOAc. The combined organic layer was then dried with sodium sulfate, filtered and condensed. Purification by column chromatography (0 to 6% MeOH/DCM) gave a colorless oil (42.4 mg, 0.131 mmol, 66%). **^{1}H NMR** (500 MHz, $CDCl_3$) δ 6.79 (s, 1H), 6.10 (s, 1H), 4.61 (dd, *J* = 8.3, 2.3 Hz, 1H), 4.58 – 4.48 (m, 1H), 3.77 (s, 2H), 3.73 (dd, *J* = 9.9, 7.0 Hz, 1H), 3.30 (dd, *J* = 9.9, 6.5 Hz, 1H), 2.76 (t, *J* = 4.6 Hz, 3H), 2.53 (ddd, *J* = 12.5, 6.3, 2.4 Hz, 1H), 2.33 – 2.22 (m, 3H), 1.85 (dt, *J* = 12.5, 8.3 Hz, 1H), 1.22 – 1.15 (m, 9H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ

171.18, 167.25, 165.34, 160.29, 104.27, 74.46, 70.06, 58.95, 54.05, 35.45, 33.42, 28.40, 26.43, 11.62. **MS** (ESI) 323.8 (M+H), 346.2 (M+Na).

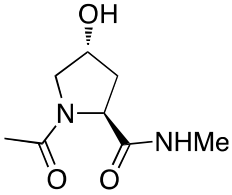
(2S,4R)-4-hydroxy-N-methyl-1-(2-(3-methylisoxazol-5-yl)acetyl)pyrrolidine-2-carboxamide (3)



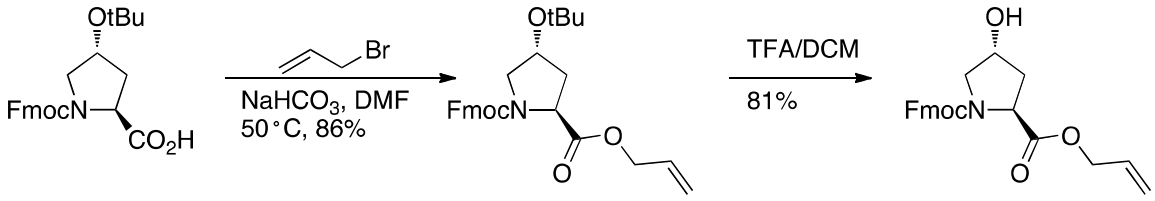
(2S,4R)-4-(tert-butoxy)-N-methyl-1-(2-(3-methylisoxazol-5-yl)acetyl)pyrrolidine-2-carboxamide (42.4 mg, 0.131 mmol, 1 eq) was dissolved in DCM (24 mL) and cooled to 4 °C in an ice bath. TFA (6 mL) was added and the solution was allowed to warm slowly to room temperature. After 12 hours, the solution was condensed and purified by column chromatography (3 to 20% MeOH/DCM) to give **3** as a colorless oil (28 mg, 0.105 mmol, 80%). **^{1}H NMR** (500 MHz, MeOD) δ 6.23 (s, 1H), 4.47 (dt, *J* = 16.3, 5.2 Hz, 2H), 3.91 (d, *J* = 5.7 Hz, 2H), 3.78 (dd, *J* = 10.9, 4.2 Hz, 1H), 3.61 (dd, *J* = 11.0, 1.8 Hz, 1H), 2.73 (s, 3H), 2.26 – 2.18 (m, 4H), 2.05 (dd, *J* = 8.3, 4.7 Hz, 1H). **^{13}C NMR** (126 MHz, MeOD) δ 174.82, 168.66, 167.67, 161.58, 105.40, 70.79, 60.67, 56.91, 39.30, 33.89, 26.35, 11.20. **MS** (ESI) 291.1 (M+Na), 268.7 (M+H).



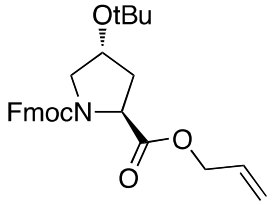
(2S,4R)-1-acetyl-4-hydroxy-N-methylpyrrolidine-2-carboxamide [NAc-Hyp-NMe]



To a solution of HN-Hyp(OtBu)-NHMe (24 mg, 0.12 mmol, 1 eq) and TEA (55 µl, 0.40 mmol, 3.3 eq) in DCM (5 ml) at 0°C was added acetic anhydride (21.5 µl, 0.22 mmol) dropwise. Upon complete addition, the reaction was warmed to RT and stirred for 2 hours. The ^{t}Bu protected product was extracted with DCM under acidic and basic conditions and dried ($MgSO_4$). The solvent was removed *in vacuo* and 95% TFA:water (3 ml) was added. The reaction was allowed to proceed for 3 hours and excess TFA blown off with nitrogen. The deprotected product **3** (5 mg, 27 µmol, 23 %) was purified using RP-HPLC on a Gilson analytical to semi-preparative HPLC system (liquid handler GX271, pump 322 and diode array detector 171) using Agilent columns (preparative: Pursuit XRs C18 (5µm; 250 21.2mm) and analytical: Pursuit XRs C18 (5µm; 250 4.6mm)). ^{1}H and ^{13}C NMR were recorded on a Bruker DPX-400. **^{1}H NMR** (400 MHz, $CDCl_3$): δ 4.61 (1H, quintet, *J*=4.7Hz), 4.56 (1H, dd, *J*=8.1Hz, *J*=5.5Hz), 3.65 (1H, dd, *J*=10.9Hz, *J*=5.3Hz), 3.39 (1H, dd, *J*=10.9Hz, *J*=3.1Hz), 2.72 (3H, d, *J*=4.8Hz), 2.49-2.55 (1H, dt, *J*=13.2Hz, *J*=5.4Hz),2.06 (3H, s), 1.92-1.96 (1H, m). **^{13}C NMR** (125 MHz, 50:50 acetonitrile-d_3:D_2O): δ 176.7, 176.6, 175.9, 175.4, 72.1, 70.8, 62.9, 61.6, 58.7, 57.1, 49.4, 42.2, 40.5, 28.4, 24.4, 23.6.

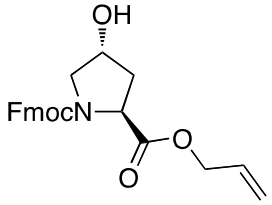


(2S,4R)-1-((9H-fluoren-9-yl)methyl) 2-allyl 4-(tert-butoxy)pyrrolidine-1,2-dicarboxylate (Fmoc-Hyp(OtBu)-OAllyl)

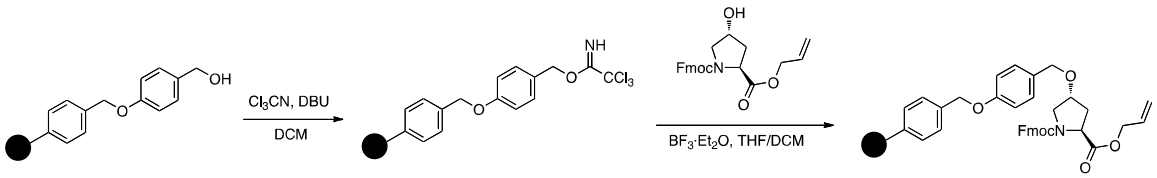


Fmoc-Hyp(OtBu)OH (24.9 g, 60.8 mmol, 1 eq) was dissolved in DMF (300 mL) at room temperature. Sodium bicarbonate (12.8 g, 152 mmol, 2.5 eq) was added, followed by allyl bromide (25.3 mL, 300 mmol, 4.9 eq). The solution was fitted with an air condenser and heated to 50 °C for 20 hours. It was then cooled to room temperature, diluted with EtOAc, washed with aqueous 1 M HCl, saturated sodium bicarbonate, water and brine. The organic layer was dried with sodium sulfate, filtered and condensed.[15] Purification by column chromatography (15 to 33% EtOAc/hexanes) gave Fmoc-Hyp(OtBu)OAllyl (23.42 g, 52.1 mmol, 86%) as a faint yellow oil. **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.76 (t, *J* = 6.3 Hz, 2H), 7.63 – 7.54 (m, 2H), 7.43 – 7.37 (m, 2H), 7.31 (t, *J* = 7.0 Hz, 2H), 5.99 – 5.79 (m, 1H), 5.39 – 5.18 (m, 2H), 4.66 (d, *J* = 5.6 Hz, 1H), 4.63 – 4.13 (m, 6H), 3.81 (ddd, *J* = 16.6, 10.7, 6.2 Hz, 1H), 3.48 – 3.33 (m, 1H), 2.31 – 2.18 (m, 1H), 2.18 – 2.08 (m, 1H), 1.21 (d, *J* = 11.6 Hz, 9H). **^{13}C NMR** (126 MHz, $CDCl_3$) (mixture of rotamers) δ 172.49, 155.01, 154.49, 144.31, 144.18, 144.06, 143.84, 141.44, 141.41, 141.36, 131.91, 131.74, 127.80, 127.76, 127.20, 127.16, 125.31, 125.28, 125.11, 120.08, 120.05, 118.93, 118.61, 74.29, 69.37, 68.48, 67.73, 65.86, 58.09, 57.79, 54.01, 53.52, 47.40, 47.28, 38.90, 37.87, 28.41, 28.37. **MS** (ESI) 450.5 (M+H).

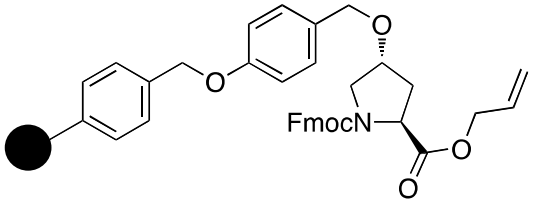
(2S,4R)-1-((9H-fluoren-9-yl)methyl) 2-allyl 4-hydroxypyrrolidine-1,2-dicarboxylate (Fmoc-Hyp(OH)-OAllyl)



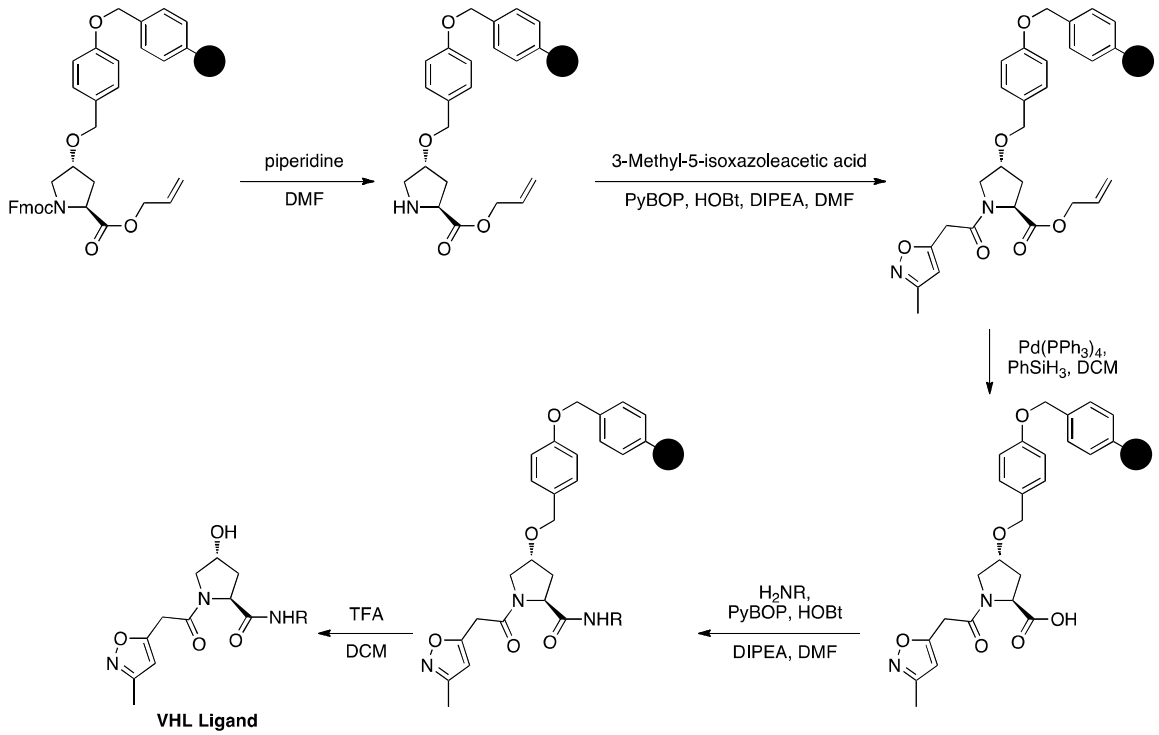
Fmoc-Hyp(OtBu)-OAllyl (23.42 g, 52.1 mmol) was dissolved in DCM (306 mL) at room temperature. TFA (54 mL, 15% vol/vol) was added and the solution was stirred for 13 hours. The solution was poured into water, neutralized by slow addition of saturated aqueous sodium bicarbonate and extracted twice with DCM and once with EtOAc. The combined organic layers were dried with sodium sulfate, filtered and condensed. Purification by column chromatography (30 to 80% EtOAc/hexanes) gave Fmoc-Hyp(OH)-OAllyl as a yellowish oil (16.7 g, 42.4 mmol, 81%). ^{1}H and ^{13}C NMR spectra matched those reported in the literature.[16]



Fmoc-Hyp(OWang)-OAllyl



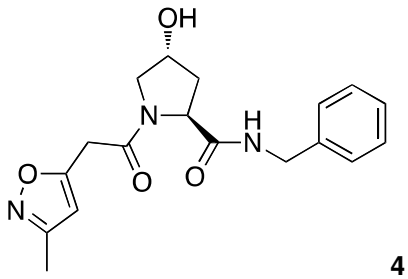
Wang Resin (12.1 g, 1.1 mmol/g loading, 13.3 mmol, 1 eq) was swelled with DCM (90 mL) in a glass reaction vessel and cooled to 4°C. Trichloroacetonitirle (20 mL, 200 mmol, 15 eq) was added, followed by the addition of DBU (3 mL, 20 mmol, 1.5 eq) in 3 portions over 3 minutes, manually shaking the reaction vessel in between additions. The reaction vessel was nutated at 4°C for 1 hour, then washed with DCM, DMSO, THF, then twice with DCM at room temperature.[17] A solution of Fmoc-Hyp(OH)-OAllyl (26.15 g, 66.5 mmol, 5 eq) in DCM (40 mL) and THF (40 mL) was then added, and shaken for 30 minutes and then washed twice with DCM, thrice with DCM and then twice with MeOH followed by DCM. The initial DCM washes were condensed, and purified by column chromatography (33% to 80% EtOAc) to recover the Fmoc-Hyp(OH)-OAllyl starting material (21.51 g, 54.67 mmol, 82%). The resin was dried in air, then dried under vacuum to give 15.5 g of Fmoc-Hyp(OWang)-OAllyl.[18] The loading of the resin was estimated to be 0.53 mmol/g based upon the increase in mass.



General Method for Solid Phase Synthesis of VHL Ligand

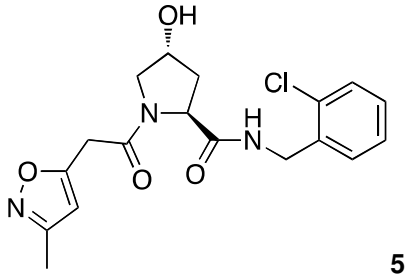
Fmoc-Hyp(OWang)-OAllyl resin (1 eq) was swelled DMF, then reacted with 20% piperidine in DMF for 30 minutes. The resin was then washed once with piperidine, and reacted again with 20% piperidine for 30 minutes to ensure complete deprotection. The resin was then washed twice with DMF and once with MeOH followed by DCM. The resulting free amine was then coupled with 3-methyl-5-isoxazoleacetic acid (4 eq), PyBOP (4 eq) HOBt (4 eq) and DIPEA (7 eq) in DMF for 4 hours. The resin was then washed thrice with DMF and twice with MeOH followed by DCM. The resin was then swelled with freshly distilled DCM, and reacted with $Pd(PPh_3)_4$ (0.1 eq) and $PhSiH_3$ (10 eq) for 30 minutes. The resin was then washed once with DCM, and reacted again with $Pd(PPh_3)_4$ (0.1 eq) and $PhSiH_3$ (10 eq) in distilled DCM for 30 minutes, after which the resin was washed twice with DMF and once with MeOH followed by DCM. The resulting carboxylic acid was then coupled with the appropriate amine (or a salt of the appropriate amine), RNH_2 (4 eq) with PyBOP (4 eq), HOBt (4 eq) and DIPEA (7 eq for free amines, 8 eq for amine salts) in DMF for 4 hours. The resin was then washed 5 times with DMF, thrice with MeOH and 5 times with DCM. The resin was then reacted with 20% TFA in DCM for 2 hours. The reaction mixture was then drained and the resin was washed with DCM. Condensation under reduced pressure, and purification by column chromatography (1% to 10% 0.5M NH_3 in MeOH/DCM or 1% to 10% MeOH in DCM) gave the desired VHL ligand. Yields are based upon the loading of the resin, which was estimated based upon its change in mass.

4



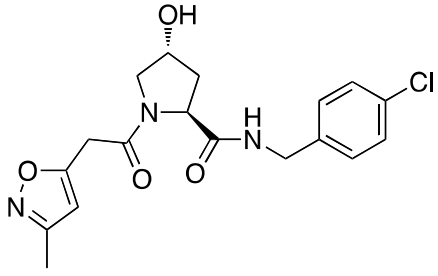
4 was synthesized from Fmoc-Hyp(OWang)-OAllyl (0.3 mmol) according to the General Method. It was isolated as a white solid (14.7 mg, 0.0428mmol, 14%). **^{1}H NMR** (400 MHz, MeOD) δ 7.31 (dd, *J* = 5.9, 5.1 Hz, 4H), 7.27 – 7.17 (m, 1H), 6.23 (s, 1H), 4.55 (t, *J* = 8.0 Hz, 1H), 4.50 (s, 1H), 4.39 (s, 2H), 3.92 (d, *J* = 1.8 Hz, 2H), 3.80 (dd, *J* = 10.9, 4.3 Hz, 1H), 3.61 (dd, *J* = 7.3, 5.5 Hz, 1H), 2.33 – 2.19 (m, 4H), 2.12 – 2.03 (m, 1H). **^{13}C NMR** (101 MHz, MeOD) δ 174.29, 168.68, 167.68, 161.60, 139.73, 129.51, 128.40, 128.14, 105.36, 70.84, 60.73, 56.97, 44.05, 39.36, 33.95, 11.21.MS (ESI) 344.3 (M+H), 366.2 (M+Na).

5



5 was synthesized from Fmoc-Hyp(OWang)-OAllyl (0.3 mmol) according to the General Method. It was isolated as a yellow solid (19.1 mg, 0.0506 mmol, 17%). **^{1}H NMR** (500 MHz, MeOD) δ 8.66 (t, *J* = 5.5 Hz, 1H), 7.48 – 7.34 (m, 2H), 7.31 – 7.21 (m, 2H), 6.23 (s, 1H), 4.58 (t, *J* = 8.0 Hz, 1H), 4.48 (qd, *J* = 15.8, 5.9 Hz, 3H), 3.99 – 3.87 (m, 2H), 3.80 (dd, *J* = 10.9, 4.3 Hz, 1H), 3.66 – 3.60 (m, 1H), 2.31 – 2.22 (m, 4H), 2.09 (ddd, *J* = 13.0, 8.2, 4.7 Hz, 1H). **^{13}C NMR** (126 MHz, MeOD) δ 174.60, 168.72, 167.65, 161.59, 136.79, 134.01, 130.29, 130.08, 129.67, 128.21, 105.37, 70.84, 60.74, 56.96, 42.08, 39.34, 33.95, 11.22. **MS** (ESI) 378.3 (M+H).

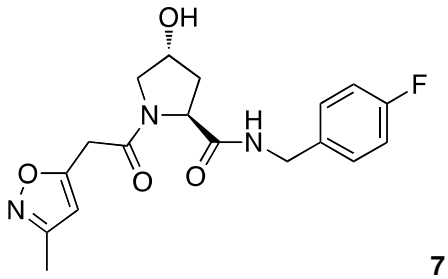
6



6 was synthesized from Fmoc-Hyp(OWang)-OAllyl (0.2 mmol) using the General Method. It was isolated as a white solid (15.9 mg, 0.0421 mmol, 21%). **^{1}H NMR** (500 MHz, MeOD) δ 8.65 (s, 1H), 7.32 – 7.26 (m, 4H), 6.22 (s, 1H), 4.58 – 4.47 (m, 2H),

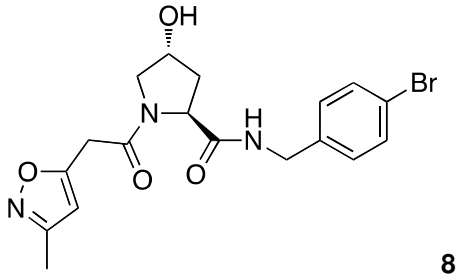
4.43 – 4.32 (m, 2H), 3.92 (d, *J* = 4.2 Hz, 2H), 3.80 (dd, *J* = 10.9, 4.3 Hz, 1H), 3.66 – 3.58 (m, 1H), 2.30 – 2.22 (m, 4H), 2.08 (dd, *J* = 8.3, 4.7 Hz, 1H). **^{13}C NMR** (126 MHz, MeOD) δ 174.48, 168.71, 167.66, 161.60, 138.67, 133.85, 129.99, 129.53, 105.37, 70.85, 60.80, 56.99, 43.45, 39.33, 33.95, 11.20. **MS** (ESI) 378.4 (M+H).

7



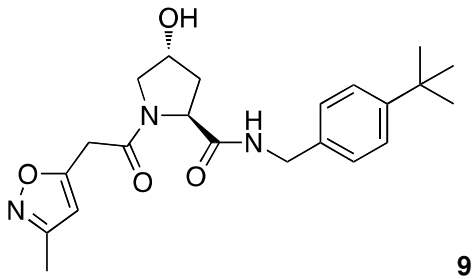
7 was synthesized from Fmoc-Hyp(OWang)-OAllyl (0.15 mmol) using the General Method. It was isolated as a white solid (9.9 mg, 0.0274mmol, 18%). **^{1}H NMR** (400 MHz, MeOD) δ 8.64 (t, *J* = 5.6 Hz, 1H), 7.37 – 7.26 (m, 2H), 7.07 – 6.99 (m, 2H), 6.23 (s, 1H), 4.57 – 4.47 (m, 2H), 4.37 (dd, *J* = 8.4, 5.8 Hz, 2H), 3.93 (d, *J* = 3.0 Hz, 2H), 3.80 (dd, *J* = 11.0, 4.2 Hz, 1H), 3.66 – 3.58 (m, 1H), 2.28 – 2.22 (m, 4H), 2.08 (dd, *J* = 8.3, 4.7 Hz, 1H). **^{13}C NMR** (126 MHz, MeOD) δ 174.31, 168.69, 167.67, 163.44 (d, *J* = 243.5 Hz), 161.60, 135.78, 130.27 (d, *J* = 8.1 Hz), 116.09 (d, *J* = 21.6 Hz), 105.37, 70.85, 60.75, 56.99, 43.32, 39.33, 33.95, 11.20. **MS** (ESI) 362.3 (M+H).

8



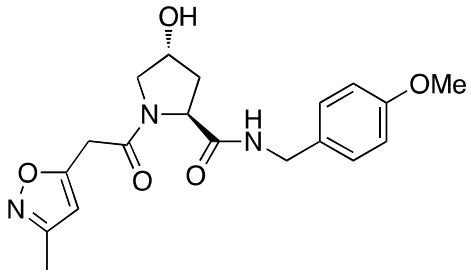
8 was synthesized from Fmoc-Hyp(OWang)-OAllyl (0.3 mmol) according to General Method. It was isolated as a light yellow solid (16.4 mg, 0.0388 mmol, 13%). **^{1}H** NMR (400 MHz, MeOD) δ 7.45 (dq, *J* = 9.0, 2.2 Hz, 2H), 7.23 (d, *J* = 8.5 Hz, 2H), 6.22 (s, 1H), 4.58 – 4.47 (m, 2H), 4.35 (dt, *J* = 18.9, 15.4 Hz, 2H), 3.92 (d, *J* = 2.6 Hz, 2H), 3.80 (dd, *J* = 10.9, 4.2 Hz, 1H), 3.63 (d, *J* = 11.0 Hz, 1H), 2.30 – 2.21 (m, 4H), 2.11 – 2.02 (m, 1H). **^{13}C NMR** (101 MHz, MeOD) δ 174.41, 168.71, 167.66, 161.61, 139.15, 132.55, 130.32, 121.77, 105.37, 70.85, 60.75, 56.99, 43.37, 39.33, 33.94, 11.22. **MS** (ESI) 424.1 (M+H).

9



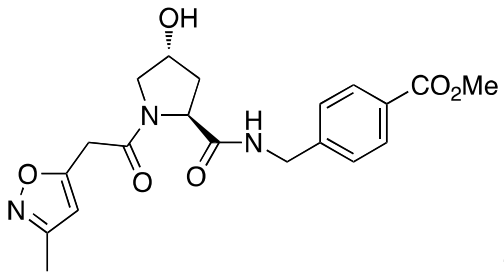
9 was synthesized from Fmoc-Hyp(OWang)-OAllyl (0.3 mmol) according to the General Method. It was isolated as a white solid (19.8 mg, 0.0496 mmol, 17%). **^{1}H NMR** (500 MHz, MeOD) δ 8.56 (s, 1H), 7.35 (d, *J* = 8.2 Hz, 2H), 7.22 (d, *J* = 8.2 Hz, 2H), 6.24 (s, 1H), 4.53 (dd, *J* = 18.3, 10.3 Hz, 2H), 4.36 (d, *J* = 5.7 Hz, 2H), 3.92 (d, *J* = 3.0 Hz, 2H), 3.80 (dd, *J* = 10.9, 4.2 Hz, 1H), 3.62 (d, *J* = 11.1 Hz, 1H), 2.29 – 2.21 (m, 4H), 2.12 – 2.02 (m, 1H), 1.29 (d, *J* = 7.9 Hz, 9H). **^{13}C NMR** (126 MHz, MeOD) δ 174.29, 168.67, 167.68, 161.59, 151.18, 136.67, 128.19, 126.39, 105.38, 70.83, 60.77, 56.97, 43.88, 39.37, 35.28, 33.95, 31.79, 31.74, 11.23. **MS** (ESI) 400.5 (M+H).

10



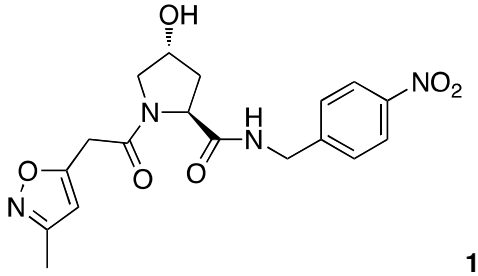
10 was synthesized from Fmoc-Hyp(OWang)-OAllyl (0.156 mmol) according to the General Method. It was isolated as a white solid (9.1 mg, 0.0244 mmol, 16%). **^{1}H NMR** (500 MHz, MeOD) δ 7.22 (dd, *J* = 8.4, 3.9 Hz, 2H), 6.86 (dd, *J* = 8.8, 2.2 Hz, 2H), 6.22 (s, 1H), 4.63 – 4.45 (m, 2H), 4.37 – 4.26 (m, 2H), 3.92 (d, *J* = 2.6 Hz, 2H), 3.83 – 3.70 (m, 4H), 3.61 (d, *J* = 11.2 Hz, 1H), 2.28 – 2.20 (m, 4H), 2.06 (ddd, *J* = 13.0, 8.1, 4.7 Hz, 1H). **^{13}C NMR** (126 MHz, MeOD) δ 174.13, 168.66, 167.68, 161.60, 160.39, 131.67, 129.76, 114.89, 105.37, 70.83, 60.74, 56.97, 55.67, 43.57, 39.34, 33.95, 11.21. **MS** (ESI) 374.5 (M+H).

11



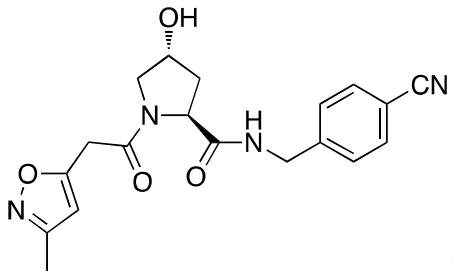
11 was synthesized from Fmoc-Hyp(OWang)-OAllyl (0.156 mmol) according to the General Method. It was isolated as a white solid (14.1 mg, 0.0351 mmol, 23%). **^{1}H NMR** (500 MHz, DMSO) δ 7.90 – 7.85 (m, 2H), 7.39 (d, *J* = 8.4 Hz, 2H), 6.23 (s, 1H), 5.17 (s, 1H), 4.37 (dd, *J* = 17.9, 10.4 Hz, 4H), 3.88 (s, 2H), 3.84 (s, 3H), 3.70 (dd, *J* = 10.5, 4.6 Hz, 1H), 3.47 (dd, *J* = 10.4, 2.5 Hz, 1H), 2.20 (d, *J* = 10.2 Hz, 3H), 2.11 – 2.03 (m, 1H), 1.92 (ddd, *J* = 12.5, 7.2, 4.9 Hz, 1H). **^{13}C NMR** (126 MHz, DMSO) δ 171.66, 166.66, 166.10, 165.66, 159.35, 145.22, 129.08, 127.99, 127.06, 103.94, 68.62, 58.76, 55.20, 52.02, 41.49, 38.17, 32.73, 10.95.**MS** (ESI) 402.6 (M+H).

12



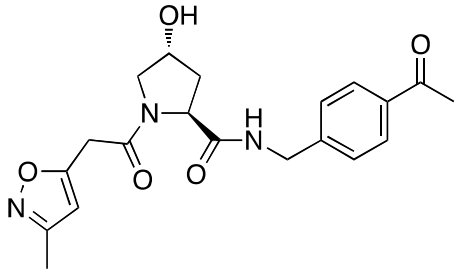
12 was synthesized from Fmoc-Hyp(OWang)-OAllyl (0.156 mmol) according to the General Method. It was isolated as a white solid (11.4 mg, 0.0294 mmol, 19%). **^{1}H NMR** (400 MHz, MeOD) δ 8.28 – 8.05 (m, 2H), 7.55 (d, *J* = 8.8 Hz, 2H), 6.23 (s, 1H), 4.64 – 4.36 (m, 4H), 3.94 (d, *J* = 3.8 Hz, 2H), 3.81 (dd, *J* = 10.9, 4.2 Hz, 1H), 3.65 (dt, *J* = 11.0, 1.7 Hz, 1H), 2.34 – 2.21 (m, 4H), 2.09 (td, *J* = 8.5, 4.2 Hz, 1H). **^{13}C NMR** (101 MHz, MeOD) δ 174.70, 168.79, 167.65, 161.63, 148.48, 147.72, 129.13, 124.56, 105.40, 70.88, 60.79, 57.03, 43.41, 39.32, 33.94, 11.20. **MS** (ESI) 389.3 (M+H), 411.4 (M+Na).

13



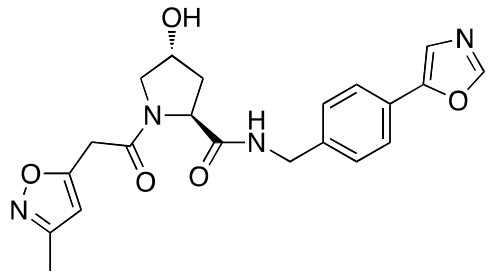
13 was synthesized from Fmoc-Hyp(OWang)-OAllyl (0.156 mmol) according to the General Method. It was isolated as a clear oil (8.0 mg, 0.0217 mmol, 14%). **^{1}H NMR** (500 MHz, MeOD) δ 8.77 (s, 1H), 7.58 (dd, *J* = 88.0, 8.1 Hz, 4H), 6.23 (d, *J* = 4.4 Hz, 1H), 4.61 – 4.33 (m, 4H), 3.93 (d, *J* = 9.7 Hz, 2H), 3.83 – 3.74 (m, 1H), 3.63 (dd, *J* = 10.4, 9.0 Hz, 1H), 2.33 – 2.27 (m, 1H), 2.26 (d, *J* = 3.7 Hz, 3H), 2.15 – 2.03 (m, 1H). **^{13}C NMR** (126 MHz, MeOD) δ 175.33, 168.76, 167.64, 161.61, 145.83, 133.39, 129.12, 119.74, 111.79, 105.28, 70.87, 70.87, 59.32, 57.02, 43.61, 38.79, 33.94, 11.21, 11.19. **MS** (ESI) 391.2 (M+Na).

14



14 was synthesized from Fmoc-Hyp(OWang)-OAllyl (0.156 mmol) according to the General Method. It was isolated as a white solid (23 mg, 0.0597 mmol, 38%). **^{1}H NMR** (400 MHz, MeOD with $CDCl_3$) δ 8.71 (s, 1H), 7.96 – 7.91 (m, 2H), 7.43 (d, *J* = 8.5 Hz, 2H), 6.22 (s, 1H), 4.56 (t, *J* = 8.0 Hz, 1H), 4.49 (ddd, *J* = 18.6, 8.6, 4.1 Hz, 3H), 3.91 (s, 2H), 3.80 (dd, *J* = 10.9, 4.2 Hz, 1H), 3.65 – 3.58 (m, 1H), 2.58 (d, *J* = 1.6 Hz, 3H), 2.28 – 2.22 (m, 4H), 2.10 (dd, *J* = 8.3, 4.7 Hz, 1H). **^{13}C NMR** (101 MHz, MeOD with $CDCl_3$) δ 200.08, 174.34, 168.43, 167.36, 161.39, 145.50, 136.96, 129.62, 128.28, 105.26, 70.65, 60.57, 56.83, 43.72, 39.14, 33.87, 26.73, 11.30. **MS** (ESI) 386.0 (M+H).

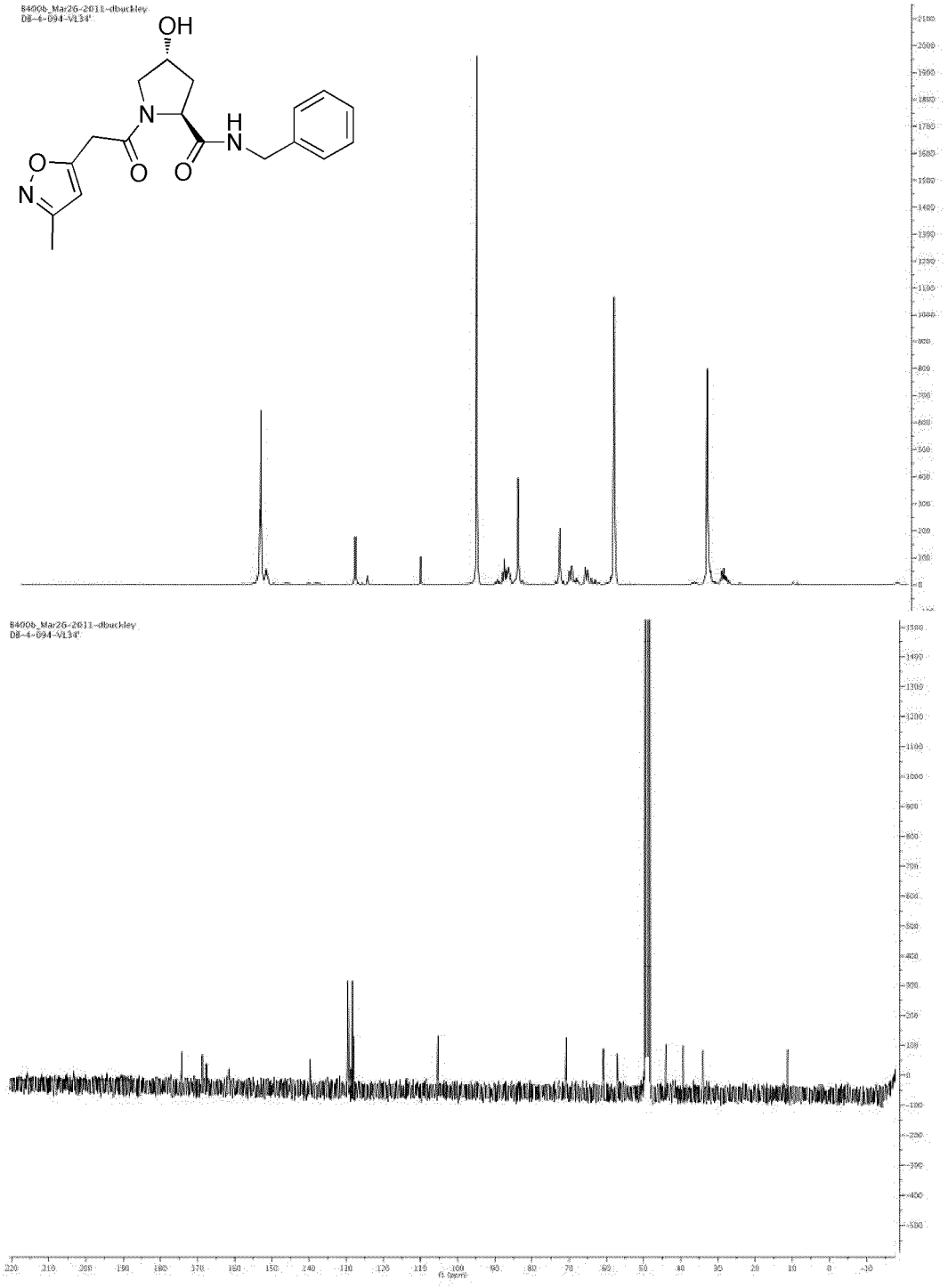
15



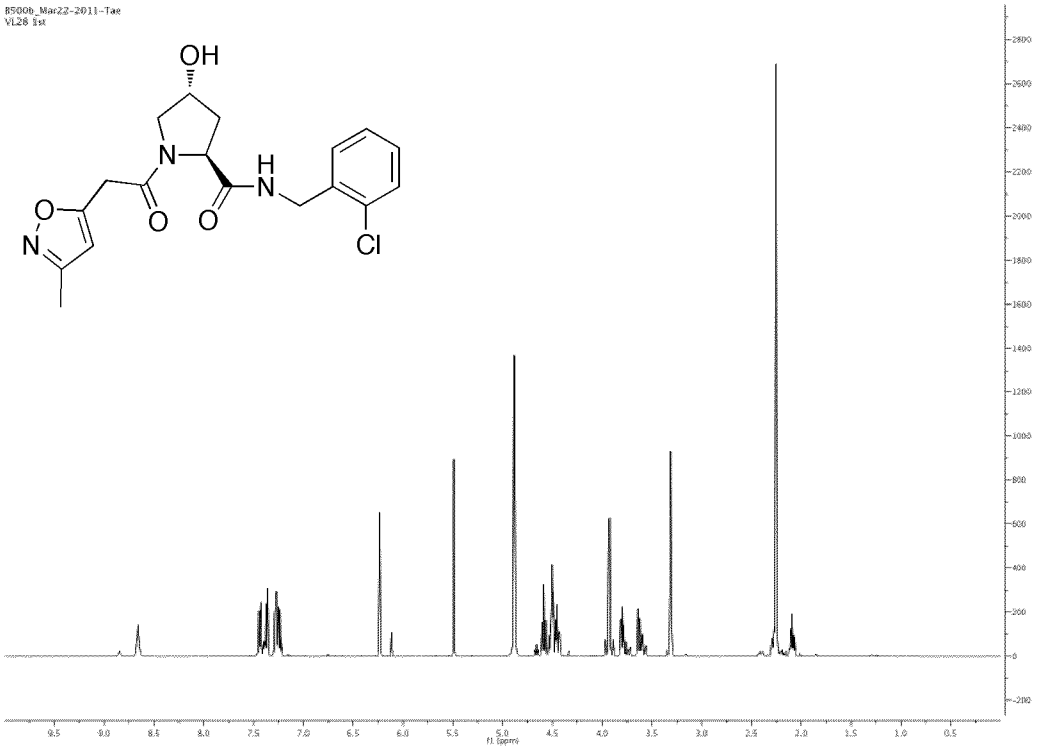
15 was synthesized from Fmoc-Hyp(OWang)-OAllyl (0.2 mmol) according to the General Method. It was isolated as a white solid (18.2 mg, 0.0443 mmol, 22%). **^{1}H NMR** (500 MHz, MeOD) δ 8.23 (s, 1H), 7.68 (d, *J* = 8.1 Hz, 2H), 7.49 (s, 1H), 7.41 (d, *J* = 8.1 Hz, 2H), 6.23 (s, 1H), 4.59 – 4.37 (m, 4H), 3.93 (d, *J* = 3.4 Hz, 2H), 3.81 (dd, *J* = 10.9, 4.1 Hz, 1H), 3.63 (d, *J* = 11.0 Hz, 1H), 2.32 – 2.17 (m, 4H), 2.09 (ddd, *J* = 13.0, 8.0, 4.6 Hz, 1H). **^{13}C NMR** (126 MHz, MeOD) δ 174.43, 168.72, 167.67, 161.60, 153.14, 152.75, 140.78, 129.06, 127.74, 125.61, 121.81, 105.37, 70.86, 60.78, 57.00, 43.72, 39.35, 33.96, 11.20. **MS** (ESI) 411.3 (M+H).

NMR Spectra

4



5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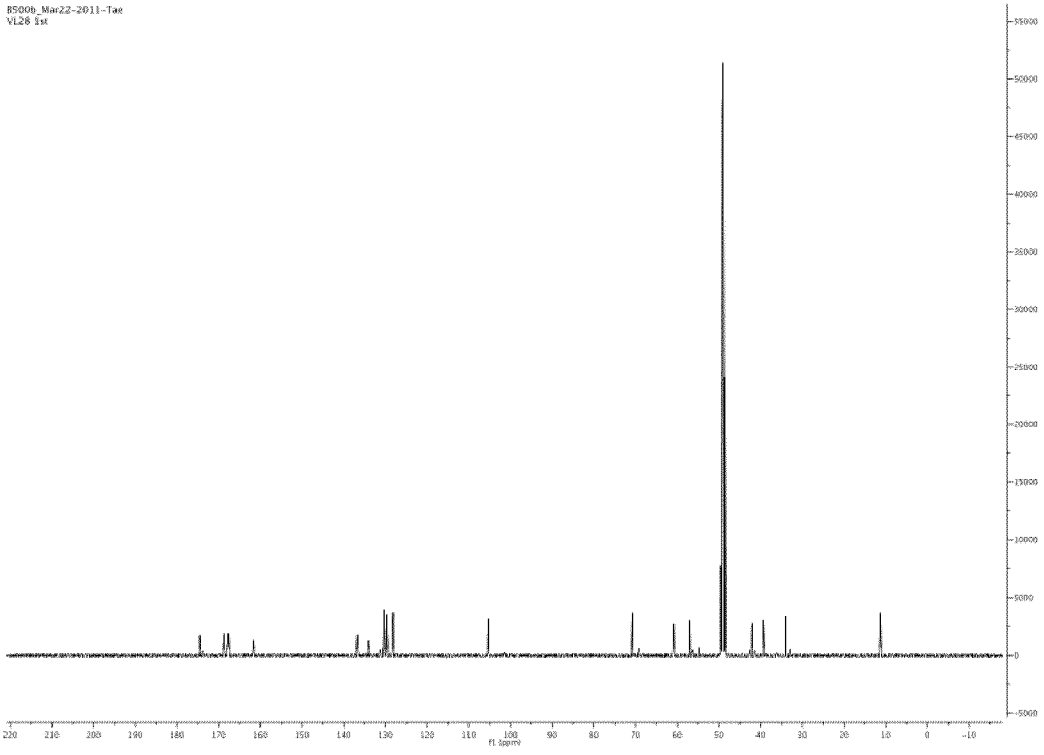
O
O
O
N
Cl
f1 (ppm)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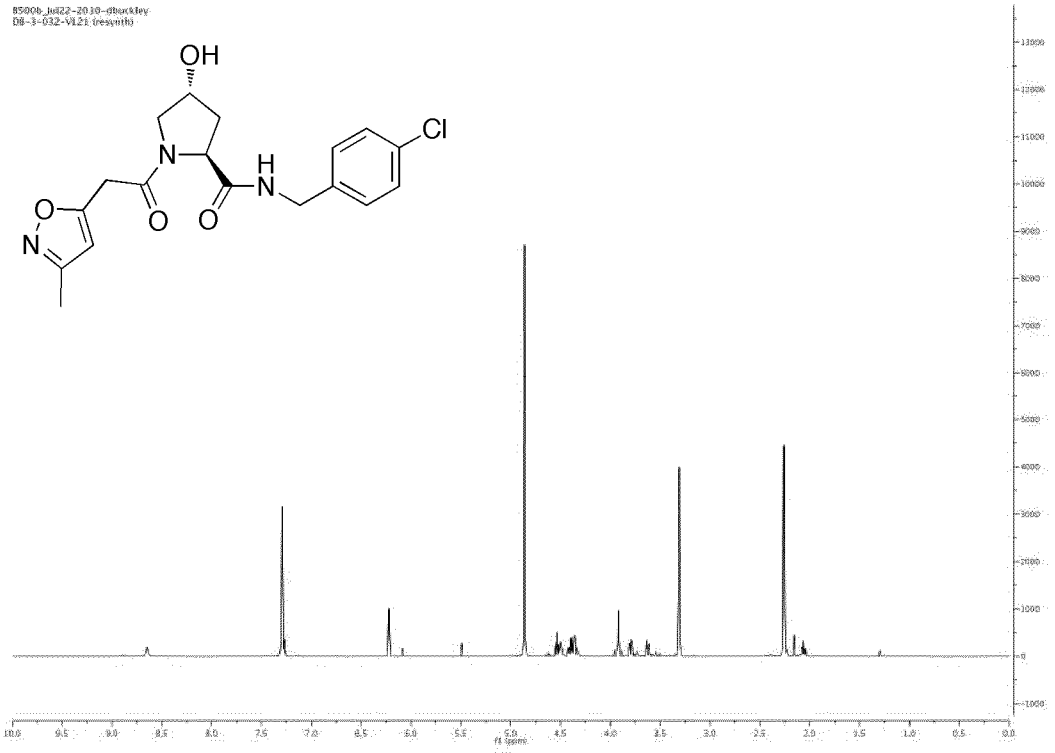
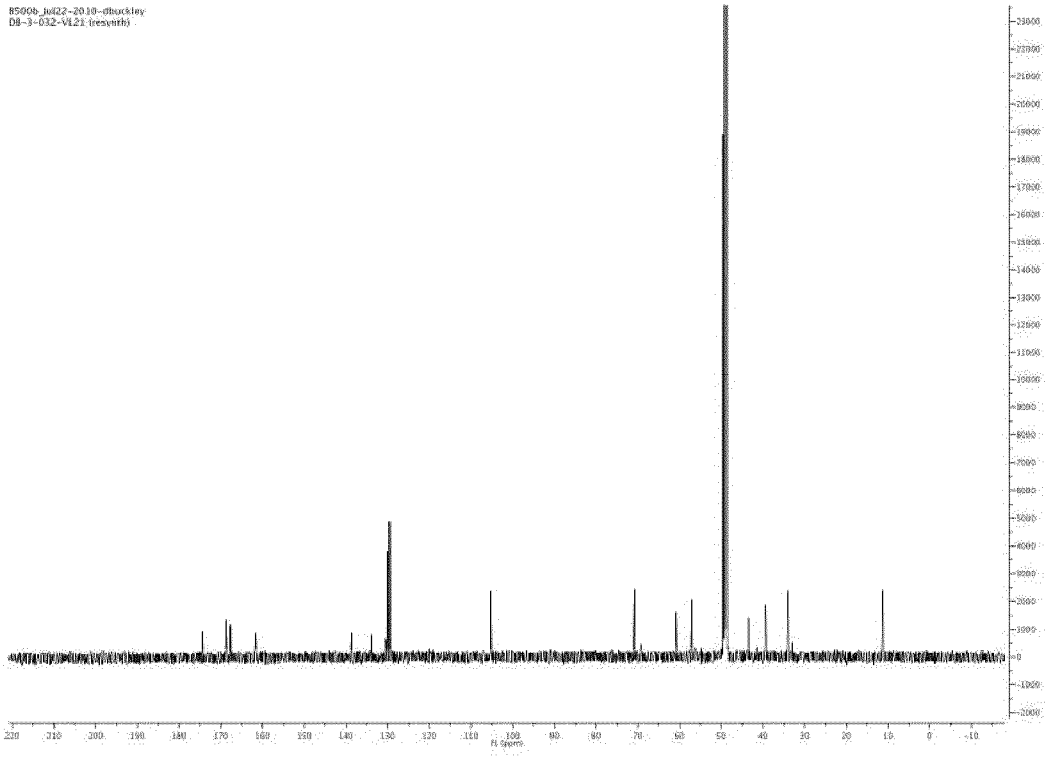
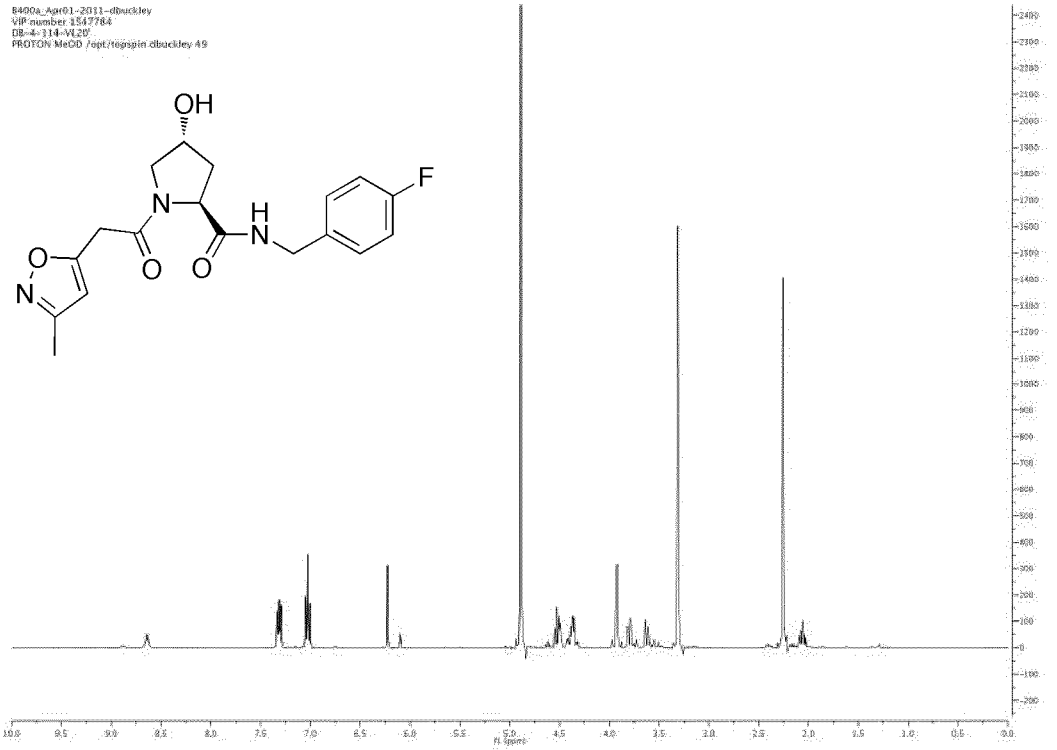
VL28 1st
f1 (ppm)

6





7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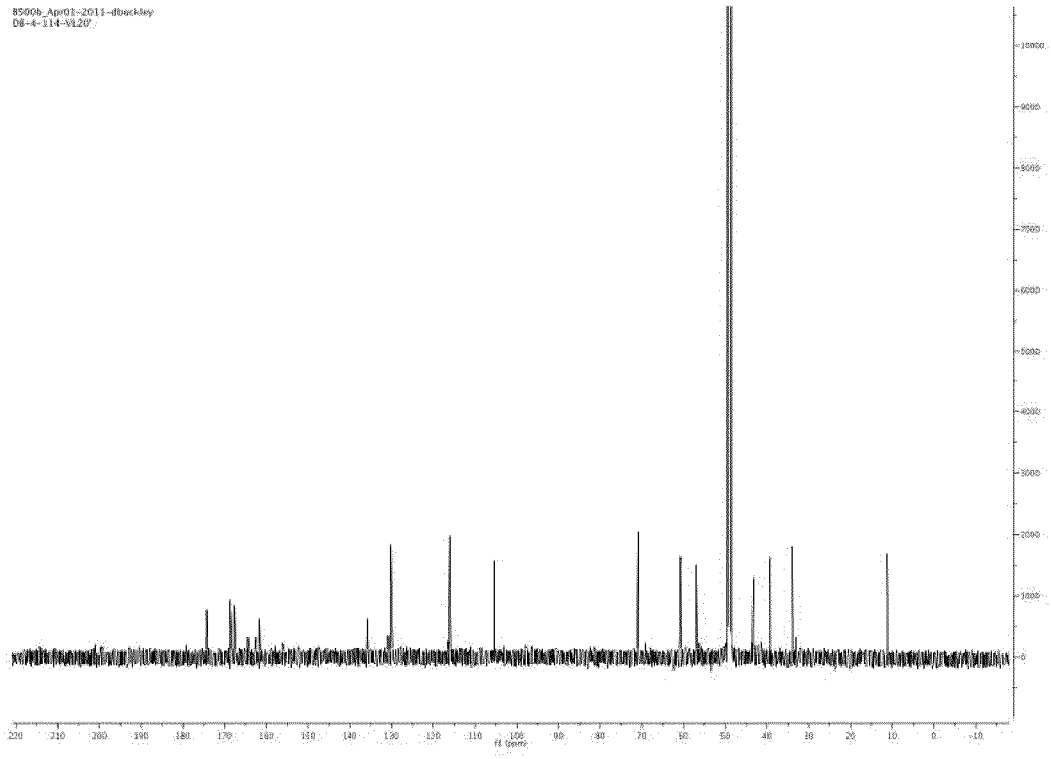
F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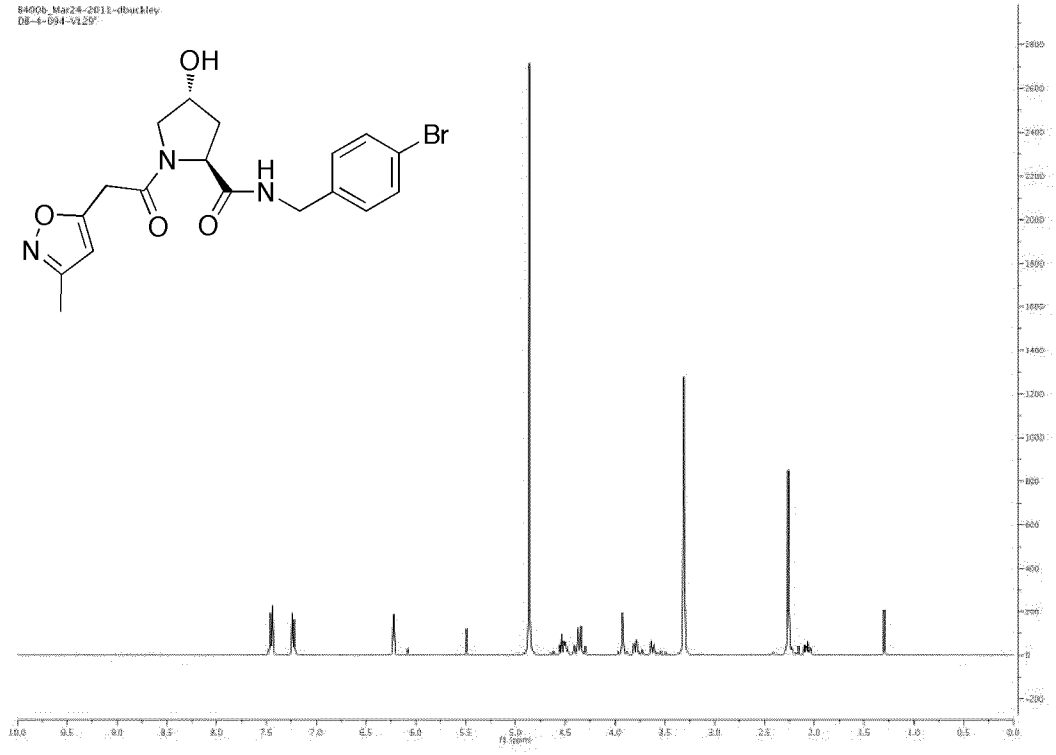
DB-4-114-VL20'

8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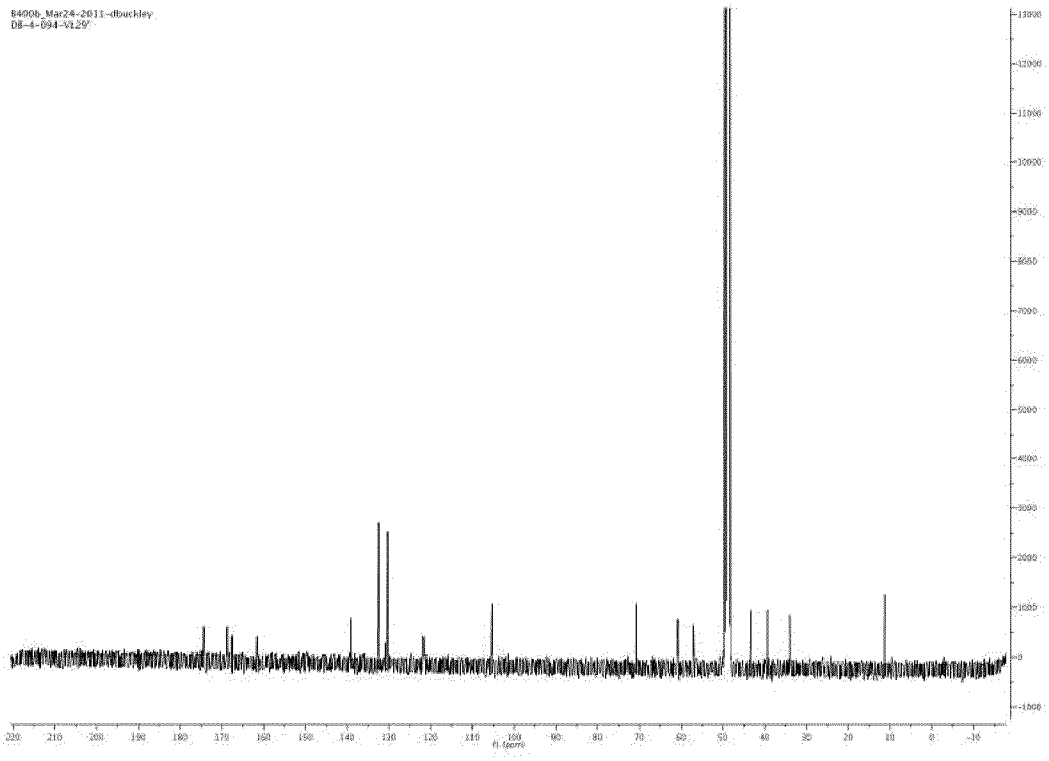
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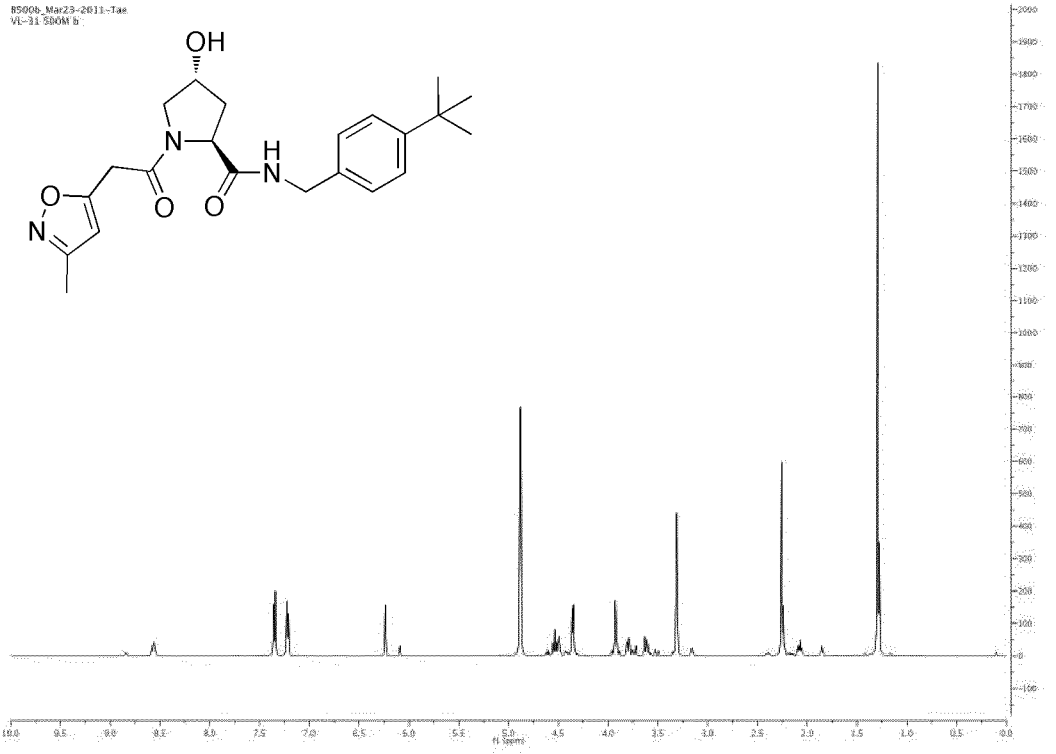
9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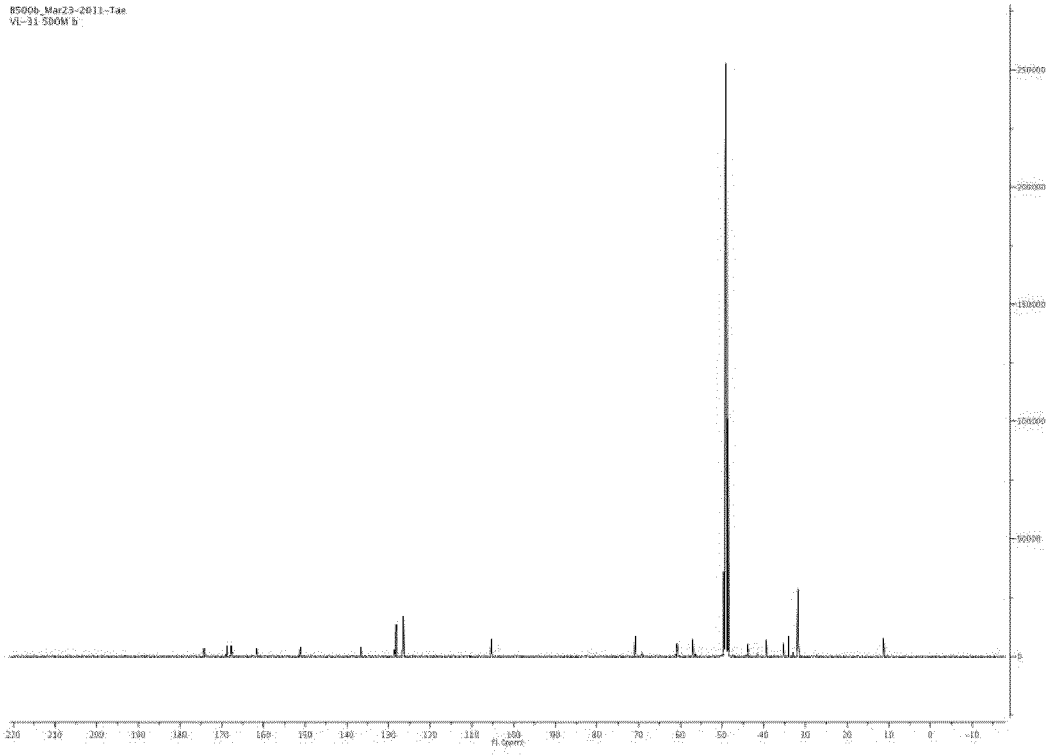
N
O
O
H
N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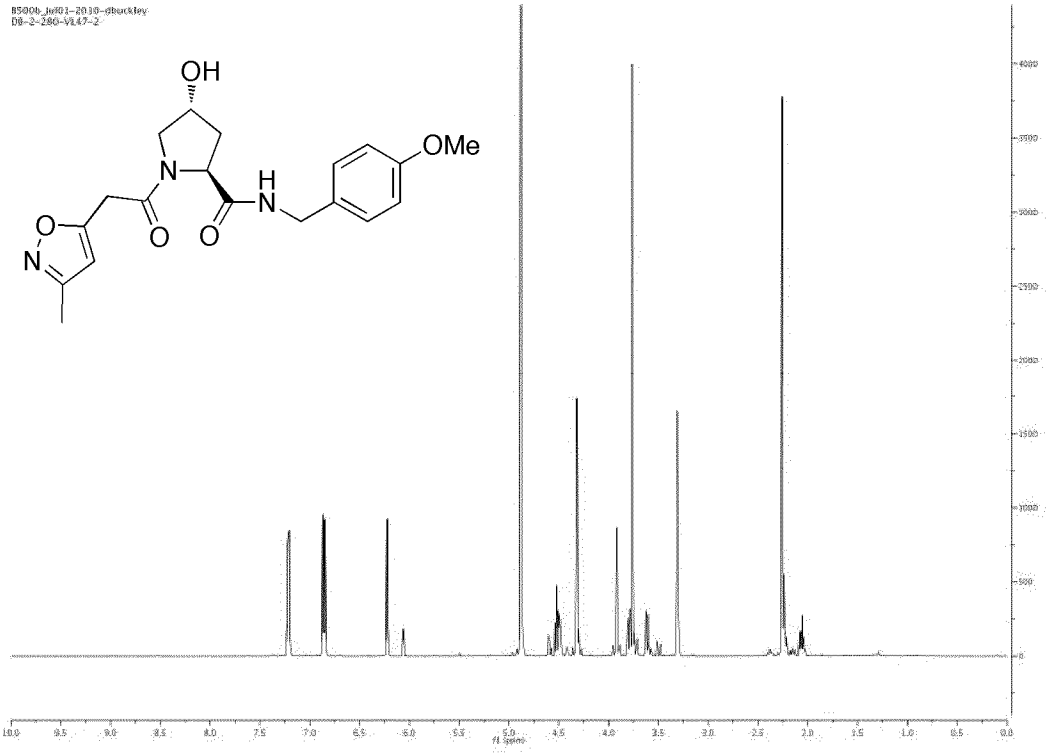
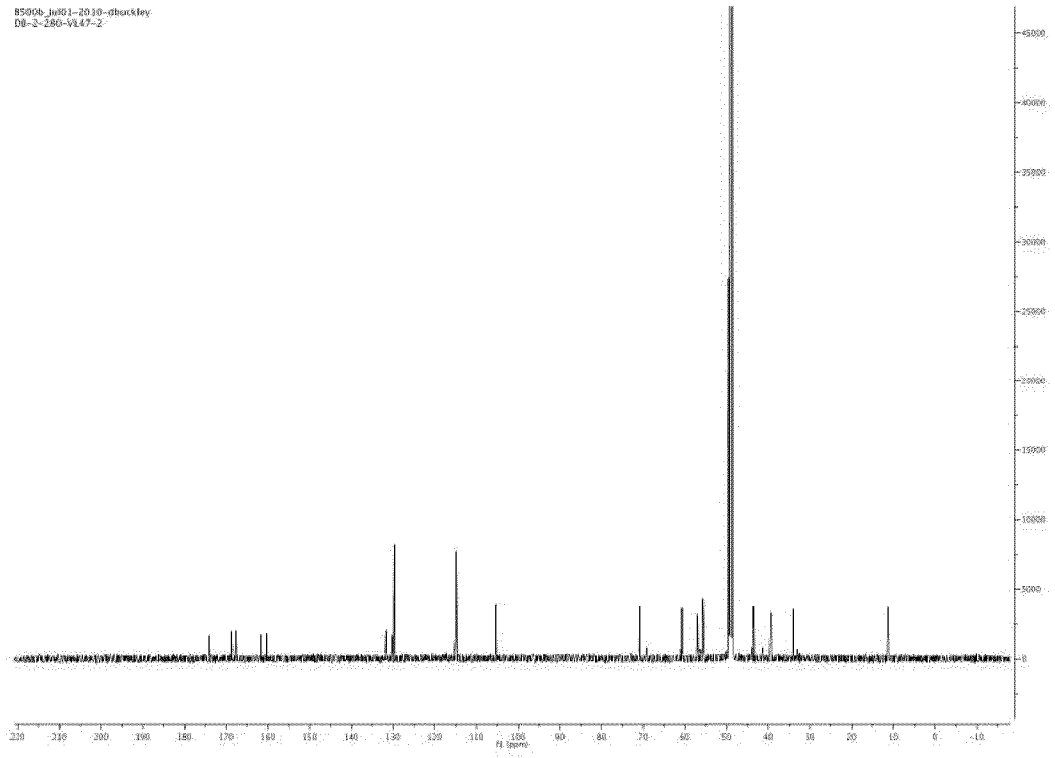
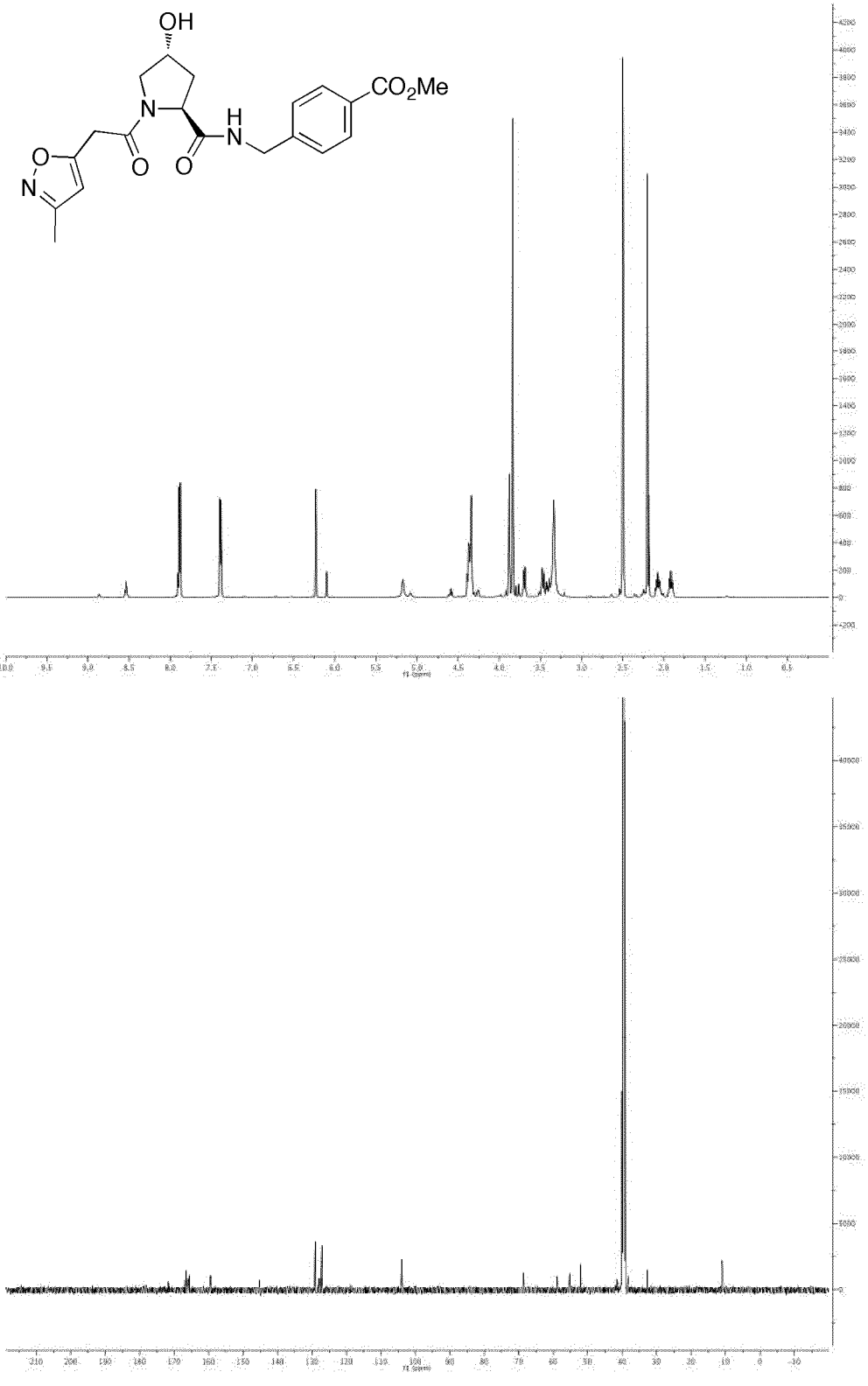
10

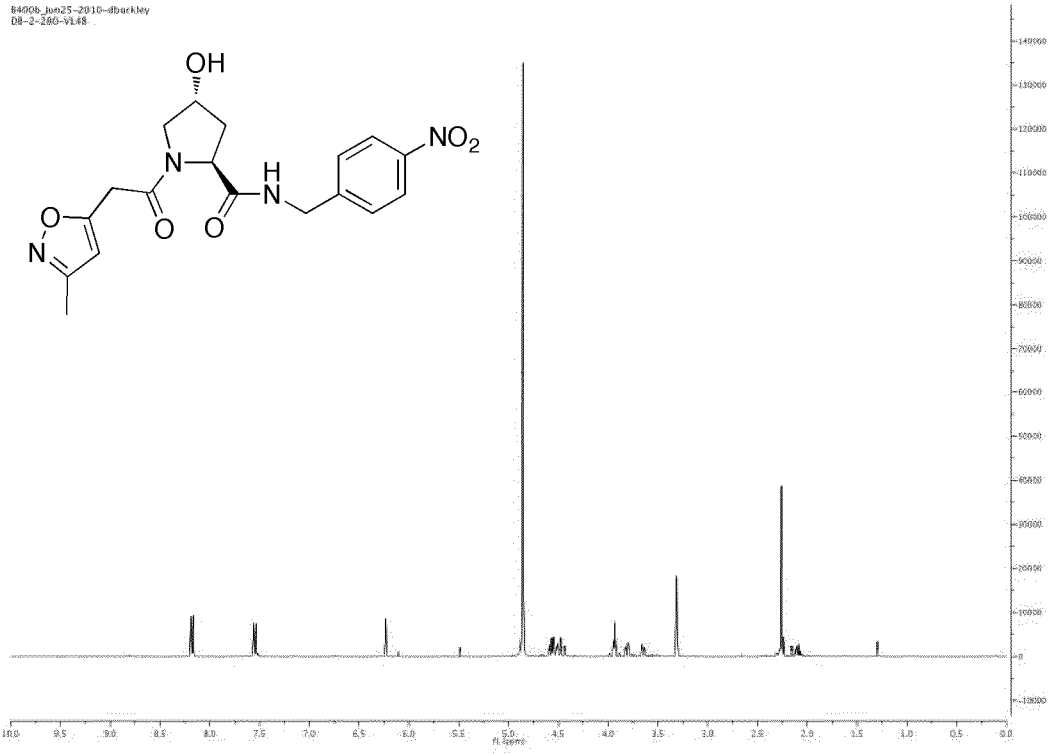




11



12


84008b_jun25-2010-dbuckley
DB-2-280-VL48
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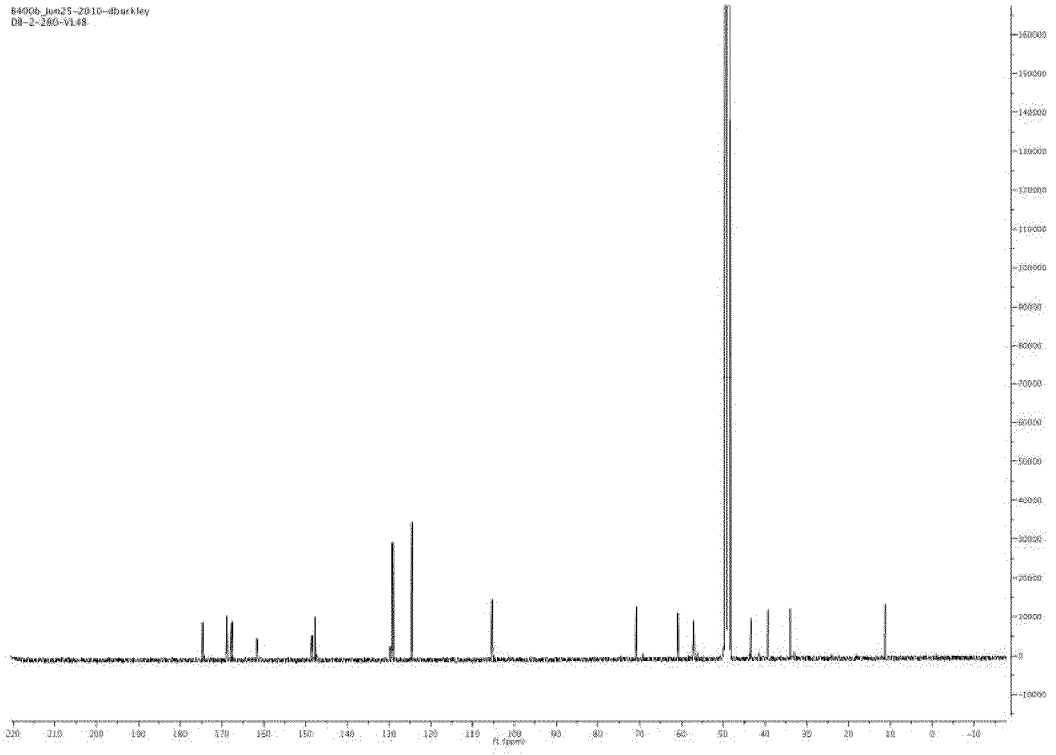
H
N
O
N
NO2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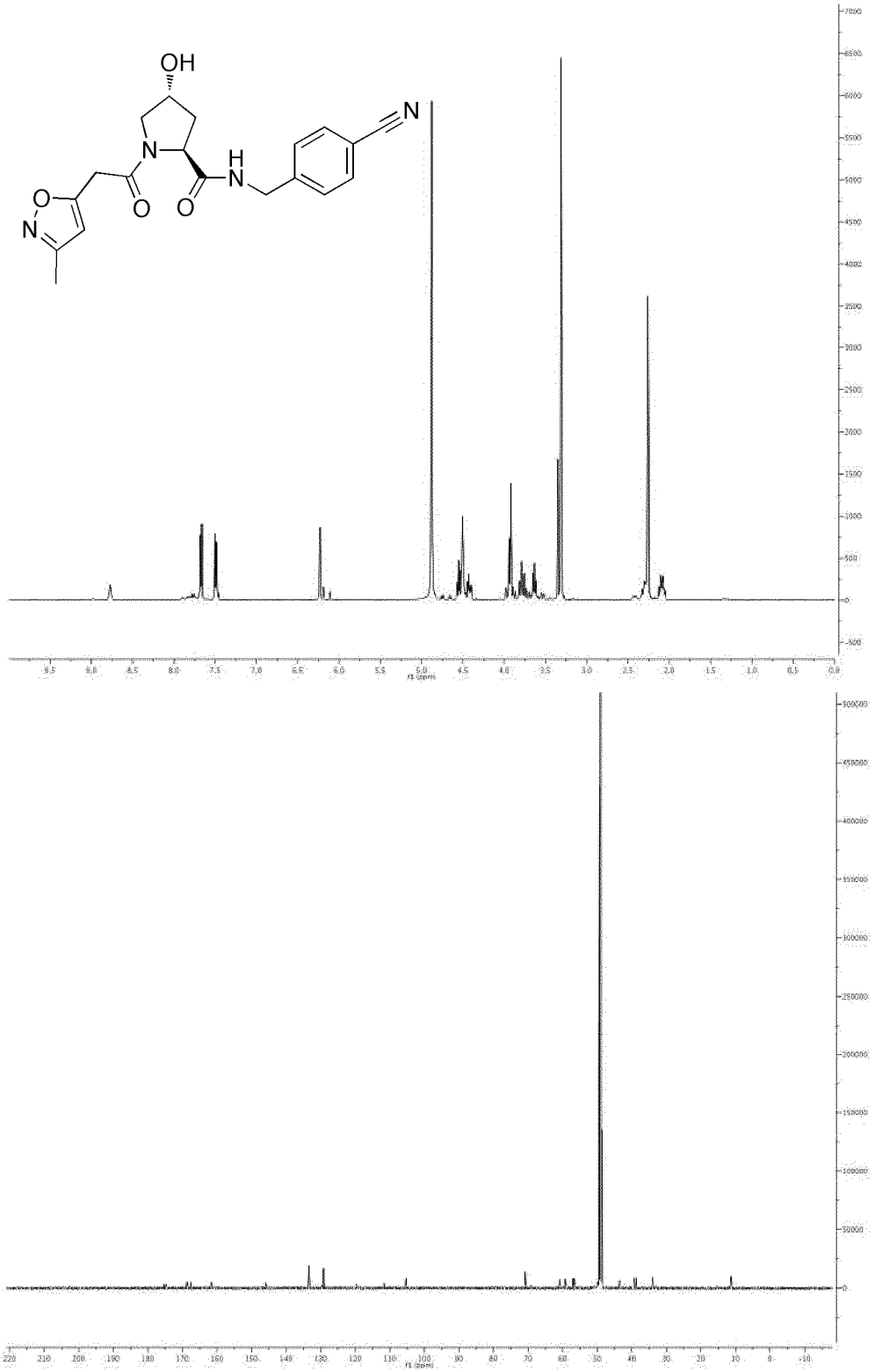
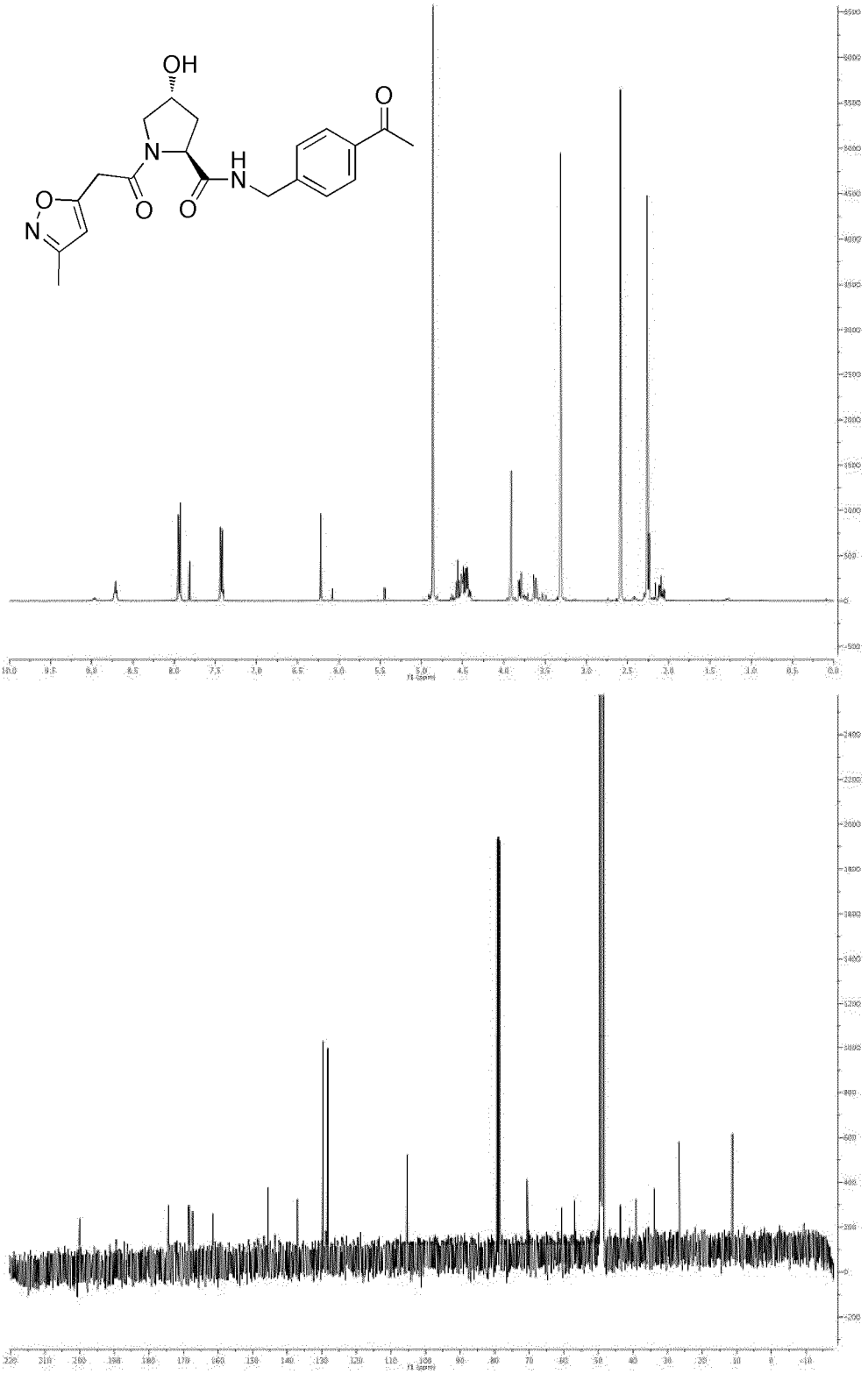
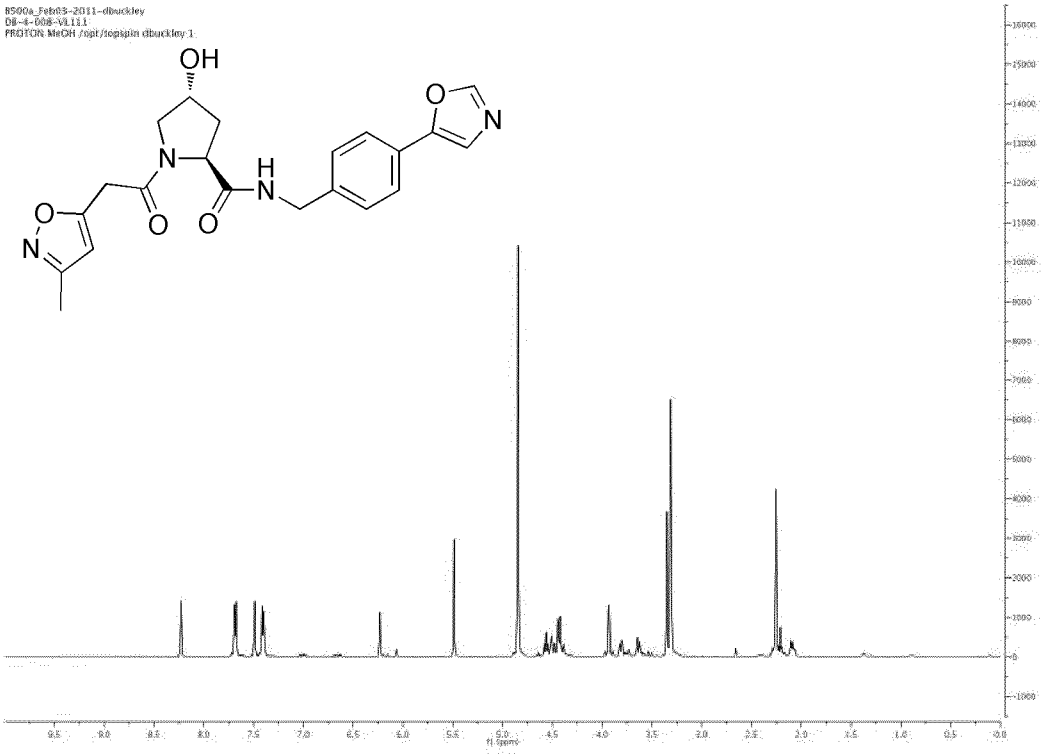


14



15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DB-4-008-VL111
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OH
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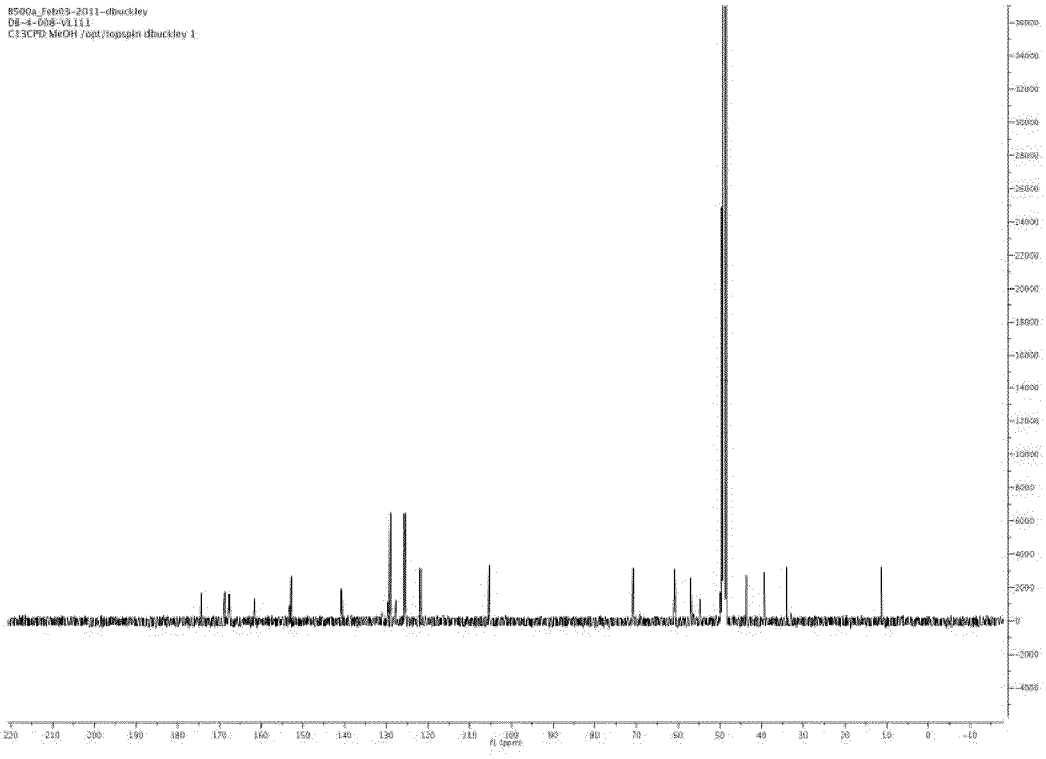
O
O
O
N
H
N
O
N


B500a_Feb03-2011-dbuckley
DB-4-008-VL111
C13CPD MeOH /opt/topspin dbuckley 1

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