ONLINE SUPPORTING INFORMATION

Fragment-Based Discovery of 6-Azaindazoles as Inhibitors of Bacterial DNA Ligase

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Contents

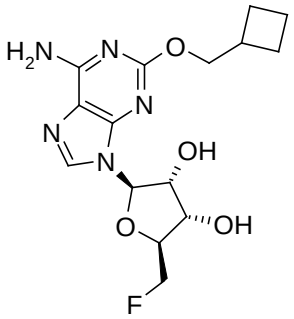
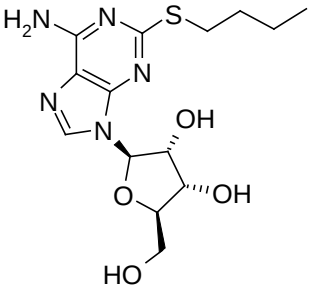
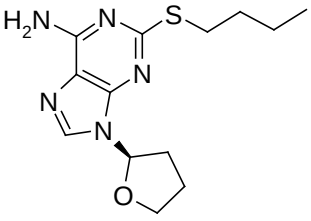
2. *S. aureus* Lig A biochemical assay (IC_{50} determination)

Recombinant full length DNA ligase from *Staphylococcus aureus* was prepared as described in section 3 (LigA protein crystallography). For the preparation of the double stranded oligonucleotide substrate, the following four 5'-labelled oligonucleotides were custom synthesised by Integrated DNA Technologies: biotin, 5′-AGTGAATTCGAGCTCG-3′ (designated as biotin-oligo1); phosphate, 5′-TAATCGAGCTCGAATTCACT-3′ (designated as phospho-oligo2); phosphate, 5′-ATTACCGTAATCATG-3′ (designated as phosphor-oligo3); Cy5, 5′-CATGATTACGG-3′ (designated as Cy5-oligo4). The four oligonucleotides were resuspended in water, combined at a final concentration of 25 µM, incubated at 100 °C for 5 min, then slowly cooled to room temperature. The annealed double stranded oligonucleotide substrate was stored in aliquots at -20 °C, protected from light.

A time-resolved fluorescence resonance energy transfer (TR-FRET) assay was used to measure *S. aureus* DNA ligase activity, based on the method described by Chen *et al.* [Chen *et al.* (2002) *Analytical Biochemistry* 309:232-240] with some modifications. Reaction mixtures (50 µL) contained 50 mM Tris pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.05 g/L BSA, 10% PEG_{8000}, 1 mM DTT, 130 nM NAD^+, 19 nM streptavidin-labelled europium (Perkin Elmer), 75 nM double stranded oligonucleotide substrate and 6 nM *S. aureus* DNA ligase in non-treated black polystyrene 96-well half-area plates (Costar). Test compounds were assayed at a final concentration of 3% DMSO. Plates were incubated for 10min at room temperature on a shaker, protected from light. Fluorescence was measured in kinetic mode over 60 min on a PHERAstar (BMG Labtech) with an HTRF optic module with simultaneous dual emission (excitation 337 nm; emissions 665 nm and 620 nm).

The 665/620 ratio was plotted against time and the linear portion of the time-course was fitted to a linear regression using MARS software (BMG Labtech). The slope of the linear fit was used to calculate percentage inhibition values relative to vehicle control, which were then plotted against compound concentration using Prism 5.0 software (GraphPad) and fitted to a four parameter logistic equation in order to determine IC_{50} values.

Reference compounds were provided by compounds **1**, **14** (see ref. 8 compound 2 and 1 respectively) and **15**. These were purchased from Sai (India).

Cpd No.	Structure	IC_{50}/μM	IC_{50}/μM (literature)
1		54% I at 0.03 μM (n=1)	0.063
14		0.080 μM (n=2) (SD = 0.015)	0.081
15		1.8 μM (n= 18) (SD = 0.8)	NA

Error limits (SD)

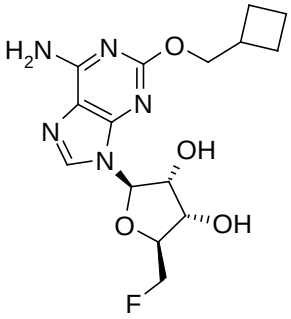
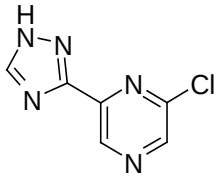
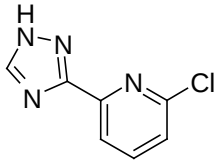
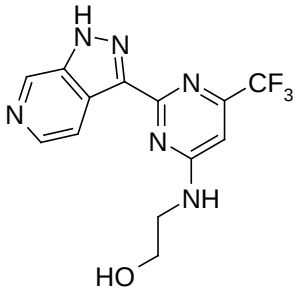
Compound number	IC_{50} (μM)	std dev
3	48%I at 300μM (n=1) 58% @ 500 μM (n=1)	

4	17	6
6	130	0
7	140	0
8	53	3
9	21	1
10	16	1
11	0.22	0.03
12	0.23	0.02
13	47%I at 0.015μM (n=1) 82%I @ 0.03 μM (n=1)	

Data based on n≥2 for all compounds

3. DNA ligase Isothermal Titration Calorimetry (ITC)

ITC experiments were performed on an Auto-ITC200 instrument at 25°C in a buffer comprising 50 mM Hepes, 100 mM NaCl, 1mM TCEP and 5% DMSO at pH 7.5. The protein used for the ITC experiments was prepared as described in Section 3 (LigA protein crystallography). All ITC experiments were set up with protein in the sample cell and compound in the injection syringe. Data were fit to a single site binding model using Origin 7.0 software. DNA ligase is purified as a mixture of adenylated and non-adenylated forms that can be quantified by LCMS. Adenylation blocks the DNA ligase active site therefore the DNA ligase protein concentration estimates used for the ITC data analysis were adjusted, using the LCMS data, to only take account of the non-adenylated protein. Using this method the binding stoichiometry values were typically in the range 0.7-1.1.

Cpd No.	Structure	K_d/μM	Replications
1		0.080 0.074	N=2
3		37 39	N=2
4		10	N=1
13		0.025	N=1

Where N=2, both K_d determinations are shown. Reference compound is provided by compound **1** (see ref. 8 compound 2).

4. LigA Protein Crystallography

DNA encoding residues 1-312 of the isolated adenylation domain of DNA Ligase from *Staphylococcus aureus* (as described by Han *et al*, 2009)[20] was cloned into a pET17 expression vector. In addition to residues 1-312, a further 6 histidine residues were incorporated directly at the c-terminal end of the construct to aid in purification. Protein expression was performed using *E.coli* BL21 (DE3) (Invitrogen). Typically, a freshly transformed colony would be transferred into 1L of Terrific Broth media and grown overnight at 37 °C, 200rpm. Aliquots of 50 mL would then be transferred into fresh 1L flasks of Terrific Broth and grown for a further 4hrs at 37 °C, 200rpm. Protein expression was induced by addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cells were grown for a further 3 hrs before harvest.

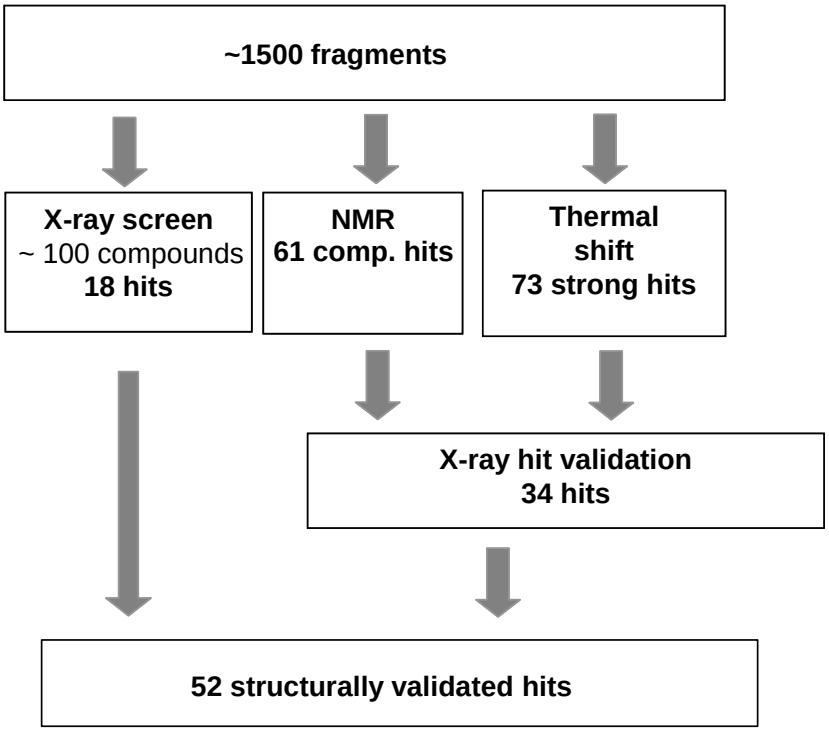
The adenylation domain of DNA Ligase was purified as follows. Bacterial cell pellets were re-suspended in 50 mM Hepes pH8.0, 250 mM NaCl and lysed by sonication. The clarified lysate was incubated with 10 mL of TALON affinity resin (Clontech) for 3 hrs at 4 °C. DNA ligase was eluted from the resin by addition of 50 mM Hepes pH8.0, 250 mM NaCl, 250 mM imidazole. Fractions containing DNA Ligase were dialysed overnight against 50 mM Hepes pH8.0, 20 mM NaCl. Sample was then applied to a Q-sepharose FF column (5 mL) attached to an AKTA system. Protein was eluted from the column by applying a linear salt gradient over 15 column volumes (from 50 mM Hepes pH8.0, 20 mM NaCl to 50 mM Hepes pH8.0, 1 M NaCl). Deadenylation was performed by incubating DNA Ligase with 20 mM nicotinamide mononucleotide (NMN) and 10 mM $MgCl_2$ for 2hrs at ambient temperature. The sample was then applied to a superdex75 (16/60) gel filtration column equilibrated in 25 mM Tis pH7.5, 150 mM NaCl, 5 mM DTT. Gel filtered protein was then concentrated to ~40 mg/mL for crystallization.

Crystallisation was performed using the hanging drop vapour diffusion method. Hanging drops (2 μL protein + 2 μL mother liquor) were set up against a screen of 1.6-2.6M ammonium sulphate, 50 mM – 150 μM $MgCl_2$, 100 mM Hepes pH7.5. Trays were incubated at 10 °C and crystals were observed after overnight incubation. Spherical/block – like crystals grew rapidly over night and continued to appear in drops containing lower concentrations of ammonium sulphate over 4-5 days. These spherical/block-like crystals belong to the monoclinic space group C2 with cell parameters of a = 170.7 b = 39.5 c = 48.9 β = 89.9. Crystals typically diffracted to 1.8-2.0 Å on and in-house X-ray source (Rigaku FR-E^+ SuperBright X-ray generator). The overall structure of the adenylation domain of LigA (*S.*

aureus) is similar to that published by Han *et al*.[20] On inspection of the structure at residue Lys211, it is clear that this side chain is not adenylated and therefore provides an empty active site to perform ligand binding studies.

Coordinates: Coordinates for the LigA complexes with fragment **3** and compound **12** have been deposited in the Protein Data Bank (PDB) under accession codes 4CC5 and 4CC6 respectively.

5. Fragment Screening using NMR, Thermal shift (T_m) Assays and X-ray Crystallography.



Approximately 1500 fragments were screened using both ligand-observed NMR and a thermal shift (T_m) assays, with a subsets (~100) of these fragments also being screened directly by high-throughput LigA X-ray crystallography.

All NMR experiments were carried out at 500 MHz, using a Bruker DRX500 instrument equipped with a TXI cryoprobe. Screening samples contained 8.0 μM de-adenylated *S. aureus* LigA and four fragments, each at 500 μM (25 mM TRIS pH7.5, 100 mM NaCl, 2% DMSO). This gave a molar ratio of fragment to target of around 60:1, a typical value for water-LOGSY experiments. At these protein and ligand concentrations, fragments that bind

with dissociation constants better than 1 mM are expected to be detected. LOGSY experiments were performed using the e-PHOGSY sequence of Dalvit *et. al.*,[1] incorporating a 1 second delay to allow cross-relaxation between fragments and water, and a 100 millisecond CPMG period to allow protein magnetization to decay before signal detection.
In order to enhance the LOGSY signal, all NMR experiments were performed at 5 °C. Lowering the temperature improves the affinities of ligands that bind exothermically and reduces the rates of exchange between bulk water and water of hydration, leading to improved LOGSY intensities.
For all fragment cocktails, LOGSY spectra were obtained in the presence and the absence of the protein, and these spectra were directly compared in order to identify fragment hits. Hits that bound at the AMP site were identified in a second step by repeating the LOGSY experiments after addition of 50 μM of the adenosine analogue **14** (K_d=1.6 μM) to the protein-ligand mixture.

Thermal shift (T_m) assay experiments were performed on a Mx3005P quantitative PCR instrument (Stratagene) that is capable of temperature control and fluorescence detection. Protein unfolding was monitored using the SYPRO orange dye (Invitrogen), which binds to hydrophobic regions on the protein during the unfolding process. The non-adenylated protein used for the T_m assay was prepared as described in Section 4 (LigA protein crystallography). Each compound was dissolved in 100% DMSO to a final experimental concentration of 100 mM. 1 μL of each compound was aliquoted into 96-well PCR plates (Starlab) for the T_m assay. The final nominal concentrations for each T_m assay consisted of 1 mM compound, 1% DMSO, 5 μM of protein, 50 mM HEPES pH 7.5, 100 mM NaCl and 2.5 X of SYPRO orange. Control experiments were performed in parallel in quartets for each 96-well plate. The plate was heated over a temperature range of 25-70 °C and the temperature was increased by 1 °C per minute. The fluorescence intensity was measured every 0.5 °C.

61 Competitive hits were identified from the NMR screen (~ 4% competitive hit rate) and 73 strong hits (defined as those with ΔT_m >4 °C) were obtained from the T_m assay (5% hit rate). 97 of these NMR/T_m hits were assayed by isothermal titration calorimetry (ITC), of which 79 demonstrated measurable affinity with dissociation constants ranging from 30 μM to 5.5 mM.

The majority of the NMR/T_m fragment hits were profiled using high throughput LigA X-ray crystallography, of which 34 were confirmed as structurally validated hits. Together with 18 hits from direct X-ray crystallographic screening of the subset, this provided a total of 52

structurally validated hits. Included in these was fragment hit **3**, described in the article, which was identified as an NMR hit and then validated as described.

[1] Dalvit, C.; Pevarello, P.; Tato, M.; Veronesi, M.; Vulpetti, A.; Sundstrom, M. Identification of compounds with binding affinity to proteins via magnetization transfer from bulk water. *J Biomol. NMR,* 2000, **18**, 65–68.

6. Bacterial strains, antibacterial susceptibility testing and cytotoxicity assays

Bacterial isolates used in antibacterial susceptibility testing were obtained from the culture collection of GlaxoSmithKline (Collegeville, Pennsylvania, USA). Whole-cell antimicrobial activity was determined by broth microdilution using the National Committee for Clinical Laboratory Standards (NCCLS) recommended procedure (Document M7-A4). The compound was tested in serial two-fold dilutions ranging from 0.125 to 128 μg/mL. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of compound that inhibited visible growth after incubation at 37 °C for 18 to 24 h, with a starting inoculum of ~5.5 X 10^5 CFU/mL. A mirror reader was used to assist in determining the MIC endpoint.

Toxicity/proliferation of these novel compounds against mammalian cell line was measured by using a mouse lymphoma line (L5178YTK +/-), and was tested in serial two-fold dilutions ranging from 0.1 to 100 μM.

7. Mode of action studies

S. aureus strains RN4220 (pYH4) and RN4220 (pYH4-YerG/LigA) from an ORF (open reading frame) expression library of the genome of *S. aureus* (Huang *et al* 2004 & Ji *et a*l 2004) were used in the mode of action studies and LigA overexpression was induced by 0.1 μg/mL of anhydro tetracycline.

E. coli TOP10 ΔTolC strains containing LigA G180E, R150S or R518H mutation were isolated by selecting spontaneous mutants resistant to LigA inhibitor **1** (see ref. 8 compound 2) at 4 times the MIC on Mueller-Hinton agar plates and followed by PCR and sequencing analysis of the *ligA* gene from the mutants to identify the LigA mutations. These mutants

were used in the mode of action studies by showing cross-resistance to the LigA inhibitors synthesized from this study using the broth microdilution MIC determination method.

REFERENCES

Huang, J., P. W. O'Toole, W. Shen, H. Amrine-Madsen, X. Jiang, N. Lobo, L. M. Palmer, L. Voelker, F. Fan, M. N. Gwynn, and D. McDevitt. 2004. Novel chromosomally encoded multidrug efflux transporter MdeA in *Staphylococcus aureus*. Antimicrob. Agents Chemother. 48:909-917.

Ji, Y., D. Yin, B. Fox, D. J. Holmes, D. Payne, and M. Rosenberg. 2004. Validation of antibacterial mechanism of action using regulated antisense RNA expression in *Staphylococcus aureus*. FEMS Microbiol Lett. 231:177-184.

8. Experimental details for the synthetic procedures and characterization data of compounds 4-13.

Chemistry. All solvents employed were commercially available "anhydrous" grade, and reagents were used as received unless otherwise noted. A Biotage Initiator™ Sixty system was used for microwave heating. Flash column chromatography was performed on a Biotage SP1 system (32–63 µm particle size, KP-Sil, 60 Å pore size). Preparative high pressure liquid chromatography (HPLC) was performed using a Waters 2525 pump with 2487 dual wavelength detector and 2767 Sample manager. Columns were Waters C18 OBD 5µm, either 50x100 mm Xbridge or 30x100 mm Sunfire. NMR spectra were recorded on a Bruker AV400 (Avance 400 MHz) or AV600 (Avance 600 MHz) instruments.

Analytical LC–MS was conducted using an Agilent 1200 series with Mass Spec Detector coupled with an Agilent 6140 single quadrupole mass detector and an Agilent 1200 MWD SLUV detector. LC retention times, molecular ion (m/z) and LC purity (by UV) based on one or more of the following methods.

Method 1: (ACIDIC)

Eluent A: 95:5 H_2O:CH_3CN + 0.1% Formic Acid
Eluent B: CH_3CN
Gradient: 5-95% eluent B over 1.1 minutes
Flow: 0.9 ml/min
Column: Waters Acquity UPLC BEH C18; 1.7 µ; 2.1x50 mm

Column T: 50°C

Method 2 (BASIC)

Eluent A: 95:5 10 mM NH_4HCO_3+NH_4OH:CH_3CN (pH = 9.2)

Eluent B: CH_3CN

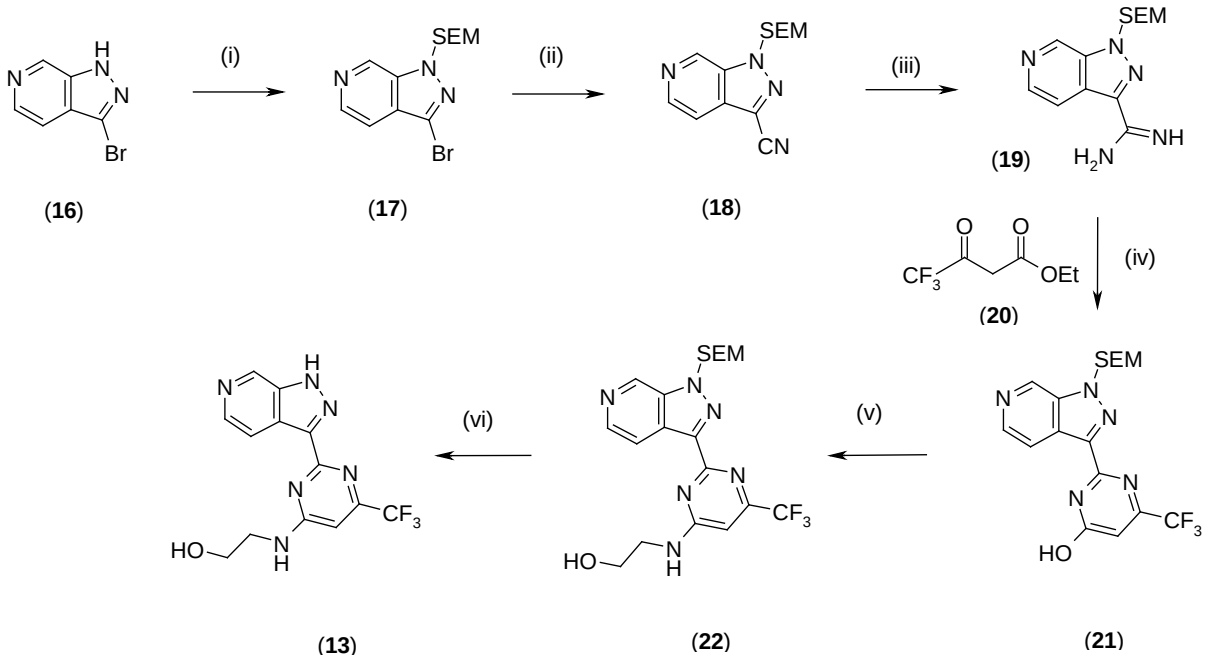
Gradient: 5-95% eluent B over 1.1 minutes

Flow: 0.9 ml/min

Column: Waters Acquity UPLC BEH C18; 1.7μ; 2.1x50 mm

Column T: 50°C

Synthesis of compound 13



Reagents and conditions: i) SEM-Cl, NaH, THF, 0°C, 46%; ii) $Zn(CN)_2$, $Pd_2(dba)_3$, dppf, DMA, 160°C, 61%; iii) NaOMe, NH_4Cl, MeOH, rt; iv) NaOMe, MeOH, 50°C, 73%, 2 steps; v) PyBOP, Cs_2CO_3, 2-aminoethanol, THF, rt, 75%; vi) 4N HCl in dioxane, MeOH, 80°C, 75%.

3-Bromo-1-((2-(trimethylsilyl)ethoxy)methyl)-1H-pyrazolo[3,4-c]pyridine (**17**). To a solution of 3-bromo-1H-pyrazolo[3,4-c]pyridine **16** (5.0 g, 25.2 mmol; Frontier Chemicals B12213) in THF was added NaH (1.21 g, 30.3 mmol., 60%) in portions. The reaction mixture was stirred at 0 °C for 30 minutes and then 2-(trimethylsilyl)ethoxymethyl chloride (5.05 g, 30.3 mmol) was added dropwise over 2 hours. The resulting mixture was stirred overnight. The reaction was quenched with aqueous NH_4Cl solution and partitioned between EtOAc and water. The organic phase was washed with brine, dried (Na_2SO_4) and concentrated *in vacuo* to give the crude product which was purified by SiO_2 chromatography (eluting with EtOAc/petrol) to give the title compound **17** (4.05 g, 11.7 mmol, 46 % yield) as a colorless oil. ^{1}H NMR (400 MHz, methanol-D4): 9.19 (1H, d), 8.40 (1H, d), 7.69 (1H, dd) 5.88 (2H, s), 3.66-3.58 (2H, m), 0.91-0.84 (2H, m), -0.07 (9H, s). MS *m/z* 328, 330 $[M+H]^+$.

1-((2-(Trimethylsilyl)ethoxy)methyl)-1H-pyrazolo[3,4-c]pyridine-3-carbonitrile (18). A mixture of bromide **17** (4.05 g, 12.3 mmol), $Zn(CN)_2$ (1.74 g, 14.8 mmol), DPPF (0.547 g, 0.987 mmol) and $Pd_2(dba)_3$ (0.452 g, 0.494 mmol) in dimethylacetamide (4 mL) was stirred in a pre-heated oil bath at 160 °C for 25 minutes. The mixture was then cooled, diluted with EtOAc and filtered. The filtrate was washed with water, brine, dried (Na_2SO_4) and concentrated *in vacuo*. Purification by SiO_2 chromatography (eluting hexanes) gave **18** (2.22 g, 7.52 mmol, 61 % yield). ^{1}H NMR (400 MHz, $CDCl_3$): 9.30 (1H, d), 8.60 (1H, d), 7.81 (1H, dd), 5.91 (2H, s), 3.65-3.54 (2H, m), 0.99-0.86 (2H, m), -0.03 (9H, s). MS *m/z* 275 $[M+H]^+$.

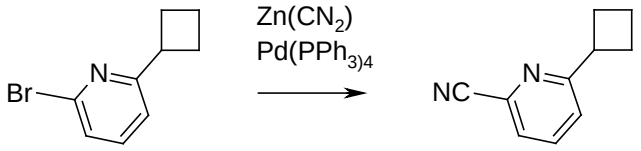
1**-((2-(Trimethylsilyl)ethoxy)methyl)-1H-pyrazolo[3,4-c]pyridine-3-carboximidamide (19**). A mixture of nitrile **18** (4.92 g, 17.9 mmol) and NaOMe (11.31 mL, 71.7 mmol, 25% wt in methanol) was stirred at 0 °C for 3 hours. Conc. aqueous NH_4OH (9.59 g, 179 mmol) was added, the mixture stirred for 5 hours and then heated to 50 °C for 1 h. The mixture was filtered, the collected solid was washed with DCM and EtOAc. The combined filtrate was concentrated *in vacuo* to give the desired amidine **19** (5.2 g) as a yellow solid. ^{1}H NMR (400 MHz, methanol-D4): 9.40 (1H, s), 8.55 (1H, d), 8.08 (1H, d), 6.08 (2H, s), 3.68 (2H, t), 0.92 (2H, t), -0.05 (9H, s). MS *m/z* 292 $[M+H]^+$.

6-(Trifluoromethyl)-2-(1-((2-(trimethylsilyl)ethoxy)methyl)-1H-pyrazolo[3,4-c]pyridin-3-yl)pyrimidin-4-ol (21). To a solution of **19** (2.0 g, 6.86 mmol) in methanol (15 mL) was added NaOMe (1.85 g, 34.3 mmol). The resulting mixture was stirred at ambient temperature for 30 minutes and then ethyl 4,4,4-trifluoro-3-oxobutanoate **20** (4.42 g, 24.0 mmol) was added dropwise. The resulting mixture was heated to 50 °C overnight. The solvent was evaporated *in vacuo* and the crude materials purified by SiO_2 chromatography (eluting with CH_2Cl_2/MeOH) to yield pyrimidine **21** (2.3 g, 5.03 mmol, 73 % yield, over 2

steps). ^{1}H NMR (400 MHz, methanol-D4): 9.43-9.21 (1H, m), 8.62-8.46 (1H, m), 8.43 (1H, br. s.), 6.86 (1H, s), 6.08 (2H, s), 3.73-3.64 (2H, m), 0.92 (2H, t), -0.06 (9H, s). MS *m/z* 412 $[M+H]^+$.

2-((6-(Trifluoromethyl)-2-(1-((2-(trimethylsilyl)ethoxy)methyl)-1H-pyrazolo[3,4-c]pyridin-3-yl)pyrimidin-4-yl)amino)ethanol (22). To a slurry of **21** (400 mg, 0.972 mmol), PyBOP (1.01 g, 1.94 mmol), 2-aminoethanol (238 mg, 3.89 mmol) in THF was added Cs_2CO_3 (633 mg, 1.94 mmol). The mixture was stirred at room temp for 1 hour and then partitioned between EtOAc and water. The organic phase was washed with brine, dried (Na_2SO_4) and concentrated *in vacuo*. The crude product was purified by SiO_2 chromatography (eluting with EtOAc/petrol) to provide pyrimidine **22** (331 mg, 0.729 mmol, 75 % yield). ^{1}H NMR (400 MHz, methanol-D4): 9.16 (1H, s), 8.45 (1H, br. s), 8.37 (1H, d), 6.86 (1H, s), 5.99 (2H, s), 3.93-3.73 (4H, m), 3.66 (2H, m), 0.89 (2H, m), -0.09 (9H, s). MS *m/z* 455 $[M+H]^+$.

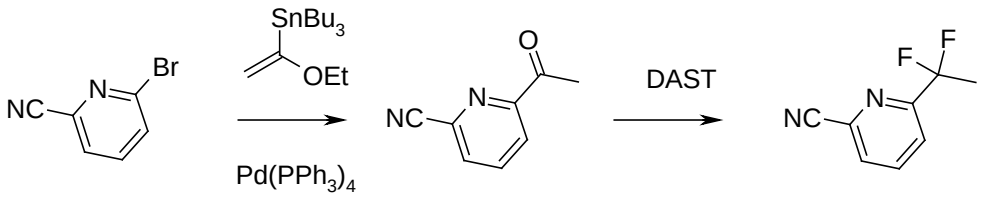
2-((2-(1H-Pyrazolo[3,4-c]pyridin-3-yl)-6-(trifluoromethyl)pyrimidin-4-yl)amino)ethanol, hydrochloride (13). To a slurry of **22** (350 mg, 0.770 mmol) in methanol (3 mL) was added 4N HCl in dioxane (2.0 mL, 0.770 mmol). The mixture was heated to 80 °C for 3 days. The reaction mixture was cooled and concentrated *in vacuo*. The residue was triturated with EtOAc give the title compound **13** (220 mg, 0.579 mmol, 75 % yield). ^{1}H NMR (400 MHz, methanol-D4): 9.71 (1H, s), 9.05 (1H, d), 8.49 (1H, d), 6.93 (1H, s), 3.84 (4H, dd). Anal. RP-LCMS, *m/z* 325 $[M+H]^+$, t_R = 0.96 min, purity > 98% (method 1); t_R = 1.09 min, purity > 98% (method 2).



(**24**)

6-Cyclobutyl-pyridine-2-carbonitrile (24). 2-Bromo-5-cyclobutylpyridine (Combi-phos CS451, 500 mg, 2.3 mmol), $Pd(PPh_3)_4$ (270 mg, 0.23 mmol) and $Zn(CN)_2$ (330 mg, 2.8 mmol) were suspended in DMF (5 mL) and heated at 90 °C for 4 hours. The mixture was cooled and partitioned between EtOAc and water. The organic layer was washed with brine, dried ($MgSO_4$) and evaporated *in vacuo*. Purification by SiO_2 chromatography (eluting with 5-

15% EtOAc/Petrol) gave the title compound **24** (269 mg, 68%) as a colourless oil. MS *m/z* 159 $[M+H]^+$



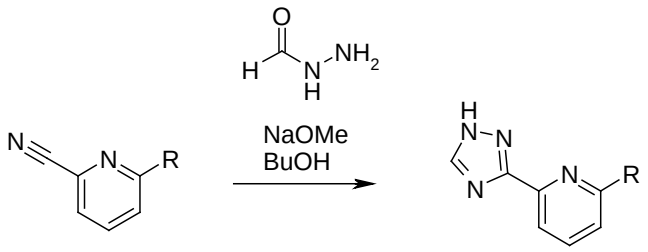
(**25**) (**26**)

6-Acetyl-pyridine-2-carbonitrile (25). 6-Bromopyridine-2-carbonitrile (3.0 g, 16.4 mmol), tributyl(1-ethoxyvinyl)tin (5.50 mL, 16.4 mmol), LiCl (2 g, 49 mmol) and $Pd(PPh_3)_4$ (900 mg, 0.82 mmol were suspended in 1,4-dioxane and heated at reflux for 5 hours. The cooled mixture was diluted with EtOAc, washed with water, dried ($MgSO_4$) and then evaporated *in vacuo*. Purification by SiO_2 chromatography (eluting with 0-5% EtOAc/Petrol) gave a white crystalline solid (2.44 g). This material was dissolved in 1,4-dioxane (15 mL) and 1N aqueous HCl (15 mL) and stirred at room temperature. After 2 hours, the mixture was neutralised by careful addition of solid $NaHCO_3$ and then partitioned between CH_2Cl_2 and water. The organic layers was dried ($MgSO_4$) and evaporated to give the title compound **25** (2.0 g, 82%) as a pale yellow crystalline solid. ^{1}H NMR (400 MHz, DMSO-D6): 8.42-8.04 (3H, m), 2.65 (3H, s). MS *m/z* 147 $[M+H]^+$.

6-(1,1-Difluoro-ethyl)-pyridine-2-carbonitrile (26). A solution of 6-acetyl-pyridine-2-carbonitrile **25** (600 mg, 4.11 mmol) in CH_2Cl_2 (9 mL) was added to a cooled (0 °C) solution of diethylaminosulfur trifluoride (DAST) (1.35 mL, 10.3 mmol) in CH_2Cl_2 (9 mL). The mixture was allowed to warm to room temperature and stirred for 48 hours. The mixture was then added slowly to an ice cold aqueous solution of $NaHCO_3$. The organic layer was separated, dried ($MgSO_4$) and evaporated. Purification by SiO_2 chromatography (eluting with 10-20% EtOAc/Petrol) gave the title compound **26** (506 mg, 55%) as a colourless liquid. ^{1}H NMR (400 MHz, DMSO-D6): 8.28-8.23 (2H, m), 8.06 (1H, dd), 2.03 (3H, t). MS *m/z* 169 $[M+H]^+$.

General procedure for synthesis of triazoles 4-10

In the case of **27a-e** the cyanopyridines were commercially available. **24** and **26** were prepared as described above.



(**27a**) R = Cl
(**27b**) R = H
(**27c**) R = OMe
(**27d**) R = Me
(**24**) R = cyclobutyl
(**26**) R = CF_2CH_3
(**27e**) R = CF_3

(**4**) R = Cl
(**5**) R = H
(**6**) R = OMe
(**7**) R = Me
(**8**) R = cyclobutyl
(**9**) R = CF_2CH_3
(**10**) R = CF_3

Cyanopyridine (**27a-e, 24, 26**) (1 equiv), NaOMe (0.1 mol. equiv) were combined in ^{n}BuOH (0.1 M) and stirred at room temperature for 1 h. Formylhydrazine (1 mol. equiv) was added and the reaction heated at reflux overnight. Solvent was removed under reduced pressure and the crude product purified by preparative LCMS to give the following products:-

2-Chloro-6-(1H-[1,2,4]triazol-3-yl)-pyridine (**4**) (34 mg, 13%) ^{1}H NMR (400 MHz, DMSO-D6): 14.64-14.51 (1H, m), 8.51-8.37 (1H, m), 8.08 (1H, d), 8.02 (1H, t), 7.60 (1H, d). Anal. RP-LCMS, *m/z* 181 $[M+H]^+$, t_R = 0.54 min, purity > 98% (method 2).

2-(1H-[1,2,4]Triazol-3-yl)-pyridine (**5**) (13 mg, 9%) ^{1}H NMR (400 MHz, DMSO-D6): 13.64 (1H, s), 8.70 (1H, d), 8.25 (1H, s), 8.09 (1H, d), 8.02-7.92 (1H, m), 7.55-7.45 (1H, m). Anal. RP-LCMS, *m/z* 147 $[M+H]^+$, t_R = 0.22 min, purity > 98% (method 2).

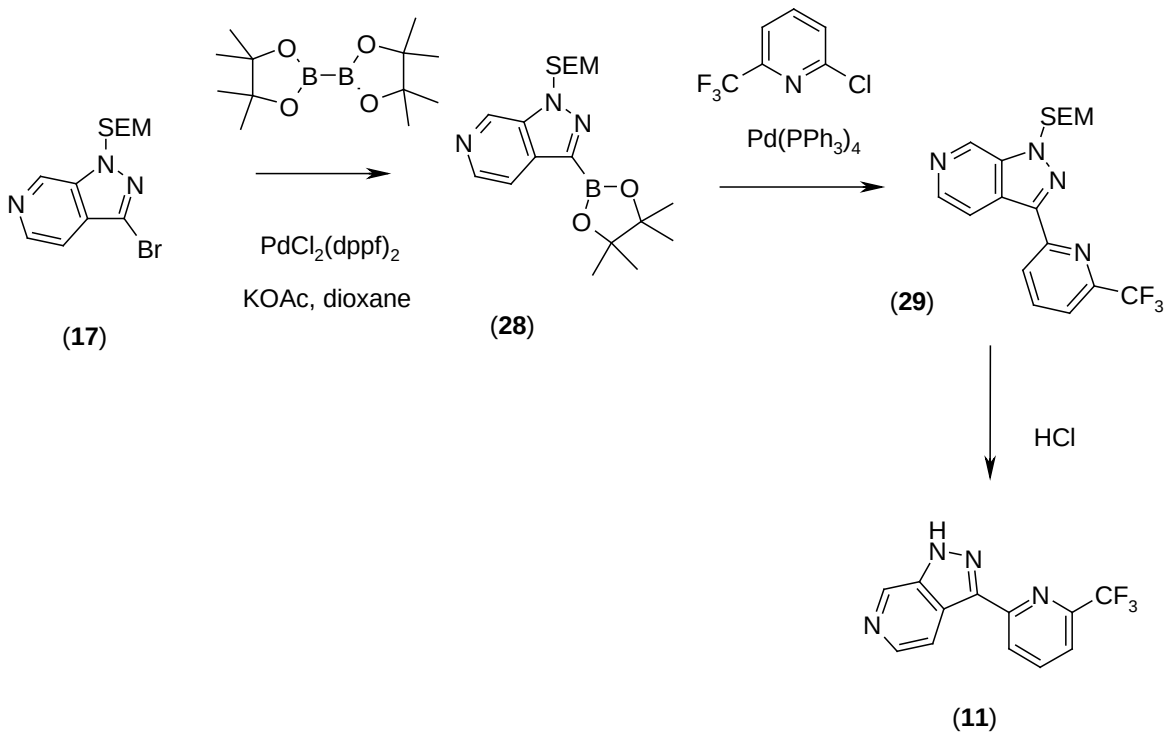
2-Methoxy-6-(1H-[1,2,4]triazol-3-yl)-pyridine (**6**) (13 mg, 5%) ^{1}H NMR (400 MHz, CDCl3): 8.11 (1H, s), 7.86-7.77 (1H, m), 7.74 (1H, t), 6.86 (1H, dd), 4.01 (3H, s). Anal. RP-LCMS, *m/z* 177 $[M+H]^+$, t_R = 0.54 min, purity > 98% (method 2).

2-Methyl-6-(1H-[1,2,4]triazol-3-yl)-pyridine (**7**) (59 mg, 22%) ^{1}H NMR (400 MHz, DMSO-D6): 14.43 (1H, s), 8.23 (1H, s), 7.93-7.76 (2H, m), 7.35 (1H, d), 2.57 (3H, s). Anal. RP-LCMS, *m/z* 161 $[M+H]^+$, t_R = 0.44 min, purity > 98% (method 2).

2-(1H-[1,2,4]Triazol-3-yl)-6-trifluoromethyl-pyridine (**10**) (41 mg, 33%) ^{1}H NMR (400 MHz, DMSO-D6): 8.51 (1H, s), 8.36 (1H, d), 8.23 (1H, t), 7.96 (1H, d). Anal. RP-LCMS, *m/z* 215 $[M+H]^+$, t_R = 0.88 min, purity = 96.5% (method 2).

2-Cyclobutyl-6-(1H-[1,2,4]triazol-3-yl)-pyridine (**8**) (14 mg, 11%) ^{1}H NMR (400 MHz, DMSO-D6): 12.10 (1H, s), 8.25 (1H, s), 7.98-7.80 (2H, m), 7.42-7.31 (1H, m), 3.79-3.64 (1H, m), 2.49-2.35 (2H, m), 2.34-2.21 (2H, m), 2.02 (1H, dd), 1.94-1.81 (1H, m). Anal. RP-LCMS, *m/z* 201 $[M+H]^+$, t_R = 1.03 min, purity = 90% (method 2).

2-(1,1-Difluoro-ethyl)-6-(1H-[1,2,4]triazol-3-yl)-pyridine (**9**) (20 mg, 15%) ^{1}H NMR (400 MHz, DMSO-D6): 14.76-14.42 (1H, m), 8.39 (1H, s), 8.26-8.06 (2H, m), 7.79 (1H, d), 2.11 (3H, t). Anal. RP-LCMS, *m/z* 211 $[M+H]^+$, t_R = 1.02 min, purity =>98 % (method 1).

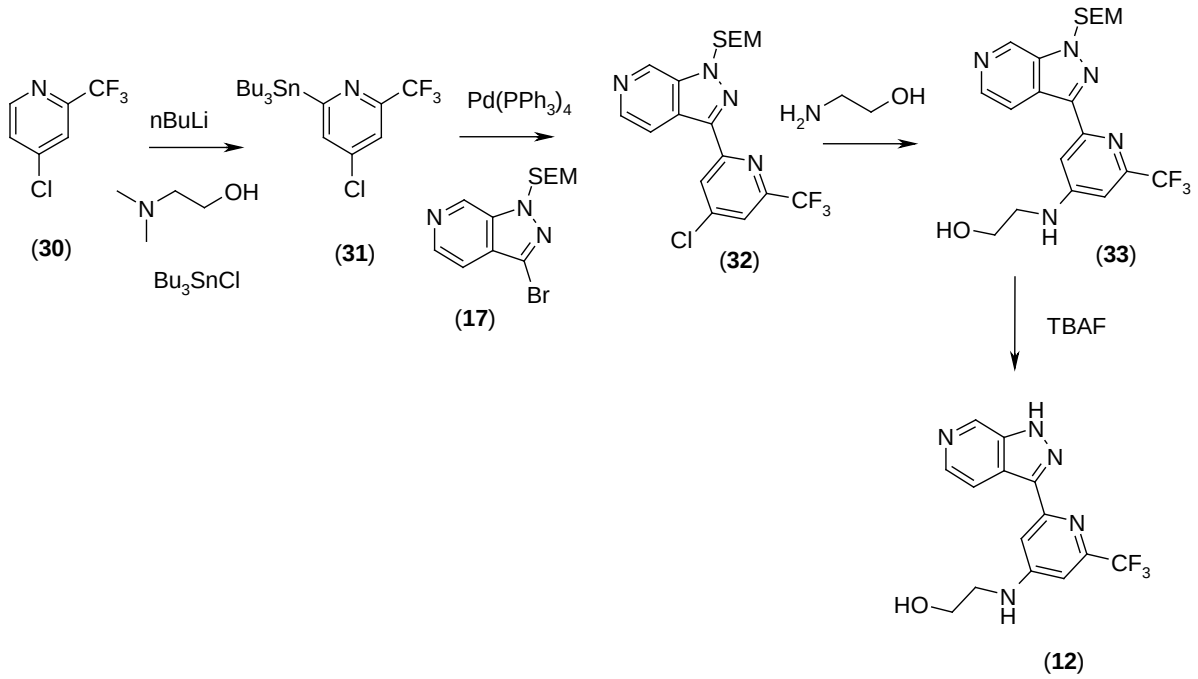


3-(4,4,5,5-Tetramethyl-[1,3,2]dioxaborolan-2-yl)-1-(2-trimethylsilanyl-ethoxymethyl)-1H-pyrazolo[3,4-c]pyridine (28). 3-Bromo-1-((2-(trimethylsilyl)ethoxy)methyl)-1H-pyrazolo[3,4-c]pyridine **17** (1.58 g, 4.83 mmol) bis(pinacolato)diboron (1.84 g, 7.25 mmol), $PdCl_2(dppf)_2$ (0.35 g, 0.48 mmol) and KOAc (1.42 g, 14.5 mmol) were suspended in 1,4-dioxane (20 mL) and heated at 95 ^{o}C for 4 hours. The mixture was cooled, evaporated and partition between

Et_2O and dil aqueous HCl. The Et_2O layer was extracted once more with dil. HCl. The combined aq. HCl fractions were basified with solid Na_2CO_3 and then the product extracted with 2 x EtOAc and then 1 x CH_2Cl_2. The combined organics fractions was dried ($MgSO_4$) and evaporated to give the boronate **28** a beige solid (0.886 g, 49%). ^{1}H NMR (400 MHz, DMSO-D6): 9.37-9.22 (1H, m), 8.36 (1H, d), 7.91-7.77 (1H, m), 5.96 (2H, s), 3.66-3.47 (2H, m), 1.37 (12H, s), 0.98-0.73 (2H, m), -0.11 (9H, s). MS *m/z* 201 $[M\text{-pinacol+H}]^+$ 294.

3-(6-Trifluoromethyl-pyridin-2-yl)-1-(2-trimethylsilanyl-ethoxymethyl)-1H-pyrazolo[3,4-c]pyridine (29). 2-Chloro-6-trifluoromethylpyridine (93 mg, 0.51 mmol), boronate **28** (194 mg, 0.52 mmol), Na_2CO_3 (220 mg, 1.0 mmol) and $Pd(PPh_3)_4$ (60 mg, 0.05 mmol were suspended in DME and water (3 mL, 2:1) and heated at 80 °C for 2 hours. The cooled mixture was diluted with EtOAc, washed with brine, dried ($MgSO_4$) and then evaporated *in vacuo*. Purification by SiO_2 chromatography (eluting with 10-50% EtOAc/Petrol) gave the title compound **29** (2.0 g, 20%). ^{1}H NMR (400 MHz, DMSO-D6): 9.39 (1H, s), 8.50 (1H, d), 8.46 (1H, d), 8.40 (1H, d), 8.27 (1H, t), 7.97 (1H, d), 6.02 (2H, s), 3.63 (2H, t), 0.85 (2H, t), -0.11 (9H, s). MS *m/z* 394 $[M+H]^+$.

3-(6-Trifluoromethyl-pyridin-2-yl)-1H-pyrazolo[3,4-c]pyridine (11). A solution of azaindazole **29** (38 mg, 96 μmol) in MeOH (1 mL) was treated with HCl (4N in 1,4-dioxane) and heated at 70 °C in a sealed tube for 2 hours. The mixture was cooled and concentrated in vacuo to ~ 50% volume. The resulting precipitate was collected by filtration and washed successively with 1,4-dioxane and Et_2O to give the product **11** (15 mg, 71%) as a colourless solid. ^{1}H NMR (400 MHz, DMSO-D6): 15.37-15.08 (1H, br), 9.63 (1H, s), 8.74 (1H, d), 8.62-8.50 (2H, m), 8.31 (1H, t), 8.01 (1H, d). Anal. RP-LCMS, *m/z* 266 $[M+H]^+$, t_R = 1.27 min, purity =>98 % (method 2).



4-chloro-2-tributylstannanyl-6-trifluoromethyl-pyridine (31). nBuLi (2.5 M in hexanes, 2.2 mL, 5.5 mmol) was added dropwise to dimethylaminoethanol (0.27 mL, 2.7 mmol) in hexane (5 mL) at -5 °C. The reaction was warmed to 0 °C for 30 min then cooled to -78 °C and 2-trifluoromethyl-4-chloro-pyridine **30** (250 mg, 1.38 mmol) in hexane (1 mL) was added dropwise. After 1.5 hours Bu_3SnCl (1.49 mL, 5.51 mmol) was added and the reaction warmed to 0 °C for 30 min. The reaction was quenched with water and extracted with CH_2Cl_2. The solvent was evaporated *in vacuo* and the crude purified by SiO_2 chromatography (eluting with 3% EtOAc/Petrol) to give 4-chloro-2-tributylstannanyl-6-trifluoromethyl-pyridine **31** (515 mg, 77%) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$): 7.66-7.55 (1H, m), 7.54-7.43 (1H, m), 1.65-1.52 (6H, m), 1.45-1.31 (6H, m), 1.27-1.15 (6H, m), 0.94-0.87 (9H, m).

3-(4-Chloro-6-trifluoromethyl-pyridin-2-yl)-1-(2-trimethylsilanyl-ethoxymethyl)-1H-pyrazolo[3,4-c]pyridine (32). Azaindazole **17** (1.50 g, 4.57 mmol), stannane **31** (2.21 g, 4.57 mmol) and toluene (46 mL) were combined and degassed for 30 min. $Pd(PPh_3)_4$ (334 mg, 0.46 mmol) was added and the reaction heated at reflux for 24 h. The solvent was evaporated *in vacuo* and the crude product purified by SiO_2 column chromatography (eluting with 0-30% EtOAc/Petrol) followed by trituration with pet. ether to give the title compound **32** (950 mg, 49%) as a white powder. ^{1}H NMR (400 MHz, $CDCl_3$): 9.27 (1H, d), 8.67 (1H, d), 8.55 (1H, d), 8.46 (1H, d), 7.70 (1H, d), 5.94 (2H, s), 3.65 (2H, t), 1.02-0.89 (2H, m), 0.02-0.13 (9H, m). MS *m/z* 429 $[M+H]^+$

2-{2-Trifluoromethyl-6-[1-(2-trimethylsilanyl-ethoxymethyl)-1H-pyrazolo[3,4-c]pyridin-3-yl]-pyridin-4-ylamino}-ethanol (33). A mixture of azaindazole **32** (80 mg, 0.19 mmol), Et_3N (160 μL, 1.1 mmol), ethanolamine (66 μL, 1.1 mmol) in MeCN (3 mL) was heated in a microwave reactor at 170 °C for 1 hour. The mixture was cooled, evaporated *in vacuo* and the residue partitioned between 20% IPA/$CHCl_3$ and water. The organic layer was washed with brine and evaporated. Purification by SiO_2 column chromatography (eluting with EtOAc/Petrol) gave the title compound **33** (78 mg, 92%). ^{1}H NMR (400 MHz, $CDCl_3$): 9.04 (1H, s), 8.60-8.48 (1H, m), 8.43 (1H, d), 7.38 (1H, s), 6.84 (1H, d), 5.79 (2H, s), 5.13 (1H, s), 3.96 (2H, t), 3.68-3.53 (2H, m), 3.52-3.40 (2H, m), 3.14 (1H, s), 1.28 (2H, m), 0.71-1.04 (9H, m).

2-[2-(1H-Pyrazolo[3,4-c]pyridin-3-yl)-6-trifluoromethyl-pyridin-4-ylamino]-ethanol (12). Compound **33** (78 mg, 0.17 mmol) was dissolved in THF (4 mL) and TBAF (1M in THF) (0.83 mL, 0.83 mmol) added. The reaction was heated to reflux overnight. The cooled reaction was quenched with water and extracted with 20% IPA in $CHCl_3$. The crude was purified by SiO_2 chromatography (eluting with 0-10% MeOH/CH_2Cl_2) followed by SCX column chromatographyto give the title compound **12** (25 mg, 46%) as a white powder. ^{1}H NMR (400 MHz, DMSO-D6): 13.92 (1H, s), 9.11 (1H, s), 8.40-8.29 (2H, m), 7.52 (1H, s), 7.34-7.23 (1H, m), 7.02 (1H, s), 4.85 (1H, s), 3.61 (2H, d). MS *m/z* 324 $[M+H]^+$. Anal. RP-LCMS, *m/z* 324 $[M+H]^+$, t_R = 1.11 min, purity = 97% (method 2).

9. Calculations of conformation preference for pyridyl-azaindazole core of compound 12.

All calculations were performed using the core of compound **12**, depicted below (Figure 1a). The torsion highlighted in red was varied from 0° to 180° in 20° increments. At each point, the geometry was optimised (with the torsion constrained to its initial value) at the HF/6-31G* level, and then a single point energy calculation was performed at the M02-6X/6-31G* level on the optimised geometry. These calculations were performed using Q-Chem version 4.0.1.[1] The results in Figure 1a show that the 180° conformation is preferred over the bound conformation (~ 0°) by 5-6 kcal/mol, at least in the gas phase. Calculations were also run with the SM8 implicit solvation model, but this did not significantly affect the results.

We also searched for relevant structures in the Cambridge Structural Database[2] (CSD) version 5.34, with updates to May 2013. The substructure query used corresponds to the SMARTS string [nH0X2r5]:c(:a(:a):[cHr6])-!@c(:[cHr6]):[nH0X2r6]. We limited the results to entries with R < 0.05, and we excluded entries with disorder or errors, polymeric entries and powder structures. The results are shown in table 1. 9 Examples were found that contained

a relevant substructure (two of which contained the substructure twice), and in all cases the 180° conformation was observed. Selected examples are shown in figure 1b.

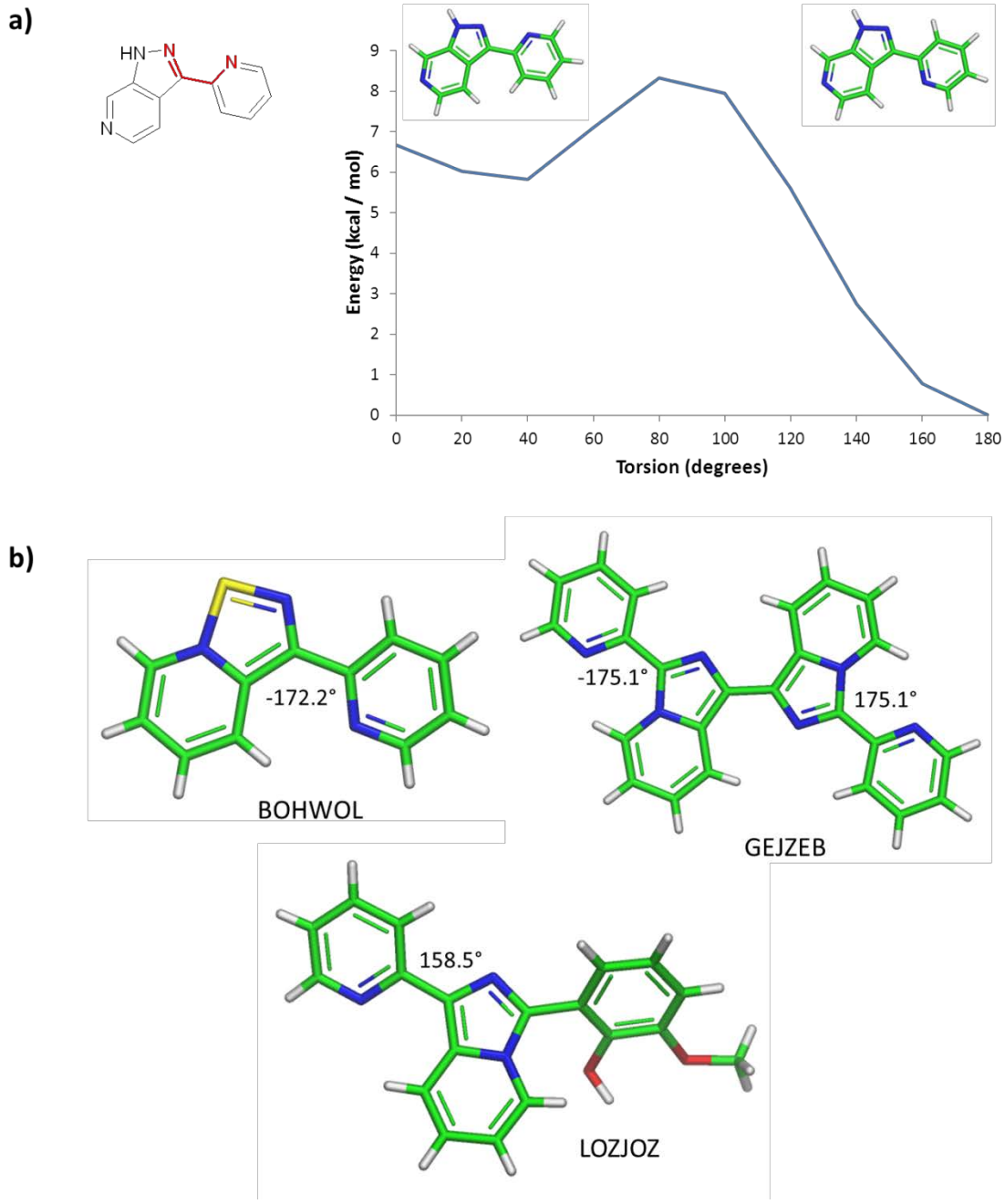


Figure 1. (a) On the left is the core of compound **12** that was used for conformational calculations, with the relevant torsion angle highlighted in red. On the right is a plot of energy vs this torsion angle. (b) Selected examples of similar compounds from the CCD.

CSD Refcode	Torsion angle (degrees)
BOHWOL	-172.2
GEJZEB	-175.1, 175.1
KEHRUM	-177.3
LOZJOZ	158.5
MOSFUW	174.6
MUNCAA	-179.9
SAXCUR	-178.3
UVEFIL	-163.0
UVEFOR	169.3, 172.5

Table S1. Entries from the CSD containing a similar substructure to molecule **12** (as described in the text), along with the torsion angle between the two nitrogen atoms.

References

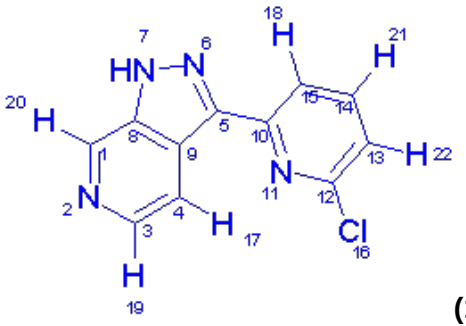
1. Advances in quantum chemical methods and algorithms in the Q-Chem 3.0 program package, Yihan Shao *et al.*, *Phys. Chem. Chem. Phys*., 8, 3172 (2006).
2. The Cambridge Structural Database: a quarter of a million crystal structures and rising , F. H. Allen, *Acta Cryst*., B58, 380-388, 2002

10. Free ligand conformation in solution by NMR Spectroscopy.

All NMR spectra were recorded at 25 °C on Bruker Avance III spectrometer operating at 500 MHz equipped with a cryoprobe. In all NMR experiments, pulsed field gradients were applied where appropriate. The data were processed and analyzed using Topspin3.0 software.

An NMR sample was produced by dissolving ~1 mg of compound **16** (IC_{50} = 0.14 μM) in 600 μL of DMSO-d_6 and in aqueous buffer at pH=7. The ^{1}H and ^{13}C resonances of each conformer were assigned from an analysis of several 2D homonuclear and heteronuclear NMR spectra. The unusual low field values of chemical shift of H17 (8.33 ppm) and of H18 (8.17 ppm) suggest the presence of hydrogen bonds with N11 and with N6, respectively.

Three-dimensional conformation was determined by measuring intramolecular rotating frame Overhauser correlations ROEs obtained from 2D ROESY spectra acquired at mixing times mix = 350 ms: the absence of ROE cross peak between H17 and H18 supports the presence of a single conformation. Similar results were determined in aqueous buffer.



(16)

Chemical shift assignment in DMSO-d_6:

^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 7.52 (d, 1 H-22) 8.01 (t, 1 H-21) 8.17 (d, 1 H-18) 8.33 (d, 1 H-17) 8.4 (d, 1 H-19) 9.13 (s, 1 H-22)

Chemical shift assignment in aqueous buffer:

^{1}H NMR (500 MHz, D_2O) δ ppm 7.48 (d, 1 H-22) 7.92 (t, 1 H-21) 8.0 (d, 1 H-18) 8.23 (d, 1 H-17) 8.24 (d, 1 H-19) 9.03 (s, 1 H-22)

11. Mechanism of NAD$^+$-dependent DNA-Ligase

Bacterial DNA ligase (LigA) is an NAD$^+$-dependent enzyme which is essential for DNA replication and has attracted interest as a novel target for antibacterial therapy.[1] LigA is responsible for ligating two strands of DNA via the formation of a phosphodiester bond between the 3'-hydroxyl end of one oligonucleotide and the 5'-phosphate end of another.[2,3] This process involves a three-step mechanism. Initially, reaction between NAD+ and an active-site lysine leads to an adenylated form of the protein. Enzyme-bound AMP is then transferred to the 5'-phosphate end of a nicked DNA strand. Finally, attack on the AMP-DNA bond by the 3'-hydroxyl of a second strand of DNA seals the phosphate backbone and releases AMP. LigA has been shown to be essential for viability in all Gram positive and

Gram negative organisms tested to date.[4,5] It is highly conserved across bacterial species and is phylogenetically quite distinct from its human, ATP-dependent, counterpart. This provides encouragement that inhibitors of LigA may exhibit both broad-spectrum antibacterial activity and selectivity over human isozymes.[6]

See main text for references