Targeting low-druggability bromodomains: Fragment based screening and inhibitor design against the BAZ2B bromodomain

Supporting Information

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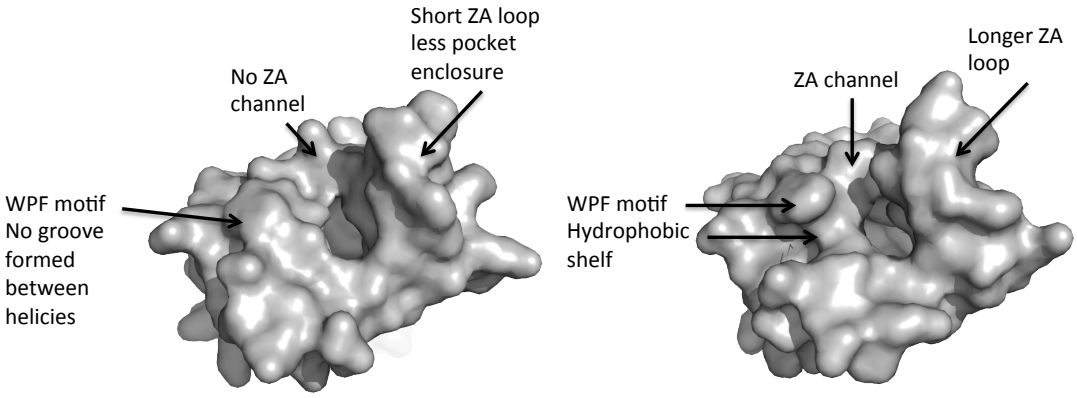
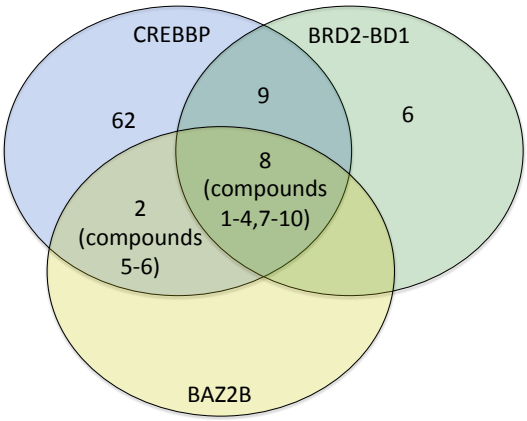
Table of Contents

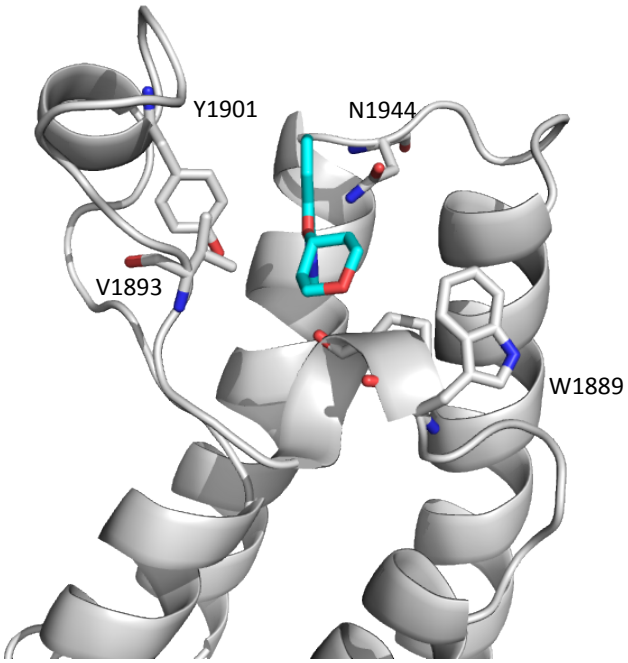
Supplementary Figures



Supplementary Figure 1: Annotated surface view of two bromodomains highlights the differences in the binding sites.
a) BAZ2B PDB 3G0L, b) BRD2-BD1, a representative member of the BET family of bromodomains, PDB 2DVQ



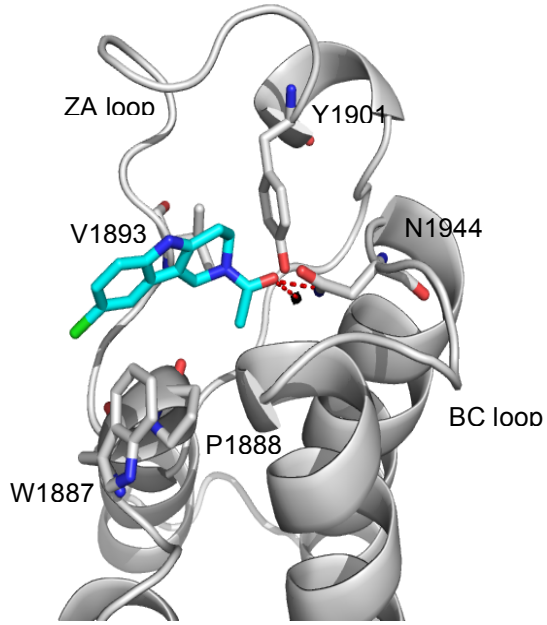
Supplementary Figure 2: Venn diagram displaying the selectivity of the fragments found for BAZ2B against BRD2 (BD1) and CREBBP.



Supplementary Figure 3: Alternative view of the crystal structure of fragment 1 bound to BAZ2B.

This view highlights the vector available for growing fragment 1 towards the left hand side of the pocket.

A



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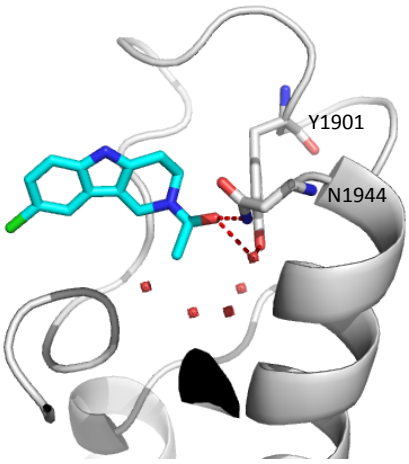
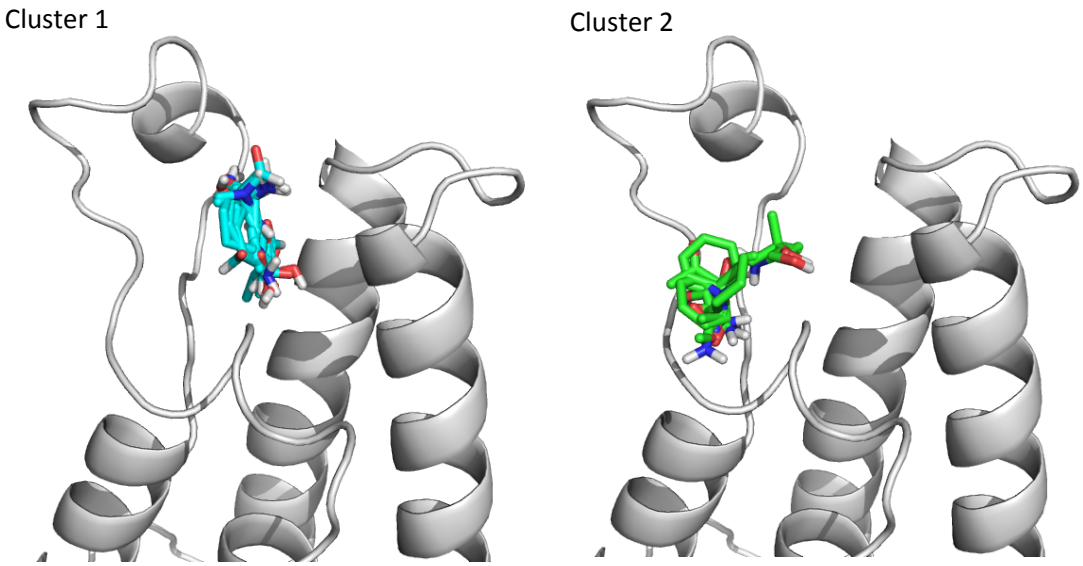


Supplementary Figure 4: Annotated crystal structure of 6 bound to BAZ2B.

(A) Enlarge annotated view
(B) Cut away view of pocket showing 5 conserved waters in the binding site



Supplementary Figure 5: FTMap clusters

Cluster 1 is in the Kac binding site, where all the crystallised fragment hits reside. Cluster 2 occupies the left hand side of the pocket, flanked by Val1893, highlighting this area as a potential hotspot to target by fragment growing.

Molecular Biology Methods

Plasmids

The plasmid used for expression of the BAZ2B bromodomain has been described here.[1]

Transformation

25 µL of chemically competent E.coli cells (DH5-α strain used for obtaining purified plasmids for DNA sequencing, BL21(DE3) strain used for protein expression) were thawed on ice. To these 1 µL of supercoiled plasmid DNA was added and incubated on ice for 20min, heat shocked at 42°C for 45s and immediately placed on ice for 2 min. Cells were diluted 15 x with Lysogeny broth (LB) and incubated at 37°C with 200 rmp shaking for 3h, plated out onto LB/agar plates containing 30 µg/mL kanamycin and incubated at 37°C for 16 h.

Protein expression

Colonies from freshly transformed cells were grown in 100mL LB containing kanamycin (50 µg/mL) at 37°C with 200 rmp shaking for 16 h. Start up cultures were diluted 1:20 with fresh medium and grown at 37°C with 200 rmp shaking to and optical density (OD_{600}) of ~0.6. The temperature was decreased to 18°C, once the system temperature had equilibrated protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 18 h. The cells were harvested by centrifugation (6,000 rpm, 30 min, 4°C) using a Beckman Coulter Avanti J-20 XP centrifuge.

Protein purification

Chromatography was carried using an Äkta FPLC (GE Heathcare). Cell pellets were re-suspended in lysis buffer, ~3mL/g cells (50 mM HEPES, pH 7.5 at 25°C, 500 mM NaCl, 5 mM imidazole, 5 % glycerol and 0.5mM TCEP) in the presence of protease inhibitor cocktail and lysed by passing through a French press (x 3) at 4°C. DNAse (10 µg/mL) and $MgCl_2$ (10mM) were added and the cells incubated on ice for 30 min. The lysate was cleared by centrifugation at (16,000 x g, 1 h, 4°C). Lysate was applied to a HisTrap™FF Column (GE Healthcare), equilibrated with 25 mL lysis buffer. Unbound sample was washed out with 50 mL lysis buffer. Protein was eluted using a gradient elution of 30mM - 250mM imidazole in lysis buffer. Where required the histidine-tag was removed by treatment with His_6-Tobacco Etch Virus (TEV) protease (20:1 ratio) in the presence of 0.5 mM EDTA and 1 mM dithiothreitol (DTT) at 4°C overnight and protease removed by running a second HisTrap column. Protein was concentrated and loaded onto Superdex 75 16/60 HiLoad gel filtration column (GE Healthcare) at 4°C equilibrated with gel filtration buffer (10mM HEPES pH 7.5, 500mM NaCl, 5% glycerol and 0.5mM TCEP). Purified protein was concentrated to ~10 mg/mL using a Vivaspin-20 concentrator with a 10,000Da MW cut-off (Vivascience) and stored at -80°C.

DNA and protein concentration

The absorbance of DNA (A_{260}) and protein (A_{280}) samples were measured on a Picodrop Microliter UV/Vis Spectrophotometer (ThermoScientific). DNA purity was verified by measuring the A_{260}/A_{280} ratio, all DNA used had $A_{260}/A_{280}>1.7$. Protein concentrations were calculated from the A_{280} using molar extinction co-efficient predicted using ProtPARAM.[2]

Protein analysis

Fractions from chromatography were monitored using SDS-PAGE, using gels containing 16% acrylamide gels, which were stained with bromophenol blue.

Nanospray-TOF MS was performed on an ABI Q-STAR mass spectrometer.

DNA purification

DNA was purified using a QIAprep Spin kit following the manufacturers protocols. Plasmids were sequenced at SourceBiosciences, Cambridge.

X-ray crystallography

Apo crystals of BAZ2B grew at 4 ˚C using sitting drop vapour diffusion method at the protein concentration of 10-20 mg/ml. Two types of the reservoir solutions were used, and comprised of i) 19% PEG 1000 and 12-15% glycerol or ii) 28-30% low molecular weight PEG smears, 0.1 M MES pH 6.5-6.7, 15% glycerol. Soaking was performed in the drop overnight using acetylated lysine at 55 mM concentration or compounds at 5 mM. Diffraction data were collected in-house using Rigaku FR-E Superbright, and subsequently processed with XDS[3] or MOSFLM[4] and scaled with Scala from CCP4 suite.[5] Structure solutions were obtained using the program PHASER[6] and the apo BAZ2B model.[7] Iterative cycles of model rebuilding and refinement were performed in COOT[8] and either REFMAC[9] or buster[10] respectively. Data collection and refinement statistics are summarized in Table 1.

Complex	BAZ2BA-Acetylated lysine	BAZ2BA-cpd#1 (N01197)
PDB accession code	4NR9	4NRB
Data Collection		
Beamline	Rigaku FR-E Superbright	Rigaku FR-E Superbright
Wavelength (Å)	1.5418	1.5418
Resolution[a] (Å)	19.67-1.98 (2.09-1.98)	29.93-2.08 (2.19-2.08)
Spacegroup	$C222_1$	$C222_1$
Cell dimensions	*a*=82.4, *b*=96.8, *c*=57.8 Å *α*=*β*=*γ*=90.0˚	*a*=81.4, *b*=96.6, *c*=57.7 Å *α*=*β*=*γ*=90.0˚
No. unique reflections[a]	16,437 (2,359)	14,018 (2,004)
Completeness[a] (%)	99.9 (100.0)	100.0 (100.0)
I/σI[a]	10.9 (2.1)	8.7 (2.0)
R_{merge}[a] (%)	0.084 (0.716)	0.154 (0.787)
Redundancy[a]	5.0 (5.0)	5.0 (4.9)
Refinement		
No. atoms in refinement (P/L/O)[b]	946/13/181	933/17/206
R_{fact} (%)	19.1	17.8
R_{free} (%)	23.3	22.3
B_f (P/His/O)[b] (Å^2)	40/47/51	28/26/44
rms deviation bond[c] (Å)	0.016	0.010
rms deviation angle[c] (°)	1.6	0.9

[a] Values in brackets show the statistics for the highest resolution shells.

[b] P/L/O indicate protein, ligand, and other (water and other molecules), respectively.

[c] rms indicates root-mean-square.

Complex	BAZ2BA-cpd#3 (N01186)	BAZ2BA-cpd#6 (E11322)
PDB accession code	4NRC	4NRA
Data Collection		
Beamline	Rigaku FR-E Superbright	Rigaku FR-E Superbright
Wavelength (Å)	1.5418	1.5418
Resolution[a] (Å)	29.93-1.86 (1.96-1.86)	19.52-1.85 (1.95-1.85)
Spacegroup	$C222_1$	$C222_1$
Cell dimensions	*a*=81.3, *b*=96.6, *c*=57.7 Å *α*=*β*=*γ*=90.0˚	*a*=81.5, *b*=96.4, *c*=57.7 Å *α*=*β*=*γ*=90.0˚
No. unique reflections[a]	19,460 (2,788)	19,728 (2,816)
Completeness[a] (%)	100.0 (100.0)	99.8 (100.0)
I/σI[a]	13.7 (2.0)	11.4 (2.1)
R_{merge}[a] (%)	0.093 (0.802)	0.078 (0.658)
Redundancy[a]	5.1 (5.0)	5.0 (5.0)
Refinement		
No. atoms in refinement (P/L/O)[b]	951/13/252	954/17/207
R_{fact} (%)	17.3	17.7
R_{free} (%)	19.1	21.2
B_f (P/His/O)[b] (Å^2)	26/42/48	37/44/48
rms deviation bond[c] (Å)	0.010	0.015
rms deviation angle[c] (°)	0.9	1.6

[a] Values in brackets show the statistics for the highest resolution shells.

[b] P/L/O indicate protein, ligand, and other (water and other molecules), respectively.

[c] rms indicates root-mean-square.

Assay Protocols

Ligand Observed NMR

All NMR experiments were carried out using a Bruker Avance 700MHz with a 5mm triple TXI cryoprobe with *z* gradients. Spectra were analyzed using TOPSPIN. Relaxation- edited NMR experiments incorporated a CPMG[11] spin-lock time of 200ms before the acquisition period. STD[12] experiments employed a 40 ms selective Gaussian 180° shaped pulse at a frequency alternating between 'on' (1.0 ppm) and 'off' (~80 ppm) resonance after every scan. In both experiments, water suppression was achieved by using a W5 Watergate gradient spin-echo pulse sequence. WaterLOGSY[13] experiments employed a 20ms selective Gaussian 180° shaped pulse at the water frequency and an NOE mixing time of 1s. Water suppression was achieved using a double-gradient echo excitation sculpting sequence with gradients. The resulting spectra were analyzed with Topspin. All samples were made up to a total volume of 200 µL. (Trimethylsilyl)-propionic acid-d4 (TSP) was present at 20 µM concentration in all samples for calibration purposes. A protein only control sample was run, and each fragment was run as 4 samples (A-D). Sample compositions were as follows:

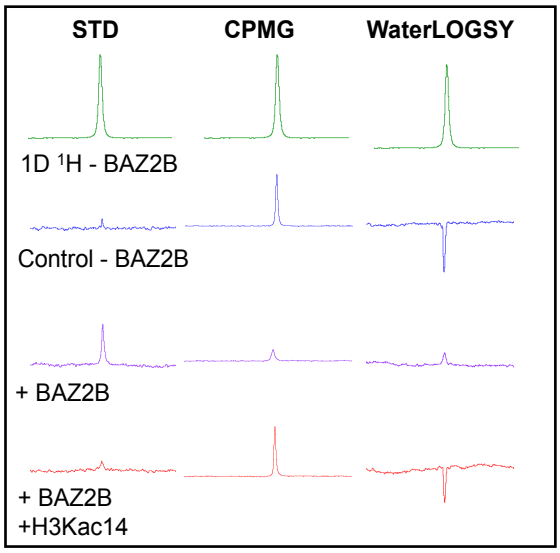
A) Fragment only: 625 µM fragment, 10% v/v D_2O, 0.825% v/v d4-MeOH, 1.65% d3-MeCN, 50mM NaH_2PO_4, 50mM NaCl, pH 7.0

B) Fragment + protein: A + 10 µM BAZ2B

C) Fragment +protein + displacer peptide: B + 100 µM H3Kac14 peptide

D) Protein only control: 10 µM BAZ2B, 10% v/v D_2O, 0.825% v/v d4-MeOH, 1.65% d3-MeCN, 50mM NaH_2PO_4, 50mM NaCl, pH 7.0

Example NMR results



AlphaScreen

AlphaScreen was performed as described previously[14], with minor modifications from the manufacturers protocol (PerkinElmer, USA). Briefly, all reagents were diluted in the recommended buffer (50 mM HEPES, 100 mM NaCl, 0.1% BSA; pH = 7.4) supplemented with 0.05% CHAPS and allowed to equilibrate to room temperature prior to addition to plates.

4 µL of His_6-tagged protein (12.5nM final) was added to low-volume 384-well plates (ProxiPlatet-384 Plus, PerkinElmer, USA), followed by 4 µL of either buffer, non-biotinylated peptide, solvent or compound and 4 µL biotinylated peptide (12.5nM final), Plates were sealed and incubated at room temperature for 30 min. 4 µL of streptavidin-coated donor beads (10ug/mL final) and 4 µL of nickel chelate acceptor beads (10 µg/ml final) were then added under low light conditions. Plates were foil sealed to protect from light, incubated at room temperature for 60 min and read on a PHERAstar FS plate reader (BMG Labtech, Germany) using an AlphaScreen 680 excitation/570 emission filter set. Fragments were dissolved in ethylene glycol to overcome sensitivity of the bromodomain assay to DMSO and allow compounds to be screened at mM concentrations. IC_{50} measurements were taken over a 12 point 1:2 serial dilution of compound. IC_{50}s were calculated in GraphPad Prism 5 (GraphPad Software, USA).

Counterscreen

The AlphaScreen assay protocol above was modified such that 8uL of His_6-biotin counterscreen peptide (PerkinElmer, USA) (12.5nM final) was used in place of 4uL His_6-tagged protein and 4uL biotinylated-peptide. False positives were defined as molecules that caused a reduction in signal (%inhibition) greater than two standard deviations away from of solvent-only controls, (20%).

Fragment hit definitions

Initial hits were defined as fragments producing >50% inhibition in the AlphaScreen assay and <20% inhibition in the counterscreen assay at 1mM compound concentration. IC_{50}s were measured for these molecules. Hits for NMR validation were chosen based on a ~1mM IC_{50} cutoff and continued commercial availability of the compounds for re-purchasing.

AlphaLISA

The assay above was re-optimised around the AlphaLISA acceptor beads according to the manufacturers instructions.

Briefly, all reagents were diluted in the recommended buffer (50 mM HEPES, 100 mM NaCl, 0.1% BSA; pH = 7.4) supplemented with 0.05% CHAPS and allowed to equilibrate to room temperature prior to addition to plates.

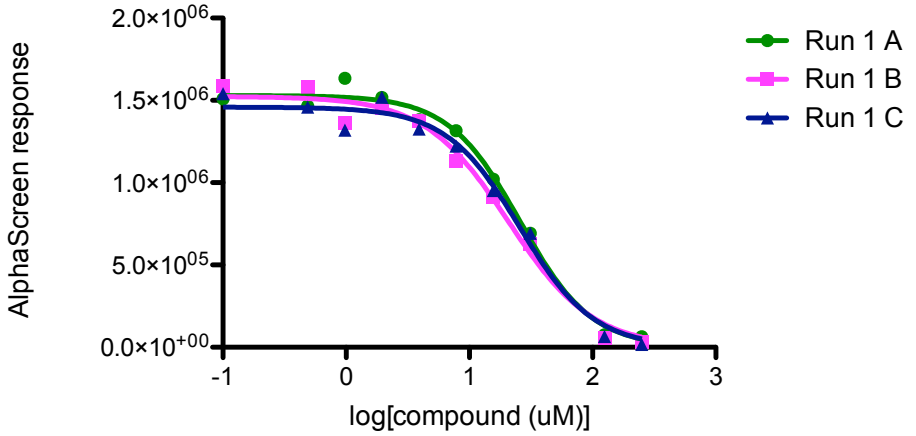
8 µL of a mixture of His_6-tagged protein (160nM final) and biotinylated peptide (40nM final) was added to low-volume 384-well plates (ProxiPlatet-384 Plus, PerkinElmer, USA), followed by 4 µL of either buffer, non-biotinylated peptide, solvent or compound. Plates were sealed and incubated at room temperature for 30 min. 8 µL of a mix containing streptavidin-coated donor beads (20 µg/mL final) and antipentahis$_6$ antibody coated AlphaLISA acceptor beads (20 µg/ml final) were then added under low light conditions. Plates were foil sealed to protect from light, incubated at room temperature for 60 min and read on a PHERAstar FS plate reader (BMG Labtech, Germany) using an AlphaScreen 680 excitation/570 emission filter set. Fragments were dissolved in ethylene glycol to overcome sensitivity of the bromodomain assay to DMSO and allow compounds to be screened at mM concentrations. IC_{50} measurements were taken over a 12 point 1:2 serial dilution of compound. IC_{50}s were calculated in GraphPad Prism 5 (GraphPad Software, USA).

Counterscreen

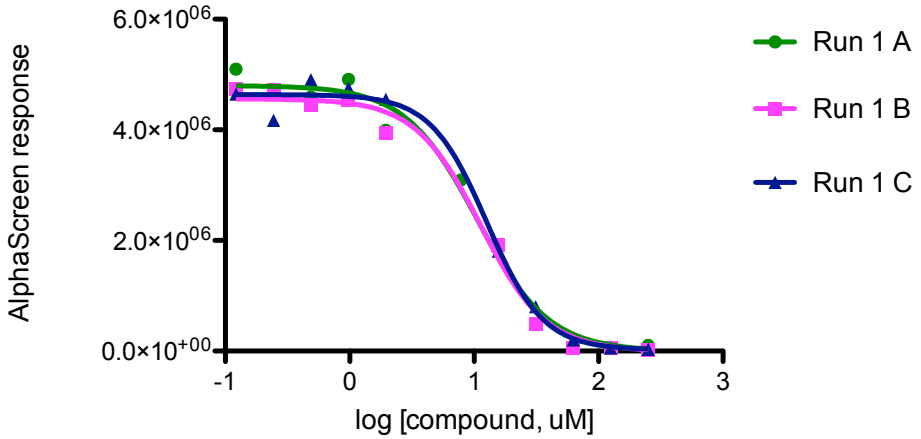
The AlphaLISA assay protocol above was modified such that 8uL of His_6-biotin counterscreen peptide (PerkinElmer, USA) (40nM final) was used in place of 8uL His_6-tagged protein and biotinylated-peptide solution. False positives were defined as molecules that caused a reduction in signal (%inhibition) greater than two standard deviations away from of solvent-only controls, (25%).

Selected IC_{50} curves

Compound 6



Compound 27



Ligand Efficiency calculation

Ligand efficiency (LE) calculated using LE = -1.4*log(IC_{50})/NHA, where NHA is number of non-hydrogen atoms in the molecule.

Supplementary Table 2: LE of reported molecules

Cmpd	MW	IC50 (M)	NHA	LE
1	235.28	3.80E-05	17	0.36
2	212.25	1.09E-04	16	0.35
3	199.22	2.41E-04	13	0.39
4	186.21	8.26E-04	14	0.31
5	153.19	1.09E-03	11	0.38
6	248.71	3.70E-05	17	0.36
7	139.18	1.90E-04	8	0.65
8	144.15	2.79E-04	9	0.55
9	203.24	4.76E-04	15	0.31
10	141.19	4.95E-04	9	0.51
6	248.71	2.40E-05	17	0.38
40	263.73	9.00E-06	18	0.39

Differential Scanning Fluorimetry

DSF was performed using a Roche LightCycler 480 (LC480) in a 96-well plate setup. The fluorescent probe was SYPRO Orange® (Invitrogen, CA), used at a 1:1000 dilution. Samples were buffered in 10mM HEPES, 500mM NaCl, 5% glycerol, 0.5mM TCEP. Final protein concentration was 2 µM, final compound concentration was 100 µM, final DMSO concentration was 0.1% and final well volume was 100 µL. Melting temperatures were defined as the minimum of the negative first derivative of the melting curve. Experiments were performed in triplicate and average values are reported.

Isothermal Titration Calorimetry

All experiments were carried out using an ITC200 micro-calorimeter (GE Healthcare). Experiments were carried out in ITC buffer (50 mM HEPES pH 7.5, 150 mM NaCl). Protein was buffer exchanged using a Pierce protein desalting spin column (Fischer Scientific, UK). All experiments were performed at 10ºC, unless otherwise stated, whilst stirring at 1000rpm. Compounds were dissolved in either a 1:2 mix of MeOH:MeCN or DMSO and diluted in ITC buffer. The final concentration of organic solvent in this solution was between 2% and 10%. This concentration of organic solvent was also added to the protein solution in order to prevent large heats of dilution from buffer mismatches occurring.

All titrations were conducted using an initial injection of 0.4 µl followed by 19 identical injections of 2 µl at a rate of of 2 sec/uL. Spacing was adjusted so that the signal returned to the baseline after each injection. Data was corrected for heats of dilution by subtracting the data from independent titrations of ligand into buffer.

Data was processed using MicroCal.™ Origin software.

Competitive ITC Titrations

In competitive titrations with H3Kac14 peptide the Kd of the ligand was calculated according to equation 1.[15]

$$Kd_{cmpd} = \frac{[cmpd]}{\left(\frac{Kd_{pep}^{app}}{Kd_{pep}}\right) - 1}$$

Equation 1

App – apparent, pep - peptide

For titrations in which DMSO was present the Kds of the ligands were calculated considering DMSO as a competing ligand according to equation 2.[16]

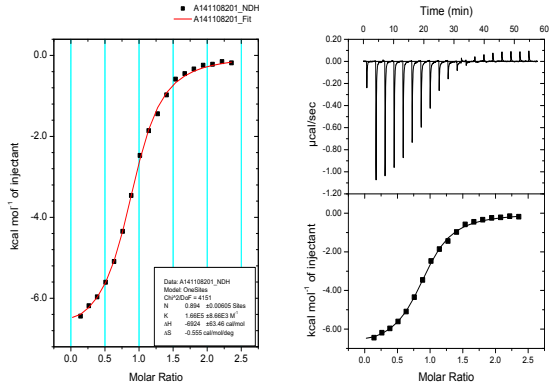
$$Kd_{cmpd} = \frac{Kd_{cmpd}^{app}}{\left(1 + \frac{[DMSO]}{Kd_{DMSO}}\right)}$$

Equation 2

App – apparent, cmpd - compound

Determination of Kd DMSO

Run 1: No DMSO



Run 2: 1.407M (10% v/v) DMSO

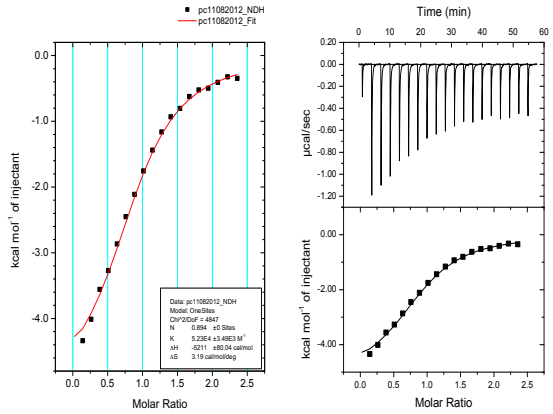


Table 3: Data for DMSO

Run	Conc DMSO (M)	app Kd (µM)	Kd DMSO (mM)
1	0.00	6.0 +/- 0.3	-
2	1.41	19.1 +/- 1.1	644.0

Competitive ITC Titrations

Table 4: Data for compounds 6, 16-17 and 22

Compound	Compound concentration (mM)	DMSO concentration (M)	K_D peptide + solvent (μM)	K_D peptide + compound (μM)	K_D compound (μM)
6	0.3	0*	8.2 +/- 0.4	50.0 +/- 5.9	65.3
17	1.0	0.14	7.0 +/- 0.6	32.5 +/- 3.1	274.0
16	1.0	0.14	7.0 +/- 0.6	27.8 +/- 2.6	335.9
22	1.0	0.28	10.0 +/- 0.8	24.4 +/- 2.5	694.4

* 10% MeCN:MeOH 1:2 mix used to dissolve compound **6**

Direct ITC Titrations

Table 5: Data for compounds 7, 27, 40 and 41

Compound	n	Temp (K)	ΔH (kcal/mol)	ΔS (kcal/mol)	ΔG (kcal/mol)	apparent K_D (μM)	DMSO concentration (M)	K_D (μM)
7	1.0	298.2	-0.7 +/-0.3	0.01	-3.7	524 +/-39	0.0	524.0
27	1.0	298.2	-7.5 +/- 0.1	0.02	-13.5	10.6 +/- 1.0	0.0	10.6
40	1.2	283.2	-0.47+/-0.01	0.02	-6.1	15.2 +/- 2.1	0.7	7.6
40	1.1	303.2	-0.36 +/-0.01	0.02	-6.4	15.0 +/- 2.1	0.7	7.5
41	1.0	298.2	-1.9 +/- 0.12	0.02	-7.9	17.0 +/- 2.6	0.0	17.0

Computational Methods

Molecular modeling

X-ray structure of BAZ2B in complex with **6** (PDB ID 4NRA) was used as the starting point for all dockings. There are 5 water molecules in the Kac binding site that form a water network and are highly conserved in the available bromodomain crystal structures.[7] These waters were retained for docking.

The protein was prepared using the protein preparation wizard in Maestro (version 9.3). The grid was generated using the receptor grid generator wizard, Glide (version 5.9). The bound ligand (**6**) was used to define the pocket. A hydrogen bond requirement to Asn1944 was defined. Default parameters were used for all other available options. Ligands were prepared using LigPrep with the default parameters. Docking was performed using Glide[17] (version 5.9) in the standard precision mode (SP). The number of output structures per ligand was set to 5, default values were used for all other parameters. Results were visualized in Maestro. (version 9.3).

FTMapping

The apo structure of BAZ2B (PDB ID 3G0L) was submitted to the FTMap webserver.[18]

Synthesis

General Methods

Commercial materials were used as received unless otherwise noted. All reactions were carried out using anhydrous solvents. Anhydrous DMF was purchased from Aldrich and used as supplied. DCM was distilled from calcium hydride, THF was distilled from sodium/benzophenone, MeCN was distilled from CaH_2. Thin-layer chromatography was performed on commercial glass backed TLC plates and visualized using UV and/or potassium permanganate (KMnO4), or ninhydrin stain. Purification via automated flash chromatography was performed using Biotage Isolera One, using Biotage SNAP Kp-Sil pre-packed columns. HPLC purification was carried out using a GX-271 Analytical to Semi-preparative HPLC System. Analytical runs were carried out using a Nucleosil C18 column (5 µm; 250 × 4.6 mm) (Machery-Nagel), run at 1 mL/min. Preparative runs were carried out using a Kromasil column (5 µm; 250 × 4.6 mm) (Hichrom), run at 21 mL/min. The solvent system used for peptide purification was A (0.1 % TFA in H_2O) and B (0.1 % TFA in MeCN). The solvent system used for small molecule purification was A (H_2O) and B (MeCN). A linear gradient of 5 % to 60 % B over 25 min was used.

Nuclear Magnetic Resonance spectra were recorded on a Bruker DPX-400 or a Bruker Avance 500. All ^{1}H and ^{13}C NMR experiments are reported in parts per million (ppm), and were measured relative to the signals for residual chloroform, methanol, D_2O, dichloromethane, or DMSO in the deuterated solvent.[19] All coupling constants are reported in Hz. The following abbreviations are used for assigning peak multiplicity; s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. All ^{13}C NMR spectra were obtained with 1H decoupling.

In most molecules multiple signals per nuclei are observed, due to restricted amide rotation. Variable temperature ^{1}H NMR is reported for compound **6**. For all molecules all signals observed are reported, along with the ratio of rotamers observed in the spectra. HRMS was performed using a Waters LCT Premier high-resolution mass spectrometer using electrospray ionization (ESI). LCMS analysis was performed with an Alliance HT Waters 2795 separations module coupled to a Waters Micromass ZQ Quadrupole Mass Analyser with accuracy no greater than 0.4 Da. A gradient of 10nM ammonium acetate containing 0.1% formic acid to 95% MeCN over 8 min was used. All peptides were characterized using MALDI-TOF-MS on an ABI 4700 Proteomics Analyzer (Applied Biosystems) using the reflectron in positive ion mode.

Peptidic reagents

H3Kac14 peptide

Peptide sequence: H-YQTARKSTGGK(Nε-ac)APRKQLATKAK-NH_2

Non-biotinylated peptides were synthesized using solid phase peptide synthesis performed on a Liberty Microwave Peptide Synthesizer (CEM Corporation, Mathews, NC). Coupling was carried out using Fmoc-protected amino acid in DMF (5 eq.), PyBOP in DMF (4.5 eq.), DIPEA in NMP (10 eq.) and reactions were heated for 5 min at 25 W with a maximum temperature of 75 °C. Fmoc-L-Arg(pbf)-OH was double coupled at RT, in the absence of microwave irradiation, to minimize Arg deletion and δ-lactam formation. Removal of the Fmoc-group was achieved using piperidine in DMF (20% v/v) for 5 min then again for 15 min, in the absence of microwave irradiation. Cleavage from the rink amide resin and global deprotection was achieved by addition of cleavage cocktail containing 88:5:5:2 (v/v) TFA:phenol:water:TIPS (5mL) at rt for 2.5 h. The cleavage reaction was filtered and resin washed with cleavage cocktail (5mL) followed by TFA (5mL). Filtrates were combined and concentrated *in vacuo*. Residual water was azeotroped with toluene (2 x 10mL). Peptide was precipitated by addition of ice-cold diethyl ether (7.5mL). Precipitate was isolated by centrifugation (7,200 rpm, 5 min, 4^{o}C), supernatant discarded and the pellet washed with ice-cold diethyl ether (3 x 10mL) to remove residual scavengers.

Expected mass: 2431.8 Da; observed mass: 2431.6 Da.

H3Kac14-biotin peptide

Sequence: H-YQTARKSTGGK(Nε-ac)APRKQLATKAK(Nε-biotin)-NH_2

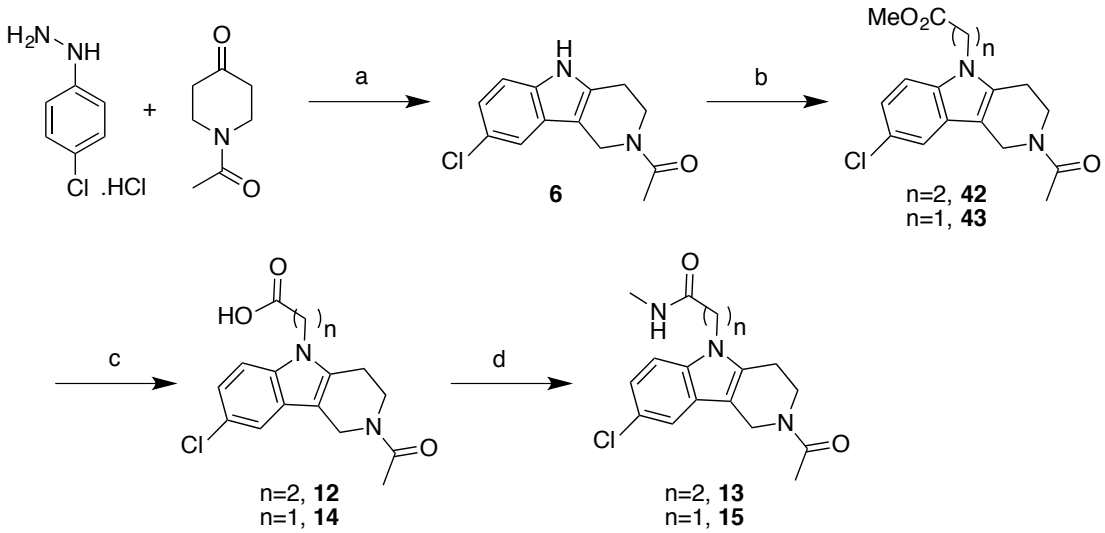
Biotinylated H3Kac14 peptide was purchased from GenScript (>95% pure) and used as supplied

Small molecules

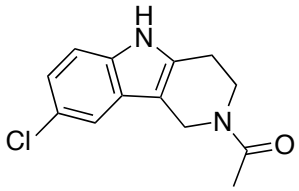
General Procedure 1

Substituted phenylhydrazine hydrochloride (1 eq) and N-acyl-4-piperidone hydrochloride monohydrate (1 eq) were refluxed in absolute EtOH for 3hrs. The reaction was cooled to rt and the solvent concentrated in vacuo. The residue was purified on silica or recrystallized from EtOH/H_2O.

Scheme 1: synthesis of N1 substituted analogs

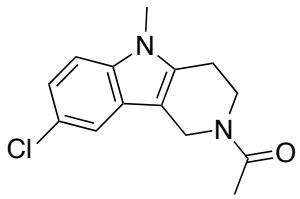


a) EtOH, reflux, 3h, b) K_2CO_3, 3-bromopropionate, DMF, 80°C, 16h c) 1M NaOH(aq), 45°C 16h d) DIPEA, CDI, NH_2M, DCM



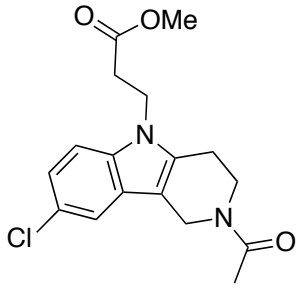
1-(8-chloro-1,3,4,5-tetrahydro-2*H*-pyrido[4,3-*b*]indol-2-yl)ethan-1-one (6)

Compound 6 was synthesised from 4-chlorophenylhydrazine hydrochloride (300mg, 1.68 mmol) and 1-acetyl-4-piperidone (0.21mL, 1.68 mmol) according to the general procedure 1. The precipitate obtained was recrystallised from EtOH to give the title compound in 32% yield as a pale yellow powder (134 mg, 0.54 mmol). Rf 0.29 (5% MeOH/DCM), **δ**H (500 MHz, d6-DMSO) (1:1.2 ratio of rotamers observed) 11.14 and 11.13 (1H, s), 7.49 and 7.47 (1H, d, J 2.0), 7.30 and 7.29 (1H, d, J 8.6), 7.03 and 7.02 (1H, dd, J 8.6, 2.1), 4.63 and 4.60 (2H, s), 3.82 ad 3.76 (2H, t, J 5.6), 2.86 and 2.74 (2H, t, J 5.6), 2.12 and 2.10 (3H, s); δC (125 MHz, d6-DMSO) 169.2, 168.9, 135.0,134.6, 134.5, 134.4, 126.5, 126.3, 123.3, 123.2, 120.5, 116.9, 116.8, 112.5, 112.4, 106.0, 105.8, 43.4, 42.8, 38.7, 38.5, 23.8, 23.0, 22.1, 21.7; HRMS(ESI) found 249.0805, $C_{13}H_{15}N_2O^{35}Cl$ [M+H] requires 249.0795, LC/MS found 249.0, purity 100%



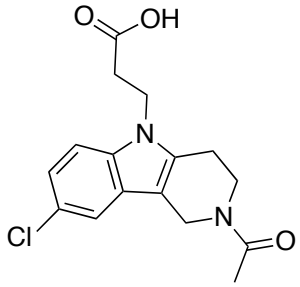
1-(8-chloro-5-methyl-1,3,4,5-tetrahydro-2*H*-pyrido[4,3-*b*]indol-2-yl)ethan-1-one (11)

Compound 6 (40 mg, 0.16 mmol) was dissolved in DMF (1ml), and KOH (26 mg, 0.64 mmol) was added. MeI (100 µl, 1.61 mmol) was added using a Gilson pipette and the reaction mixture was then stirred at room temperature overnight. The reaction mixture was then diluted with H_2O and EtOAc and the aqueous layer extracted three times with EtOAc. The organic layers were combined, dried over $MgSO_4$, filtered and concentrated in vacuo and finally washed with MeOH to give a pale yellow solid (18 mg, 0.07 mmol) which was confirmed as the product in 43 % yield. **δ**H (500 MHz, d6-DMSO) (1:1.2 ratio of rotamers observed) 7.57 and 7.53 (1H, d, J 2.1), 7.48 and 7.47 (1H, d, J 8.7), 7.14 and 7.13 (1H, dd, J 8.6, 2.1), 4.68 and 4.65 (2H, s), 3.88 and 3.83 (2H, t, J 5.8), 3.67 and 3.66 (3H, s), 2.93 and 2.82 (2H, t, J 5.7), 2.17 and 2.14 (3H, s); **δ**C (125 MHz, d6-DMSO) 169.5, 169.2, 136.9, 136.5, 135.7, 135.6, 126.3, 126.0, 123.9, 123.8, 120.9, 117.5, 117.4, 111.4, 111.3, 106.3, 106.1, 43.7, 43.2, 39.0, 38.8, 29. 8 and 29.2, 23.0, 22.4, 22.2, 22.0; HRMS(ESI) found 263.1011 $C_{14}H_{16}N_2O^{35}Cl$ [M+H] requires 263.0951; LC/MS [M+H] found 263.0, purity 100%



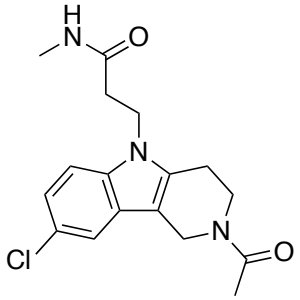
methyl 3-(2-acetyl-8-chloro-1,2,3,4-tetrahydro-5*H*-pyrido[4,3-*b*]indol-5-yl)propanoate (42)

Compound 6 (165mg, 0.67mmol) was dissolved in DMF (3mL), and K_2CO_3 (463mg, 3.35 mmol) was added. The flask was sealed with a septum, methyl bromopropionate (110µL, 1 mmol) was added via syringe, and the reaction was heated at 80°C overnight under an atmosphere of N_2. The crude reaction mixture was concentrated in vacuo and diluted with H_2O and EtOAc and the aqueous layer extracted three times with EtOAc. The organics were combined, washed with brine, dried over $MgSO_4$, filtered and concentrated in vacuo. The crude product was purified on silica (MeOH/DCM) to afford the title compound (175mg, 0.52mmol) as a white powder in 78% yield. Rf 0.3 (5% MeOH/DCM); **δ**H (500 MHz, $CDCl_3$) (1:1.4 ratio of rotamers observed) 7.42-7.40 (m, 1H), 7.25-7.13 (m, 2H), 4.76 and 4.62 (s, 2H), 4.33 (t, 2H, J 6.5), 4.00 and 3.82 (t, 2H, J 5.0), 3.65 (s, 3H), 2.94-2.90 and 2.86-2.82 (m, 2H), 2.75-2.71 (m, 2H), 2.22-2.21 (m, 3H); **δ**C (125 MHz, $CDCl_3$) 171.2, 169.9, 169.4, 135.4, 134.5, 126.1, 125.4, 121.7, 117.7, 117.1, 110.2, 109.9, 106.1, 52.1, 43.8, 43.3, 39.0, 38.8, 34.4, 23.2, 22.2; HRMS (ESI) found 335.1146 $C_{17}H_{20}N_2O_3{}^{35}Cl$ [M+H] requires 335.1157; LC/MS found 321.5 (15%, product hydrolysed on column) and 335.1 (85%)



3-(2-acetyl-8-chloro-1,2,3,4-tetrahydro-5*H*-pyrido[4,3-*b*]indol-5-yl)propanoic acid (12)

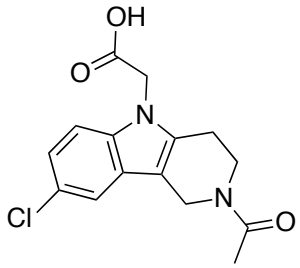
Compound (40) (150mg, 0.45 mmol)was dissolved in 1M NaOH (aq, 5 mL) and MeCN (2 mL) and heated at 45°C overnight. The reaction mix was cooled to room temperature, acidified with 2N HCl (aq) and extracted three times with DCM. The organics were combined, dried over $MgSO_4$, filtered and concentrated in vacuo to afford the product as an off white powder in 71% yield (102mg, 0.32 mmol). **δ**H (500 MHz, $CDCl_3$/ CD_3OD) (1:1.3 ratio of rotamers observed) 7.35-7.32 (m, 1H), 7.21-7.16 (m, 1H), 7.08-7.05 (m, 1H), 4.67 and 4.57 (s, 2H), 4.27-4.24 (m ,2H), 3.92-3.90 and 3.78-3.76 (m, 2H), 3.30-3.29 (m, 2H), 2.87-2.86 and 2.80-2.78 (m, 2H), 2.15-2.14 (m, 3H); **δ**C (125 MHz, $CDCl_3$/CD_3OD) 173.0, 172.9, 170.7, 170.3, 135.3, 134.6, 133.7, 126.5, 126.1, 125.3, 121.7, 117.5, 117.0, 110.3, 110.1, 106.6, 105.6, 43.9, 43.5, 34.4, 39.3, 38.9, 23.0, 22.1, 21.9, 21.4; HRMS (ESI) found 321.1004 $C_{16}H_{18}N_2O_3{}^{35}Cl$ [M+H] requires 321.1000; LC/MS [M+H] found 320.9, 323.0, purity 100%



3-(2-acetyl-8-chloro-1,2,3,4-tetrahydro-5*H*-pyrido[4,3-*b*]indol-5-yl)-*N*-methylpropanamide (13)

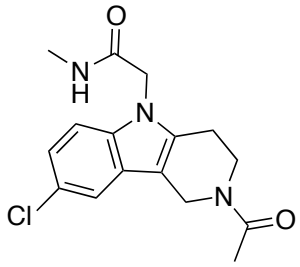
Compound 12 (90mg, 0.28 mmol) was dissolved in DCM (2 mL) and 1,1-carbonyldiimidazole (55mg, 0.34 mmol) was added. The reaction was stirred at room temperature for 4h under N_2. A 2M solution of methylamine in THF (0.28mL, 0.56 mmol) was added via syringe. DIPEA (79µL, 0.45 mmol) was added via syringe and the reaction stirred at room temperature overnight. The reaction was diluted with DCM and washed with H_2O and brine. The organics were dried over $MgSO_4$, filtered and concentrated in vacuo. The crude reaction product was purified on silica (MeOH/DCM) to afford the title product (43mg, 0.13 mmol) as a white powder in 46% yield. Rf 0.38 10%MeOH/DCM; **δ**H (500 MHz, $CDCl_3$) 7.34 (s, 1H), 7.18-7.16 (m, 1H), 7.08-7.06 (m, 1H), 5.13 (br s, 1H), 4.65 and 4.57 (br s, 2H), 4.31 (t, 2H, J 8.0), 3.91 and 3.74 (br s, 2H), 2.80 (br s, 2H), 2.61 (s, 3H), 2.47 (t, 2H, J 8), 2.14 (s, 3H); HRMS found 334.1310

$C_{17}H_{21}N_3O_2{}^{35}Cl$ [M+H] requires 334.1317; LC/MS [M+H] found 333.9, 335.9, purity 100%



2-(2-acetyl-8-chloro-1,2,3,4-tetrahydro-5*H*-pyrido[4,3-*b*]indol-5-yl)acetic acid (14)

Compound 6 (250mg, 1.01 mmol) was dissolved in DMF (3mL), and K_2CO_3 (695mg, 5.03 mmol) was added. The flask was sealed with a septum, methyl bromoacetate (145µL, 1.51 mmol) was added via syringe, and the reaction was heated at 80°C overnight under an atmosphere of N_2 to generate intermediate **43**, which was not isolated. The reaction was exposed to the air, H_2O (3mL) was added and the reaction heated at 80°C overnight. The crude reaction mixture was concentrated in vacuo and diluted with 1N HCl (aq) and EtOAc and the aqueous layer extracted three times with EtOAc. The organic layers were combined, dried over $MgSO_4$, filtered and concentrated in vacuo. The residue was purified on silica (AcOH:MeOH:DCM) to afford the title compound as a white powder (80mg, 0.26 mmol) in 26% yield. Rf 0.28 4%AcOH/16%MeOH/80%DCM. **δ**H (500 MHz, $CDCl_3$) (1:1.3 ratio of rotamers observed) 7.38-7.33 (m, 1H), 7.11-7.04 (m, 2H), 4.69 and 4.60 (s, 2H), 4.65 (d, 2H, J 3.0), 3.93 and 3.78 (t, 2H, J 5.5), 2.79-2.75 and 2.72-2.69 (m, 2H), 2.15 (s, 3H); **δ**C (125 MHz, $CDCl_3$) 170.7, 170.4, 170.2, 135.7, 135.4, 134.0, 126.5, 126.0, 125.6, 122.1, 122.0, 121.9, 117.6, 117.3, 117.1, 110.0, 109.9, 109.7, 107.2, 106.5, 106.2, 44.4, 44.3, 43.4, 39.2, 39.1, 22.8, 21.8, 21.4. HRMS (ESI) found 307.825, $C_{15}H_{16}N_2O_3{}^{35}Cl$ [M+H] requires 307.0844. LC/MS [M+H] found 307, 308.2, purity 100%

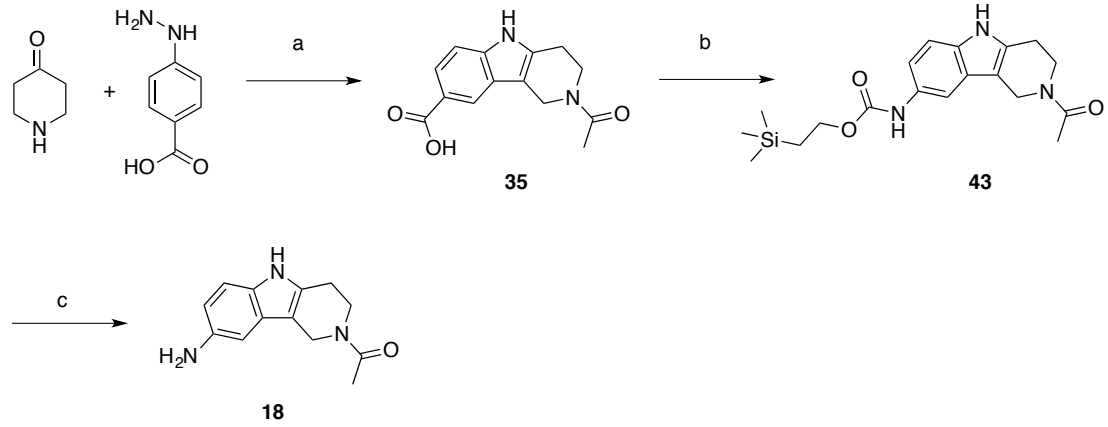


2-(2-acetyl-8-chloro-1,2,3,4-tetrahydro-5*H*-pyrido[4,3-*b*]indol-5-yl)-*N*-methylacetamide (15)

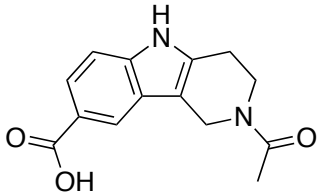
Compound 14 (50mg, 0.16 mmol) was dissolved in DCM (2 mL) and 1,1-carbonyldiimidazole (31mg, 0.19 mmol) was added. The reaction was stirred at room temperature for 4h under N_2. A 2M solution of methylamine in THF (0.21mL, 0.42 mmol) was added via syringe. DIPEA (46µL, 0.26 mmol) was added via syringe and the reaction stirred at room temperature overnight. The reaction was diluted with DCM and

washed with H_2O and brine. The organics were dried over $MgSO_4$, filtered and concentrated in vacuo. The crude reaction product was purified on silica (MeOH/DCM) to afford the title product (37.5mg, 0.12 mmol) as a white powder in 73% yield. Rf 0.3 7%MeOH/DCM; δH (500 MHz, $CDCl_3$) 7.47 (d, 1H, J 1.5), 7.11-7.04 (m, 2H), 5.28 (br s, 1H), 4.78 and 4.67 (br s, 2H), 4.66 (s, 2H), 4.01 and 3.85 (br s, 2H), 2.77 (br s, 2H), 2.75 (d, 3H, J 4.5), 2.22 (s, 3H); **δ**C (125 MHz, $CDCl_3$) 169.7, 169.6, 168.1, 134.9, 126.5, 122.8, 118.1, 117.8, 110.0, 46.6, 43.3, 43.4, 38.9, 22.7, 21.8; HRMS (ESI) found 320.1157, $C_{16}H_{19}N_3O_2{}^{35}Cl$ [M+H] requires 320.1160; LC/MS [M+H] found 320.0, 321.9, purity 95%

Scheme 2: Synthesis of 8-NH2 analog



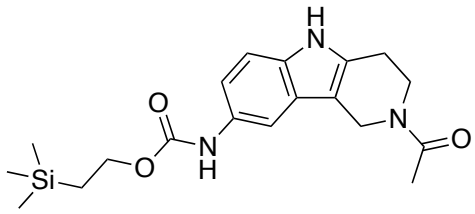
a) 4N HCl/dioxane, reflux, 3h b) Et_3N, DPPA, 2-TMS EtOH, toluene, reflux, 16h c) TFA, rt, 16h



2-acetyl-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indole-8-carboxylic acid (37)

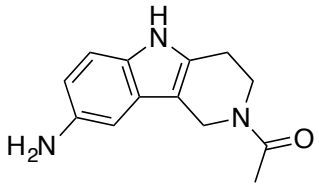
4-hydrazinobenzoic acid hydrochloride (3.0g, 19.7 mmol) and 4-piperidone monohydrate hydrochloride (3.3g, 2.17 mmol) were suspended in a 4N solution of HCl in dioxane (25mL) and heated at reflux for 3 h. The reaction was cooled to room temperature and filtered. The solids were suspended in DCM (25mL) and basified with Et_3N (6mL, 5.36 mmol). Acetic anhydride (3mL, 3.18 mmol) was added and the reaction stirred at room temperature overnight. The reaction mixture was concentrated in vacuo and the residue diluted with saturated sodium bicarbonate solution (aq) and extracted with DCM. The aqueous layer was acidified with conc. HCl (aq) and the precipitate filtered and dried to afford the title product as a pale yellow powder (2.172g, 8.4 mmol) in 43% yield. **δ**H (500 MHz, d6-DMSO) (1:1.07 ratio of rotamers observed) 11.29 (s, 1H), 8.14 and 8.07 (br s, 1H), 7.70-7.67 (m, 1H), 7.358 and 7.34 (d, 1H, J 1 Hz), 4.71 and 4.66 (s, 2H), 3.84 and 3.78 (t, 2H, J 7.5), 2.88 and 2.76 (t, 2H, J 7.5), 2.13 and 2.14 (s, 3H); **δ**C (125 MHz, d6-DMSO) 169.3, 168.9, 168.5, 167.5, 150.0, 147.5, 138.5, 138.4, 134.7, 134.4, 122.2, 122.1, 121.2, 120.1, 119.8, 119.7, 111.3, 110.7, 107.3, 107.1, 43.4, 43.8, 38.5, 38.4,

23.7, 22.9, 22.0, 21.6; HRMS (ESI) found 259.1039 $C_{14}H_{15}N_2O_3$ [M+H] requires 259.1077; LC/MS[M+H] found 259.1, purity 95%



2-(trimethylsilyl)ethyl (2-acetyl-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indol-8-yl)carbamate (44)

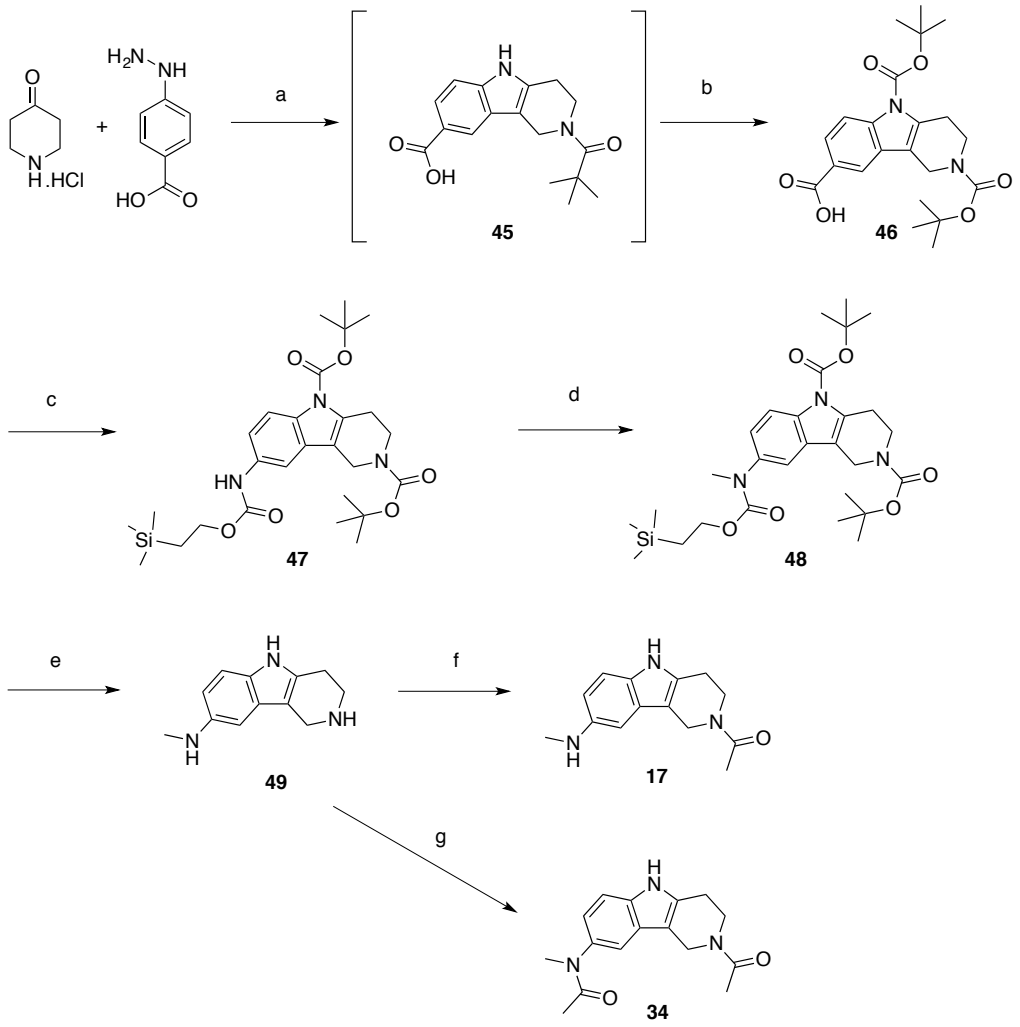
Compound 37 (500mg, 1.93 mmol) was suspended in toluene (5mL). Et_3N (300µL, 2.13 mmol), diphenylphosphoryl azide (458µL, 2.13 mmol) and 2-(trimethylsilyl)ethanol (557µL, 3.86 mmol) were added via asyringe and the reaction refluxed under N_2 overnight. The reaction was cooled to room temperature and concentrated in vacuo. The residue was diluted with diluted with saturated sodium bicarbonate solution (aq) and extracted three times with DCM. The organics were combined, dried over $MgSO_4$, filtered and concentrated in vacuo. The crude product was purified on silica to afford the title compound (412mg, 1.10 mmol) as a yellow oil in 57% yield. Rf 0.3 5% MeOH/DCM; **δ**H (500 MHz, CD_3OD) (1:1.05 ratio of rotamers observed) 7.21 (m, 3H), 4.73 (m, 2H), 4.28 (m, 2H), 3.98 and 3.90 (t, 2H, J 7.5), 2.93 and 2.84 (t, 2H, J 7.5), 2.25 (m, 3H,), 1.09 (t, 2H, J 10.5), 0.10 (s, 9H); **δ**C (125 MHz, MeOD) 172.6, 172.3, 134.8, 134.7, 132.1, 126.9, 111.8, 106.9, 106.5, 63.8, 45.3, 44.7, 24.9, 24.1, 21.5, 18.7, -1.45; LC/MS [M+H] found 374.1, purity 100%



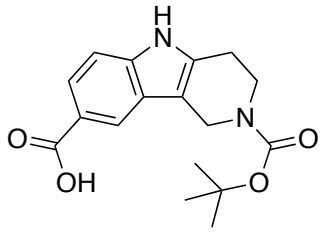
1-(8-amino-1,3,4,5-tetrahydro-2*H*-pyrido[4,3-*b*]indol-2-yl)ethan-1-one (16)

Compound 44 (was dissolved in a 7:3 mixture of TFA and H_2O and stirred overnight at room temperature. The reaction mixture was concentrated in vacuo and the residue diluted with a saturated aqueous solution of sodium bicarbonate and extracted with DCM three times. The organics were combined, dried over $MgSO_4$, filtered and concentrated in vacuo. The residue was purified on silica (MeOH/DCM) to afford the title product (660mg, 2.88 mmol) as a pale brown powder in 51% yield. Rf 0.37 (10% MeOH:DCM); **δ**H (500 MHz, d6-DMSO) (1:1.2 ratio rotamers observed)10.41 (s, 1H), 6.98-6.96 (m, 1H), 6.55-6.53 (m,1H), 6.44-6.41 (m,1H), 4.52 and 4.50 (s, 2H), 3.80 and 3.73 (t, 2H, J 5.5), 2.79 and 2.67 (t, 2H, J 5.5), 2.11 and 2.10 (s, 3H); **δ**C (125 MHz, $CDCl_3$) 169.1, 168.7, 132.6, 132.2, 126.1, 125.9, 111.2, 104.6, 104.2, 101.3, 43.5, 43.1, 23.8, 22.0, 21.6; HRMS (ESI) found 230.1288 $C_{13}H_{16}N_3O$ [M+H] requires 230.1288, LC/MS [M+H] found 230.1, purity 100%

Scheme 3: Synthesis of 8-NR_1R_2 analogs



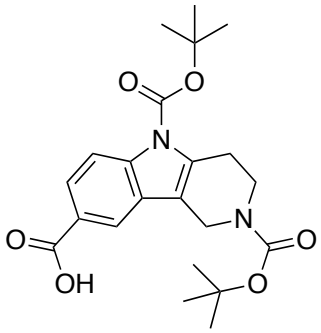
a) 4N HCl/dioxane, reflux, 3h, Boc_2O, Et_3N, DCM b) Boc_2O, 4-DMAP, THF c) Et_3N, DPPA, 2-TMS EtOH, toluene, reflux, 16h d) DMF, NaH, MeI, rt, 3h e) TFA, rt, 16h f) AcOSu, DIPEA, $CHCl_3$ 22h g) Ac_2O, Et_3N, DCM, 2h



2-(*tert*-butoxycarbonyl)-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indole-8-carboxylic acid (45)

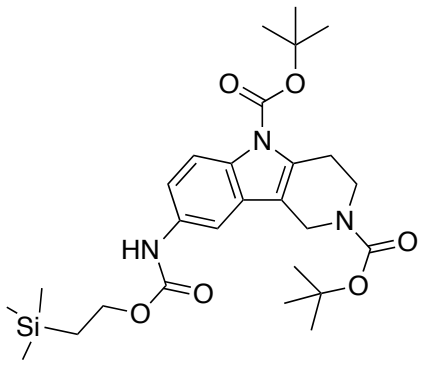
4-hydrazinobenzoic acid hydrochloride (0.5g, 3.30 mmol) and 4-piperidone monohydrate hydrochloride (0.56g, 3.63 mmol) were suspended in a 4N solution of HCl in dioxane (6mL) and heated at refluxed for 3h. The solution was cooled to RT and concentrated in vacuo. The residue was dissolved in MeOH (6 mL) and the pH adjusted to 9-10 with 5N NaOH (aq). Di-tert-butyl dicarbonate (0.72g, 3.30 mmol) was added and the reaction stirred at room temperature overnight. The reaction was concentrated in

vacuo and the residue diluted with H_2O and and extracted with DCM. The aqueous phase was acidified with acetic acid to pH 5 and the solids were collected by filtration to give the title compound (624mg, 1.97 mmol) as a pale beige powder in 60% yield, which was used without further purification. **δ**H (500 MHz, d6-DMSO) 7.69 (dd, 1H, J 1.5, 10.5), 7.35 (d, 1H, J 10.5), 4.57 (s, 2H), 3.72 (t, 2H, J 7.0), 2.79 (t, 2H, J 7.0), 1.45 (s, 9H); HRMS (ESI) found 317.1457 $C_{17}H_{21}N_2O_4$ [M+H] requires 317.1496; LC/MS found 317.4, purity 95%



2,5-bis(*tert*-butoxycarbonyl)-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indole-8-carboxylic acid (46)

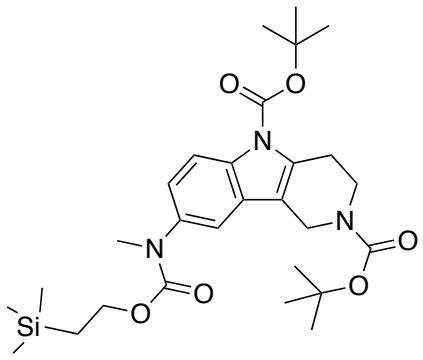
Compound 45 (322mg, 1.02 mmol) was dissolved in THF (3mL) and di-tert-butyl dicarbonate (445mg, 2.04 mmol) and 4-DMAP (187mg, 1.53 mmol) was added. The reaction was stirred at room temperature for 3h. the reaction was diluted with 2N HCl (aq) and extracted with DCM three times. The organics were combined, dried over $MgSO_4$ and concentrated in vacuo. The crude residue was recrystallized from EtOH to afford the title compound (165mg, 0.48 mmol) as a white powder in 48% yield. **δ**H (500 MHz, CD_3OD) 8.16 (d, 1H, J 11), 8.07 (d, 1H, J 2), 7.90 (dd, 1H, J 11, 2), 4.56 (s, 2H), 3.69 (t, 2H, J 6.5), 3.04 (t, 2H, J 6.5), 1.64 (s, 9H), 1.45 (s, 9H); **δ**C (125 MHz, CD_3OD) 167.7, 167.5, 154.2, 149.4, 133.0, 129.4, 128.7, 125.3, 119.6, 114.9, 84.6, 79.4, 28.2, 27.8; HRMS (ESI) found 417.1958 $C_{22}H_{29}N_2O_6$ [M+H] requires 417.2020; LC/MS [M-H] found 415.2, purity 92%



di-*tert*-butyl 8-(((2-(trimethylsilyl)ethoxy)carbonyl)amino)-3,4-dihydro-1*H*-pyrido[4,3-*b*]indole-2,5-dicarboxylate (47)

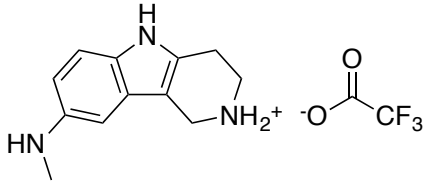
Compound 46 (1.9g, 4.58 mmol) was suspended in toluene. Et_3N (700µL, 5.03 mmol), diphenylphosphoryl azide (1.08mL, 5.03 mmol) and 2-(trimethylsilyl)ethanol

(1.32mL, 9.16 mmol) were added via syringe and the reaction refluxed under N_2 for 2 h. The reaction mix was cooled to RT and concentrated in vacuo. The residue was diluted with a saturated solution of sodium bicarbonate (aq) and extracted with DCM three times. The organics were cmbined, dried over $MgSO_4$ and concentrated in vacuo. The crude product was purified on silica (EtOAc/DCM) to afford the title product (1.97g, 3.71 mmol) as a pale yellow oil in 81% yield . Rf 0.5 1% EtOAc/DCM; **δ**H (500 MHz, CD_3OD) 8.03 (d, 1H, J 11), 7.57 (br s, 1H), 7.24 (br s, 1H), 4.55 (s, 2H), 4.28 (t, 2H, J 10.5), 3.77 (t, 2H, J 7.0), 3.09 (t, 2H, J 7.0), 1.69 (s, 9H), 1.53 (s, 9H), 1.10 (t, 2H, J 10.5), 0.11 (s, 9H); **δ**C (125 MHz, CD_3OD) 156.7, 156.4, 151.4, 135.6, 135.1,133.3, 116.9, 116.6, 114.8, 108.3, 84.9, 81.6, 64.0, 43.0, 41.9, 28.9, 28.6, 27.4, 18.7, -1.32.



di-*tert*-butyl 8-(methyl((2-(trimethylsilyl)ethoxy)carbonyl)amino)-3,4-dihydro-1*H*-pyrido[4,3-*b*]indole-2,5-dicarboxylate (48)

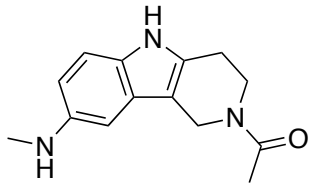
Compound 47 (90mg, 0.17 mmol) was dissolved in DMF. 60% NaH in mineral oil (10.4mg, 0.26 mmol) was added under N_2 in one portion, at 0°C. The reaction was stirred at room temperature for 30 min. MeI (16.2µL, 0.26 mmol) was added via syringe and the reaction stirred at room temperature for 3h. The reaction was concentrated in vacuo, diluted with H_2O, and extracted with DCM three times. The organics were combined, dried over $MgSO_4$, filtered and concentrated in vacuo to afford the title product as a yellow powder (89mg, 0.16 mmol) in 94% yield. **δ**H (500 MHz, d6-DMSO) 8.03 (d, 1H, J 9) 7.41 (d, 1H, J 2), 7.18 (dd, 1H, J 9, 2) 4.48 (s, 2H), 4.10 (t, 2H, J 8), 3.67 (t, 2H, J 6), 3.24 (s, 3H), 3.01 (t, 2H, J6), 1.62 (s, 9H), 1.44 (t, 9H), 0.90 (br s, 2H), -0.04 (s, 9H); **δ**C (125 MHz, d6-DMSO) 155.2, 154.1, 149.6, 115.2, 84.1, 79.3, 63.1, 28.1, 27.8, 17.3, -1.4; HRMS (ESI) found 546.2992 $C_{28}H_{44}N_3O_6Si$ [M+H] requires 546.2994; LC/MS [M+H] found 460.2 (19% carbamate hydrolysed by MeOH in injection solvent) and 546.2 (81%).



***N*-methyl-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indol-8-amine (48)**

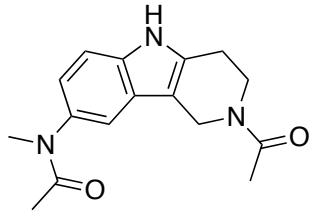
Compound 47 (1.00g, 1.83 mmol) was dissolved in TFA and stirred at room temperature for 1 hour. The solvent was concentrated in vacuo and residual TFA was

azeotroped with THF to afford the title compound (554mg, 1.75 mmol), which was used without further purification. **δ**H (400 MHz, D_2O) 7.28 (m, 1H), 6.83 (m, 1H), 6.73 (m, 1H), 4.30 (s, 2H), 3.58 (m, 2H), 3.09 (m, 2H), 2.71 (s, 3H); LC/MS [M+H] found 202.2, purity 100%



1-(8-(methylamino)-1,3,4,5-tetrahydro-2*H*-pyrido[4,3-*b*]indol-2-yl)ethan-1-one (17)

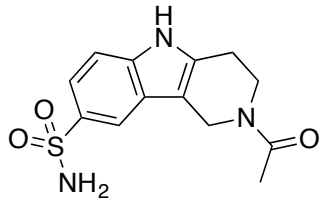
Acetic acid (63µL, 1.00 mmol) was dissolved in $CHCl_3$ (4mL) under Ar. *N*-Hydroxysuccinimide (150mg, 1.30 mmol) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (211mg, 1.10 mmol) were added under Ar and the reaction stirred at room temperature for 3 h. Compound 49 (344mg, 1.09 mmol) was dissolved in $CHCl_3$ (1mL) and added via syringe, DIPEA (716µL, 4.1 mmol) was added via syringe and the reaction stirred for 22 h. The reaction was quenched with H_2O and extracted with $CHCl_3$ three times. The organics were combined, dried over $MgSO_4$, filtered and concentrated in vacuo. The crude residue was purified on silica (Et_3N, MeOH, DCM), followed by trituration with DCM to afford the title compound (115mg, 0.47 mmol) as a white powder in 47% yield. Rf (0.5% Et_3N, 4.5% MeOH, 95% DCM) 0.16; **δ**H (500 MHz, d6-DMSO) (1:1.3 ratio rotamers observed) 10.41 and 10.39 (s, 1H), 7.0 (dd, 1H, J 3, 9.5), 6.42-6.39 (m, 2H), 5.00 (br s, 1H), 4.55 (d, 2H, J11), 3.80 and 3.73 (t, 2H, J 5.5), 2.80 and 2.69 (t, 2H, J 5.5), 2.67 (d, 3H, J 4), 2.11 (s, 3H); **δ**C (125 MHz, d6-DMSO) 169.1, 168.7, 143.5, 132.2, 131.7, 129.2, 129.1, 126.1, 125.9, 111.2, 110.0, 104.9, 104.5, 97.2, 43.5, 43.1, 61.0, 23.8, 22.0, 21.6; HRMS (ESI) found 244.1425 $C_{15}H_{17}N_3O$ [M+H] requires 244.1444; LC/MS [M+H] found 244.4, purity 96%



***N*-(2-acetyl-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indol-8-yl)-*N*-methylacetamide (33)**

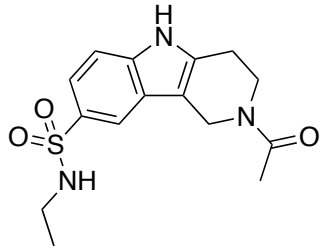
Compound 48 (36mg, 0.18 mmol) was dissolved in THF, Et_3N (51µL, 0.36 mmol) and acetic anhydride (186µL, 0.18 mmol) were added and the reaction stirred at room temperature for 30 min. The reaction was quenched with H_2O and concentrated in vacuo. The residue was diluted with H_2O and extracted with DCM three times. The organics were combined, dried over $MgSO_4$, filtered and concentrated in vacuo. The residue was purified on silica to yield the title compound as a yellow oil. Rf 0.3 3% MeOH/DCM; **δ**H (500 MHz, CD_3OD) (1:1.1 ratio rotamers observed) 7.34-7.30 (m, 2H), 6.94-6.91 (m, 1H), 4.63-4.60 (m, 2H), 3.97 and 3.89 (t, 2H, J 6), 3.29 and 3.28 (s, 3H),

2.95 and 2.86 (t, 2H, J6), 2.24 and 2.23 (s, 3H), 1.85 and 1.83 (s, 3H); **δ**C (125 MHz, d6-DMSO) 172.1, 169.6, 169.2, 168.8, 136.1, 136.0, 134.8, 134.7, 134.2, 125.8, 125.6, 119.9, 119.8, 115.9, 115.8, 111.7, 111.7, 106.6, 106.2, 43.4, 42.8, 38.7, 38.4, 37.3, 37.2, 23.8, 23.0, 22.3, 22.2, 22.0, 21.6, 21.1; HRMS (ESI) found 286.1537, $C_{16}H_{20}N_3O_2$ [M+H] requires 286.1550; LC/MS [M+H] found 286.1, purity 93%



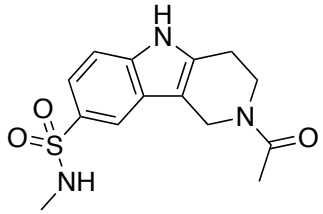
2-acetyl-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indole-8-sulfonamide (18)

The title compound was synthesized from 4-hydrazinobenzenesulfonamide hydrochloride (4.47, 20 mmol) and N-acyl-4-piperidone hydrochloride monohydrate (2.82g, 20 mmol) according to general procedure 1. The crude product was purified by trituration with MeOH. Yield 27% (1.58g, 5.4 mmol). **δ**H (500 MHz, d6-DMSO) (1:1.2 ratio of rotamers observed) 11.38 (br s, 1H), 7.97 and 7.93 (d, 1H, J 2), 7.55-7.51 (m, 1H), 7.45-7.44 and 7.43-7.42 (m, 1H), 7.07 (br s, 2H), 4.71 and 4.67 (s, 2H), 3.85 and 3.80 (t, 2H, J 6), 2.91 and 2.80 (t, 2H, J6), 2.14 and 2.13 (s, 3H); **δ**C (125 MHz, d6-DMSO) 169.2, 168.9, 137.1, 137.0, 135.4, 135.0, 134.6, 134.5, 124.2, 124.0, 118.3, 118.2, 115.8, 115.6, 111.0, 110.9, 107.1, 106.9, 43.2, 42.6, 38.3, 38.3, 23.6, 22.8; HRMS (ESI) found 294.0905, $C_{13}H_{16}N_3O_3S$ [M+H] requires 294.0907 LC/MS [M+H] found 294.4, purity 96%



2-acetyl-*N*-ethyl-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indole-8-sulfonamide (19)

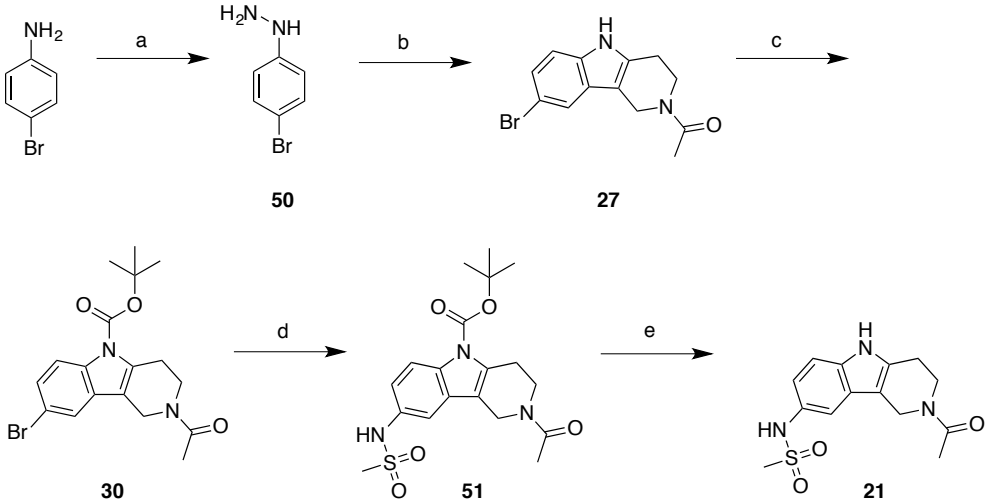
Compound 18 (250mg, 0.85 mmol) and acetaldehyde (100µL, 2.27 mmol) were dissolved in DCE. Sodium triacetoxyborohydride (505mg, 2.38 mmol) was added under N_2. Et_3N was added via syringe and the reaction stirred at room temperature for 5 days. The reaction was quenched with saturated solution of sodium bicarbonate (aq) and extracted with EtOAc three times. The organics were combined, dried over $MgSO_4$, filtered and concentrated in vacuo. The residue was purified by preparatory thin layer chromatography (7% MeOH/DCM) to afford the title compound (49mg, 0.15 mmol) in 18% yield. Rf 0.4 10% MeOH/DCM; **δ**H (500 MHz, $CDCl_3/CD_3OD$) (1:1.3 ratio of rotamers observed) 7.95 and 7.94 (d, 1H, J 1.5), 7.56-7.55 (m, 1H), 7.39-7.37 (m, 1H), 4.78 and 4.72 (s, 2H), 3.96 and 3.84 (t, 2H, J 6), 2.93 and 2.88 (t, 2H, J 6), 2.86 (m, 2H), 2.21 and 2.22 (s, 3H), 1.03-1.01 (m, 3H); **δ**C (125 MHz, $CDCl_3/CD_3OD$) 171.5, 171.1, 138.7, 136.0, 134.6, 130.3, 129.2, 129.1, 125.5, 125.0, 120.1, 120.0, 118.1, 117.9, 112.0, 111.7, 108.0, 107.2, 44.4, 44.0, 39.9, 39.8, 38.4, 38.3, 24.3, 23.5, 22.1, 21.6; HRMS (ESI) found 322.1223, $C_{15}H_{20}N_3O_3S$ [M+H] requires 322.1220; LC/MS [M+H] found 322.0, purity 96%



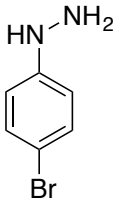
2-acetyl-*N*-methyl-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indole-8-sulfonamide (20)

Compound 18 (50mg, 0.17 mmol) was dissolved in DMF. K_2CO_3 (45mg, 0.34 mmol) was added and the reaction was sealed and evacuated and backfilled with N_2 three times. MeI (21.4µL, 0.34 mmol) was added via syringe and the reaction was stirred at room temperature for 4d. The reaction was diluted with H_2O and extracted with EtOAc three times. The organics were combined, washed with brine, dried over $MgSO_4$, filtered and concentrated in vacuo. The crude residue was purified by HPLC to afford the title compound (12mg, 0.04 mmol) as a white powder in 24% yield. **δ**H (500 MHz, d6-DMSO) (ratio of rotamers 1:1.2) 11.43 (s, 1H), 7.91-7.84 (m, 1H), 7.45-7.44 (m, 2H), 7.10 (br s, 1H), 4.69 and 4.64 (s, 2H), 3.81 and 3.76 (t, 2H, J 5.5), 2.87 and 2.76 (t, 2H, J 5.5), 2.33 (br s, 3H), 2.11 and 2.10 (s, 3H); **δ**C (125 MHz, d6-DMSO) 170.1, 169.7, 137.9, 137.9, 136.1, 135.8, 129.5, 129.4, 125.0, 124.7, 119.6, 119.5, 117.9, 117.4, 111.9, 111.8, 107.7, 107.5, 43.7, 43.1, 38.9, 38.8, 29.3, 29.2, 24.1, 23.3, 22.4, 21.9; HRMS (ESI) found 308.1061, $C_{14}H_{18}N_3O_3$ [M+H] requires 308.1063; LC/MS [M+H] found 308.4, purity 97%

Scheme 4: Synthesis of 8-NHSO$_2$Me analog

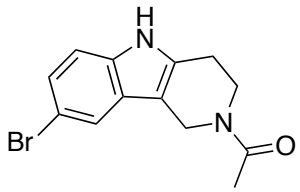


a) $NaNO_3$, $SnCl_2$, HCl (aq) 0-25ºC 3h b) EtOH, reflux, 3h c)Boc_2O, DMAP, THF, rt, 2h d) K_2CO_3, tBuXPhos, $Pd[(allyl)Cl]_2$, 2-Me-THF, 75ºC, 5h, e) K_2CO_3, MeOH, reflux, 3h



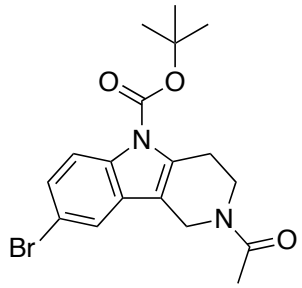
4-bromophenyl)hydrazine[20]

Sodium nitrite (120 mg, 1.74 mmol) was dissolved in H_2O (1.2 ml) and added over a period of 30 minutes to a stirred solution of 4-bromoaniline (300 mg, 1.74 mmol) in concentrated HCl (3.4 ml) and H2O (1.2 ml) at 0°C. The reaction mixture was stirred for a further 30 minutes then added dropwise to a solution of tin chloride hydrate (1.97 g, 8.71 mmol) in concentrated HCl (3 ml) at 0°C. After addition the reaction was stirred at room temperature for 3 hours, cooled to 0°C and filtered, giving the product as the white hydrochloride salt (200 mg, 1.07 mmol) in 61 % yield. Rf 0.48, 5% MeOH/DCM; δH (400 MHz, d6-DMSO) 10.10 (3H, br s), 8.36 (1H, br, NH), 7.50 (2H, d, J 8.9), 6.95 (2H, d, J 8.9).



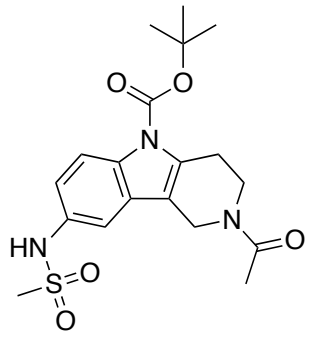
1-(8-bromo-1,3,4,5-tetrahydro-2*H*-pyrido[4,3-*b*]indol-2-yl)ethan-1-one (27)

Compound 27 was synthesised from 4-bromophenylhydrazine hydrochloride (4g, 21.36 mmol) and 1-acetyl-4-piperidone monohydrate hydrochloride (2.64mL, 21.36 mmol) according to general procedure 1. The reaction mixture was concentrated in vacuo and purified on silica (MeOH/DCM) followed by recrystallisation from EtOH to give the title compound as yellow crystals (1.99g, 6.83 mmol) in 32% yield. Rf 0.38 3% MeOH/DCM; **δ**H (400 MHz, d6-DMSO)(1:1.2 ratio of rotamers observed) 11.20 and 11.20 (1H, s), 7.68 and 7.65 (1H, d, J 1.9), 7.30 and 7.29 (1H, d, J 8.5), 7.18 and 7.17 (1H, dd, J 8.6 and 1.9), 4.67 and 4.64 (2H, s), 3.86 and 3.79 (2H, t, J 5.8), 2.90 and 2.78 (2H, t, J 5.7), 2.16 and 2.14 (3H, s); **δ**C (100 MHz, d6-DMSO) 169.5, 169.2, 135.2, 135.0, 134.9 , 134.8, 127.5, 127.3, 123.4, 120.3, 120.1, 113.0, 113.0, 111.6, 111.5, 106.3, 106.1, 43.7 , 38.7, 43.1, 39.0, 24.1, 23.3, 22.4 and 22.0; HRMS(ESI) found 293.0294, $C_{13}H_{15}N_2OBr$ [M+H] requires 293. 0289, LC/MS found 293.0, purity 100%



***tert*-butyl 2-acetyl-8-bromo-1,2,3,4-tetrahydro-5*H*-pyrido[4,3-*b*]indole-5-carboxylate (30)**

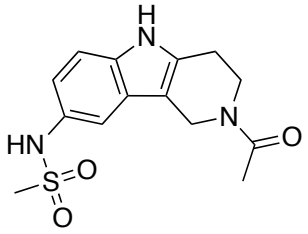
Compound 27 (280mg, 0.95 mmol) was dissolved in THF (3mL). 4-Dimethylaminopyridine (187mg, 1.53 mmol) and di-*tert*-butyl dicarbonate (445.2 mg, 2.04 mmol) was added. The reaction was stirred at room temperature for 1h. The reaction was treated with dilute HCl (aq) and extracted with DCM three times. The organics were combined, dried over $MgSO_4$, filtered and concentrated in vacuo to afford the title compound (327mg, 0.83 mmol) as a white powder in 87% yield. **δ**H (400 MHz, CD_3OD) (1:1.4 ratio of rotamers observed) 8.05 and 8.03 (d, 1H, J 6), 7.65 and 7.59 (d, 1H, J 1.6), 7.38-7.37 (m, 1H), 4.67 and 4.68 (s, 2H), 3.92 and 3.86 (t, 2H, J 6), 3.20 and 3.11 (t, 2H, J6), 2.24 and 2.22 (s, 2H), 1.68 and 1.67 (s, 9H); **δ**C (100 MHz, CD_3OD) 162.9, 162.7, 1411.7, 127.0, 126.6, 126.2, 121.0,120.7,118.3, 111.9, 111.8, 108.6, 107.6, 104.6, 104.3, 76.2, 70.1, 69.8, 69.4, 35.6, 34.5, 31.0, 30.4, 21.7, 19.0, 18.6, 18.4, 17.7, 12.4, 12.0; HRMS (ESI) found 393.0814 , $C_{18}H_{22}N_2O_3Br$ requires 393.0808. LC/MS found 394.9, purity 100%



***tert*-butyl 2-acetyl-8-(methylsulfonamido)-1,2,3,4-tetrahydro-5*H*-pyrido[4,3-*b*]indole-5-carboxylate (51)**

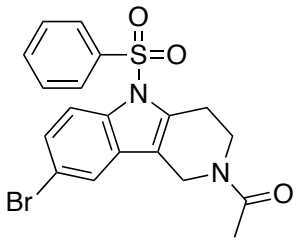
A round-bottomed flask was charged with compound 50 (260mg, 0.66 mmol), K_2CO_3 (210mg, 1.52 mmol), tBu_2XPhos (6.4mg, 0.015 mmol), $[Pd(allyl)Cl]_2$ (1.8mg, 0.005 mmol) and a stirbar. The flask was sealed with a subaseal and evacuated and backfilled with N_2 three times. 2-Methyltetrahydrofuran (3mL) was added and the reaction heated at 75°C for 5h. The reaction was cooled to room temperature, diluted with saturated ammonium chloride solution (aq) and extracted with EtOAc three times. The organics were combined, washed with brine, dried over $MgSO_4$, filtered and concentrated in vacuo. The crude residue was purified by preparatory thin layer chromatography to yield the title compound (119mg, 0.29 mmol) as a yellow solid in 44% yield. **δ**H (500 MHz, CD_3OD) (1:1.4 ratio rotamers observed) 8.07 and 8.09 (d, 1H, J 8.5), 7.37 and 7.31

(d, 1H, J 2), 7.17 and 7.15 (dd, 1H, J 8.5, 2), 4.67-4.68 (m, 2H), 3.90 and 3.84 (t, 2H, J 6), 3.18 and 3.08 (t, 2H, J 6), 2.92 and 2.91 (s, 3H), 2.22 (s, 3H), 1.67 and 1.66 (s, 9H); **δ**C (125 MHz, CD_3OD) 172.5, 172.3, 151.4, 151.3, 136.3, 136.5, 135.0, 134.9, 134.6, 134.5, 129.4, 129.2, 119.6, 119.5, 117.2, 114.6, 114.3, 111.8, 111.5, 85.5, 85.4, 45.2, 44.0, 40.5, 39.9, 38.9, 38.8, 28.5, 28.4, 27.9, 27.8, 21.8, 21.6, 21.4; HRMS (ESI) found 408.1585, $C_{19}H_{26}N_3O_5S$ [M+H] requires 408.1588; LC/MS found 408.0, purity 100%



_N_-(2-acetyl-2,3,4,5-tetrahydro-1_H_-pyrido[4,3-_b_]indol-8-yl)methanesulfonamide (21)

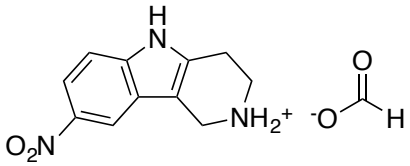
Compound 51 (70mg, 0.17 mmol) was dissolved in MeOH (1mL) and K_2CO_3 (76mg, 0.55 mmol) added. The reaction was refluxed for 3 hours, cooled to room temperature and concentrated in vacuo. The residue was diluted with brine and extracted with EtOAc three times. The organics were combined, dried over $MgSO_4$, filtered and concentrated in vacuo. The crude product was purified on silica (MeOH/DCM) to afford the title compound (57mg, 0.19 mmol) as a white solid in 94% yield. Rf (7.5% MeOH/DCM) 0.33; **δ**H (500 MHz, CD_3OD) (1:1.2 ratio rotamers observed) 7.37 and 7.32 (d, 1H, J 2.0) 7.29 and 7.27 (d, 1H, J 2.8), 7.05-7.00 (m, 1H), 4.72-4.73 (m, 2H), 3.96 and 3.88 (t, 2H, J 6.0), 2.92 and 2.83 (t, 2H, J 6.0), 2.89 and 2.88 (s, 3H), 2.23 and 2.22 (s, 3H); **δ**C (125 MHz, CD_3OD) 171.2, 170.9, 134.7, 134.7, 134.0, 133.1, 129.1, 129.1, 125.9, 125.6, 117.4, 117.4, 111.9, 111.7, 111.0, 110.9, 105.7, 105.4, 53.4, 43.8, 43.3, 39.2, 39.1, 37.0, 36.9, 23.4, 22.6, 20.5, 20.1; HRMS (ESI) found 308.1069, $C_{14}H_{18}N_3O_3S$ [M+H] requires 308.1063; LC/MS [M+H] found 308.0, purity 94%



1-(8-bromo-5-tosyl-1,3,4,5-tetrahydro-2_H_-pyrido[4,3-_b_]indol-2-yl)ethan-1-one (29)

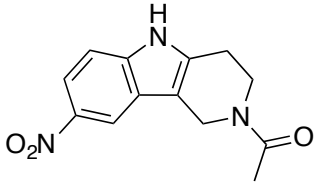
Compound 27 (300mg, 0.87mmol) was dissolved in DMF (2mL) and a 60% dispersion of NaH in mineral oil (16.1mg, 0.67mmol) was added under N_2. Once H_2 gas evolution had ceased the reaction mix was stirred for a further 10min followed by addition of benzenesulfonylchloride (0.12mL, 0.90mmol). The reaction was poured into saturated aqueous solution of ammonium chloride and extracted with EtOAc x 3. The organics were combined, washed with brine, treated with active charcoal and filtered through celite. The filtrate was dried over $MgSO_4$, filtered, concentrated in vacuo and

purified on silica (DCM/MeOH) to afford the title compound (176mg, 0.41mmol) as a white solid in 47% yield. **δ**H (400 MHz, d6-DMSO) 8.07-8.02 (m, 1H), 7.98-7.94 (m, 2H), 7.86-7.85 (m, 1H), 7.79-7.75 (m, 1H), 7.68-7.63 (m, 2H), 7.57-7.52 (m, 1H), 4.67 and 4.61 (s, 2H), 3.88 and 3.83 (t, 2H, J 5.6), 3.27-3.23 and 3.15-3.11 (m, 2H), 2.20 and 2.14 (s, 3H); **δ**C (125 MHz, d6-DMSO) 169.5, 169.3, 137.6, 137.6, 135.4, 135.3, 135.3, 1135.2, 134.6, 134.5, 130.5, 130.5, 130.0, 129.8, 127.6, 127.5, 126.9, 126.9, 122.0, 121.9, 117.0, 116.8, 116.2, 116.1, 116.0, 115.8, 43.4, 43.4, 42.4, 42.3, 38.5, 38.5, 25.7, 24.8, 22.3, 21.9; HRMS found 433.0238, $C_{19}H_{17}N_2O_3SBr$ [M+H] requires 433.0216; LC/MS [M+H] found 434.8, purity 96%



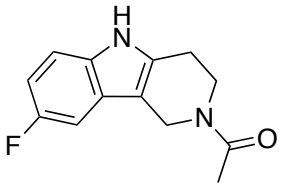
8-nitro-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indole (52)

4-nitrophenylhydrazine (200 mg, 1.31 mmol) and 4-piperidone monohydrate hydrochloride (201 mg, 1.31 mmol) were dissolved in formic acid (1.3 ml) and heated to 120°C in a sealed microwave tube for 6 hours. After cooling to room temperature 1,4-dioxane (2.5 ml) was added and after 12 hours the dark brown precipitate was collected, giving the product as the formic acid salt (20 mg, 0.08 mmol) in 6 % yield. **δ**H (500 MHz, d6-DMSO) 12.10 (1H, s, NH), 9.61 (2H, br), 8.61 (1H, d, J 2.3), 8.04 (1H, dd, J 9.0, 2.3), 7.57 (1H, d, J 9.1), 4.43 (2H, t, J 4.3), 3.51-3.49 (2H, m), 3.11 (2H, t, J 5.9); **δ**C (125 MHz, d6-DMSO) 140.7, 139.2, 135.0, 124.4, 117.0, 115.2, 111.6, 104.8, 40.6, 39.9, 20.2; HRMS(ESI) found 218.0935, $C_{11}H_{13}N_3O_2$ [M+H] requires 218.0930, LC/MS [M+H] found 218.0, purity 100%



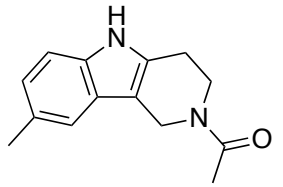
1-(8-nitro-1,3,4,5-tetrahydro-2*H*-pyrido[4,3-*b*]indol-2-yl)ethan-1-one (22)

Compound 52 (81 mg, 0.31) was dissolved in DCM (2mL) and acetic anhydride (0.7mL, 6.2 mmol) was added. The reaction was stirred at room temperature for 1h. The reaction was diluted with a saturated solution of sodium bicarbonate and extracted with DCM three times. The organics were combined, washed with brine, dried over $MgSO_4$, filtered and concentrated in vacuo. The crude residue was recrystallized from EtOH two times to yield the title compound (6.5mg, 0.03 mmol) in 10% yield. **δ**H (400 MHz, d6-DMSO) 11.74 (s, 1H), 8.50 and 8.45 (d, 1H, J 2.4), 7.99-7.95 (m, 1H), 7.49-7.46 (m, 1H), 4.75 and 4.70 (s, 2H), 3.84 and 3.78 (t, 2H, J 6), 2.90 and 2.78 (t, 2H, J 6), 2.13 and 2.12 (s, 3H); **δ**C (100 MHz, d6-DMSO) 169.2, 169.0, 140.4, 140.3, 139.3, 139.2, 137.3, 136.9, 124.5, 124.7, 116.3, 116.3, 114.8, 114.6, 111.3, 111.3, 108.9, 108.8, 42.6, 43.2, 38.5, 38.3, 23.7, 22.9, 22.1, 21.6; HRMS (ESI) found 260.1034, $C_{13}H_{14}N_3O_3$ [M+H] requires 260.1030; LC/MS [M+H] found 260.0, purity 90%



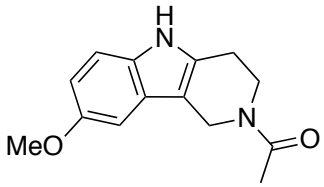
1-(8-fluoro-1,3,4,5-tetrahydro-2*H*-pyrido[4,3-*b*]indol-2-yl)ethan-1-one (23)

4-Fluoro phenylhydrazine (250mg, 1.54 mmol) and 1-acetyl-4-piperidone (0.19mL, 1.54 mmol) were suspended in a 4N solution of HCl in dioxane (3mL) and heated at reflux for 16 h. The reaction was cooled to room temperature, concentrated in vacuo and the crude residue purified on silica (MeOH/DCM) followed by trituration with petroleum ether (boiling point 40-60°C) to afford the title compound (35mg, 0.15 mol) as a white solid in 10% yield. **δ**H (400 MHz, $CDCl_3$) (1:1.2 ratio rotamers observed) 7.88-7.83 (m, 1H), 7.26-7.07 (m, 1H), 6.94-6.89 (m, 1H), 4.77 and 4.63 (s, 2H), 4.00 and 3.82 (t, 2H, J 5.6), 2.90 and 2.85 (t, 2H, J 5.6), 2.22 (s, 3H); HRMS (ESI) found 233.1113, $C_{13}H_{14}N_2OF$ [M+H] requires 233.1085; LC/MS [M+H] found 215.3, purity 100%



1-(8-methyl-1,3,4,5-tetrahydro-2*H*-pyrido[4,3-*b*]indol-2-yl)ethan-1-one (24)

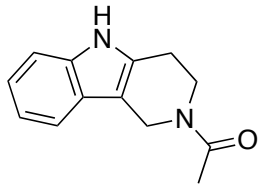
Compound 24 was synthesised from p-tolylhydrazine hydrochloride (400 mg, 2.52 mmol) and 1-acetyl-4-piperidone (0.31 ml, 2.52 mmol) according to general proceedure 1. The reaction mixture was concentrated in vacuo and purified by flash column chromatography (5% MeOH/DCM) to afford the title compound as a pale yellow solid (400 mg, 1.75 mmol) in 67% yield. Rf 0.3 5%MeOH/DCM; **δ**H (500 MHz, d6-DMSO) (1:1.3 ratio of rotamers observed) 10.76 and 10.74 (1H), 7.2 – 7.16 (2H, m), 6.86 (1H, dd, J 8.4 and 1.0), 4.61 and 4.59 (2H, s), 3.82 and 3.75 (2H, t, J 5.7), 2.84 and 2.73 (2H, t, J 5.5), 2.36 and 2.35 (3H, s) 2.12 and 2.11 (3H, s); **δ**C (125 MHz, d6-DMSO) 169.1, 168.7, 134.3, 134.2, 132.9, 132.4, 126.9, 126.9, 125.5, 125.3, 122.1, 117.0, 116.9, 110.6, 105.4, 105.1, 43.3, 38.9, 43.0, 39.2, 23.7, 22.8, 21.9, 21.5, 21.2; HRMS(ESI) found 229.1339, $C_{14}H_{17}N_2O$ [M+H] requires 229.1341; LC/MS [M+H] found 229.0, purity 100%



1-(8-methoxy-1,3,4,5-tetrahydro-2*H*-pyrido[4,3-*b*]indol-2-yl)ethan-1-one (25)

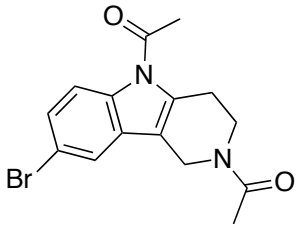
Compound 11 was synthesised from 4-methoxyphenylhydrazine hydrochloride (300 mg, 1.72 mmol) and 1-acetyl-4-piperidone (0.21 ml, 1.72 mmol) according to the general preceedure 1. The yellow precipitate obtained was recrystallised from EtOH to give the title compound as pale yellow powder (273 mg, 1.12 mmol) in 32% yield. δH

(400 MHz, d6-DMSO) (1:1.3 ratio of rotamers observed) 10.78 and 10.75 (1H, s), 7.21 and 7.20 (1H, d, J 8.7), 6.98 and 6.96 (1H, d, 2.4), 6.71 and 6.70 (1H, dd, J 8.7, 2.1), 4.65 and 4.63 (2H, s), 3.85 and 3.79 (2H, t, J 5.8), 3.79 and 3.78 (3H, s), 2.87 and 2.76 (2H, t, J 5.6), 2.16 and 2.15 (3H, s); δC (100 MHz, d6-DMSO) 169.5 and 169.2, 153.5, 134.0 and 133.5, 131.4 and 131.3, 126.1 and 125.8, 112.0 and 111.9, 110.8 and 110.7, 106.2 and 106.0, 100.0 and 99.9, 55.8 and 55.7, 43.9 and 39.3, 43.5 and 38.9, 24.2 and 23.4, 22.5 and 22.0; HRMS(ESI) found 245.1293, $C_{14}H_{18}N_2O_2$ [M+H] requires 245.1290



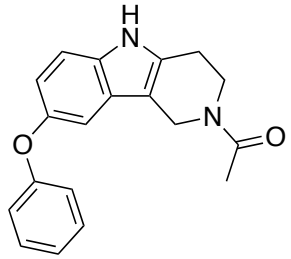
1-(1,3,4,5-tetrahydro-2*H*-pyrido[4,3-*b*]indol-2-yl)ethan-1-one (26)

Compound 26 was synthesised from hydrazine hydrochloride (300 mg, 2.07 mmol) and 1-acetyl-4-piperidone (0.29 ml, 2.07mmol) according to general procedure 1. The precipitate was washed with 50:50 H2O/EtOH and filtered to give the title compound as a cream solid (259 mg, 1.2 mmol) in 58% yield. **δ**H (400 MHz, d6-DMSO) (1:1.4 ratio of rotamers observed) 10.91 and 10.90 (1H, s, NH), 7.42 – 7.39 (1H, m), 7.30 – 7.28 (1H, m), 7.06 – 7.01 (1H, m), 6.99 – 6.93 (1H, m), 4.64 and 4.62 (2H, s), 3.83 and 3.76 (2H, t, J 5.7), 2.86 and 2.75 (2H, t, J 5.6), 2.13 and 2.12 (3H, s); **δ**C (100 MHz, d6- DMSO) 169.1, 168.8, 135.9, 135.8, 132.9, 132.4, 125.3, 125.1, 120.7, 120.6, 118.5, 117.3, 117.2, 111.0, 110.9, 105.9, 105.6, 43.4, 38.5, 42.9, 38.8, 23.7, 22.9, 22.0, 21.6; HRMS (ESI) found 215.1167, $C_{13}H_{15}N_2O$ [M+H] requires 215.1179; LRMS found 215.18, purity 100%



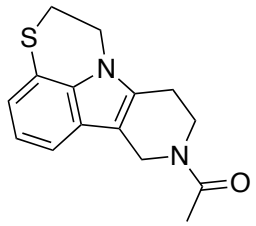
1,1'-(8-bromo-3,4-dihydro-1*H*-pyrido[4,3-*b*]indole-2,5-diyl)bis(ethan-1-one) (28)

Compound 27 (300mg, 0.97 mmol) and DMAP (22mg, 0.18 mmol) were dissolved in DCE (3mL). Et_3N (533µL, 3.80 mmol) and acetic anhydride (140µL, 1.46 mmol) were added and the reaction refluxed for 24 h. The reaction was cooled to room temperature, EtOAc (3mL) and saturated ammonium chloride solution (10mL) were added. The mixture was extracted with EtOAc three times. The organics were combined, washed with dilute HCl, dried over $MgSO_4$, filtered and concentrated in vacuo. The crude residue was purified on silica (EtOAc) and triturated with MeOH to afford the title compound (180mg, 0.54 mmol) as a white solid in 56% yield. Rf 0.25 EtOAc; **δ**H (500 MHz, $CDCl_3$) 8.00-7.81 (M, 1h), 7.52 (S, 1H), 7.40-7.38 (m, 1H), 4.69-4.59 (m, 2H), 3.93-3.76 (m, 2H), 3.19-3.11 (m, 2H), 2.80 (s, 3H), 2.22 (s, 3H); **δ**C (125 MHz, $CDCl_3$) 169.6, 169.3, 134.5, 134.1, 127.3, 121.0, 120.2, 116.8, 43.9, 42.8, 38.8, 27.6, 27.0, 21.9, 21.6; HRMS (ESI) found 335.0405, $C_{15}H_{16}N_2O_2Br$ [M+H] requires 335.0390; LC/MS found 337.3, purity 94%



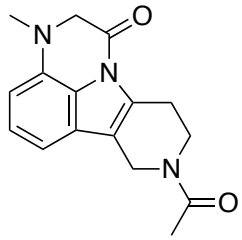
1-(8-phenoxy-1,3,4,5-tetrahydro-2*H*-pyrido[4,3-*b*]indol-2-yl)ethan-1-one (36)

The title compound was synthesized from 4-phenoxyphenylhydrazine (312mg, 1.32 mmol) and 1-acetyl 4-piperidone (162µL, 1.32 mmol) according to general procedure 1. The crude residue was purified on silica (MeOH/DCM) to yield the title compound (378mg, 1.24 mmol) in 93% yield. Rf 0.43 (5%MeOH/DCM); **δ**H (400 MHz, d6-DMSO) (1:1.2) 10.99 and 10.97 (s, 1H), 7.34-7.29 (m, 3H), 7.13 and 7.10 (d, 1H, J 2.4), 7.04-7.00 (m, 1H), 6.92-6.88 (m, 2H), 6.82-6.76 (m, 1H), 4.60 and 4.57 (s, 2H), 3.83 and 3.77 (t, 2H, J 5.6), 2.87 and 2.75 (t, 2H, J 5.6), 2.12 and 2.09 (s, 3H); **δ**C (125 MHz, d6- DMSO) 169.3, 168.9, 159.3, 159.3, 148.6, 134.7, 134.2, 133.1, 133.0, 129.8, 126.0, 125.8, 122.0, 116.9, 116.9, 114.1, 114.1, 112.1, 108.3, 108.2, 106.3, 106.0, 48.7, 43.5, 43.0, 23.9, 23.0, 22.0, 21.6; HRMS (ESI) found 307.1429, $C_{19}H_{19}N_2O_2$ [M+H] requires 307.1441; LC/MS found 307.0, purity 98%.



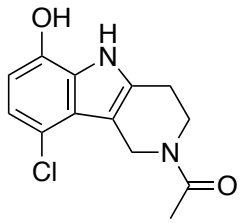
1-(1,2,9,10-tetrahydropyrido[4,3-*b*][1,4]thiazino[2,3,4-*hi*]indol-8(7*H*)-yl)ethan-1-one (31)

This compound was a gift from DuPont and was used as supplied without further characterization.



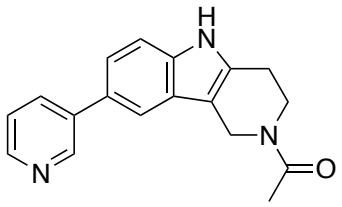
8-acetyl-3-methyl-2,3,7,8,9,10-hexahydro-1*H*-pyrido[3',4':4,5]pyrrolo[1,2,3-*de*]quinoxalin-1-one (32)

This compound was a gift from DuPont and was used as supplied without further characterization.



1-(9-chloro-6-hydroxy-1,3,4,5-tetrahydro-2*H*-pyrido[4,3-*b*]indol-2-yl)ethan-1-one (33)

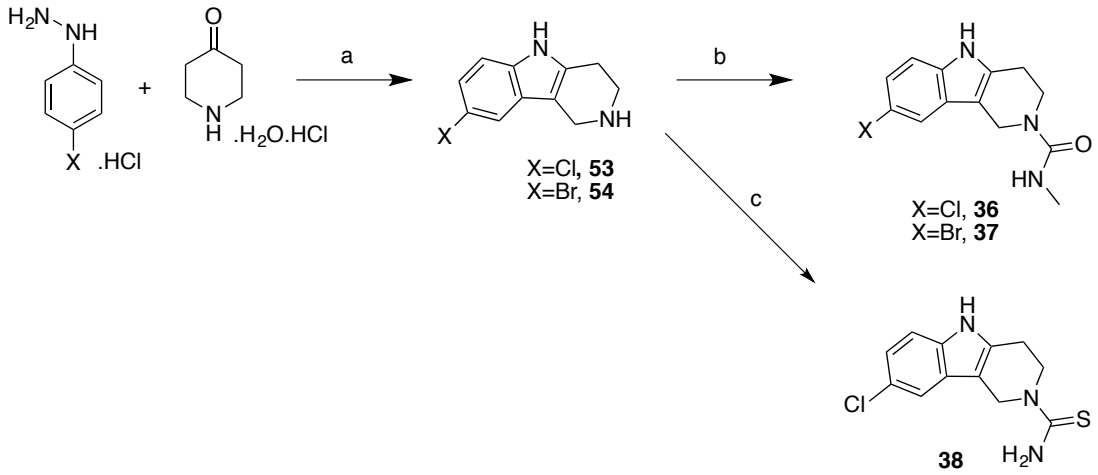
This compound was a gift from DuPont and was used as supplied without further characterization.



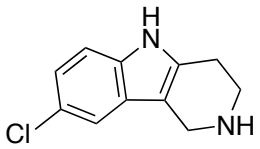
1-(8-(pyridin-3-yl)-1,3,4,5-tetrahydro-2*H*-pyrido[4,3-*b*]indol-2-yl)ethan-1-one (35)

Compound 27 (50 mg, 0.17 mmol) and 3-pyridinylboronic acid (23 mg, 0.19 mmol) were placed in a microwave vial to which DME (1.5 ml) and K2CO3 (71 mg, 5.1 mmol) in H2O (0.75 ml) were added. The solution was purged with N_2 for 5 min then Pd(dppf)Cl_2.CH_2Cl_2 (0.1 eq by weight) was added and the system purged again. The tube was sealed and heated in the microwave at 120 °C for 30 min. After cooling to room temperature the solution was concentrated in vacuo and purified by column chromatography (2% - 10% MeOH/DCM) to give the desired product as a thick yellow oil (35 mg, 0.12 mmol) in 71 % yield. Rf 0.29, 5% MeOH/DCM δ**H** (500 MHz, CDCl3) (amide rotamers) 9.01 – 8.84 (2H, m), 8.53 (1H, m), 7.91 and 7.88 (1H, d, J 7.7), 7.61 (1H, m), 7.42 – 7.33 (2H, m), 4.85 and 4.72 (2H, s), 4.00 and 3.81 (2H, t, J 5.0), 2.91 and 2.85 (2H, t, J 5.0), 2.23 and 2.21 (3H, s); δ**C** (125 MHz, CDCl3) 170.2, 169.8, 148.4, 148.3, 147.5, 147.4, 138.0, 136.1, 134.7, 134.6, 134.2, 132.2, 129.6, 129.5, 126.5, 126.0, 123.8, 123.7, 121.2, 121.2, 116.6, 116.1, 111.8, 111.5, 108.0, 106.8, 44.0, 39.3, 43.8, 39.6, 24.4, 23.4, 22.3, 21.9; HRMS (ESI) found 292.1436, $C_{19}H_{17}N_3O$ requires 292.1444; LCMS found 292.2, purity 95%

Scheme 5: Synthesis of N2 substituted analogs

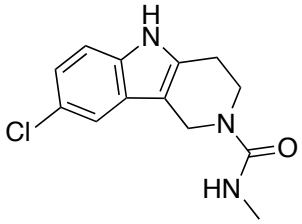


a) EtOH, reflux, 3h b) $MeNH_4Cl$, 4-nitrophenylchloroformate, MeCN, Et_3N c) Fmoc-NCS, DCM, piperidine.



8-chloro-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indole (53) [21]

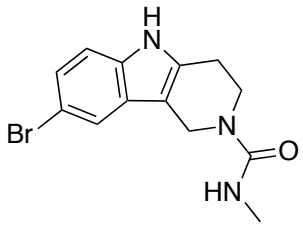
Intermediate 53 was synthesized from 4-chlorohydrazine hydrochloride (200 mg, 1.12 mmol) and 4- piperidone monohydrate hydrochloride (172 mg, 1.12 mmol) were reacted according to general procedure 1. The precipitate obtained was recrystallised from EtOH to give the title compound 19 as a pale yellow powder (44 mg, 2.13 mmol) in 19 % yield, which was used without purification. **δ**H (500 MHz, d6-DMSO) 10.92 (1H, s), 7.34 (1H, d, J 1.9), 7.25 (1H, d, J 8.5), 6.97 (1H, dd, J 8.5, 2.1), 3.80 (2H, s), 2.99 (2H, t, J 5.7), 2.65 (2H, t, J 2.65), 2.34 (br s, 1H); HRMS (ESI) found 207.0690, $C_{11}H_{12}N_2{}^{35}Cl$ [M+H] requires 207.0689; LC/MS [M+H] found 207.3,s purity 90%



8-chloro-*N*-methyl-1,3,4,5-tetrahydro-2*H*-pyrido[4,3-*b*]indole-2-carboxamide (40)

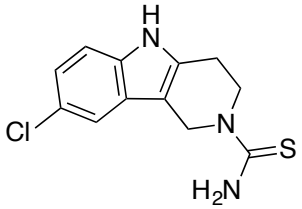
Methylamine hydrochloride (34mg, 0.53 mmol), sodium bicarbonate (110mg, 1.03 mmol) and 4-nitrophenyl chloroformate (83mg, 0.42 mmol) were stirred in MeCN (2mL) at room temperature for 3 h. Compound 53 (85mg, 0.41 mmol) and Et_3N (145µL, 1.03 mmol) were added and the reaction stirred at room temperature for 24 h. The

reaction was diluted with water and extracted three times with ethyl acetate. The organics were combined, dried over $MgSO_4$, filtered and concentrated in vacuo. The crude residue was purified by preparatory thin layer chromatography (8% MeOH in DCM) to afford the title compound (20.5mg, 0.08 mmol) as an orange solid in 20% yield. Rf 0.3 (8% MeOH/DCM); **δ**H (500 MHz, d6-DMSO) (1:0.1 ratio rotamers observed, major rotamer only reported) 7.34 (d, 1H, J 8.5), 7.32 (dd, 1H, J 0.5, 2.0), 6.97 (dd, 1H, J 2.0, 8.5), 6.55 (q, 1H, J 4.5), 4.46 (s, 2H), 3.66 (t, 2H, J 5.5), 2.73 (t, 2H, J 5.5), 2.60 (d, 3H, J 4.5); **δ**C (125 MHz, d6-DMSO) 158.3, 136.3, 134.3, 118.8, 118.3, 110.7, 106.7, 55.0, 40.7, 40.0, 29.0, 27.3, 23.1; HRMS (ESI) found 264.0923, $C_{13}H_{15}N_3O^{35}Cl$ [M+H] requires 264.0898, LC/MS found 264.4, purity 97%



8-bromo-*N*-methyl-1,3,4,5-tetrahydro-2*H*-pyrido[4,3-*b*]indole-2-carboxamide (41)

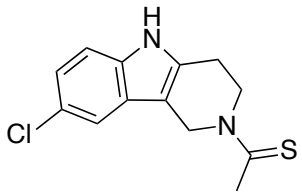
$MeNH_3Cl$ (169mg, 2.5 mmol), sodium carbonate (742mg, 7.0 mmol) and 4-nitrophenylchloroformate (523mg, 2.6 mmol) were suspended in MeCN and stirred at room temperature under N_2 for 5h. Compound 54 (500mg, 2.0mmol) and Et_3N (421µL, 3.0 mmol) were added and the reaction stirred for 48 h. The reaction was diluted with dilute aqueous HCl and extracted with DCM three times. The organics were combined, dried over $MgSO_4$, filtered and concentrated in vacuo. The crude residue was purified on silica (DCM/MeOH) to afford the title compound (46mg, 0.15 mmol) as a pale yellow solid in 8% yield. **δ**H (500 MHz, d6-DMSO) 11.08 (s, 1H), 7.50 (d, 1H, J 2.0), 7.25 (d, 1H, J 9.0), 7.13 (dd, 1H, J 9.0, 2.0), 6.52 (q, 1H, J 4.5), 4.46 (s, 2H), 3.67 (t, 2H, J 5.5), 2.74 (t, 2H, J 5.5), 2.60 (d, 3H, J 4.5); **δ**C (125 MHz, d6-DMSO) 158.2, 134.9, 134.5, 127.0, 122.8, 119.4, 112.9, 110.9, 106.2, 40.7, 40.6, 27.2, 23.1; HRMS (ESI) found 308.0378, $C_{13}H_{15}N_3OBr$ [M+H] requires 308.0393; LC/MS [M+H] found 308.1, purity 98%



8-chloro-*N*-methyl-1,3,4,5-tetrahydro-2*H*-pyrido[4,3-*b*]indole-2-carbothioamide (36)

Compound 53 (290mg, 1.40 mmol) was dissolved in DCM (2mL). Fmoc isothiocyanate (640mg, 2.28 mmol) was dissolved in DCM (2mL) and added under N_2. The reaction was stirred at room temperature for 2.5 h. Piperidine (1.15mL, 11.4 mmol) was added via syringe and the reaction stirred at room temperature for 2 h. The reaction was diluted with H_2O and extracted with DCM. The organics were combined, washed with dilute HCl (aq), dried over $MgSO_4$, filtered and concentrated in vacuo. The crude residue was purified by preparatory thin layer chromatography (5% MeOH/DCM)

to afford the title compound as a white solid (17mg, 0.06 mmol) in 5% yield. Rf 0.3 5% MeOH/DCM. **δ**H (500 MHz, $CDCL_3$) 7.21 (br s, 1H), 7.20 (br, 1H), 7.19 (br s, 1H), 4.73 (br s, 2H), 4.18 (br s, 2H), 2.81 (t, 2H, J 5.2); **δ**C (125 MHz, $CDCL_3$) 181.3, 136.9, 133.4, 127.2, 123.9, 122.0, 119.8, 119.5, 118.0, 111.1, 109.8, 105.4, 60.75, 29.8, 23.1, 22.7, 21.0; HRMS (ESI) found 266.0547, $C_{12}H_{13}N_3S^{35}Cl$ [M+H] requires 266.0513; LC/MS [M+H] found 266.4, 90% purity.



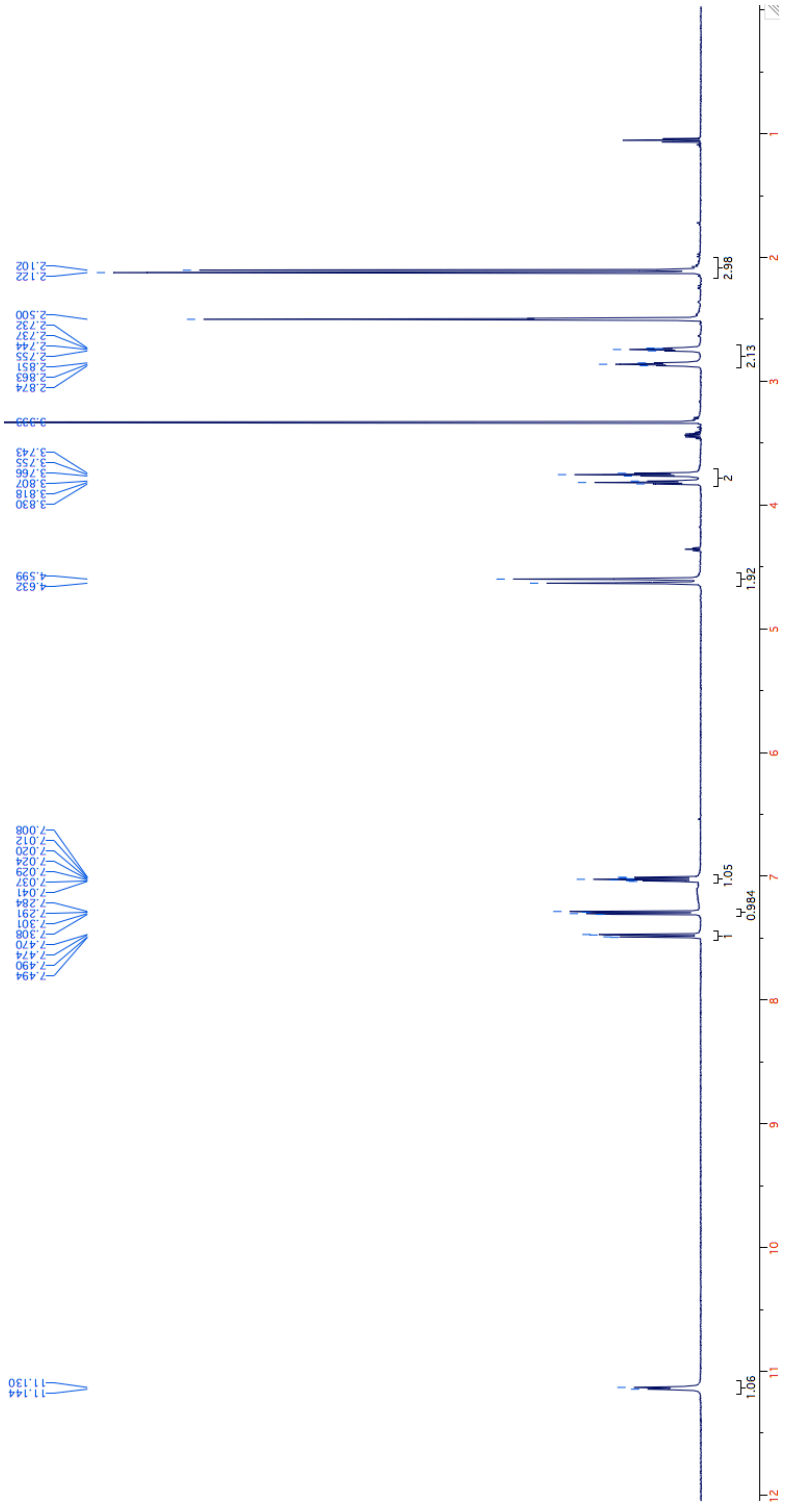
1-(8-chloro-1,3,4,5-tetrahydro-2*H*-pyrido[4,3-*b*]indol-2-yl)ethane-1-thione (37)

Compound 6 (300mg, 1.20 mmol) was suspended in toluene (5mL) and heated at reflux. Lawessons reagent (360mg, 0.9 mmol) was added portion-wise and the reaction refluxed for 1 h. The reaction was cooled to room temperature and the solvent concentrated in vacuo. The residue was diluted with H_2O and extracted with DCM three times. The crude residue was purified on silica (DCM) to afford the title compound (173mg, 0.66 mmol) as a yellow powder in 55% yield. Rf 0.25 DCM; **δ**H (500 MHz,$CDCl_3$) 7.35 (m, 1H), 7.18 (m,1H), 7.04 (m, 1H), 5.33 and 4.84 (t, 2H, J 5.5), 4.62 and 4.03 (t, 2H, J 5.5), 3.38 and 2.94 (m, 2H), 2.72 and 2.71 (s, 3H); **δ**C (125 MHz,$CDCl_3$) 199.7, 199.2, 134.6, 134.3, 131.9, 126.4, 125.9, 125.1, 125.1, 121.8, 121.7, 117.3, 116.6, 112.1, 111.8, 106.2, 104.7, 47.9, 47.7, 47.4, 47.3, 32.8, 32.2, 23.8, 22.4; HRMS found 265.0559, $C_{13}H_{14}N_2S^{35}Cl$ [M+H] requires 265.0561; LC/MS [M+H] found 265.0, purity 95%

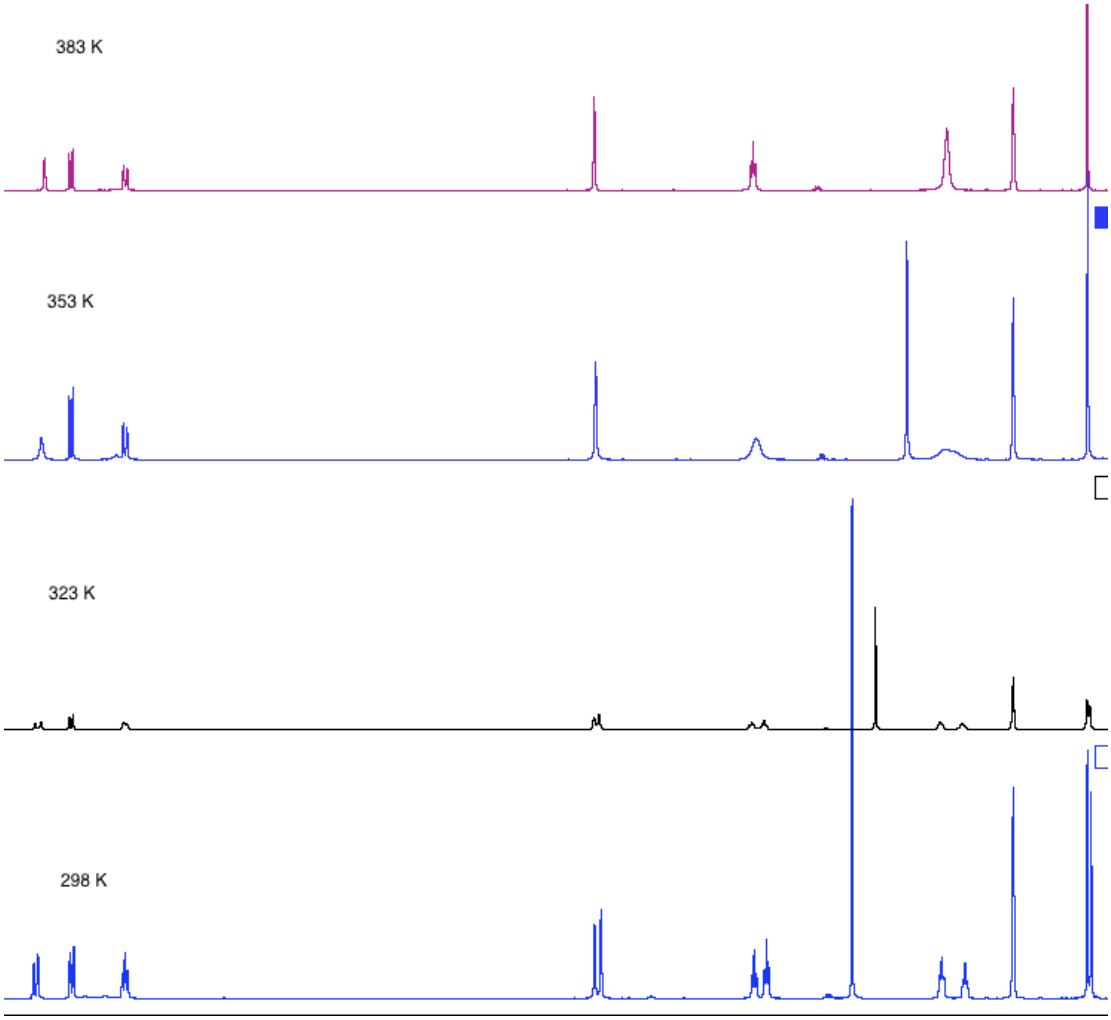
Selected Spectra

8-chloro-*N*-methyl-1,3,4,5-tetrahydro-2*H*-pyrido[4,3-*b*]indole-2-carbothioamide (6)

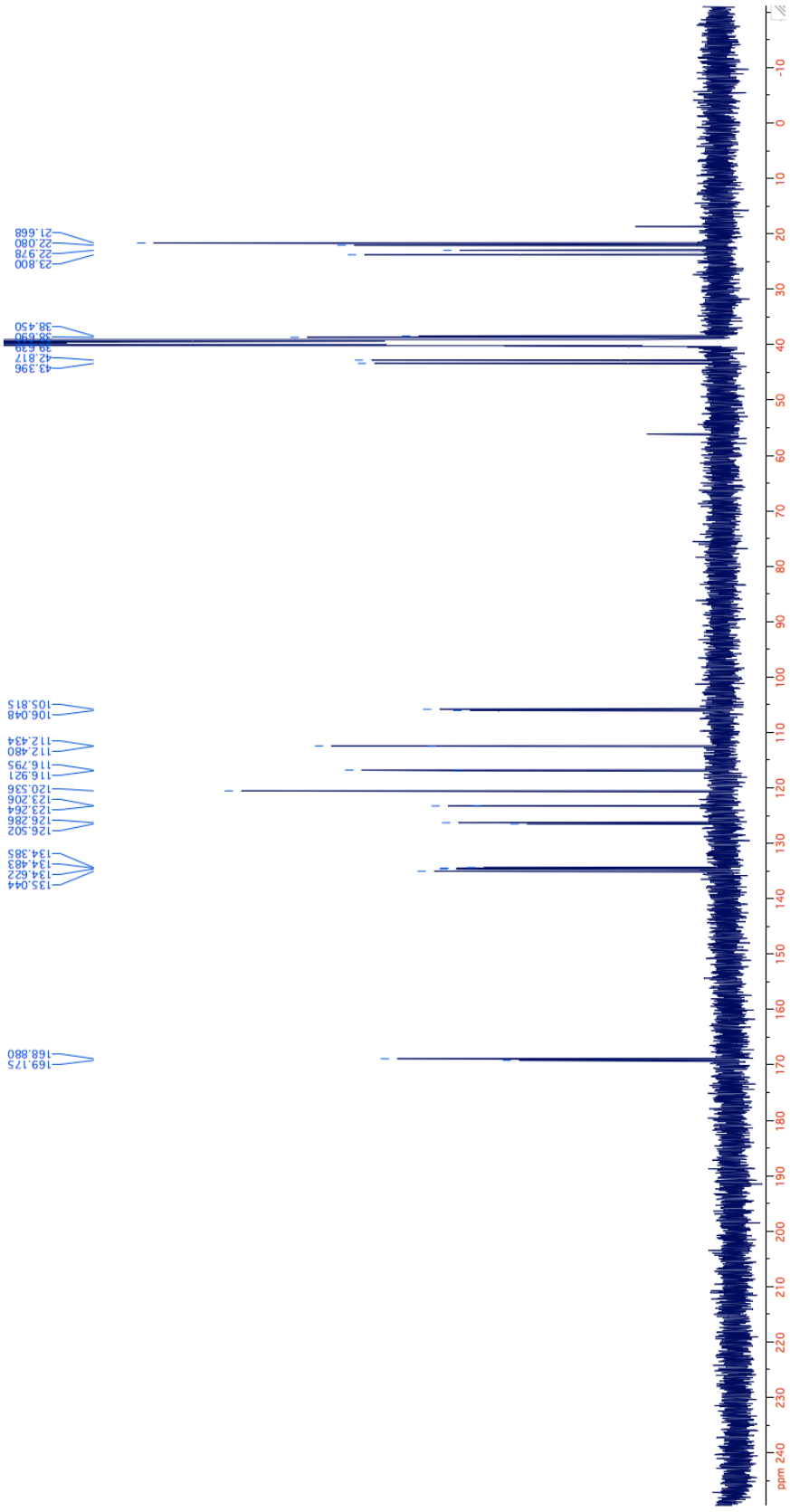
^{1}H NMR



VT-^{1}H NMR



^{13}C NMR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116.921
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123.206
123.264
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126.502
134.385
134.483
134.622
135.044
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169.175
-10
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220
230
ppm 240

LC/MS trace

Openlynx Report Open Access LC-MS Report A Ciulli Page 1

Sample: 1 Vial Number:4:42 ID:A Ciulli677-1
File Name:A Ciulli677-1 Date:22-Oct-2012 Time:08:54:20
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Printed: Mon Oct 22 09:13:27 2012

Sample Report:

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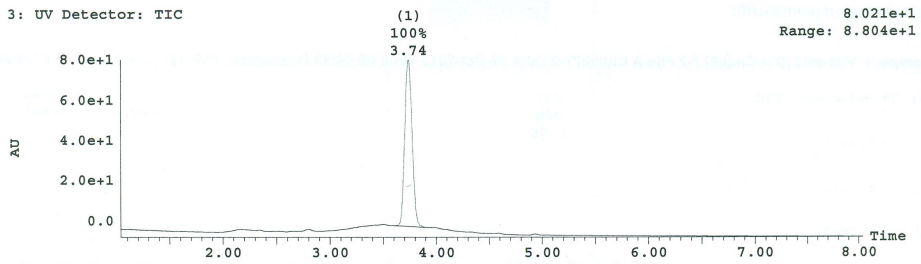

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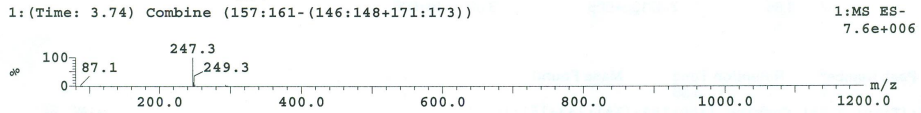

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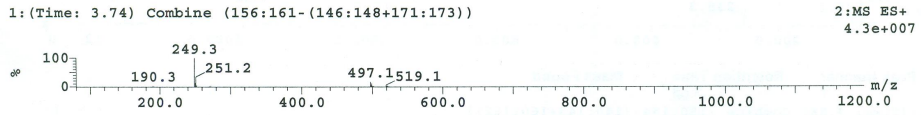

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