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Identification and Structure-Guided Development of Pyrimidinone Based USP7 Inhibitors

Colin R. O'Dowd[†]*, Matthew D. Helm[†], J. S. Shane Rountree[†], Jakub T. Flasz[‡§], Elias Arkoudis[‡§], Hugues Miel[†], Peter R. Hewitt[†], Linda Jordan[†§], Oliver Barker[†], Caroline Hughes[†], Ewelina Rozycka[†], Eamon Cassidy[†], Keeva McClelland[†§], Ewa Odrzywol[†§], Natalie Page[†], Stephanie Feutren-Burton[†], Scarlett Dvorkin[‡], Gerald Gavory[†], Timothy Harrison[†‡].

[†]Almac Discovery Ltd, Centre for Precision Therapeutics, 97 Lisburn Road, Belfast, Northern Ireland BT9 7AE, United Kingdom. [‡]Centre for Cancer Research and Cell Biology, Queen's University Belfast, Northern Ireland BT9 7AE, United Kingdom.

ABSTRACT: Ubiquitin specific protease 7 (USP7, HAUSP) has become an attractive target in drug discovery due to the role it plays in modulating Mdm2 levels and consequently p53. Increasing interest in USP7 is emerging due to its potential involvement in oncogenic pathways as well as possible roles in both metabolic and immune disorders in addition to viral infections. Potent, novel and selective inhibitors of USP7 have been developed using both rational and structure-guided design enabled by high resolution cocrystallography. Initial hits were identified *via* fragment-based screening, scaffold-hopping and hybridization exercises. Two distinct sub-series are described along with associated SAR trends, as are initial efforts aimed at developing compounds suitable for *in vivo* experiments. Overall these discoveries will enable further research into the wider biological role of USP7.

The process of protein ubiquitination is a central tenet of the ubiquitin proteasome system (UPS) and is crucial in many fundamental cellular processes such as proteolysis, cell-cycle control, DNA repair and apoptosis.[1,2] The importance of this pathway in controlling such key cellular processes cannot be understated and increasing evidence linking the UPS to human diseases such as cancer[3] and neurodegenerative disorders[4] is emerging. The approved proteasome inhibitor Velcade® (bortezimib) has demonstrated that the UPS is a viable target for small molecule therapeutic intervention.[5] Targeting the UPS upstream of the proteasome may therefore yield new opportunities for targeted therapeutics with improved specificity and toxicity profiles.

Ubiquitination is a post-translational modification *via* covalent attachment of the 76 amino acid protein ubiquitin to lysine side chains of target substrates. This elegant and complex molecular "tagging" of target proteins is effected by E1 (activating), E2 (conjugating) and E3 (ligase) enzymes and has multiple functions including targeted substrate degradation, activation for further processing and cellular localization.[6]

The process of ubiquitination is reversed by deubiquitinase enzymes (DUBs) of which there are around 100 encoded by human genes.[7] Ubiquitin specific proteases (USPs) are cysteine proteases that comprise the largest (>50) sub-class of DUBs and are gaining interest as an emerging target class for pharmaceutical intervention.[8] There are sparingly few validated small molecule inhibitors reported for USPs and as such there is an acute need to develop robust probe compounds for use in deciphering the biological pathways associated with the USP target class.

USP7 represents one of the most studied USPs from a target class that remains largely underexplored and as such has gained attention in recent years due to its association with cancer.[9,10] USP7 is involved in the regulation of the stability of the tumor suppressor p53 *via* deubiquitination of the oncoprotein Mdm2.[11] USP7 mediated stabilization of Mdm2 reduces cellular p53 and may protect damaged cells from apoptosis. In addition, USP7 has also been implicated in the regulation of several other key signaling proteins linked to tumorigenesis.[12-17] Targeting USP7 with small molecules has therefore been of great interest over recent years but has until recently met with limited success due to several factors including poor compound specificity, low potency and/or poor compound properties.[18] Very recent publications have described the characterization of more drug-like USP7 inhibitors that further reinforce the potential druggability of this target class.[19-22]

Recently we published the detailed *in vitro* biological profiling and co-crystal structures of highly potent non-covalent USP7 inhibitors in a variety of biochemical and cellular assays.[22] These compounds have proven to be highly valuable tools for interrogating the complex biology of USP7 and will enable further studies aimed at delineating USP7 biology. Herein we describe the hit-finding and medicinal chemistry efforts towards these tool compounds, summarize the SAR and outline the identification of a novel sub-series of USP7 inhibitors. The binding mode (by way of X-ray co-crystallography) of this sub-series is highlighted as is the pharmacokinetic profiling of early leads from both series.

Our USP7 hit-finding strategy involved initial fragment screening using surface plasmon resonance (SPR) coupled with

the parallel *in vitro* benchmarking of published USP7 inhibitors. Briefly, SPR screening of 1.9k fragments versus immobilized USP7 catalytic domain afforded a range of primary USP7 binding fragments including thieno-pyrimidinone **1** (**Figure 1**).
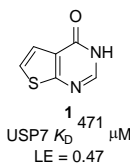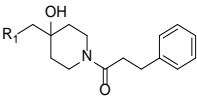

1
USP7 K_D 471 μM
LE = 0.47

Figure 1. Primary USP7 SPR binding fragment **1**.

Compound **1** was found to be a high ligand efficiency USP7 binder (LE = 0.47) with an SPR equilibrium binding constant (K_D) of 471 μM (**Figure S1**, SI). Binding of compound **1** to USP7 was subsequently confirmed by orthogonal techniques (ligand observed STD, CPMG and WaterLOGSY NMR experiments) (**Figure S2**, SI). Further profiling revealed that **1** had excellent aqueous kinetic solubility (>200 μM) and was free from redox cycling activity – a liability that has the potential to lead to false positive readouts in biochemical assays. **1** and analogues thereof were then incorporated into our wider USP7 medicinal chemistry program which involved scaffold-hopping as well as hybridization with known literature USP binding motifs (e.g. 4-hydroxypiperidines[23]). From this program of work compounds **2** and **3** were synthesized and found to have modest USP7 biochemical activity (**Table 1**).

Table 1. Early USP7 hits and associated physicochemical properties.

Cmpd	R$_1$	USP7 IC$_{50}$ (μM)	KSol (μM)	LE	MW/LogD$_{7.4}$
2		23	>200	0.23	397.5/1.9
3		78	186	0.21	397.5/1.7

Compounds **2** and **3** were deemed suitable for further SAR studies due to their reasonable ligand efficiencies (LE) and favorable physicochemical properties. A range of analogues derived by scaffold-hopping to other fused 5-membered ring pyrimidinones were explored in parallel to substitution on the bicyclic pyrimidinone core (**Table 2**). Furano, pyrazolo and thiazolo-pyrimidinone analogues **4**-**8** were largely equipotent with thieno-pyrimidinones **2** and **3**. Substitution of a lipophilic bromine atom at either the *C*-5 or *C*-6 positions of the thiophene ring in compounds **9** and **10** did not lead to marked USP7 potency gains whereas substitution at the *C*-7 position led to an appreciable (*ca.* 5-fold) potency enhancement as demonstrated by compound **11**. Pleasingly, substitution at this position with other lipophilic groups such as cyclopropyl **12**, alkynyl **13** and phenyl **14** also afforded noticeable potency gains versus the non-substituted analogue **3**. Substitution at the 2-position of the pyrimidinone ring with a methyl substituent was not tolerated (compound **15**).

Table 2. Early SAR of 5-membered heterocyclic pyrimidinones.

| Cmpd	R$_1$	USP7 IC$_{50}$ (μM)	Cmpd	R$_1$	USP7 IC$_{50}$ (μM)
4		87	**10**		34
5		115	**11**		13
6		44	**12**		20
7		32	**13**		8
8		130	**14**		10
9		60	**15**		>200

With potency gains of *ca.* 5-10 fold attainable *via* modification of the bicyclic thienopyrimidinone ring, our attention turned to SAR analysis of the phenethylamide moiety. During the course of our studies a key breakthrough was the observation that methyl substitution at the benzylic position of compounds such as **11** led to a significant (>40-fold) increase in USP7 potency. This large increase in potency was fully dependent on the stereochemistry of the newly installed chiral methyl group as exemplified by compounds **16** and ***ent*-16** (**Figure 2**).

16 (*R*)-Me, USP7 IC$_{50}$ = 0.3 μM
***ent*-16** (*S*)-Me, USP7 IC$_{50}$ = 46.0 μM
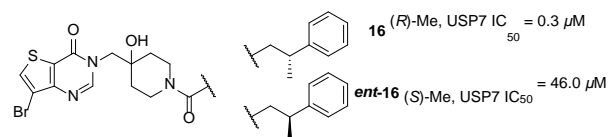
Figure 2. Effects of benzylic substitution.

Interestingly, the (*R*)-enantiomer **16** was found to be >150 times more potent than the corresponding (*S*)-enantiomer ***ent*-16**. With potent USP7 compounds such as **16** now in hand we initiated co-crystallization studies with the catalytic domain (amino acids 207-560) of USP7. We subsequently obtained a 2.3 Å resolution X-ray co-crystal structure of compound **16** and USP7 that revealed the ligand bound in the catalytic cleft between the β-sheet of the palm subdomain and the helices of the thumb subdomain.[22] Removal of key ligand hydrogen-bonding heteroatoms (such as the amide carbonyl, tertiary alcohol or pyrimidinone *N*-1 nitrogen from the bicyclic core) was highly deleterious to USP7 binding (data not shown) highlighting the importance of each individual interaction.

Aided by the X-ray co-crystal structure, we were able to use structure-based design to further optimize our USP7 inhibitors with the aim of enhancing target affinity (whilst retaining favorable physicochemical properties). Our initial focus centered on the thiophene ring of compound **16** which contains a bromine substituent at *C*-7 that is orientated towards the protein surface with a high degree of solvent exposure and was thus viewed as a promising vector for new analogue design and optimization. Encouragingly, scaffold-hopping from the thieno-pyrimidinone core of compound **16** to the more drug-like *N*-methyl pyrazolo-pyrimidine core (compound **17**, **Table 3**) not only lowered overall lipohilicity (logD$_{7.4}$ 1.5 vs 2.3) but was

also not detrimental to USP7 activity. Given this result, we decided to concentrate our efforts on the pyrazolo-pyrimidinone core and a summary of the SAR is presented in **Table 3**.

Table 3. USP7 biochemical potencies of *N*-methyl pyrazolo-pyrimidinones.
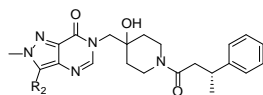
Cmpd	R$_2$	USP7 IC$_{50}$ (μM)	Cmpd	R$_2$	USP7 IC$_{50}$ (μM)
17	(Br)	0.3	26	(CN)	0.15
18	(alkyne)	0.24	27	(NH$_2$)	0.024
19	(HO alkyne)	0.3	28	(OH)	0.039
20	(isopropenyl)	0.33	29	(CONH$_2$)	0.03
21	(isobutyl)	0.56	30	(NMe$_2$)	0.075
22	(pyrazole NH)	0.035	31	(morpholine)	0.25
23	(phenyl)	0.03	32	(H$_2$NOC)	0.05
24	(F phenyl)	0.09	33	(OH)	0.04
25	(NH$_2$ phenyl)	1.4	34	(NH$_2$)	0.006

Our strategy (guided by docking studies using the co-crystal structure) was centered on targeting both hydrogen-bond and hydrophobic interactions *via* selective substitution at the pyrazole *C*-3 position. Incorporation of alkynyl, alkenyl or isopropyl substituents at the *C*-3 position (compounds **18**–**21**) did not prove fruitful, howev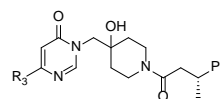er, when pyrazole or phenyl groups were incorporated at this position a *ca.* 10-fold increase in USP7 potency was observed (e.g. compound **23**, IC$_{50}$ 0.03 μM). This marked increase in potency may involve a CH-π interaction between the pendant pyrazole or phenyl groups of **22** or **23** and Gln351 as postulated in the published co-crystal structure.[22] Addition of an *ortho*-fluoro group to the pendant phenyl in compound **23** led to a slight decrease in potency (compound **24**, IC$_{50}$ 0.09 μM) in contrast to *ortho*-anilino substituted compound **25** which was around 40-fold less potent than **23** – possibly due to the larger *ortho*-NH$_2$ substituent increasing the dihedral angle between the phenyl ring and the core, causing an unfavorable steric clash between the phenyl ring and the protein

surface. Addition of hydrogen-bond acceptors and donors at the *meta* and *para* positions on the pendant phenyl group (compounds **26**-**30**) largely maintained potency in relation to **23** with the exception of compound **31** which showed a marked decrease in potency, most likely due to the added steric bulk of the morpholine group. Interestingly, the *para*-carboxamide or benzylic alcohol groups in compounds **32** and **33** did not lead to potency increases whereas the *para*-benzylic amine moiety in compound **34** (IC$_{50}$ = 6 nM) provided a *ca.* 5-fold increase in potency over compound **23**. The (*S*)-Me enantiomer of **34** was found to have an IC$_{50}$ of 2.4 μM, representing a 400-fold decrease in USP7 potency.[22]

In addition to identifying the promising pyrazolo-pyrimidinones described above, we also investigated how truncation of the bicyclic core would affect USP7 binding. Hence, a series of monocyclic pyrimidinones such as compound **35** were synthesized (**Table 4**). Although only moderately active, compound **35** (IC$_{50}$ = 23 μM) represented a promising and ligand efficient (LE = 0.25) starting point for further analogue work with respect to its low molecular weight and logD$_{7.4}$ (355 and 0.9 respectively). A range of analogues were subsequently prepared substituted at the *C*-6 position of the pyrimidinone ring with representative examples shown in **Table 4**.

Table 4. USP7 biochemical potencies of monocyclic pyrimidinones.

Cmpd	R$_3$	USP7 IC$_{50}$ (μM)	Cmpd	R$_3$	USP7 IC$_{50}$ (μM)
35	(H)	23	41	(N-O)	7.2
36	(NHMe)	4.2	42	(N-N)	0.39
37	(NH-Ph)	1.9	43	(O-NH)	12
38	(CH$_2$-Ph)	90	44	(morpholine-NH)	1.4
39	(alkynyl-Ph)	2.8	45	(N-N-NH)	1.1
40	(N-NH)	0.12	46	(pyrrolidine-NH)	0.09

Overall SAR at this position suggested that substitution was largely beneficial for USP7 potency. Substitution with simple amines such as methylamine or aniline (compounds **36** and **37**) increased potency by 5 to 10-fold versus the unsubstituted analogue **35**. Switching the –NH linker in compound **37** to –CH$_2$ in compound **38** resulted in a significant USP7 potency dropoff (IC$_{50}$ = 1.9 μM versus 90 μM). Potency was regained when the methylene phenyl linker in compound **38** was switched to an alkynyl linker group (compound **39**, IC$_{50}$ = 2.8 μM). Ethylenediamine analogue **40** (IC$_{50}$ = 0.12 μM) represented a >190-fold improvement in USP7 biochemical potency over the simple analogue **35** suggestive of new hydrogen-bonding interactions. Indeed, close analogues of compound **40** in which the hydrogen-bonding interaction potential of the ethylenediamine side-chain was modified led to decreases in potency (compounds **41**-**45**). Pyrrolidine analogue **46** represented the first

sub-100 nM compound from this series and suggested that the basic pyrrolidine nitrogen was making a critical hydrogen-bond with USP7.

Intrigued by the observed SAR in which the precise nature of the side-chain linker and the pK_a of the amine both seemed crucial, molecular modelling suggested that a close interaction between the protonated amine of **46** and Asp295 was potentially achievable. This was subsequently confirmed *via* a high resolution (2.2 Å) X-ray co-crystal structure of compound **46** bound to USP7 that demonstrates a similar binding mode to that of compound **16** reported previously (**Figures 3A** and **3B**).[22]
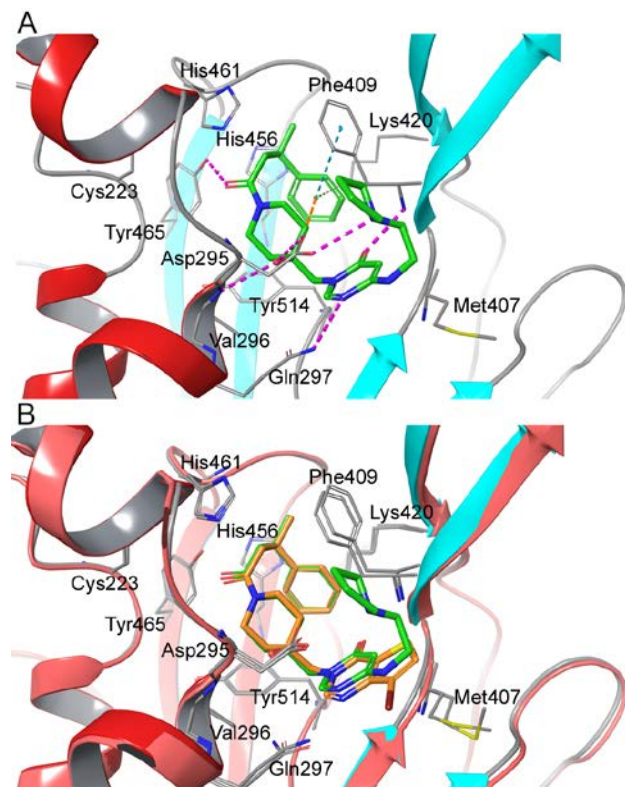

Figure 3. A. High-resolution X-ray co-crystal structure of USP7 in complex with **46** (PDB code: 6F5H). **B**. Overlay of compounds **46** (green) and **16**[22] (orange, PDB code: 5N9R) bound to USP7.

In addition to the important hydrogen bond interaction network observed previously with **16**, we also observed the postulated additional hydrogen bond between the protonated nitrogen of the pyrrolidine side-chain of **46** and Asp295 demonstrating a unique bidentate binding interaction pattern with Asp295 which had not been previously reported. This extra interaction appears to be crucial for improving affinity within the monocyclic series. As with compound **16**, the amide carbonyl in **46** interacts with the Tyr465 hydroxyl group and the ligand tertiary alcohol and forms hydrogen bonds with both Asp295 and Val296. With regards to the pyrimidinone ring of **46**, the carbonyl oxygen atom forms a hydrogen bond with the backbone NH of Phe409 whereas the *N*-4 ring atom forms a hydrogen bond with Gln297 effectively stabilizing the monocyclic ring with interactions above and below the ring system (**Figure 3A**). The folded bioactive conformation of the phenethylamide side chain of **46** may partly be induced by allylic 1,3-strain between the benzylic CH and the phenyl ring as well as stabilization *via* a CH-π intramolecular interaction between the piperidine *C*-3 axial hydrogen and the phenyl ring. These intramolecular conformational drivers may in part be responsible for inducing binding site side-chain movements that create the overall binding

pocket that accommodates this portion of the ligand. The resulting conformation is stabilized by a cation-π interaction between Lys420 and the ligand phenyl ring in addition to an edge-to-face π-π interaction with Phe409. Additional contacts between the phenethylamide methylene hydrogen atoms of **46** and the π system of His461 may also contribute to overall binding efficiency.

With highly potent USP7 inhibitors (from two distinct subseries) such as **34** and **46** in hand, we performed extensive *in vitro* profiling and demonstrated that **34** shows potent target engagement of endogenous USP7 in cells as well as excellent selectivity for USP7 in panels of deubiquitinases, proteases and kinases.[22] In addition, we also identified cancer cell lines that are hyper-sensitive to our USP7 inhibitors. Similar to compound **34**, monocyclic analogue **46** shows excellent selectivity versus a panel of USPs (n = 21) when screened at a fixed concentration of 10 μM as well as potent intracellular USP7 target engagement (EC_{50} = 0.32 μM) in cells (**Figures 4A** and **4B**).
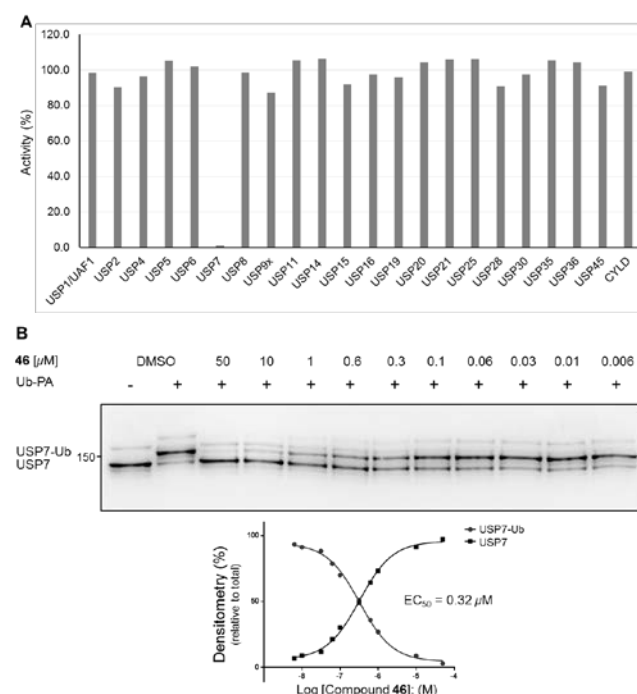
Figure 4. A. Selectivity profile of **46** against a panel of 21 USPs. Screening was performed at a fixed concentration of 10 μM (Ubiquigent). **B**. USP7 target engagement of **46** in HCT116 cells. See Supporting Information for assay conditions.

In parallel to the in-depth cellular profiling outlined above, potent compounds were assessed in a range of *in vitro* assays in order to determine their suitability for *in vivo* studies. Generally, non-basic analogues were unstable in both human and mouse liver microsomes and had poor caco-2 permeability if they contained >1 hydrogen bond donor (HBD), thus precluding them from *in vivo* PK studies (e.g. compounds **23** and **33**, **Table 5**). Likewise, benzylic amine **34** was found to have high *in vitro* metabolic turnover in both human and mouse liver microsomes (with predicted hepatic clearances of 18 mL/min/kg and 62 mL/min/kg respectively), whereas monocycle **46** had moderate metabolic stability in human and mouse liver microsomes (11 mL/min/kg and 34 mL/min/kg respectively). The aqueous kinetic solubility of both **34** and **46** was high (KSol > 190 μM) but the caco-2 A:B permeability of each at pH 6.5 was found to be low (P_{app} < 0.3 x 10^{-6} cm/s) limiting their potential for oral dosing. The low predicted *in vivo* hepatic stability of

4

the highly potent benzylic amine **34** encouraged us to carry out further chemical optimization in order to identify compounds with improved metabolic stability to facilitate *in vivo* proof-of-concept studies. As part of the chemistry program aimed at improving metabolic stability of compound **34**, trifluoromethyl analogue **47** (**Figure S3**, SI) was prepared via an asymmetric hydrogenation route described previously.[22]

Table 5. Biochemical and ADME profiling of compounds 23, 33, 34, 46 and 47.

Cmpd	USP7 IC_{50} (μM)	HLM / MLM CL_{hep}	$logD_{7.4}$ / KSol	Caco-2 P_{app} A:B
23	0.030	19 / 84	2.3 / 175	1.93
33	0.040	19 / 86	1.7 / 200	0.15
34	0.006	18 / 62	-0.1 / 191	0.28
46	0.087	11 / 34	0 / 200	0.26
47	0.022	7 / 28	0.1 / 179	0.14

Table notes: See Supporting Information for assay conditions. HLM/MLM units: mL/min/kg. KSol units: μM. Caco-2 units: 10^{-6} cm/s

Compound **47** was found to have improved *in vitro* microsomal stability in both human and mouse microsomes compared to compound **34** (HLM CL_{hep} 7 mL/min/kg vs 18 mL/min/kg and MLM CL_{hep} 28 mL/min/kg *vs*. 62 mL/min/kg) (**Table 5**). Furthermore, **47** largely maintained USP7 biochemical potency when contrasted with methyl analogue **34** (IC_{50} = 22 nM vs 6 nM).

The pharmacokinetic profiles of compounds **46** and **47** were subsequently assessed in male CD-1 mice (**Table 6**). As expected from their low caco-2 permeabilities (A:B P_{app} < 0.3 x 10^{-6} cm/s) both compounds exhibited poor oral bioavailability (*F* < 1%). However, when dosed intraperitoneally (*i.p.*) both compounds exhibited reasonable bioavailability (*F* = 44% and 64% for **46** and **47** respectively). Volume of distribution for each compound was low (V_{ss} ≤ 1 L/kg), in line with their low lipophilicities ($logD_{7.4}$ ≤ 0). Compound **46** demonstrated low plasma clearance (CL = 13 mL/min/kg) whereas compound **47** had moderate clearance (CL = 32 mL/min/kg). Further optimization studies aimed at improving PK profiles based on these encouraging preliminary *in vivo* results are underway and will be reported in due course.

Table 6. Pharmacokinetic profile of compounds 46 and 47 in male CD-1 mice

Route[a]	Cmpd	C_{max}[b]	AUC_{all}[c]	$t_{1/2}$[d]	CL[e]	V_{ss}[f]
i.v.	**46**	4035	1700	1.1	13	0.4
	47	2808	516	1.0	32	0.9
i.p	**46**	3430	3002	1.1	-	-
	47	2876	1904	0.8	-	-

[a] *i.v.* **46** 1.3 mg/kg, **47** 0.9mg/kg in 2% DMSO in 20% aq. 2-hydroxypropyl-β-cyclodextrin, *i.p.* **46** 6.2 mg/kg, **47** 5.7 mg/kg in saline. [b] ng/mL. [c] ng.h/mL. [d] hr. [e] mL/min/kg. [f] L/kg.

In conclusion, we have identified and optimized highly potent USP7 inhibitors based on two different core chemotypes. Key compounds have a well understood mode of binding as evidenced by the high resolution co-crystal structures obtained. These USP7 inhibitors have been highly valuable in validating the druggability of USP7 as well as enabling studies towards a deeper understanding of the underlying biology of USP7 and its potential as a therapeutic target. Efforts are continuing towards the further development of these inhibitors into compounds suitable for *in vivo* proof-of-concept studies.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental details and characterization data for all compounds, biochemical, cellular & selectivity assay protocols, crystallization conditions and methodology, *in vitro* ADME assay protocols, *in vivo* procedures, computational methods and supplementary Figures (PDF).

AUTHOR INFORMATION

Corresponding Author

*Email: colin.odowd@almacgroup.com

Present Addresses

§ E. A., Randox laboratories, 55 Diamond road, Crumlin, County Antrim, BT29 4QY UK; J. F., UCB Celltech, 208 Bath Road, Slough, SL1 3WE, UK; K. M. The European Bioinformatics Institute (EMBL-EBI), Wellcome Genome Campus, CB10 1SD, UK; E. O., Medivir AB, 141 22 Huddinge, Sweden; L. J., Redag Crop Protection, BioHub, Alderley Park, Mereside, Macclesfield SK10 4TG, UK.

Author Contributions

TH conceived the concept and directed the research. COD and GG helped conceive and develop the concept and designed and supervised medicinal chemistry and biology experiments. MH, EA, JF, HM, JSSR and LJ carried out the design, synthesis and characterization of compounds. NP performed SPR experiments. ER, CH, KMcC, EO, EC, and NP carried out compound screening, target validation and biochemical and cellular profiling studies. OB carried out computational modelling and structural analysis. SFB carried out ADME profiling. SD performed NMR experiments. The manuscript was written through contributions of all authors.

ABBREVIATIONS

CPMG, Carr-Purcell-Meiboom-Gill sequence NMR experiment; DUB, deubiquitinase; ee, enantiomeric excess; HAC, heavy atom count; HLM, human liver microsomes; KSol, kinetic solubility; LE, ligand efficiency (-1.4 x pK_D or pIC_{50}/HAC); MLM, mouse liver microsomes; PK, pharmacokinetics; SAR, structure-activity relationship; STD, saturation transfer difference; UPS, ubiquitin proteasome system; USP, ubiquitin specific protease; Water-LOGSY, Water-Ligand Observed via Gradient SpectroscopY.

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Identification and Structure-Guided Development of Pyrimidinone Based USP7 Inhibitors

Colin R. O'Dowd[†]*, Matthew D. Helm[†], J. S. Shane Rountree[†], Jakub T. Flasz[‡§], Elias Arkoudis[‡§], Hugues Miel[†], Peter R. Hewitt[†], Linda Jordan[†§], Oliver Barker[†], Caroline Hughes[†], Ewelina Rozycka[†], Eamon Cassidy[†], Keeva McClelland[†§], Ewa Odrzywol[†§], Natalie Page[†], Stephanie Feutren-Burton[†], Scarlett Dvorkin[‡], Gerald Gavory[†], Timothy Harrison[†‡].

[†]Almac Discovery Ltd, Centre for Precision Therapeutics, 97 Lisburn Road, Belfast, Northern Ireland BT9 7AE, United Kingdom. [‡]Centre for Cancer Research and Cell Biology, Queen's University Belfast, Northern Ireland BT9 7AE, United Kingdom.

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1. Materials and reagents:

Common organic solvents that were used in reactions (e.g. THF, DMF, DCM, and methanol) were purchased anhydrous from Sigma-Aldrich® in Sure/Seal™ bottles and were handled appropriately under nitrogen. Water was deionised using an Elga PURELAB Option-Q. All other solvents used (i.e. for work-up procedures and purification) were generally HPLC grade and were used as supplied from various commercial sources. Unless otherwise stated, all starting materials used were purchased from commercial suppliers and used as supplied.

For assay purposes, all reagents and chemicals were purchased from Sigma-Aldrich® unless otherwise stated. All inhibitors were prepared as 10 mM DMSO stocks for cell culture experiments and stored in a controlled environment using the MultiPod system. CellTiter-Glo® was purchased from Promega (#G7571). The ubiquitin-propargylamine (Ub-PA) probe was purchased from UbiQ (#UbiQ-057). Unless otherwise stated, all other reagents were obtained from commercial sources and used without further purification.

2. Experimental Procedures and Spectroscopic Data:

Abbreviations and Acronyms

aq: aqueous; Boc: *tert*-butyloxycarbonyl; DCM: dichloromethane; br: broad; d: doublet; DIPEA: diisopropylethylamine; DMAP: 4-(dimethylamino)pyridine; DMF: *N,N*-dimethylformamide; DMSO: dimethylsulfoxide; dppf: 1,1'-bis(diphenylphosphino)ferrocene; EDC: *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride; EtOAc: ethyl acetate; ESI: electrospray ionisation; h: hour; HATU: *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HPLC: high pressure liquid chromatography; LC: liquid chromatography; LCMS: liquid chromatography mass spectrometry; *m/z*: mass-to-charge ratio; MeCN: acetonitrile; MeOH: methanol; min: minutes; MS: mass spectrometry; m: multiplet (spectral); OAc: acetate; PE: petroleum ether (40-60 °C); R_T: retention time; rt: room temperature; s: singlet; t: triplet; TFA: trifluoroacetic acid; THF: tetrahydrofuran.

1

General Experimental Conditions

Microwave synthesis

Microwave experiments were carried out using a Biotage Initiator™ Eight system or a CEM Discover™/Explorer24™ system controlled by Synergy 1.5 software. Both machines give good reproducibility and control at temperature ranges from 60-250 °C and pressures of up to a maximum of 20 bar.

Flash chromatography

Purification of compounds by flash chromatography was achieved using a Biotage Isolera Four system. Unless otherwise stated, Biotage KP-Sil SNAP cartridge columns (10-340 g) were used along with the stated solvent system and an appropriate solvent gradient depending on compound polarity. In the case of more polar and basic compounds, Biotage KP-NH SNAP cartridge columns (11 g) were used.

NMR spectroscopy

^{1}H NMR spectra were recorded at ambient temperature using a Bruker Avance (300 MHz), Bruker Avance III (400 MHz) or Bruker Ascend (500 MHz) spectrometer. All chemical shifts (δ) are expressed in ppm. Residual solvent signals were used as an internal standard and the characteristic solvent peaks were corrected to the reference data outlined in *J. Org. Chem.*, 1997, 62, p7512-7515; in other cases, NMR solvents contained tetramethylsilane, which was used as an internal standard.

Liquid Chromatography Mass Spectrometry (LCMS)

Liquid Chromatography Mass Spectrometry (LCMS) experiments to determine retention times (R_T) and associated mass ions were performed using the following method:

Method A: The system consisted of an Agilent Technologies 6130 quadrupole mass spectrometer linked to an Agilent Technologies 1290 Infinity LC system with UV diode array detector and autosampler. The spectrometer consisted of an electrospray ionization source operating in positive and negative ion mode. LCMS experiments were performed on each sample submitted using the following conditions: LC Column: Agilent Eclipse Plus C18 RRHD, 1.8 µm, 50 x 2.1 mm maintained at 40 °C. Mobile phases: A) 0.1% (v/v) formic acid in water; B) 0.1% (v/v) formic acid in acetonitrile.

Gradient Time (min)	Flow (mL/min)	%A	%B
0.00	0.5	80	20
1.80	0.5	0	100
2.20	0.5	0	100
2.50	0.5	80	20
3.00	0.5	80	20

Method B: The system consisted of an Agilent Technologies 6140 single quadrupole mass spectrometer linked to an Agilent Technologies 1290 Infinity LC system with UV diode array detector and autosampler. The spectrometer consisted of a multimode ionization source (electrospray and atmospheric pressure chemical ionizations) operating in positive and negative ion mode. LCMS experiments were performed on each sample submitted using the following conditions: LC Column: Zorbax Eclipse Plus C18 RRHD, 1.8 µm, 50 x 2.1 mm maintained at 40 °C. Mobile phases: A) 0.1% (v/v) formic acid in water; B) 0.1% (v/v) formic acid in acetonitrile.

Gradient Time (min)	Flow (mL/min)	%A	%B
0.00	1.0	95	5
1.80	1.0	0	100
2.20	1.0	0	100
2.21	1.0	95	5
2.50	1.0	95	5

Preparative High Pressure Liquid Chromatography

Method A: This system consisted of an Agilent Technologies 6120 single quadrupole mass spectrometer linked to an Agilent Technologies 1200 Preparative LC system with multiple wavelength detector and autosampler. The mass spectrometer used a multimode ionization source (electrospray and atmospheric pressure chemical ionizations) operating in

positive and negative ion mode. Fraction collection was mass-triggered (multimode positive and negative ion). Purification experiments, unless otherwise stated, were performed under basic conditions at an appropriate solvent gradient that was typically determined by the retention time found using the LCMS method. In cases where the basic conditions were unsuccessful, acidic conditions were employed.

Basic conditions: LC Column: Waters XBridge™ Prep C18 5 μm OBDTM 19 x 50 mm column at rt. Mobile phase: A) 0.1% (v/v) ammonium hydroxide in water; B) 0.1% (v/v) ammonium hydroxide in 95:5, acetonitrile/water. Total experiment time was *ca*. 10 min and an example method is given:

Gradient Time (min)	Flow (mL/min)	%A	%B
0.00	20.0	50	50
3.00	20.0	12	88
5.00	20.0	12	88
7.00	20.0	0	100
8.0	20.0	0	100
8.20	20.0	50	50

Acidic conditions: LC Column: Waters XBridge™ Prep C18 5μm OBDTM 19 x 50 mm column at rt. Mobile phase: A) Water 0.1% (v/v) formic acid in water; B) 0.1% (v/v) formic acid in 95:5, acetonitrile/water. Total experiment time was *ca*. 10 min and an example method is given:

Gradient Time (min)	Flow (mL/min)	%A	%B
0.00	20.0	95	5
7.00	20.0	0	100
9.00	20.0	0	100
9.20	20.0	95	5

Method B: This system consisted of a Waters Autopurification HPLC/MS, with a Gemini NX-C18 column from Phenomenex, 5 µm, 50 mm x 30 mm i.d., running at a flow rate of 60 mL/min, 25 °C with UV diode array detection (210–400 nm) and mass-directed collection. A typical gradient was 5-50% HPLC grade acetonitrile (mobile phase B) in HPLC grade water + 0.1% (v/v) ammonia solution (mobile phase A) over 10 min, or modified as necessary. The mass spectrometer used was a Waters Micromass ZQ2000 spectrometer, operating in positive or negative ion electrospray ionisation modes, with a molecular weight scan range of 150 to 1000.

The pure fractions were combined and concentrated using a Genevac EZ-2 Elite, unless stated otherwise.

Final compound purity analysis
The purity of final compounds was assessed by LCMS and ^{1}H NMR. The HPLC purity of each compound was measured using the method stated compared to a blank.

High Resolution Mass Spec
High resolution mass spectra were acquired on a Thermo ScientificLTQ Orbitrap XL spectrometer at the EPSRC UK National Mass Spectrometry Facility (University of Swansea).

General procedures

General procedure 1: Epoxide opening with a pyrimidinone
A suspension of the pyrimidinone (1 equiv.), epoxide (1-3 equiv.) and Cs_2CO_3 (1-3 equiv.) in DMF were heated at 80 °C for 10-24 h. The reaction was allowed to cool to rt, saturated $NH_4Cl_{(aq)}$ was added and the resulting mixture was extracted with DCM (x3) using a Biotage phase separator. The combined organic phases were concentrated under reduced pressure and the residue was purified by flash chromatography (Biotage KP-Sil and KP-NH, 0-100% EtOAc in cyclohexane or PE, then 0-30% MeOH in EtOAc) to give the product.

General procedure 2: *N*-Boc deprotection
A solution of the *N*-Boc piperidine in DCM/TFA was stirred for 1-24 h at rt before being concentrated under reduced pressure. The residue was then dissolved in triethylamine and DCM before being purified by flash chromatography

(Biotage KP-NH, 0-100% EtOAc in cyclohexane or PE, then 0-30% MeOH in EtOAc or 0-100% DCM in cyclohexane or PE, then 0-30% MeOH in DCM) to give the product.

General procedure 3: EDC coupling

A solution of amine (1 equiv.), carboxylic acid (1 equiv.) and EDC (3 equiv.) was stirred in (DCM) for 1-24 h at rt. The reaction was quenched by the addition of water and the resulting mixture was extracted with DCM (x3) using a Biotage phase separator. The combined organic phases were concentrated under reduced pressure and the residue was purified by flash chromatography (Biotage KP-Sil and KP-NH, 0-100% EtOAc in cyclohexane or PE, then 0-30% MeOH in EtOAc) to give the product.

General procedure 4: HATU coupling in DCM

To a suspension of the amine (1 equiv.), carboxylic acid (1-1.5 equiv.) and HATU (1-1.5 equiv.) in DCM was added DIPEA (1-4 equiv.). The reaction was stirred for 1-24 h at rt before being quenched by the addition of saturated Na-$HCO_{3(aq)}$ and the resulting mixture was extracted with DCM (x3) using a Biotage phase separator. The combined organic phases were concentrated under reduced pressure and the residue was purified by flash chromatography (Biotage KP-Sil and KP-NH, 0-100% EtOAc in cyclohexane or PE, then 0-30% MeOH in EtOAc) to give the product.

### General procedure 5: Suzuki-Miyaura coupling

A reaction vial was charged with a mixture of the bromide (1 equiv.), the organoboron reagent (1-3 equiv.), a Pd catalyst (0.05-0.1 equiv.) and an inorganic base (2-5 equiv.) in 1,4-dioxane/water and the O_2 was removed by evacuating and refilling with N_2 three times or by bubbling N_2 through the mixture before the reaction tube was sealed. The reaction was heated under the indicated conditions for the indicated time before being cooled to rt and saturated $NH_4Cl_{(aq)}$ added. The resulting mixture was extracted with DCM (x3) using a Biotage phase separator. The combined organic phases were concentrated under reduced pressure and the residue was purified by flash chromatography (Biotage KP-Sil and/or KP-NH, 0-100% EtOAc in cyclohexane or PE, then 0-30% MeOH in EtOAc) to give the product.

General procedure 6: Sonagashira coupling

A reaction tube was charged with the bromide (1 equiv.), a Cu catalyst (0.2-0.4 equiv.) and a Pd catalyst (0.1-0.2 equiv.) and then evacuated and refilled with N_2 three times. To this was added toluene, triethylamine (20-40 equiv.) and the alkyne (1-4 equiv.) before the mixture was again evacuated and refilled with N_2 three times. The reaction tube was sealed and the reaction was heated under the indicated conditions for the indicated time. The reaction was cooled to rt and quenched by the addition of saturated $NH_4Cl_{(aq)}$. The mixture was extracted with DCM (x 3) using a Biotage phase separator, the combined organic phases were concentrated under reduced pressure and the residue was purified by flash chromatography (Biotage KP-Sil and KP-NH, 0-100% EtOAc in cyclohexane or PE, then 0-30% MeOH in EtOAc) to give the product.

Compounds 11, **16**, *ent*-**16**, **17**, **34**, *ent*-**34** and **47** were prepared as described previously.[1]

Compound 2: 3-((4-Hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)thieno[2,3-*d*]pyrimidin-4(3*H*)-one
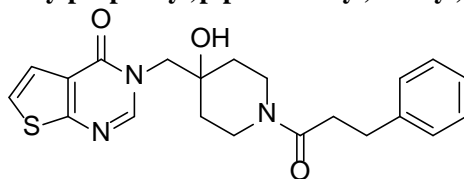
General procedure 1 using 3-phenyl-1-(1-oxa-6-azaspiro[2.5]octan-6-yl)propan-1-one[1] (63 mg, 0.257 mmol), thieno[2,3-*d*]pyrimidin-4(3*H*)-one (36 mg, 0.237 mmol), Cs_2CO_3 (91 mg, 0.279 mmol) and DMF (1.5 ml) gave title compound (48 mg, 51%) as a colourless solid. LCMS (Method A, ES⁺): R_T = 1.08 min (purity >97% at 254 nm), *m/z* Calcd for $C_{21}H_{24}N_3O_3S$ [M+H]⁺ 398, found 398. ¹H NMR (400 MHz, CDCl₃): δ 8.07 (s, 1H), 7.45 (d, 1H), 7.34-7.23 (m, 3H), 7.22-7.11 (m, 3H), 4.32 (d, 1H), 4.09 (d, 1H), 3.98 (d, 1H), 3.58 (d, 1H), 3.42-3.21 (m, 1H), 3.10-2.98 (m, 1H), 2.96-2.84 (m, 2H), 2.69-2.49 (m, 2H), 1.68-1.29 (m, 4H).

Compound 3: 3-((4-Hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one
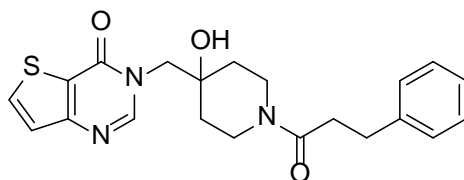
Step 1: *tert*-Butyl 4-hydroxy-4-((4-oxothieno[3,2-*d*]pyrimidin-3(4*H*)-yl)methyl)piperidine-1-carboxylate: General procedure 1 using *tert*-butyl 1-oxa-6-azaspiro[2.5]octane-6-carboxylate[1] (640 mg, 3.00 mmol), thieno[3,2-*d*]pyrimidin-4(3*H*)-one (502 mg, 3.30 mmol), Cs_2CO_3 (977 mg, 3.00 mmol) and DMF (6 mL) gave the title compound (889 mg, 81%) as a pale yellow foam. LCMS (Method A, ES⁺): R_T = 1.07 min, *m/z* Calcd for $C_{17}H_{24}N_3O_4S$ [M+H]⁺ 366, found 366. ¹H NMR (300 MHz, CDCl₃): δ 8.18 (s, 1H), 7.70 (d, 1H), 7.18 (d, 1H), 4.25-3.87 (m, 2H), 3.76 (br s, 2H), 3.08 (t, 2H), 1.67-1.39 (m, 4H), 1.33 (s, 9H).

Step 2: 3-((4-Hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one: A solution of *tert*-butyl 4-hydroxy-4-((4-oxothieno[3,2-*d*]pyrimidin-3(4*H*)-yl)methyl)piperidine-1-carboxylate (15 mg, 41.0 µmol) was stirred in TFA (1 mL) for 16 h at rt. The resulting mixture was concentrated under reduced pressure and to the residue were successively added DCM (0.33 mL), DIPEA (13.8 µL), 3-phenylpropanoic acid (4.75 mg, 0.032 mmol), EDC (10.1 mg, 0.053 mmol), and HOBt (8 mg, 0.053 mmol). The reaction mixture was stirred for 30 min at rt before being diluted with water (10 mL) and the resulting mixture was extracted with DCM (3 x 10 mL) using a Biotage phase separator. The combined organic phases were concentrated under reduced pressure and the residue was purified by flash chromatography (Biotage KP-Sil 10 g cartridge, 0-100% EtOAc in PE, then 0-30% MeOH in EtOAc) to give the title compound (4 mg, 24%) as a colourless solid. LCMS (Method A, ES⁺): R_T = 1.00 min (purity >95% at 254 nm), *m/z* Calcd for $C_{21}H_{24}N_3O_3S$ [M+H]⁺ 398, found 398. ¹H NMR (300 MHz, CDCl₃): δ 8.05 (s, 1H), 7.84 (d, 1H), 7.35 (d, 1H), 7.32-7.19 (m, 5H), 4.41 (d, 1H), 4.13-4.00 (m, 2H), 3.62 (s, 1H), 3.60 (d, 1H), 3.33 (t, 1H), 3.08-2.94 (m, 3H), 2.66-2.59 (m, 2H), 1.69-1.25 (m, 4H).

Compound 4: 3-((4-Hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)furo[2,3-*d*]pyrimidin-4(3*H*)-one
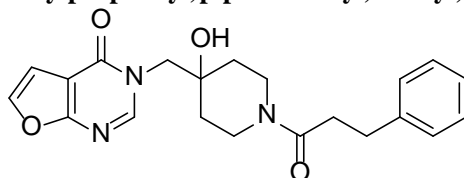
Step 1: *tert*-Butyl 4-hydroxy-4-((2-iodofuran-3-carboxamido)methyl)piperidine-1-carboxylate: General procedure 3 using *tert*-butyl 4-(aminomethyl)-4-hydroxypiperidine-1-carboxylate (100 mg, 0.434 mmol), 2-iodofuran-3-carboxylic acid (103 mg, 0.434 mmol), EDC (166 mg, 0.868 mmol) and DCM (5.4 mL) gave the title compound (123 mg, 62%) as a yellow solid. LCMS (Method A, ES⁺): R_T = 1.22 min, *m/z* Calcd for $C_{16}H_{23}IN_2NaO_5$ [M+Na]⁺ 473, found 473.

Step 2: *tert*-Butyl 4-hydroxy-4-((4-oxofuro[2,3-*d*]pyrimidin-3(4*H*)-yl)methyl)piperidine-1-carboxylate: A suspension of *tert*-butyl 4-hydroxy-4-((2-iodofuran-3-carboxamido)methyl)piperidine-1-carboxylate (400 mg, 0.888 mmol), formamidine hydrochloride (358 mg, 4.44 mmol), CuI (17 mg, 89.2 µmol) and K_2CO_3 (368 mg, 2.67 mmol) in DMF (6 mL) was heated in a microwave at 150 °C for 8 h. The reaction mixture was partitioned between 1:1 brine/water (40 mL) and EtOAc (10 mL) and the mixture was filtered through a plug of Celite®. The aqueous layer was separated and extracted into EtOAc (3 x 10 mL). The combined organic layers were washed with 1:1 brine/water (40 mL), dried over Na_2SO_4, concentrated under reduced pressure and the residue was purified by flash chromatography (Biotage KP-Sil 50 g cartridge, 0-100% EtOAc in PE) to give the title compound (34 mg, 10%) as a pale yellow solid. LCMS (Method A, ES⁺): R_T = 1.17 min, *m/z* Calcd for $C_{17}H_{23}N_3NaO_5$ [M+Na]⁺ 372, found 372.

Step 3: 3-((4-Hydroxypiperidin-4-yl)methyl)furo[2,3-*d*]pyrimidin-4(3*H*)-one: General procedure 2 using *tert*-butyl 4-hydroxy-4-((4-oxofuro[2,3-*d*]pyrimidin-3(4*H*)-yl)methyl)piperidine-1-carboxylate (34 mg, 97.3 µmol), TFA (0.6 mL) and DCM (0.6 mL) gave the title compound (23 mg, 94%) as a pale yellow solid. LCMS (Method A, ES⁺): R_T = 0.23 min, *m/z* Calcd for $C_{12}H_{16}N_3O_3$ [M+H]⁺ 250, found 250.

Step 4: 3-((4-Hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)furo[2,3-*d*]pyrimidin-4(3*H*)-one: General procedure 3 using 3-((4-hydroxypiperidin-4-yl)methyl)furo[2,3-*d*]pyrimidin-4(3*H*)-one (23 mg, 92.3 µmol), 3-phenylpropanoic acid (14 mg, 92.3 µmol), EDC (53 mg, 0.277 mmol) and DCM (0.9 mL) gave the title compound (13 mg,
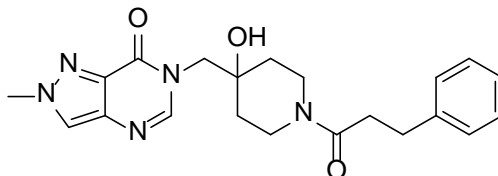
36%) as a colourless solid. LCMS (Method A, ES$^+$): R$_T$ = 0.91 min (purity >95% at 254 nm), m/z Calcd for C$_{21}$H$_{24}$N$_3$O$_4$ [M+H]$^+$ 382, found 382. ^{1}H NMR (300 MHz, CDCl$_3$): δ 7.99 (s, 1H), 7.49 (d, 1H), 7.33-7.21 (m, 5H), 6.92 (d, 1H), 4.41 (d, 1H), 4.16-4.00 (m, 2H), 3.56 (d, 1H), 3.32 (t, 1H), 3.08-2.94 (m, 4H), 2.66-2.60 (m, 2H), 1.65-1.27 (m, 4H).

Compound 5: 3-((4-Hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)furo[3,2-*d*]pyrimidin-4(3*H*)-one
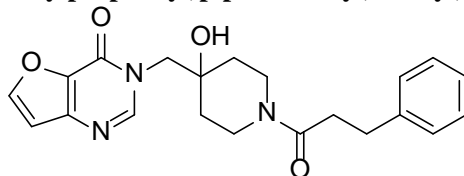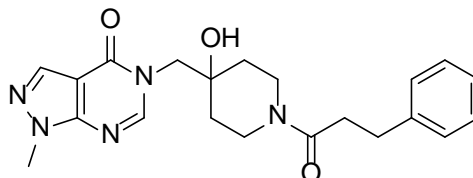

General procedure 1 using 3-phenyl-1-(1-oxa-6-azaspiro[2.5]octan-6-yl)propan-1-one[1] (81 mg, 0.331 mmol), furo[3,2-*d*]pyrimidin-4(3*H*)-one (45 mg, 0.331 mmol), Cs$_2$CO$_3$ (323 mg, 0.992 mmol) and DMF (0.6 mL) gave the title compound (100 mg, 79%) as a colourless solid. LCMS (Method A, ES$^+$): R$_T$ = 0.94 min (purity >98% at 254 nm), m/z Calcd for C$_{21}$H$_{24}$N$_3$O$_4$ [M+H]$^+$ 382, found 382. ^{1}H NMR (300 MHz, CDCl$_3$): δ 8.10 (s, 1H), 7.81 (d, 1H), 7.32-7.19 (m, 5H), 6.80 (d, 1H), 4.35 (d, 1H), 4.17-4.00 (m, 2H), 3.92 (s, 1H), 3.60 (d, 1H), 3.33 (t, 1H), 3.09-2.92 (m, 3H), 2.65-2.58 (m, 2H), 1.61-1.26 (m, 4H).

Compound 6: 6-((4-Hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one
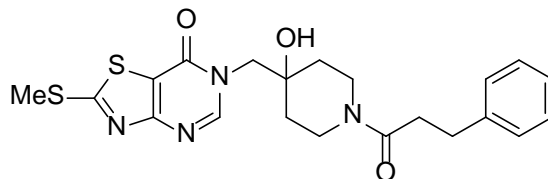

General procedure 1 using 3-phenyl-1-(1-oxa-6-azaspiro[2.5]octan-6-yl)propan-1-one[1] (65 mg, 0.266 mmol), 2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one (Oslob, J. D.; Yu, C. H. WO 2007/013964 A1; Feb 01, 2007) (40 mg, 0.266 mmol) and Cs$_2$CO$_3$ (260 mg, 0.799 mmol) gave the title compound (54 mg, 51%) as a colourless solid. LCMS (Method A, ES$^+$): R$_T$ = 0.85 min (purity >96% at 254 nm), m/z Calcd for C$_{21}$H$_{26}$N$_5$O$_3$ [M+H]$^+$ 396, found 396. ^{1}H NMR (300 MHz, CDCl$_3$): δ 7.91 (s, 1H), 7.78 (s, 1H), 7.31-7.18 (m, 5H), 4.32 (d, 1H), 4.21-3.95 (m, 5H), 3.60 (d, 1H), 3.38-3.30 (m, 1H), 3.10-3.01 (m, 1H), 2.92 (t, 2H), 2.66-2.55 (m, 2H), 1.62-1.37 (m, 4H).

Compound 7: 5-((4-Hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)-1-methyl-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one

General procedure 1 using 3-phenyl-1-(1-oxa-6-azaspiro[2.5]octan-6-yl)propan-1-one[1] (48 mg, 0.196 mmol), 1-methyl-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one (27 mg, 0.180 mmol), Cs$_2$CO$_3$ (70 mg, 0.213 mmol) and DMF (1.5 mL) gave the title compound (43 mg, 60%) as a colourless solid. LCMS (Method A, ES$^+$): R$_T$ = 0.93 min (purity >98% at 254 nm), m/z Calcd for C$_{21}$H$_{26}$N$_5$O$_3$ [M+H]$^+$ 396, found 396. ^{1}H NMR (300 MHz, CDCl$_3$): δ 8.05 (s, 1H), 8.03 (s, 1H), 7.36-7.24 (m, 2H), 7.24-7.10 (m, 3H), 4.31 (d, 1H), 4.13-4.07 (m, 1H), 3.97 (s, 3H), 3.96-3.88 (m, 1H), 3.58 (d, 1H), 3.41-3.21 (m, 1H), 3.12-2.97 (m, 1H), 2.97-2.84 (m, 2H), 2.71-2.48 (m, 2H), 1.65-1.29 (m, 4H).

Compound 8: 6-((4-Hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)-2-(methylthio)thiazolo[4,5-*d*]pyrimidin-7(6*H*)-one

General procedure 1 using 3-phenyl-1-(1-oxa-6-azaspiro[2.5]octan-6-yl)propan-1-one[1] (55 mg, 0.224 mmol), 2-(methylthio)thiazolo[4,5-*d*]pyrimidin-7(6*H*)-one (Prepared according to *Liebigs Annalen der Chemie*, 1989, p409-411) (41

6

mg, 0.206 mmol), Cs_2CO_3 (80 mg, 0.246 mmol) and DMF (1.5 mL) gave the title compound (36 mg, 39%) as a colourless solid. LCMS (Method A, ES$^+$): R_T = 1.12 min (purity >99% at 254 nm), m/z Calcd for $C_{21}H_{25}N_4O_3S_2$ [M+H]$^+$ 445, found 445. ^{1}H NMR (300 MHz, CDCl$_3$): δ 8.20 (s, 1H), 7.47-7.06 (m, 5H), 4.69-4.28 (m, 2H), 4.17-3.90 (m, 2H), 3.62 (d, 1H), 3.49-3.26 (m, 1H), 3.19-2.87 (m, 3H), 2.74 (s, 3H), 2.69-2.50 (m, 2H), 1.87-1.32 (m, 4H).

Compound 9: 6-Bromo-3-((4-hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one
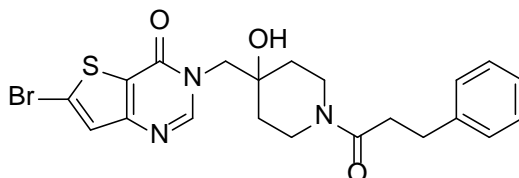
Step 1: 6-Bromothieno[3,2-*d*]pyrimidin-4(3*H*)-one: To a solution of 6-bromo-4-chlorothieno[3,2-*d*]pyrimidine (600 mg, 2.40 mmol) in THF (5 mL) was added 1 M NaOH$_{(aq)}$ (3.61 mL, 3.61 mmol). The reaction was stirred at rt for 17 h before the reaction was heated to 50 °C for 3 h. The reaction was cooled to rt and 1 M HCl$_{(aq)}$ was added until a neutral pH was achieved. The product was collected by filtration and subsequently three additional batches of the product were collected from the mother liquor after allowing it to stand for ~1 h. All the batches were dried in vacuo to give the title compound (471 mg, 84%) as a yellow solid. LCMS (Method A, ES$^+$): R_T = 0.75 min, m/z Calcd for $C_6H_4BrN_2OS$ [M+H]$^+$ 231, 233, found 231, 233. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.15 (s, 1H), 7.61 (s, 1H).

Step 2: 6-Bromo-3-((4-hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one: General procedure 1 using 3-phenyl-1-(1-oxa-6-azaspiro[2.5]octan-6-yl)propan-1-one[1] (45 mg, 0.183 mmol), 6-bromothieno[3,2-*d*]pyrimidin-4(3*H*)-one (39 mg, 0.169 mmol), Cs_2CO_3 (65 mg, 0.199 mmol) and DMF (1.5 mL) gave, after purification by preparative HPLC, the title compound (4.2 mg, 5%) as a colourless solid. LCMS (Method A, ES$^+$): R_T = 1.29 min (purity >98% at 254 nm), m/z Calcd for $C_{21}H_{23}BrN_3O_3S$ [M+H]$^+$ 476, 478, found 476, 478. ^{1}H NMR (300 MHz, CDCl$_3$): δ 8.03 (s, 1H), 7.45-7.15 (m, 6H), 4.40 (d, 1H), 4.04 (dd, 2H), 3.61 (d, 1H), 3.44-3.19 (m, 1H), 3.14-2.90 (m, 4H), 2.78-2.51 (m, 2H), 1.65-1.39 (m, 2H), 1.39-1.14 (m, 2H).
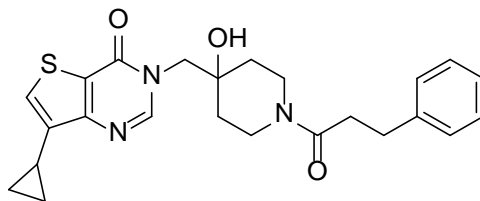
Compound 10: 5-Bromo-3-((4-hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)thieno[2,3-*d*]pyrimidin-4(3*H*)-one
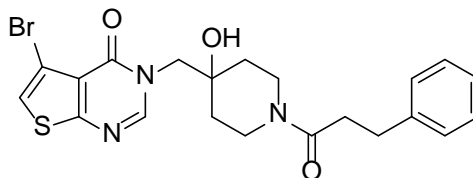
Step 1: 5-Bromothieno[2,3-*d*]pyrimidin-4(3*H*)-one: To a stirred suspension of thieno[2,3-*d*]pyrimidin-4(3*H*)-one (250 mg, 1.64 mmol) in acetic acid (1.6 mL) in a reaction tube was dropwise added Br$_2$ (0.254 mL, 4.93 mmol). The reaction tube was sealed and the mixture was heated at 95 °C for 16 h before the reaction was allowed to cool to rt. To this mixture was added MeOH (7 mL) and Et$_2$O (7 mL). The resulting precipitate was collected by filtration and washed with Et$_2$O (50 mL) before being dried under high vacuum to give the title compound (352 mg, 93%) as a beige solid. LCMS (Method A, ES$^+$): R_T = 0.84 min, m/z Calcd for $C_6H_4BrN_2OS$ [M+H]$^+$ 231, 233, found 231, 233. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.67 (br s, 1H), 8.15 (s, 1H), 7.55 (s, 1H).

Step 2: 5-Bromo-3-((4-hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)thieno[2,3-*d*]pyrimidin-4(3*H*)-one: General procedure 1 using 3-phenyl-1-(1-oxa-6-azaspiro[2.5]octan-6-yl)propan-1-one[1] (49 mg, 0.200 mmol), 5-bromothieno[2,3-*d*]pyrimidin-4(3*H*)-one (42 mg, 0.182 mmol), Cs_2CO_3 (71 mg, 0.218 mmol) in DMF (1.5 ml) gave the title compound (26 mg, 30%) as a colourless solid. LCMS (Method A, ES$^+$): R_T = 1.34 min (purity >96% at 254 nm), m/z Calcd for $C_{21}H_{23}BrN_3O_3S$ [M+H]$^+$ 476, 478, found 476, 478. ^{1}H NMR (300 MHz, CDCl$_3$): δ 8.00 (s, 1H), 7.48 (s, 1H), 7.42-7.14 (m, 5H), 4.36 (d, 1H), 4.11 (d, 1H), 3.97 (d, 1H), 3.73-3.54 (m, 2H), 3.46-3.21 (m, 1H), 3.18-2.87 (m, 3H), 2.79-2.49 (m, 2H), 1.69-1.30 (m, 4H).

**Compound 12: 7-Cyclopropyl-3-((4-hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one**

General procedure 5 using 7-bromo-3-((4-hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one[1] (23 mg, 48.3 µmol), cyclopropylboronic acid (12 mg, 0.145 mmol), PdCl$_2$(dppf) (3.5 mg, 4.83 µmol) and K$_3$PO$_4$ (46 mg, 0.217 mmol) in 1,4-dioxane (0.5 mL) and water (0.1 mL) at 110 °C for 16 h gave the title compound (9.5 mg, 44%) as a colourless solid. LCMS (Method A, ES$^+$): R$_T$ = 1.29 min (purity >98% at 254 nm), *m/z* Calcd for C$_{24}$H$_{28}$N$_3$O$_3$S [M+H]$^+$ 438, found 438. ¹H NMR (300 MHz, CDCl$_3$): δ 8.07 (s, 1H), 7.40-7.14 (m, 6H), 4.43 (d, 1H), 4.18-4.06 (m, 2H), 3.75-3.48 (m, 1H), 3.44-3.24 (m, 1H), 3.14-2.89 (m, 3H), 2.72-2.53 (m, 2H), 2.33-2.14 (m, 1H), 1.70-1.30 (m, 5H), 1.12-0.92 (m, 2H), 0.92-0.70 (m, 2H).

Compound 13: 7-Ethynyl-3-((4-hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one
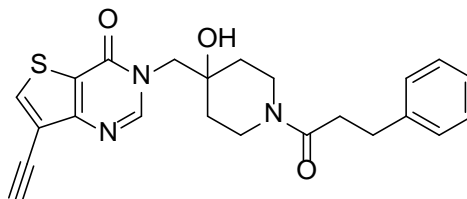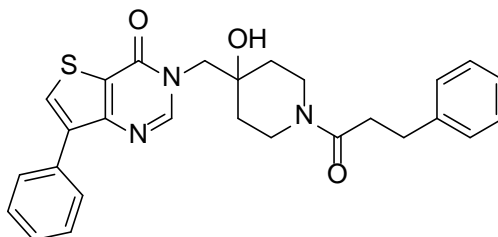

Step 1: *tert*-Butyl 4-hydroxy-4-((4-oxo-7-((trimethylsilyl)ethynyl)thieno[3,2-*d*]pyrimidin-3(4*H*)-yl)methyl)piperidine-1-carboxylate: General procedure 6 using *tert*-butyl 4-((7-bromo-4-oxothieno[3,2-*d*]pyrimidin-3(4*H*)-yl)methyl)-4-hydroxypiperidine-1-carboxylate[1] (102 mg, 0.230 mmol), CuI (8.7 mg, 45.7 µmol), Pd(PPh$_3$)$_2$Cl$_2$ (16 mg, 22.8 µmol), triethylamine (0.640 mL, 4.59 mmol), ethynyltrimethylsilane (0.127 mL, 0.917 mmol) and toluene (2 mL) at 110 °C for 16 h gave the title compound (100 mg, 94%) as a yellow oil. LCMS (Method A, ES$^+$): R$_T$ = 1.76 min, *m/z* Calcd for C$_{22}$H$_{32}$N$_3$O$_4$SSi [M+H]$^+$ 462, found 462. ¹H NMR (400 MHz, CDCl$_3$): δ 8.21 (s, 1H), 7.96 (s, 1H), 4.27-4.03 (m, 2H), 3.97-3.75 (m, 2H), 3.15 (t, 2H), 3.06 (s, 1H), 1.78-1.49 (m, 4H), 1.45 (s, 9H), 0.29 (s, 9H).

Step 2: 7-Ethynyl-3-((4-hydroxypiperidin-4-yl)methyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one: General procedure 2 using *tert*-butyl 4-hydroxy-4-((4-oxo-7-((trimethylsilyl)ethynyl)thieno[3,2-*d*]pyrimidin-3(4*H*)-yl)methyl)piperidine-1-carboxylate (112 mg, 0.243 mmol), TFA (2 mL) and DCM (2 mL) gave a mixture of the TMS and desilated alkyne. This material was treated with K$_2$CO$_3$ (101 mg, 0.731 mmol) in MeOH for 45 min before the reaction mixture was purified directly by flash chromatography (Biotage KP-NH 11 g cartridge, 0-100% DCM in PE, then 0-40% MeOH in DCM) to give the title compound (51 mg, 72%) as a colourless solid. LCMS (Method A, ES$^+$): R$_T$ = 0.23 min, *m/z* Calcd for C$_{14}$H$_{16}$N$_3$O$_2$S [M+H]$^+$ 290, found 290. ¹H NMR (400 MHz, CDCl$_3$): δ 8.27 (s, 1H), 7.98 (s, 1H), 4.14 (s, 2H), 3.41 (s, 1H), 3.01-2.84 (m, 4H), 1.91-1.44 (m, 6H).

Step 3: 7-Ethynyl-3-((4-hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one: General procedure 4 using 7-ethynyl-3-((4-hydroxypiperidin-4-yl)methyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (18 mg, 62.2 µmol), 3-phenylpropanoic acid (14 mg, 93.3 µmol), HATU (35 mg, 93.3 µmol), DIPEA (42 µL, 0.243 mmol) and DCM (0.5 mL) gave the title compound (24 mg, 91%) as a colourless solid. LCMS (Method A, ES$^+$): R$_T$ = 1.15 min (purity >95% at 254 nm), *m/z* Calcd for C$_{23}$H$_{24}$N$_3$O$_3$S [M+H]$^+$ 422, found 422. ¹H NMR (400 MHz, CDCl$_3$): δ 8.19 (s, 1H), 7.99 (s, 1H), 7.33-7.25 (m, 2H), 7.24-7.16 (m, 3H), 4.34 (d, 1H), 4.12 (d, 1H), 4.02 (d, 1H), 3.59 (d, 1H), 3.52 (s, 1H), 3.41-3.25 (m, 2H), 3.11-2.99 (m, 1H), 2.99-2.89 (m, 2H), 2.70-2.52 (m, 2H), 1.67-1.28 (m, 4H).

Compound 14: 3-((4-Hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)-7-phenylthieno[3,2-*d*]pyrimidin-4(3*H*)-one

Step 1: *tert*-Butyl 4-hydroxy-4-((4-oxo-7-phenylthieno[3,2-*d*]pyrimidin-3(4*H*)-yl)methyl)piperidine-1-carboxylate: General procedure 5 using *tert*-butyl 4-((7-bromo-4-oxothieno[3,2-*d*]pyrimidin-3(4*H*)-yl)methyl)-4-hydroxypiperidine-1-carboxylate[1] (50 mg, 0.113 mmol), phenylboronic acid (41 mg, 0.338 mmol), PdCl$_2$(dppf) (8.2 mg, 11.2 µmol) and K$_3$PO$_4$ (108 mg, 0.506 mmol) in 1,4-dioxane (1 mL) and water (0.2 mL) at 110 °C for 16 h gave the title compound (48 mg, 96%) as a beige solid. LCMS (Method A, ES$^+$): R$_T$ = 1.59 min, *m/z* Calcd for C$_{23}$H$_{28}$N$_3$O$_4$S [M+H]$^+$ 442, found 442. ^{1}H NMR (300 MHz, CDCl$_3$): δ 8.17 (s, 1H), 7.87 (s, 1H), 7.82-7.72 (m, 2H), 7.54-7.34 (m, 3H), 4.28-3.99 (m, 2H), 3.89 (br s, 2H), 3.41 (s, 1H), 3.15 (t, 2H), 1.72-1.48 (m, 4H), 1.45 (s, 9H).

Step 2: 3-((4-Hydroxypiperidin-4-yl)methyl)-7-phenylthieno[3,2-*d*]pyrimidin-4(3*H*)-one: General procedure 2 using *tert*-butyl 4-hydroxy-4-((4-oxo-7-phenylthieno[3,2-*d*]pyrimidin-3(4*H*)-yl)methyl)piperidine-1-carboxylate (48 mg, 0.109 mmol), TFA (1 mL) and DCM (1 mL) gave the title compound (30 mg, 80%) as a colourless solid. LCMS (Method A, ES$^+$): R$_T$ = 0.73 min, *m/z* Calcd for C$_{18}$H$_{20}$N$_3$O$_2$S [M+H]$^+$ 342, found 342. ^{1}H NMR (300 MHz, CDCl$_3$): δ 8.21 (s, 1H), 7.85 (s, 1H), 7.83-7.73 (m, 2H), 7.65-7.32 (m, 3H), 4.12 (s, 2H), 3.12-2.74 (m, 4H), 2.19 (br s, 2H), 1.83-1.47 (m, 4H).

Step 3: 3-((4-Hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)-7-phenylthieno[3,2-*d*]pyrimidin-4(3*H*)-one: General procedure 4 using 3-((4-hydroxypiperidin-4-yl)methyl)-7-phenylthieno[3,2-*d*]pyrimidin-4(3*H*)-one (30 mg, 87.9 µmol), 3-phenylpropanoic acid (20 mg, 0.132 mmol), HATU (50 mg, 0.132 mmol), DIPEA (61 µL, 0.351 mmol) and DCM (1 mL) gave the title compound (22 mg, 52%) as a colourless solid. LCMS (Method A, ES$^+$): R$_T$ = 1.47 min (purity >97% at 254 nm), *m/z* Calcd for C$_{27}$H$_{28}$N$_3$O$_3$S [M+H]$^+$ 474, found 474. ^{1}H NMR (300 MHz, CDCl$_3$): δ 8.09 (s, 1H), 7.91 (s, 1H), 7.87-7.76 (m, 2H), 7.59-7.45 (m, 2H), 7.44-7.37 (m, 1H), 7.35-7.16 (m, 5H), 4.44 (d, 1H), 4.14 (d, 1H), 4.05 (d, 1H), 3.63 (d, 1H), 3.45 (br s, 1H), 3.42-3.24 (m, 1H), 3.18-2.88 (m, 3H), 2.76-2.55 (m, 2H), 1.77-1.34 (m, 4H).

Compound 15: 3-((4-Hydroxy-1-(3-phenylpropanoyl)piperidin-4-yl)methyl)-2-methylthieno[3,2-*d*]pyrimidin-4(3*H*)-one
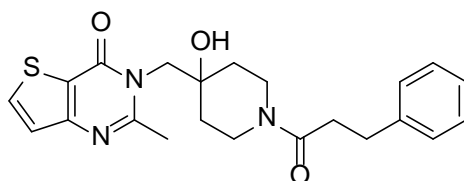
General procedure 1 using 3-phenyl-1-(1-oxa-6-azaspiro[2.5]octan-6-yl)propan-1-one[1] (70 mg, 0.285 mmol), 2-methylthieno[3,2-*d*]pyrimidin-4(3*H*)-one (43 mg, 0.259 mmol), Cs$_2$CO$_3$ (101 mg, 0.311 mmol) in DMF (1.5 ml) gave the title compound (29 mg, 27%) as a pale yellow solid. LCMS (Method A, ES$^+$): R$_T$ = 1.08 min (purity >98% at 254 nm), *m/z* Calcd for C$_{22}$H$_{26}$N$_3$O$_3$S [M+H]$^+$ 412, found 412. ^{1}H NMR (300 MHz, CDCl$_3$): δ 7.82-7.66 (m, 1H), 7.29-7.08 (m, 6H), 4.45-4.34 (m, 1H), 4.26 (br s, 1H), 4.19 (d, 1H), 4.07 (d, 1H), 3.59-3.47 (m, 1H), 3.34-3.18 (m, 1H), 2.95-2.80 (m, 3H), 2.60 (s, 3H), 2.58-2.44 (m, 2H), 1.61-1.21 (m, 4H).

Compound 18: (*R*)-3-Ethynyl-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one
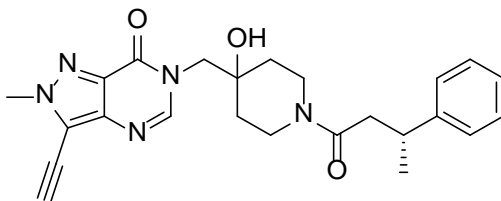

Step 1: *tert*-Butyl 4-hydroxy-4-((2-methyl-7-oxo-3-((trimethylsilyl)ethynyl)-2,7-dihydro-6*H*-pyrazolo[4,3-*d*]pyrimidin-6-yl)methyl)piperidine-1-carboxylate: General procedure 6 using *tert*-butyl 4-((3-bromo-2-methyl-7-oxo-2,7-dihydro-6*H*-pyrazolo[4,3-*d*]pyrimidin-6-yl)methyl)-4-hydroxypiperidine-1-carboxylate[1] (60 mg, 0.136 mmol), CuI (5.2 mg, 27.3 µmol), Pd(PPh$_3$)$_2$Cl$_2$ (9.5 mg, 13.5 µmol), triethylamine (0.378 mL, 2.71 mmol), ethynyltrimethylsilane (75 µL, 0.918 mmol) and toluene (1.4 mL) at 110 °C for 16 h gave the title compound (41 mg, 65%) as a yellow oil. LCMS (Method A, ES$^+$): R$_T$ = 1.60 min, *m/z* Calcd for C$_{22}$H$_{34}$N$_5$O$_4$Si [M+H]$^+$ 460, found 460. ^{1}H NMR (300 MHz, CDCl$_3$): δ 7.94 (s, 1H), 4.27-4.00 (m, 5H), 4.00-3.77 (m, 2H), 3.27-3.06 (m, 3H), 1.75-1.39 (m, 13H), 0.31 (s, 9H).

Step 2: 3-Ethynyl-6-((4-hydroxypiperidin-4-yl)methyl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one: General procedure 2 using *tert*-butyl 4-hydroxy-4-((2-methyl-7-oxo-3-((trimethylsilyl)ethynyl)-2,7-dihydro-6*H*-

pyrazolo[4,3-*d*]pyrimidin-6-yl)methyl)piperidine-1-carboxylate (32 mg, 69.6 µmol), TFA (1 mL) and DCM (1 mL) gave a mixture of the TMS and desilated alkyne. This material was treated with K_2CO_3 (29 mg, 0.209 mmol) in MeOH (1 mL) for 45 min before the reaction mixture was purified directly by flash chromatography (Biotage KP-NH 11 g cartridge, 0-100% DCM in PE then 0-40% MeOH in DCM) affording the title compound (16 mg, 79%) as a colourless solid. LCMS (Method A, ES$^+$): R_T = 0.33 min, *m/z* Calcd for $C_{14}H_{18}N_5O_2$ [M+H]$^+$ 288, found 288.
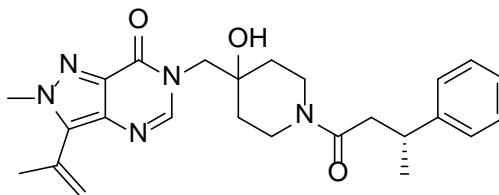
Step 3: (*R*)-3-Ethynyl-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one: General procedure 3 using 3-ethynyl-6-((4-hydroxypiperidin-4-yl)methyl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one (12 mg, 41.8 µmol), (*R*)-3-phenylbutanoic acid (7 mg, 41.8 µmol), EDC (24 mg, 0.125 mmol) and DCM (0.4 mL) gave the title compound (9 mg, 49%) as a colourless solid. LCMS (Method A, ES$^+$): R_T = 1.06 min (purity >99% at 254 nm), *m/z* Calcd for $C_{24}H_{28}N_5O_3$ [M+H]$^+$ 434, found 434. ^{1}H NMR (300 MHz, CDCl$_3$, this molecule appears conformers in a 2:3 ratio): δ 7.94 (s, 0.4H), 7.82 (s, 0.6H), 7.39-7.16 (m, 5H), 4.42-4.32 (m, 1H), 4.17 (s, 3H), 4.01-3.88 (m, 2H), 3.61-2.87 (m, 5H), 2.71-2.41 (m, 2H), 1.67-1.23 (m, 7.4H), 0.89-0.75 (m, 0.6).

Compound 19: (*R*)-6-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-3-(3-hydroxy-3-methylbut-1-yn-1-yl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one
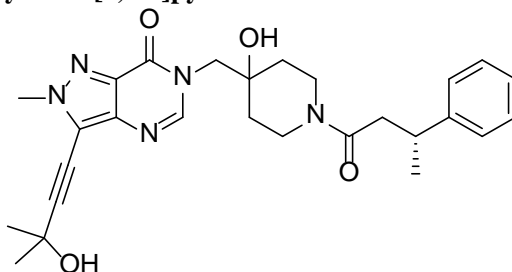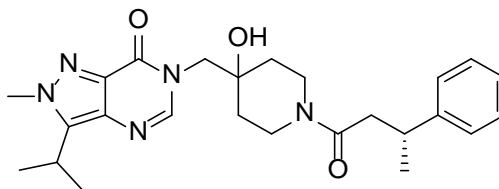

General procedure 6 using (*R*)-3-bromo-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one[1] (40 mg, 81.9 µmol), CuBr·SMe$_2$ (0.7 mg, 3.28 µmol), Pd(PPh$_3$)$_4$ (1.9 mg, 1.64 µmol), triethylamine (0.46 mL, 3.28 mmol) and 2-methylbut-3-yn-2-ol (8 mg, 98.3 µmol) at 70 °C for 1 h gave the title compound (27 mg, 67%) as a colourless solid. LCMS (Method A, ES$^+$): R_T = 1.06 min (purity >99% at 254 nm), *m/z* Calcd for $C_{27}H_{34}N_5O_4$ [M+H]$^+$ 492, found 492. ^{1}H NMR (300 MHz, CDCl$_3$, this molecule appears as conformers in a 2:3 ratio): δ 7.94 (s, 0.4H), 7.86 (s, 0.6H), 7.37-7.15 (m, 5H), 4.45-3.83 (m, 6H), 3.63-3.46 (m, 1H), 3.39-3.19 (m, 2H), 3.12-2.90 (m, 1H), 2.71-2.41 (m, 2H), 2.08 (br s, 2H), 1.76-1.21 (m, 12.4H), 1.01-0.82 (m, 0.6H).

Compound 20: (*R*)-6-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-3-(prop-1-en-2-yl)-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one
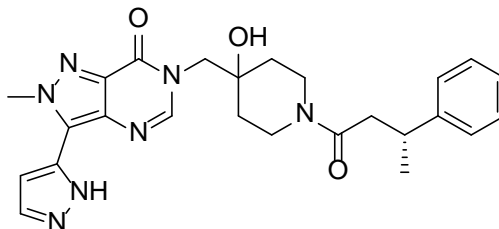

General procedure 5 using (*R*)-3-bromo-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one[1] (75 mg, 0.154 mmol), potassium isopropenyltrifluoroborate (68 mg, 0.461 mmol), K_3PO_4 (98 mg, 0.461 mmol), Pd(PPh$_3$)$_4$ (18 mg, 15.4 µmol), 1,4-dioxane (1.2 mL) and water (0.3 mL) at 130 °C under microwave irradiation for 45 min gave the title compound (56 mg, 81%) as a colourless solid. LCMS (Method A, ES$^+$): R_T = 1.12 min (purity >96% at 254 nm), *m/z* Calcd for $C_{25}H_{32}N_5O_3$ [M+H]$^+$ 450, found 450. ^{1}H NMR (300 MHz, CDCl$_3$, this molecule appears as conformers in a 2:3 ratio): δ 7.85 (s, 0.4H), 7.74 (s, 0.6H), 7.37-7.13 (m, 5H), 5.66-5.55 (m, 1H), 5.32-5.25 (m, 1H), 4.44-3.88 (m, 6H), 3.77-3.46 (m, 2H), 3.40-3.14 (m, 2H), 3.11-2.85 (m, 1H), 2.71-2.57 (m, 1H), 2.57-2.41 (m, 1H), 2.27 (d, 3H), 1.66-1.21 (m, 6.4H), 0.93-0.74 (m, 0.6H).

Compound 21: (*R*)-6-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-3-isopropyl-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one
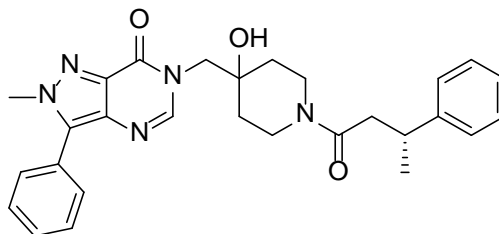
A solution of (*R*)-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-3-(prop-1-en-2-yl)-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one (50 mg, 0.111 mmol) in MeOH (10 mL) was hydrogenated in an H-Cube® (10% Pd/C CatCart®, 1 mLmin⁻¹, 50 °C, 60 bar H₂). The resulting solution was concentrated under reduced pressure and the residue was purified by flash chromatography (Biotage KP-Sil 10 g cartridge, 0-100% EtOAc in PE, then 0-30% MeOH in EtOAc) to give the title compound (20 mg, 39%) as a colourless solid. LCMS (Method A, ES⁺): R_T = 1.02 min (purity >98% at 254 nm), *m/z* Calcd for $C_{25}H_{34}N_5O_3$ [M+H]⁺ 452, found 452. ¹H NMR (300 MHz, CDCl₃, this molecule appears as conformers in a 2:3 ratio): δ 7.70 (s, 0.4H), 7.59 (s, 0.6H), 7.38-7.15 (m, 5H), 4.48-4.26 (m, 1H), 4.19-3.83 (m, 5H), 3.65-2.84 (m, 6H), 2.72-2.58 (m, 1H), 2.58-2.42 (m, 1H), 1.64-1.21 (m, 12.4H), 0.79-0.63 (m, 0.6H).

Compound 22: (*R*)-6-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-3-(1*H*-pyrazol-5-yl)-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one

General procedure 5 using (*R*)-3-bromo-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one[1] (40 mg, 81.9 µmol), (1*H*-pyrazol-5-yl)boronic acid (27 mg, 0.246 mmol), K₃PO₄ (52 mg, 0.246 mmol), Pd(PPh₃)₄ (9 mg, 8.19 µmol), 1,4-dioxane (0.65 mL) and water (0.16 mL) at 150 °C under microwave irradiation for 10 min gave the title compound (26 mg, 66%) as a colourless solid. LCMS (Method A, ES⁺): R_T = 0.97 min (purity >95% at 254 nm), *m/z* Calcd for $C_{25}H_{30}N_7O_3$ [M+H]⁺ 476, found 476. ¹H NMR (300 MHz, CDCl₃, this molecule appears as conformers in a 2:3 ratio): δ 7.87 (s, 0.4H), 7.81-7.71 (m, 1.6H), 7.39-7.15 (m, 6H), 6.95 (br s, 1H), 4.46-3.90 (m, 6H), 3.77-3.47 (m, 2H), 3.44-2.88 (m, 3H), 2.72-2.42 (m, 2H), 1.79-1.24 (m, 6.4H), 0.97-0.74 (m, 0.6H).

Compound 23: (*R*)-6-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-3-phenyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one
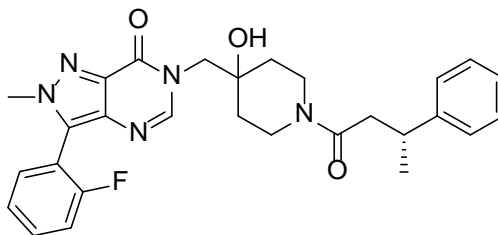

General procedure 5 using (*R*)-3-bromo-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one[1] (40 mg, 81.9 µmol), phenylboronic acid (30 mg, 0.246 mmol), K₃PO₄ (52 mg, 0.246 mmol), Pd(PPh₃)₄ (9.5 mg, 8.19 µmol), 1,4-dioxane (0.7 mL) and water (0.15 mL) at 150 °C under microwave irradiation for 10 min gave the title compound (31 mg, 77%) as a colourless solid. LCMS (Method A, ES⁺): R_T = 1.23 min (purity >98% at 254 nm), *m/z* Calcd for $C_{28}H_{32}N_5O_3$ [M+H]⁺ 486, found 486. ¹H NMR (300 MHz, CDCl₃, this molecule appears as conformers in a 2:3 ratio): δ 7.84 (s, 0.4H), 7.73 (s, 0.6H), 7.65-7.44 (m, 5H), 7.39-7.14 (m, 5H), 4.43-3.88 (m, 6H), 3.68-3.44 (m, 2H), 3.40-2.85 (m, 3H), 2.71-2.41 (m, 2H), 1.66-1.23 (m, 6.4H), 0.93-0.73 (m, 0.6H).

Compound 24: (*R*)-3-(2-Fluorophenyl)-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one
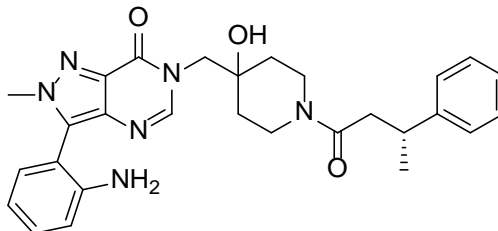
General procedure 5 using (*R*)-3-bromo-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-di-hydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one[1] (33 mg, 67.6 µmol), (2-fluorophenyl)boronic acid (28 mg, 0.203 mmol), K_3PO_4 (43 mg, 0.203 mmol), Pd(PPh$_3$)$_4$ (7.8 mg, 6.76 µmol), 1,4-dioxane (0.5 mL) and water (0.13 mL) at 150 °C under microwave irradiation for 10 min gave the title compound (24 mg, 70%) as a colourless solid. LCMS (Method A, ES$^+$): R$_T$ = 1.23 min (purity >97% at 254 nm), *m/z* Calcd for $C_{28}H_{31}FN_5O_3$ [M+H]$^+$ 504, found 504. ^{1}H NMR (300 MHz, CDCl$_3$, this molecule appears as conformers in a 2:3 ratio): δ 7.86 (s, 0.4H), 7.74 (s, 0.6H), 7.61-7.44 (m, 2H), 7.41-7.15 (m, 7H), 4.44-3.89 (m, 6H), 3.62-2.86 (m, 5H), 2.70-2.41 (m, 2H), 1.63-1.23 (m, 6.4H), 0.98-0.71 (m, 0.6H).

Compound 25: (*R*)-3-(2-Aminophenyl)-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one
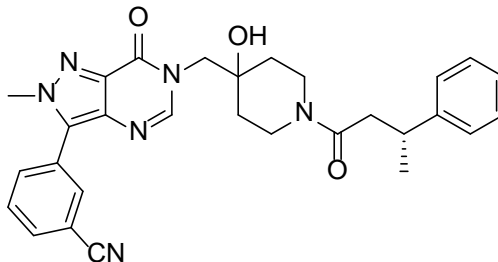

General procedure 5 using (*R*)-3-bromo-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-di-hydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one[1] (40 mg, 81.9 µmol), 2-aminophenylboronic acid hydrochloride (36 mg, 0.205 mmol), K_3PO_4 (70 mg, 0.328 mmol), Pd(PPh$_3$)$_4$ (9.5 mg, 8.19 µmol), 1,4-dioxane (0.7 mL) and water (0.16 mL) at 150 °C under microwave irradiation for 10 min gave the title compound (33 mg, 80%) as a colourless solid. LCMS (Method A, ES$^+$): R$_T$ = 1.14 min (purity >97% at 254 nm), *m/z* Calcd for $C_{28}H_{33}N_6O_3$ [M+H]$^+$ 501, found 501. ^{1}H NMR (300 MHz, CDCl$_3$, this molecule appears as conformers in a 2:3): δ 7.82 (s, 0.4H), 7.72 (s, 0.6H), 7.39-7.13 (m, 5H), 7.13-7.05 (m, 1H), 6.93-6.57 (m, 3H), 4.40-3.84 (m, 6H), 3.62-2.84 (m, 5H), 2.70-2.41 (m, 2H), 1.64-1.24 (m, 6.4H), 0.92-0.77 (m, 0.6H).

Compound 26: (*R*)-3-(6-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-7-oxo-6,7-dihydro-2*H*-pyrazolo[4,3-*d*]pyrimidin-3-yl)benzonitrile
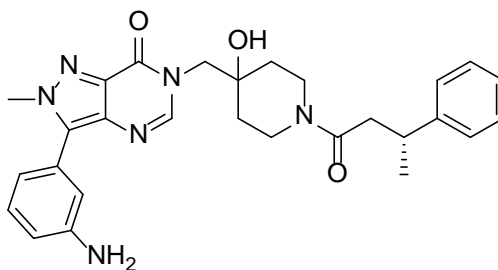

General procedure 5 using (*R*)-3-bromo-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-di-hydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one[1] (33 mg, 67.6 µmol), (3-cyanophenyl)boronic acid (30 mg, 0.203 mmol), K_3PO_4 (43 mg, 0.203 mmol), Pd(PPh$_3$)$_4$ (7.8 mg, 6.76 µmol), 1,4-dioxane (0.5 mL) and water (0.13 mL) at 150 °C under microwave irradiation for 10 min gave the title compound (24 mg, 69%) as a colourless solid. LCMS (Method A, ES$^+$): R$_T$ = 1.20 min (purity >95% at 254 nm), *m/z* Calcd for $C_{29}H_{31}N_6O_3$ [M+H]$^+$ 511, found 511. ^{1}H NMR (300 MHz, CDCl$_3$, this molecule appears as conformers in a 2:3 ratio): δ 7.92-7.67 (m, 5H), 7.33-7.18 (m, 5H), 4.43-3.90 (m, 6H), 3.65-2.86 (m, 5H), 2.72-2.41 (m, 2H), 1.66-1.23 (m, 6.4H), 0.92-0.77 (m, 0.6H).

Compound 27: (*R*)-3-(3-Aminophenyl)-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one
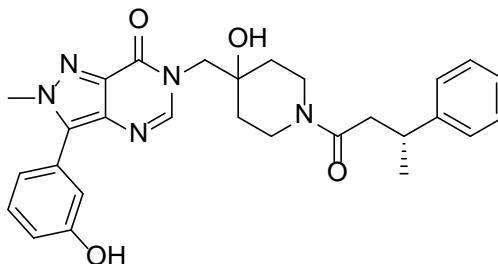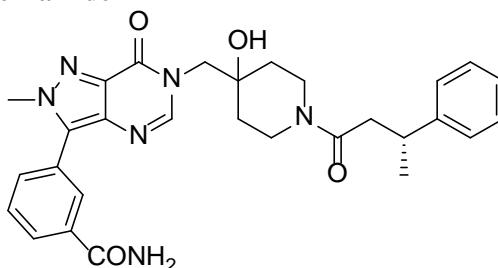
General procedure 5 using (*R*)-3-bromo-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-di-hydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one[1] (40 mg, 81.9 µmol), (3-aminophenyl)boronic acid (34 mg, 0.246 mmol), K_3PO_4 (52 mg, 0.246 mmol), $Pd(PPh_3)_4$ (9.5 mg, 8.19 µmol), 1,4-dioxane (0.65 mL) and water (0.16 mL) at 150 °C under microwave irradiation for 10 min gave the title compound (35 mg, 85%) as a colourless solid. LCMS (Method A, ES⁺): R_T = 0.99 min (purity >96% at 254 nm), *m/z* Calcd for $C_{28}H_{33}N_6O_3$ [M+H]⁺ 501, found 501. ¹H NMR (300 MHz, CDCl₃, this molecule appears as conformers in a 2:3 ratio): δ 7.83 (s, 0.4H), 7.73 (s, 0.6H), 7.38-7.15 (m, 6H), 6.87-6.71 (m, 3H), 4.42-3.71 (m, 8H), 3.59-3.43 (m, 1H), 3.42-3.11 (m, 2H), 3.09-2.82 (m, 1H), 2.71-2.40 (m, 2H), 1.93 (s, 1H), 1.63-1.23 (m, 6.4H), 0.96-0.73 (m, 0.6H).

Compound 28: (*R*)-6-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-3-(3-hydroxyphenyl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one
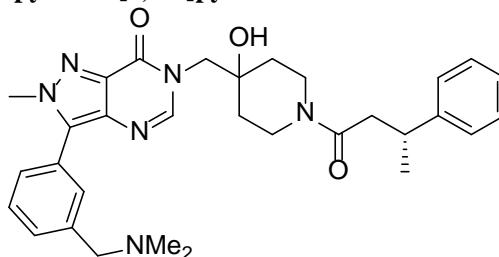

General procedure 5 using (*R*)-3-bromo-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-di-hydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one[1] (32 mg, 65.5 µmol), (3-hydroxyphenyl)boronic acid (27 mg, 0.197 mmol), K_3PO_4 (42 mg, 0.197 mmol), $Pd(PPh_3)_4$ (7.6 mg, 6.55 µmol), 1,4-dioxane (0.5 mL) and water (0.13 mL) at 150 °C under microwave irradiation for 10 min gave the title compound (21 mg, 63%) as a colourless solid. LCMS (Method A, ES⁺): R_T = 1.09 min (purity >98% at 254 nm), *m/z* Calcd for $C_{28}H_{32}N_5O_4$ [M+H]⁺ 502, found 502. ¹H NMR (300 MHz, methanol-d_4, this molecule appears as conformers in a 2:3 ratio): δ 7.89 (s, 0.4H), 7.83 (s, 0.6H), 7.34-7.01 (m, 6H), 7.00-6.89 (m, 2H), 6.89-6.78 (m, 1H), 4.19-3.70 (m, 6H), 3.66-3.47 (m, 1H), 3.26-2.31 (m, 5H), 1.60-1.08 (m, 6.4H), 0.85-0.69 (m, 0.6H).

Compound 29: (*R*)-3-(6-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-7-oxo-6,7-dihydro-2*H*-pyrazolo[4,3-*d*]pyrimidin-3-yl)benzamide

General procedure 5 using (*R*)-3-bromo-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-di-hydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one[1] (40 mg, 81.9 µmol), (3-carbamoylphenyl)boronic acid (41 mg, 0.246 mmol), K_3PO_4 (52 mg, 0.246 mmol), $Pd(PPh_3)_4$ (9.5 mg, 8.19 µmol), 1,4-dioxane (0.65 mL) and water (0.16 mL) at 150 °C under microwave irradiation for 10 min gave the title compound (29 mg, 66%) as a colourless solid. LCMS (Method A, ES⁺): R_T = 0.96 min (purity >96% at 254 nm), *m/z* Calcd for $C_{29}H_{33}N_6O_4$ [M+H]⁺ 529, found 529. ¹H NMR (300 MHz, CDCl₃, this molecule appears as conformers a 2:3 ratio): δ 8.48-8.43 (m, 1H), 8.19 (s, 0.4H), 8.13-8.05 (m, 1.6H), 7.99-7.93 (m, 1H), 7.63-7.54 (m, 1H), 7.39-7.17 (m, 5H), 6.97 (s, 1H), 4.51-3.79 (m, 3H), 3.66-3.48 (m, 1H), 3.45-2.81 (m, 3H), 2.75-2.44 (m, 2H), 1.75-1.24 (m, 8.4H), 0.76-0.60 (m, 0.6H).
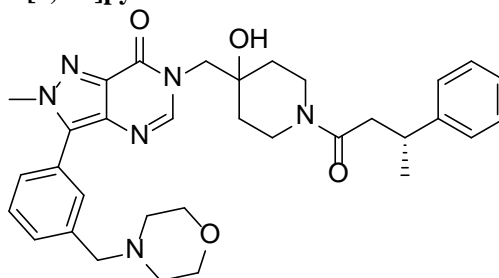
Compound 30: (*R*)-3-(3-((Dimethylamino)methyl)phenyl)-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one

Step 1: (*R*)-3-(3-(Aminomethyl)phenyl)-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one: General procedure 5 using (*R*)-3-bromo-6-((4-hydroxy-1-(3-phenyl-butanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one[1] (90 mg, 0.184 mmol), (3-(aminomethyl)phenyl)boronic acid hydrochloride (86 mg, 0.461 mmol), K_3PO_4 (156 mg, 0.737 mmol), Pd(PPh$_3$)$_4$ (21 mg, 18.4 µmol), 1,4-dioxane (1.5 mL) and water (0.5 mL) at 150 °C under microwave irradiation for 10 min gave the title compound (30 mg, 31%) as a colourless solid. LCMS (Method B, ES$^+$): R$_T$ = 0.75 min, *m/z* Calcd for $C_{29}H_{35}N_6O_3$ [M+H]$^+$ 515, found 515.
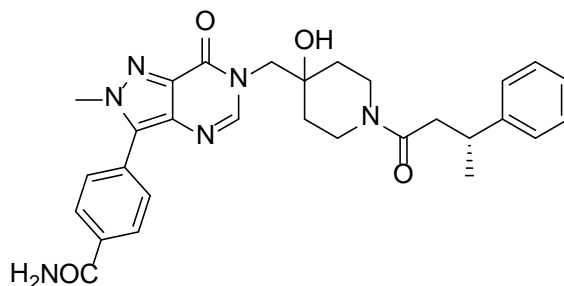
Step 2: (*R*)-3-(3-((Dimethylamino)methyl)phenyl)-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one: To a mixture of (*R*)-3-(3-(aminomethyl)phenyl)-6-((4-hy-droxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one (30 mg, 58.3 µmol) and paraformaldehyde (8.75 mg, 0.291 mmol) in ethanol (0.3 mL) was added NaBH$_4$ (6.6 mg, 0.175 mmol) and the mixture was heated at reflux for 190 min. The reaction mixture was allowed to cool to rt before being diluted with brine (20 mL) and extracted with DCM (20 mL) using a Biotage phase separator. The organic phase was concentrated under reduced pressure and the residue was purified by flash chromatography (Biotage KP-NH 11 g cartridge, 0-100% EtOAc in PE, then 0-30% MeOH in EtOAc) to give the title compound (25 mg 79%) as a pale yellow solid. LCMS (Method B, ES$^+$): R$_T$ = 0.77 min (purity >95% at 254 nm), *m/z* Calcd for $C_{31}H_{39}N_6O_3$ [M+H]$^+$ 543, found 543. ^{1}H NMR (500 MHz, DMSO-d_6, this molecule appears as conformers in a 1:1 ratio): δ 8.00 (s, 0.5H), 7.97 (s, 0.5H), 7.64-7.56 (m, 2H), 7.56-7.51 (m, 1H), 7.46-7.41 (m, 1H), 7.31-7.20 (m, 4H), 7.18-7.13 (m, 1H), 4.86 (s, 0.5H), 4.85 (s, 0.5H), 4.10 (s, 3H), 4.06-3.87 (m, 4H), 3.68-3.61 (m, 1H), 3.50 (s, 2H), 3.25-3.12 (m, 2H), 2.93-2.83 (m, 1H), 2.61-2.55 (m, 1H), 2.19 (s, 6H), 1.59-1.23 (m, 4H), 1.21 (d, 3H).

Compound 31: (*R*)-6-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-3-(3-(morpholinome-thyl)phenyl)-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one

General procedure 5 using (*R*)-3-bromo-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-di-hydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one[1] (25 mg, 51.2 µmol), 4-(3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)ben-zyl)morpholine (39 mg, 0.128 mmol), K_3PO_4 (33 mg, 0.154 mmol), Pd(PPh$_3$)$_4$ (6 mg, 5.12 µmol), 1,4-dioxane (0.4 mL) and water (0.1 mL) at 130 °C under microwave irradiation for 15 min gave the title compound (19 mg, 63%) as a col-ourless solid. LCMS (Method B, ES$^+$): R$_T$ = 0.76 min (purity >95% at 254 nm), *m/z* Calcd for $C_{33}H_{41}N_6O_4$ [M+H]$^+$ 585, found 585. ^{1}H NMR (500 MHz, DMSO-d_6, this molecule appears as conformers in a 1:1 ratio): δ 8.00 (s, 0.5H), 7.98 (s, 0.5H), 7.64 (s, 1H), 7.60 (d, 1H), 7.54 (t, 1H), 7.46 (d, 1H), 7.30-7.21 (m, 4H), 7.18-7.12 (m, 1H), 4.89 (s, 0.5H), 4.87 (s, 0.5H), 4.11 (s, 3H), 4.14-3.86 (m, 4H), 3.70-3.53 (m, 6H), 3.27-3.13 (m, 2H), 2.92-2.83 (m, 1H), 2.66-2.55 (m, 2H), 2.44-2.35 (m, 4H), 1.58-1.25 (m, 4H), 1.21 (d, 3H).

Compound 32: (*R*)-4-(6-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-7-oxo-6,7-dihydro-2*H*-pyrazolo[4,3-*d*]pyrimidin-3-yl)benzamide
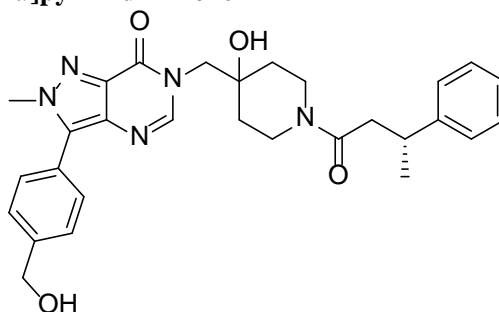
General procedure 5 using (*R*)-3-bromo-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-di-hydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one[1] (25 mg, 51.2 µmol), (4-carbamoylphenyl)boronic acid (21 mg, 0.128 mmol), K_3PO_4 (33 mg, 0.154 mmol), $Pd(PPh_3)_4$ (6 mg, 5.12 µmol), 1,4-dioxane (0.4 mL) and water (0.1 mL) at 130 °C under microwave irradiation for 15 min gave the title compound (22 mg, 81%) as a colourless solid. LCMS (Method A, ES+): R_T = 0.94 min (purity >95% at 254 nm), *m/z* Calcd for $C_{29}H_{33}N_6O_4$ [M+H]+ 529, found 529. ^{1}H NMR (500 MHz, DMSO-d_6, this molecule appears as conformers in a 1:1 ratio): δ 8.11 (s, 1H), 8.06 (d, 2H), 8.03 (s, 0.5H), 8.00 (s, 0.5H), 7.82 (d, 2H), 7.49 (s, 1H), 7.30-7.21 (m, 4H), 7.19-7.13 (m, 1H), 4.88 (s, 0.5H), 4.87 (s, 0.5H), 4.15 (s, 3H), 4.05-3.89 (m, 3H), 3.69-3.61 (m, 1H), 3.28-3.12 (m, 3H), 2.90-2.84 (m, 1H), 2.62-2.56 (m, 1H), 1.55-1.29 (m, 4H), 1.21 (d, Hz, 3H).

Compound 33: (*R*)-6-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-3-(4-(hydroxymethyl)phenyl)-2-methyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one

General procedure 5 using (*R*)-3-bromo-6-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-2-methyl-2,6-di-hydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one[1] (25 mg, 51.2 µmol), (4-(hydroxymethyl)phenyl)boronic acid (19 mg, 0.128 mmol), K_3PO_4 (33 mg, 0.154 mmol), $Pd(PPh_3)_4$ (6 mg, 5.12 µmol), 1,4-dioxane (0.4 mL) and water (0.1 mL) at 130 °C under microwave irradiation for 15 min gave the title compound (19 mg, 71%) as a colourless solid. LCMS (Method A, ES+): R_T = 1.01 min (purity >95% at 254 nm), *m/z* Calcd for $C_{29}H_{34}N_5O_4$ [M+H]+ 516, found 516. ^{1}H NMR (500 MHz, DMSO-d_6, this molecule appears as conformers in a 1:1 ratio): δ 7.99 (s, 0.5H), 7.97 (s, 0.5H), 7.69-7.65 (m, 2H), 7.54-7.49 (m, 2H), 7.29-7.23 (m, 4H), 7.17-7.14 (m, 1H), 5.32 (t, 1H), 4.87 (s, 0.5H), 4.86 (s, 0.5H), 4.60 (d, 2H), 4.10 (s, 3H), 4.07-3.87 (m, 3H), 3.69-3.61 (m, 1H), 3.27-3.13 (m, 2H), 2.92-2.83 (m, 1H), 2.66-2.55 (m, 2H), 1.57-1.28 (m, 4H), 1.21 (d, 3H).

Compound 35: (*R*)-3-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)pyrimidin-4(3*H*)-one
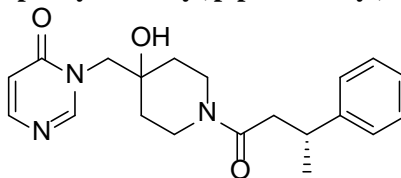
Step 1: (*R*)-1-(3-Phenylbutanoyl)piperidin-4-one: To freshly prepared piperidin-4-one hydrochloride (Young, J., *et al.* WO 2011/084402 A1, Jul 14, 2011) (1.70 g, 12.6 mmol) was added EDC (2.89 g, 15.1 mmol), DMAP (153 mg, 1.26 mmol), DCM (15 mL) and DIPEA (11 mL, 62.7 mmol). After 10 min a solution of *(R)*-3-phenylbutanoic acid (2.47 g, 15.1 mmol) in DCM (10 mL) was added. After 20 h, EDC (2.89 g, 15.1 mmol) was added and the reaction stirred for a further 4 h. The reaction was quenched by the addition of saturated $NaHCO_{3(aq)}$ (150 mL) and the resulting mixture was extracted with EtOAc (3 x 50 mL). The combined organic phases were washed with water (50 mL) and brine (50 mL) before being dried over $MgSO_4$, concentrated under reduced pressure and the residue was purified by flash chromatography (Biotage KP-Sil 50 g cartridge, 0-60% EtOAc in PE) to give the title compound (2.93 g, 95%) as a colourless oil. LCMS (Method A, ES+): R_T = 1.07 min, *m/z* Calcd for $C_{15}H_{20}NO_2$ [M+H]+ 246, found 246. ^{1}H NMR (300 MHz,

CDCl$_3$): δ 7.44-7.13 (m, 5H), 4.30-4.03 (m, 1H), 3.77-3.58 (m, 1H), 3.46 (tdd, 2H), 3.11-2.93 (m, 2H), 2.82-2.61 (m, 4H), 1.86 (m, 1H), 1.77-1.62 (m, 1H), 1.54-1.33 (m, 2H).

Step 1: (*R*)-3-Phenyl-1-(1-oxa-6-azaspiro[2.5]octan-6-yl)butan-1-one: To a solution of trimethylsulfonium iodide (6.09 g, 29.9 mmol) in DMSO (30 mL) was added NaH (1.19 g, 29.9 mmol). The resulting mixture was stirred at rt for 1 h before a solution of *(R)*-1-(3-phenylbutanoyl)piperidin-4-one (2.93 g, 11.9 mmol) in DMSO (15 mL) was added. The reaction mixture was stirred at 50 °C for 2 h before it was allowed to cool to rt, quenched by the addition of water (100 mL) and the resulting mixture was extracted with Et$_2$O (3 x 50 mL). The combined organic phases were washed with brine (50 mL), dried over Na$_2$SO$_4$, concentrated under reduced pressure and the residue was purified by flash chromatography (Biotage KP-Sil 50 g cartridge, 0-70% EtOAc in PE) to give the title compound (2.68 g, 87%) as a colourless oil. LCMS (Method A, ES$^+$): R$_T$ = 1.16 min, *m/z* Calcd for C$_{16}$H$_{22}$NO$_2$ [M+H]$^+$ 260, found 260. ^{1}H NMR (300 MHz, CDCl$_3$): δ 7.43-7.14 (m, 5H), 4.30-3.95 (m, 1H), 3.69-3.18 (m, 4H), 2.84-2.47 (m, 4H), 1.87-1.66 (m, 2H), 1.51-1.31 (m, 2H), 1.37 (d, 3H).

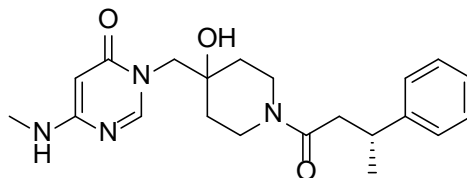**Step 3: (*R*)-3-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)pyrimidin-4(3*H*)-one:** General procedure 1 using pyrimidin-4(3*H*)-one (22 mg, 0.229 mmol), Cs$_2$CO$_3$ (94 mg, 0.289 mmol), (*R*)-3-phenyl-1-(1-oxa-6-azaspiro[2.5]octan-6-yl)butan-1-one (50 mg, 0.193 mmol) and DMF (1 mL) gave the title compound (27 mg, 39%) as a white solid. LCMS (Method A, ES$^+$): R$_T$ = 0.88 min (purity >99% at 254 nm), *m/z* Calcd for C$_{20}$H$_{26}$N$_3$O$_3$ [M+H]$^+$ 356, found 356. ^{1}H NMR (300 MHz, CDCl$_3$): δ 7.92-8.10 (m, 2H), 7.25 (m, 5H), 6.52 (d, 1H), 4.25-4.50 (m, 1H), 3.48-4.06 (m, 4H), 2.80-3.46 (m, 3H), 2.70 (m, 1H), 2.51 (m, 1H), 1.50 (m, 2H), 1.28 (m, 4H), 0.70 (m, 1H).
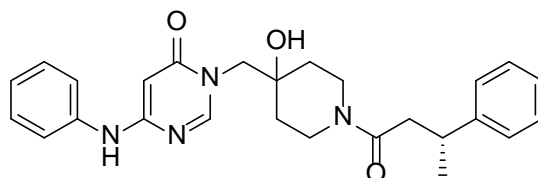
Compound 36: (*R*)-3-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-6-(methylamino)pyrimidin-4(3*H*)-one

Step 1: (*R*)-6-Chloro-3-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)pyrimidin-4(3*H*)-one: A suspension of 6-chloropyrimidin-4(3*H*)-one (200 mg, 1.53 mmol), (*R*)-3-phenyl-1-(1-oxa-6-azaspiro[2.5]octan-6-yl)butan-1-one (397 mg, 1.53 mmol) and DIPEA (401 μl, 2.30 mmol) in DMF (3 mL) was heated at 80 °C for 16 h. The reaction mixture was allowed to cool to rt and quenched by the addition of saturated NH$_4$Cl$_{(aq)}$ (20 mL). The resulting mixture was extracted with EtOAc (3 x 20 mL), the combined organic extracts were dried over Na$_2$SO$_4$, concentrated under reduced pressure and the residue was purified by flash chromatography (Biotage KP-Sil 25 g cartridge, 0-100% EtOAc in PE, then 0-30% MeOH in EtOAc) to give the title compound (350 mg, 59%) as a pale yellow solid. LCMS (Method A, ES$^+$): R$_T$ = 1.11 min, *m/z* Calcd for C$_{20}$H$_{25}$ClN$_3$O$_3$ [M+H]$^+$ 390, 392, found 390, 392. ^{1}H NMR (300 MHz, methanol-*d$_4$*, this molecule appears as conformers in a 2:3 ratio): δ 8.28 (s, 0.4H), 8.24 (s, 0.6H), 7.38-7.13 (m, 5H), 6.58 (s, 1H), 4.27-4.09 (m, 1H), 4.00 (dd, 0.8H), 3.84 (dd, 1.2H), 3.74-3.57 (m, 1H), 3.39-2.86 (m, 3H), 2.86-2.67 (m, 1H), 2.66-2.43 (m, 1H), 1.66-1.20 (m, 6.4H), 0.93-0.79 (m, 0.6H).

Step 2: (*R*)-3-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-6-(methylamino)pyrimidin-4(3*H*)-one: A solution of (*R*)-6-chloro-3-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)pyrimidin-4(3*H*)-one (30 mg, 76.9 μmol) in 2 M MeNH$_2$ in THF (0.5 mL, 1.00 mmol) was heated at 130 °C under microwave irradiation for 1 h. The reaction mixture was concentrated under reduced pressure and the residue was purified by flash chromatography (Biotage KP-NH 11 g cartridge, 0-100% EtOAc in PE, then 0-30% MeOH in EtOAc) to give the title compound (23 mg, 77%) as a colourless solid. LCMS (Method A, ES$^+$): R$_T$ = 0.84 min (purity >96% at 254 nm), *m/z* Calcd for C$_{21}$H$_{29}$N$_4$O$_3$ [M+H]$^+$ 385, found 385. ^{1}H NMR (300 MHz, CDCl$_3$, this molecule appears as conformers in a 2:3 ratio): δ 7.73 (s, 0.4H), 7.62 (s, 0.6H), 7.37-7.15 (m, 5H), 5.30-5.23 (m, 1H), 5.11 (d, 1H), 4.49-4.30 (m, 1H), 4.03-3.47 (m, 3H), 3.42-3.16 (m, 2H), 3.07-2.84 (m, 1H), 2.84 (d, 3H), 2.71-2.58 (m, 1H), 2.57-2.42 (m, 1H), 1.92 (br s, 1H), 1.60-1.10 (m, 6.4H), 0.67-0.52 (m, 0.6H).

Compound 37: (*R*)-3-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-6-(phenylamino)pyrimidin-4(3*H*)-one

A solution of (*R*)-6-chloro-3-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)pyrimidin-4(3*H*)-one (30 mg, 76.9 µmol) in aniline (175 µL, 1.92 mmol) was heated at 130 °C under microwave irradiation for 1 h before the reaction mixture was purified directly by flash chromatography (Biotage KP-NH 11 g cartridge, 0-100% EtOAc in PE then 0-30% MeOH in EtOAc) to give the title compound (22 mg, 64%) as a colourless solid. LCMS (Method A, ES+): R_T = 1.20 min (purity >95% at 254 nm), *m/z* Calcd for $C_{26}H_{31}N_4O_3$ [M+H]+ 447, found 447. ¹H NMR (300 MHz, methanol-*d₄*, this molecule appears as conformers in a 2:3 ratio): δ 8.23 (d, 0.4H), 8.08 (d, 0.6H), 7.43-6.92 (m, 10H), 6.56 (s, 0.4H), 5.65 (s, 0.6H), 4.26-3.51 (m, 4H), 3.35-2.85 (m, 3H), 2.85-2.65 (m, 1H), 2.64-2.41 (m, 1H), 1.63-1.20 (m, 6.4H), 0.95-0.73 (m, 0.6H).

Compound 38: (*R*)-6-Benzyl-3-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)pyrimidin-4(3*H*)-one
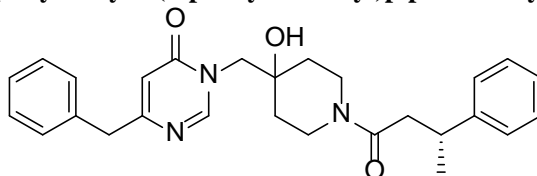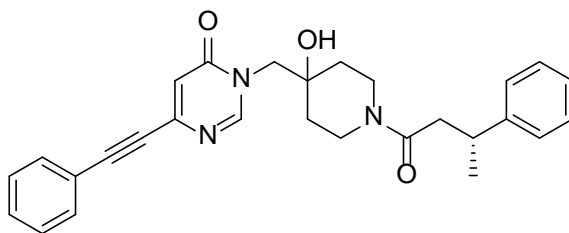

Step 1: *tert*-Butyl 4-((4-chloro-6-oxopyrimidin-1(6*H*)-yl)methyl)-4-hydroxypiperidine-1-carboxylate: A solution of 6-chloropyrimidin-4(3*H*)-one (3.72 g, 28.5 mmol), *tert*-butyl 1-oxa-6-azaspiro[2.5]octane-6-carboxylate[1] (6.08 g, 28.5 mmol) and DIPEA (7.47 mL, 42.7 mmol) in DMF (35 mL) was heated at 80 °C for 16 h. The reaction mixture was allowed to cool to rt before it was quenched by the addition of saturated $NH_4Cl_{(aq)}$ (100 mL) and the resulting mixture was extracted with EtOAc (3 x 50 mL). The combined organic extracts were dried over $MgSO_4$, concentrated under reduced pressure and the residue was purified by flash chromatography (GraceResolv silica 120 g cartridge, 0-100% EtOAc in cyclohexane) to give the title compound (5.87 g, 60%) as an off-white solid. LCMS (Method B, ES+): R_T = 0.99 min, *m/z* Calcd for $C_{15}H_{23}ClN_3O_4$ [M+H]+ 344, 346, found 344, 346.

Step 2: 6-Benzyl-3-((4-hydroxypiperidin-4-yl)methyl)pyrimidin-4(3*H*)-one: A solution of *tert*-butyl 4-((4-chloro-6-oxopyrimidin-1(6*H*)-yl)methyl)-4-hydroxypiperidine-1-carboxylate (60 mg, 0.174 mmol), potassium benzyltrifluoroborate (38 mg, 0.192 mmol) and triethylamine (36 µL, 0.26 mmol) in toluene (2 mL) and water (0.2 mL) was purged with N₂ before PdCl₂(dppf) (12.8 mg, 17.4 µmol) was added. The reaction tube was sealed and the mixture was heated at 110 °C for 16 h. The reaction was allowed to cool to rt, diluted with water (20 mL) and extracted into EtOAc (3 x 20 mL). The combined organic phases were washed with brine (20 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash chromatography (GraceResolv silica 12 g cartridge; 10-100% EtOAc in cyclohexane, then 0-15% MeOH in EtOAc) to give a colourless glass. This was dissolved in DCM (1 mL) and TFA (1 mL) and stirred for 10 min before being concentrated under reduce pressure. The residue was dissolved in methanol and added to a 2 g SCX-2 cartridge. The column was flushed with MeOH before being eluted with 2 M NH₃ in MeOH. The NH₃ fractions were concentrated under reduced pressure to give the title compound (11 mg, 21%) as a colourless glass. LCMS (Method A, ES+): R_T = 0.55 min, *m/z* Calcd for $C_{17}H_{22}N_3O_2$ [M+H]+ 300, found 300.

Step 3: (*R*)-6-Benzyl-3-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)pyrimidin-4(3*H*)-one: General procedure 4 using 6-benzyl-3-((4-hydroxypiperidin-4-yl)methyl)pyrimidin-4(3*H*)-one (11 mg, 36.7 µmol), (*R*)-3-phenylbutanoic acid (6.6 mg, 40.4 µmol), HATU (15.4 mg, 40.4 µmol), DIPEA (7.7 µL, 44.1 µmol) and DCM (1 mL) gave the title compound (11 mg, 67%) as a white solid. LCMS (Method B, ES+): R_T = 1.14 min (purity >95% at 254 nm), *m/z* Calcd for $C_{27}H_{32}N_3O_3$ [M+H]+ 446, found 446. ¹H NMR (300 MHz, DMSO-*d₆*): δ 8.20 (d, 1H), 7.27 (m, 9H), 7.22 (m, 1H), 6.21 (m, 1H), 4.92 (m, 1H), 3.86 (m, 2H), 3.76 (s, 3H), 3.60 (m, 1H), 3.14 (m, 2H), 2.80 (m, 1H), 2.55 (m, 2H), 1.10-1.50 (m, 7H).
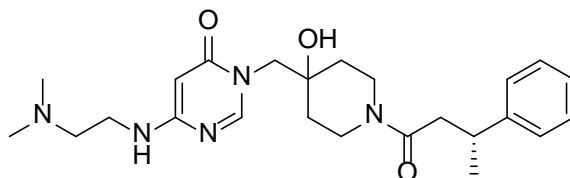
Compound 39: (*R*)-3-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-6-(phenylethynyl)pyrimidin-4(3*H*)-one

Step 1: 3-((4-Hydroxypiperidin-4-yl)methyl)-6-(phenylethynyl)pyrimidin-4(3H)-one: A solution of *tert*-butyl 4-((4-chloro-6-oxopyrimidin-1(6H)-yl)methyl)-4-hydroxypiperidine-1-carboxylate (100 mg, 0.291 mmol), phenylacetylene (70 µL, 0.640 mmol) and trimethylamine (0.24 mL, 1.75 mmol) in DMF (1 mL) was purged with N_2 before AuCl(PPh$_3$) (7.2 mg, 14.5 µmol) and PdCl$_2$(PPh$_3$)$_2$ (10.2 mg, 14.5 µmol) were added. The reaction tube was sealed and the reaction was heated at 60 °C for 16 h before being allowed to cool to rt. The mixture was diluted with water (20 mL) and extracted with EtOAc (3 x 20 mL). The combined organic phases were washed with brine (20 mL), dried over MgSO$_4$ and concentrated under reduced pressure. The residue was purified by flash chromatography (GraceResolv 12 g cartridge, 0-100% EtOAc in cyclohexane) to give an orange syrup. This was dissolved in DCM (1 mL) and TFA (1 mL) and stirred for 5 min before being concentrated under reduced pressure. The residue was dissolved in MeOH and added to a 2 g SCX-2 cartridge. The column was flushed with MeOH before being eluted with 2 M NH$_3$ in MeOH. The NH$_3$ fractions were concentrated under reduced pressure to give the title compound (60 mg, 66%). LCMS (Method A, ES$^+$): R_T = 0.73 min, *m/z* Calcd for C$_{18}$H$_{20}$N$_3$O$_2$ [M+H]$^+$ 310, found 310.
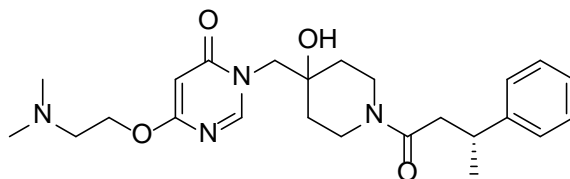
Step 2: (R)-3-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-6-(phenylethynyl)pyrimidin-4(3H)-one: General procedure 4 using 3-((4-hydroxypiperidin-4-yl)methyl)-6-(phenylethynyl)pyrimidin-4(3H)-one (58 mg, 0.187 mmol), (R)-3-phenylbutanoic acid (33.9 mg, 0.206 mmol), DIPEA (0.043 mL, 0.244 mmol), HATU (82 mg, 0.216 mmol) and DCM (2 mL) gave the title compound (9 mg, 10%) as a colourless glass. LCMS (Method B, ES$^+$): R_T = 1.25 min (purity >95% at 254 nm), *m/z* Calcd for C$_{28}$H$_{30}$N$_3$O$_3$ [M+H]$^+$ 456, found 456. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.28 (d, 1H), 7.60 (d, 2H), 7.50 (m, 3H), 7.26 (m, 4H), 7.22 (m, 1H), 6.68 (m, 1H), 4.96 (m, 1H), 4.00 (m, 1H), 3.94 (m, 2H), 3.71 (m, 1H), 3.21 (m, 2H), 2.90 (m, 1H), 2.55 (m, 2H), 1.05-1.55 (m, 7H).

Compound 40: (R)-6-((2-(Dimethylamino)ethyl)amino)-3-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)pyrimidin-4(3H)-one

A mixture of (R)-6-chloro-3-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)pyrimidin-4(3H)-one (25 mg, 64.1 µmol), N,N-dimethylethylenediamine (85 mg, 0.962 mmol) and ethanol (0.5 mL) was heated at 120 °C under microwave irradiation for 20 min before the reaction mixture was purified directly by flash chromatography (Biotage KP-NH 11 g cartridge, 0-100% EtOAc in PE, then 0-30% MeOH in EtOAc) to give the title compound (22 mg, 77%) as a pale yellow solid. LCMS (Method A, ES$^+$): R_T = 0.60 min (purity >96% at 254 nm), *m/z* Calcd for C$_{24}$H$_{36}$N$_5$O$_3$ [M+H]$^+$ 442, found 442. ^{1}H NMR (300 MHz, CDCl$_3$, this molecule appears as conformers in a 2:3 ratio): δ 7.71 (s, 0.4H), 7.60 (s, 0.6H), 7.36-7.15 (m, 5H), 5.68-5.58 (m, 1H), 5.29-5.23 (m, 1H), 4.49-4.32 (m, 1H), 4.00-3.48 (m, 3H), 3.43-3.07 (m, 4H), 3.05-2.84 (m, 1H), 2.71-2.59 (m, 1H), 2.56-2.42 (m, 3H), 2.25 (s, 6H), 1.61-1.11 (m, 6.4H), 0.62-0.49 (m, 0.6H).

Compound 41: (R)-6-(2-(Dimethylamino)ethoxy)-3-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)pyrimidin-4(3H)-one

A mixture of (R)-6-chloro-3-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)pyrimidin-4(3H)-one (25 mg, 64.1 µmol) and 2-(dimethylamino)ethanol (200 µL, 1.99 mmol) was heated at 150 °C under microwave irradiation for 15 min before the reaction mixture was purified directly by flash chromatography (Biotage KP-NH 11 g cartridge, 0-

100% EtOAc in PE, then 0-30% MeOH in EtOAc) to give the title compound (17 mg, 59%) as a pale yellow solid. LCMS (Method A, ES$^+$): R$_T$ = 0.58 min (purity >96% at 254 nm), m/z Calcd for C$_{24}$H$_{35}$N$_4$O$_4$ [M+H]$^+$ 443, found 443. ^{1}H NMR (300 MHz, CDCl$_3$, this molecule appears as conformers in a 2:3 ratio): δ 7.95 (s, 0.4H), 7.83 (s, 0.6H), 7.37-7.16 (m, 5H), 5.78 (s, 1H), 4.47-4.23 (m, 3H), 4.11-4.02 (m, 0.6H), 3.90-3.69 (m, 1.4H), 3.64-3.49 (m, 1H), 3.42-3.16 (m, 2H), 3.10-2.85 (m, 1H), 2.75-2.58 (m, 3H), 2.56-2.42 (m, 1H), 2.33 (s, 3H), 2.31 (s, 3H), 1.58-1.16 (m, 6.4H), 0.68-0.52 (m, 0.6H).
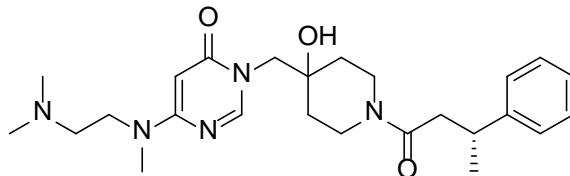
Compound 42: (*R*)-6-((2-(Dimethylamino)ethyl)(methyl)amino-3-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)pyrimidin-4(3*H*)-one

A mixture of (*R*)-6-chloro-3-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)pyrimidin-4(3*H*)-one (25 mg, 64.1 µmol), *N*,*N*,*N'*-trimethylethylenediamine (83 µL, 0.641 mmol) and 1,4-dioxane (0.5 mL) was heated at 150 °C under microwave irradiation for 15 min before the reaction mixture was purified directly by flash chroma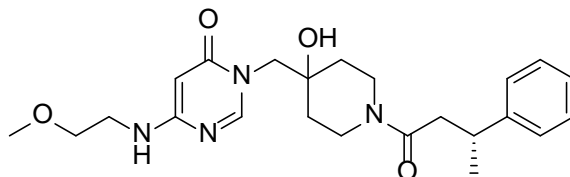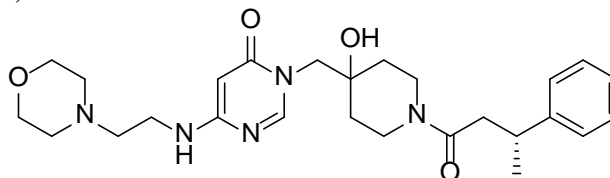tography (Biotage KP-NH 11 g cartridge, 0-100% EtOAc in PE, then 0-30% MeOH in EtOAc) to give the title compound (24 mg, 82%) as a pale yellow solid. LCMS (Method A, ES$^+$): R$_T$ = 0.64 min (purity >96% at 254 nm), m/z Calcd for C$_{25}$H$_{38}$N$_5$O$_3$ [M+H]$^+$ 456, found 456. ^{1}H NMR (300 MHz, CDCl$_3$, this molecule appears as conformers in a 2:3 ratio): δ 7.74 (s, 0.4H), 7.63 (s, 0.6H), 7.36-7.15 (m, 5H), 5.38-5.30 (m, 1H), 4.50-4.33 (m, 1H), 4.00-3.17 (m, 8H), 3.08-2.84 (m, 4H), 2.72-2.59 (m, 1H), 2.57-2.40 (m, 3H), 2.28 (d, 6H), 1.62-1.10 (m, 6.4H), 0.63-0.50 (m, 0.6H).

Compound 43: (*R*)-3-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-6-((2-methoxyethyl)amino)py-rimidin-4(3*H*)-one

A mixture of (*R*)-6-chloro-3-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)pyrimidin-4(3*H*)-one (25 mg, 64.1 µmol), 2-methoxyethanamine (56 µL, 0.641 mmol) and 1,4-dioxane (0.5 mL) was heated for 15 min at 150 °C under microwave irradiation. The reaction mixture was diluted with brine (15 mL) and extracted with DCM (10 mL) using a Biotage phase separator. The organic layer was concentrated under reduced pressure and the residue was purified by flash chromatography (Biotage KP-NH 11 g cartridge, 0-100% EtOAc in PE, then 0-30% MeOH in EtOAc) to afford the title compound (17 mg, 61%) as colourless solid. LCMS (Method A, ES$^+$): R$_T$ = 0.85 min (purity >96% at 254 nm), m/z Calcd for C$_{23}$H$_{33}$N$_4$O$_4$ [M+H]$^+$ 429, found 429. ^{1}H NMR (500 MHz, DMSO-d_6, this molecule appears as conformers in a 1:1 ratio): δ 7.98 (s, 0.5H), 7.96 (s, 0.5H), 7.31-7.21 (m, 4H), 7.20-7.12 (m, 1H), 6.95 (s, 1H), 5.09 (s, 1H), 4.99 (s, 0.5H), 4.99 (s, 0.5H), 4.03-3.94 (m, 1H), 3.84-3.58 (m, 4H), 3.42 (t, 2H), 3.25 (s, 3H), 3.28-3.12 (m, 3H), 2.93-2.86 (m, 1H), 2.62-2.53 (m, 2H), 1.48-1.22 (m, 4H), 1.20 (d, 3H).

Compound 44: (*R*)-3-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-6-((2-morpho-linoethyl)amino)pyrimidin-4(3*H*)-one

A mixture of (*R*)-6-chloro-3-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)pyrimidin-4(3*H*)-one (25 mg, 64.1 µmol), 2-morpholinoethan-1-amine (84 µL, 0.641 mmol) and 1,4-dioxane (0.5 mL) was heated for 15 min at 150 °C under microwave irradiation. The reaction mixture was diluted with brine (15 mL) and extracted with DCM (10 mL) using a Biotage phase separator. The organic layer was concentrated under reduced pressure and the residue was purified by flash chromatography (Biotage KP-NH 11 g cartridge, 0-100% EtOAc in PE, then 0-30% MeOH in EtOAc) to afford the title compound (25 mg, 80%) as pale yellow solid. LCMS (Method B, ES$^+$): R$_T$ = 0.66 min (purity >98% at 254 nm), m/z Calcd for C$_{26}$H$_{38}$N$_5$O$_4$ [M+H]$^+$ 484, found 484. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.01-7.93 (m, 1H), 7.31-7.21

(m, 4H), 7.20-7.13 (m, 1H), 6.74 (br s, 1H), 5.12-5.03 (m, 1H), 5.03-4.92 (m, 1H), 4.04-3.92 (m, 1H), 3.85-3.68 (m, 2H), 3.66-3.58 (m, 1H), 3.59-3.52 (m, 4H), 3.28-3.09 (m, 4H), 2.95-2.85 (m, 1H), 2.62-2.53 (m, 2H), 2.46-2.42 (m, 2H), 2.41-2.36 (m, 4H), 1.48-1.22 (m, 4H), 1.20 (d, 3H).

Compound 45: (*R*)-6-((3-(Dimethylamino)propyl)amino-3-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)pyrimidin-4(3*H*)-one

A mixture of (*R*)-6-chloro-3-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)pyrimidin-4(3*H*)-one (25 mg, 64.1 µmol), N^1,N^1-dimethylpropane-1,3-diamine (81 µL, 0.641 mmol) and 1,4-dioxane (0.5 mL) was heated for 15 min at 150 °C under microwave irradiation. The reaction mixture was diluted with brine (15 mL) and extracted with DCM (10 mL) using a Biotage phase separator. The organic layer was concentrated under reduced pressure and the residue was purified by flash chromatography (Biotage KP-NH 11 g cartridge, 0-100% EtOAc in PE, then 0-30% MeOH in EtOAc) to afford the title compound (24 mg, 82%) as pale yellow solid. LCMS (Method B, ES$^+$): R$_T$ = 0.66 min (purity >97% at 254 nm), *m/z* Calcd for $C_{25}H_{38}N_5O_3$ [M+H]$^+$ 456, found 456. ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.99-7.90 (m, 1H), 7.34-7.19 (m, 4H), 7.20-7.13 (m, 1H), 7.03-6.93 (m, 1H), 5.08-4.93 (m, 2H), 4.03-3.93 (m, 1H), 3.84-3.69 (m, 2H), 3.68-3.58 (m, 1H), 3.26-2.96 (m, 4H), 2.94-2.85 (m, 1H), 2.61-2.52 (m, 2H), 2.27-2.17 (m, 2H), 2.13-2.07 (m, 6H), 1.68-1.57 (m, 2H), 1.55-1.23 (m, 4H), 1.20 (d, 3H).
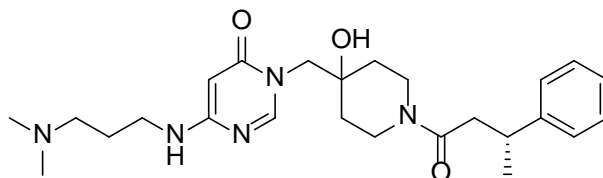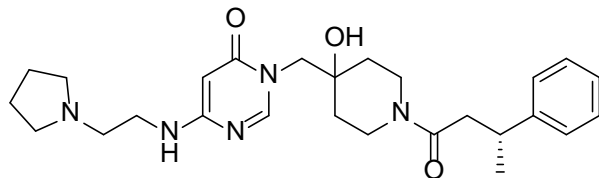
Compound 46: (*R*)-3-((4-Hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)-6-((2-(pyrrolidin-1-yl)ethyl)amino)pyrimidin-4(3*H*)-one

A mixture of (*R*)-6-chloro-3-((4-hydroxy-1-(3-phenylbutanoyl)piperidin-4-yl)methyl)pyrimidin-4(3*H*)-one (25 mg, 64.1 µmol), 2-(pyrrolidin-1-yl)ethanamine (81 µL, 0.641 mmol) and 1,4-dioxane (0.5 mL) was heated at 150 °C under microwave irradiation for 15 min before the reaction mixture was purified directly by flash chromatography (Biotage KP-NH 11 g cartridge, 0-100% EtOAc in PE, then 0-30% MeOH in EtOAc) to give the title compound (22 mg, 73%) as a pale yellow solid. LCMS (Method A, ES+): R$_T$ = 0.61 min (purity >98% at 254 nm), *m/z* Calcd for $C_{26}H_{38}N_5O_3$ [M+H]$^+$ 468, found 468. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.06-7.93 (m, 1H), 7.37-7.19 (m, 4H), 7.19-7.13 (m, 1H), 6.79 (br s, 1H), 5.05 (s, 1H), 5.00 (s, 1H), 4.02-3.94 (m, 1H), 3.86-3.68 (m, 2H), 3.66-3.58 (m, 1H), 3.25-3.10 (m, 4H), 2.94-2.86 (m, 1H), 2.65-2.52 (m, 4H), 2.48-2.35 (m, 4H), 1.77-1.61 (m, 4H), 1.50-1.22 (m, 4H), 1.20 (d, 3H). ^{13}C NMR (126 MHz, DMSO-d_6): δ 169.0 + 169.0 (conformers), 161.9, 161.5, 152.2, 146.6 + 146.6 (conformers), 128.2 + 128.2 (conformers), 126.8, 125.9 + 125.9 (conformers), 85.6 (very broad), 69.0 + 68.9 (conformers), 53.5, 52.8, 41.1 + 41.0 (conformers), 40.3, 37.0, 36.1 + 36.0 (conformers), 35.0 + 34.9 (conformers), 34.3 + 34.2 (conformers), 23.1, 22.0 + 21.8 (conformers). HRMS (FTMS ES$^+$): *m/z* Calcd for $C_{26}H_{38}N_5O_3$ [M+H]$^+$ 468.2969, found 468.2963.

3. USP7 surface plasmon resonance (SPR)
Fragment Library Screening:
SPR fragment library screening was performed by Beactica (Sweden) using Biacore 4000, S51 and T200 instruments (GE Healthcare/Biacore, Uppsala, Sweden). USP7 (His$_6$-USP7CD (HAUSP cat domain), catalytic domain, aa 213-548, Boston Biochem, Lot # DBCW0111101) was immobilized by amine coupling using materials provided by GE Healthcare. The protein was prepared as a 0.1–0.2 mg/mL solution in coupling buffer (USP7: 50 mM acetate pH 5.5 supplemented with 2 mM DTT, 0.5 mM EDTA) and injected for 5 min over activated surfaces (50 mM N-hydroxy-succinimide, 200 mM N-ethyl-N´-((dimethylamino)propyl)carbodiimide) of CM7 or CM5 chips. The surfaces were subsequently deactivated by 1 M ethanolamine, pH 8.0.

Compound characterization: All interaction experiments were performed at 25 °C. Experiments were conducted in HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl), supplemented with 0.05% Pluronic 127, 2 mM DTT, 0.5 mM EDTA and 5 % (v/v) DMSO (all Sigma). The test compounds were diluted in the running buffer in two- or three-fold

dilution series up to 300 μM and injected for 15-25 s over the immobilized Target Proteins as well as three Reference Targets (GST (Schistosoma japonicum, Sigma), anti-His antibody (Pierce), serine protease (Sprint™)).

Data analysis: Report points from sensorgrams were extracted 5-15 s after initiation of the association phase. Responses were referenced with responses from unmodified reference surfaces, corrected for bulk shifts arising from differences in DMSO concentrations between samples and running buffer (solvent correction) and blank-referenced. For competition analysis, responses of competitor-Test Compound mixtures were compared to the sum of responses of Test Compound and competitor alone. Competition values were determined as normalised deviation from theoretical responses for independent interaction (additivity of responses). Responses of mixtures $3 \times \sigma$ lower or higher than predicted responses were classified as potential competitive or synergistic, respectively. For K_D determination, the data was globally fitted to the sum of a Langmuir term and a linear term to compensate for non-specific binding using the Sprint™ evaluation software.

SPR K_D evaluation of compound 1:

SPR K_D evaluations were carried out on a SensíQ Pioneer instrument using COOH5 sensor chips, Qdat™ software and coupling reagents from Pall ForteBio. EDC (1-ethyl 3-(3-dimethylaminopropyl)-carbodiimide hydrochloride), sulfo-NHS (sulfo-N-hydroxysuccinimide), sodium acetate pH4.5 and ethanolamine pH8.9 were from GE Health Sciences. Catalytic domain USP7 was procured from Boston Biochem.

A COOH5 sensor chip was installed in the SensíQ Pioneer system, normalized with air, followed by DMSO, and primed with running buffer (10 mM HEPES, 150 mM NaCl, pH 7.4; HBS). Catalytic domain of USP 7 was immobilized at 25 ℃ onto a COOH5 sensor chip using standard amine-coupling methods and HBS as the running buffer. The surface was activated with 0.5 mM EDC and 0.2 mM sulfo-NHS for 5 min, followed by a 12-min injection of USP7 CD at 100 μg/mL in 10 mM sodium acetate, pH 4.5, and finally a 5-min blocking step of 1 M ethanolamine, pH 8.9. This coupling method resulted in a density of 9,000 RU (resonance units) of USP7 on one flow cell of the COOH5 chip. OneStep screening using Taylor dispersion concentration gradient of compound **1** at concentrations in a three-fold dilution series starting at 5 mM were completed at a flow rate of 75 μL/min for 60 s. The response data were processed using Qdat™ software using a reference surface to correct for any bulk refractive index changes and blank injections for double referencing. The binding profiles were fitted globally to a 1:1 interaction model.

4. USP7 NMR-binding studies:

Human USP7 catalytic domain (amino acids 208-555, Crelux GmbH) was delivered in PBS (137 mM NaCl, 2.7 mM KCl, 1 mM NaH_2PO_4/Na_2HPO_4), pH 7.4 at a concentration of 13.9 mg/mL (331 μM). The protein was cloned and expressed in *E. coli* using an *N*-terminal 6-histidine tag. Purification of cell lysate was performed by Ni-NTA column, followed by Superdex size exclusion chromatography (SEC). Purity was determined to be >85% using SDS page.

Reference ^{1}H spectrum for compound **1** was collected at 1 mM concentration, formulated in PBS buffer identical to that the protein was supplied in. The compound tested negative for self-aggregation using the WaterLOGSY pulse sequence with no macroscopically visible precipitate or diminished signals in the proton spectrum. Compound **1** was included in a cocktail of other compounds, including a positive and negative control. The cocktail was comprised of 10 μM USP7 and a total of 6 compounds at a concentration of 200 μM each, resulting in a 1:20 USP7:compound ratio. Each cocktail was subjected to a trio of ^{1}H, ligand-observed experiments, namely saturation transfer difference (STD), Carr-Purcell-Meiboom-Gill (CPMG) T2, and WaterLOGSY experiments.

NMR Hardware and Processing: All experiments were performed in-house on a 500 MHz Bruker AVANCE spectrometer equipped with TCI Cryoprobe, using standard pulse programs included in Bruker's experiment library. Spectra were collected at 25 °C, and processed using Bruker's TopSpin software program.

5. Biochemical and cellular assays:

The USP7 biochemical assay was performed using materials and conditions outlined in reference 1.

Target Engagement Assay in HCT116 Cells

HCT116 cells were treated with vehicle (DMSO) or USP7 inhibitor for 2 h. Following incubation, cells were washed extensively with 1 x PBS and harvested in TE lysis buffer containing 50 mM TRIS-HCl (pH7.4), 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.5% NP40, 10% Glycerol, 2 mM DTT and clarified cell lysates (40 μg) incubated with the ubiquitin-propargylamine probe (Ub-PA; 8 μg/ml final concentration) in assay buffer containing 50 mM TRIS-HCl (pH7.6), 5 mM $MgCl_2$, 250 mM Sucrose, 0.5 mM EDTA, 2 mM DTT for 30 min. The reaction was terminated by the addition of LDS sample buffer (Life Technologies) and heated to 70 °C. Samples were then analyzed by western blotting using the Cell Signalling anti-USP7 Ab (#4833; 1/1000 dilution). EC_{50} values were determined upon densitometry analysis. Band intensities were quantified using ImageJ software where the upper bands (USP7-Ub) and lower bands (USP7) were calculated as a percentage of the corresponding DMSO controls (-/+ Ub-PA) and values were then normalized to the sum of the lower and upper bands for each concentration.

6. DUB selectivity assay on compound 46:

Selectivity assays were performed against all USPs included in the DUBprofiler™ panel (Ubiquigent Ltd). Screening was performed at a fixed inhibitor concentration of 10 μM. Data generated is displayed as a percentage inhibition of total enzyme activity for each enzyme. Under the conditions of this screen, **46** exhibited an IC_{50} value of 140 ± 22 nM. Data reported as the mean of 2 independent experiments.

7. Protein production, crystallization, data collection and structure determination:

The USP7 catalytic domain (residues 207-560), genetically fused with a C-terminal hexa-histidine tag, was expressed in E.coli. BL21 cells were transformed with the corresponding expression plasmid and grown in Terrific broth (TB) and protein expression induced with 0.25 mM IPTG overnight at 16°C. After harvesting by centrifugation, cell pellets were resuspended in Lysis Buffer (40 mM TRIS-HCl, 500 mM NaCl, 1 mM AEBSF, 2 mM TCEP, 5 mM Imidazole, 0.1% Tween 20, pH 7.5) and lysed by sonication on ice. The soluble fraction was then loaded directly onto an IMAC column (5 mL HisTrap HP) pre-equilibrated with Lysis Buffer and the protein eluted with IMAC Buffer B (40 mM TRIS-HCl, 500 mM NaCl, 1 mM AEBSF, 2 mM TCEP, 300 mM Imidazole, 0.1% Tween 20, pH 7.5). Fractions containing the desired protein were pooled and buffer exchanged by disalysis (MWCO 8,000-10,000 Da) against anion exchange (AEX) Buffer A (20 mM TRIS-HCl, 30 mM NaCl, 1 mM EDTA, 4 mM DTT, pH 8.0).

The protein was then loaded onto a YMC-BioPro ion exchange column (15 x 120, 7.4 mL) pre-equilibrated with AEX Buffer A and eluted over 30 CV with a gradient of 0-50% AEX Buffer B (20 mM TRIS-HCl, 1M NaCl, 1 mM EDTA, 4 mM DTT, pH 8.0).

Fractions were analyzed by SDS-PAGE and those containing the desired protein were pooled and then further purified by SEC (HighLoad Superdex 75 column) using a running buffer of 10 mM TRIS-HCl, 100 mM NaCl, 4 mM DTT, pH 8. SEC fractions were analyzed by SDS-PAGE and the pure fractions pooled and concentrated (Vivaspin column, MWCO 12KDa) to 5.3 mg / mL as measured by UV A_{280nm}.

Crystals of USP7 in complex with **46** were grown by hanging drop vapour diffusion. USP7 (14.2 mg/ml in 10 mM TRIS-HCl, 100 mM NaCl, 4mM TCEP, pH 8.0) was pre-incubated with an 8.9-fold molar excess of **46** (150 mM in DMSO) for 2 h. 0.7 µl of the protein solution was then mixed with 0.7 µl of reservoir solution containing 100 mM TRIS-HCl (pH 7.75), 200 mM Li_2SO_4, 25% (w/v) PEG4000 and equilibrated at 20°C over 0.4 mL of reservoir solution. Crystals appeared within 4 days.

Diffraction data at 2.2 Å resolution for a USP7/**46** crystal was collected at the ESRF synchrotron radiation source, id30a1, Grenoble. The structure was solved via molecular replacement using the PDB structure 5N9R as a template. Iterative manual modelling in Coot and refinement using REFMAC5 resulted in the final model. 97.5% of backbone torsions for the final model are within the Ramachandran favoured regions, with 2.5% in the allowed regions. The crystallography data collection and refinement statistics are provided in **Supplementary Table 1** below.

Data collection statistics	
Space group	$P2_1$
Unit cell a, b, c (Å) α, β, γ, (°)	74.9. 67.3, 80.7 90, 105.1, 90
# molecules per au	2
Resolution (Å)	28.22-2.16 (2.28-2.16)
# unique reflections	39952 (5872)
Completeness (%)	95.9 (96.8)
Redundancy	2.4 (2.4)
R_{merge}	0.110 (0.670)
I/σ	6.4 (1.4)
Refinement statistics	
Resolution (Å)	29.95-2.16 (2.22-2.16)
R_{work} / R_{free}	0.202 / 0.262

Supplementary Table 1. Crystallography data collection and refinement statistics for compound 46 and USP7

8. *In vitro* ADME & physicochemical methods:

Kinetic Solubility:

Test compounds (5 μL; 10 mM DMSO stock) were added to 245 μL of PBS buffer pH 7.4 (Dulbecco A) in a Millipore MultiScreen® Solubility Filter plate and mixed at 300 rpm at rt on a plate shaker for 90 min. Meanwhile 5-points calibration curves for each compound were established in a mixture of acetonitrile/PBS buffer (top concentration 200 μM). After filtration and matrix match, the calibration and assay plates were analyzed on a Bioteck Synergy 4 plate reader (240-400 nm). Final concentration of the test compound in the filtrate was calculated using the slope of the calibration curve. Two markers were used as controls: hydrocortisone (high solubility KSol >180 μM) and reserpine (low solubility KSol < 25 μM).

Caco-2 permeability:

Caco-2 permeability measurements were performed at Cyprotex Ltd. Caco-2 cells obtained from the ATCC are used between passage numbers 40-60. Cells are seeded on to Millipore Multiscreen Caco-2 plates at 1 x 10^5 cells/cm^2. They are cultured for 20 days in DMEM and media is changed every two or three days. On day 20 the permeability study is performed.

Hanks Balanced Salt Solution (HBSS) pH 7.4 buffer with 25 mM HEPES and 4.45 mM glucose at 37 °C is used as the medium in the permeability studies. Incubations are carried out in an atmosphere of 5% CO_2 with a relative humidity of 95% at 37 °C.

On day 20, the monolayers are prepared by rinsing both basolateral and apical surfaces twice with HBSS at 37 °C. Cells are then incubated with HBSS in both apical and basolateral compartments for 40 min to stabilise physiological parameters.

HBSS is then removed from the apical compartment and replaced with test compound dosing solutions. The solutions are made by diluting 10 mM test compound in DMSO with HBSS to give a final test compound concentration of 10 μM (final DMSO concentration 1%). The fluorescent integrity marker Lucifer yellow is also included in the dosing solution. Analytical standards are made from dosing solutions. The apical compartment inserts are then placed into 'companion' plates containing fresh HBSS. For basolateral to apical (B-A) permeability determination the experiment is initiated by replacing buffer in the inserts then placing them in companion plates containing dosing solutions. At 120 min, the companion plate is removed and apical and basolateral samples diluted for analysis by LC-MS/MS. Test compound permeability is assessed in duplicate. On each plate compounds of known permeability characteristics are run as controls.

Test and control compounds are quantified by LC-MS/MS cassette analysis using a 5-point calibration with appropriate dilution of the samples. Cyprotex generic analytical conditions are used. The starting concentration (C_0) is determined from the dosing solution and experimental recovery calculated from C_0 and both apical and basolateral compartment concentrations.

The integrity of the monolayers throughout the experiment is checked by monitoring Lucifer yellow permeation using fluorimetric analysis. Lucifer yellow permeation is low if monolayers have not been damaged. If a Lucifer yellow P_{app} value is above QC limits in one individual test compound well, then an n=1 result is reported. If Lucifer yellow P_{app} values are above QC limits in both replicate wells for a test compound, the compound is re-tested. If on repeat, high Lucifer yellow permeation is observed in both wells then toxicity or inherent fluorescence of the test compound is assumed. No further experiments are performed in this instance.

Data Analysis:

The permeability coefficient for each compound (P_{app}) is calculated from the following equation:

$$P_{app} = \left(\frac{dQ/dt}{C_0 \times A} \right)$$

Where dQ/dt is the rate of permeation of the drug across the cells, C_0 is the donor compartment concentration at time zero and A is the area of the cell monolayer. C_0 is obtained from analysis of dosing solution at the start of the experiment. An efflux ratio (ER) is derived as follows:

$$ER = \frac{P_{app(B-A)}}{P_{app(A-B)}}$$

An efflux ratio greater than two shows efflux from the Caco-2 cells, which indicates that the compound may have potential absorption problems *in vivo*.

The apparent permeability ($P_{app\,(A-B)}$) values of test compounds are compared to those of control compounds, atenolol and propranolol, which have human absorption of approximately 50 and 90% respectively. Talinolol (a known P-gp substrate[2]) is also included as a control compound to assess whether functional P-gp is present in the Caco-2 cell monolayer.

LogD$_{7.4}$:

Test compounds (8 μL; 10 mM DMSO stock) was added to 392 μL of PBS buffer pH 7.4 (Dulbecco A pre-saturated with octanol) and 400 μL of octanol (pre-saturated with PBS buffer). The plate was shaken at rt for 4 h. The layers were allowed to separate before being analyzed by HPLC.

Samples were analyzed on an Agilent 1260 HPLC fitted with a Phenomenex Kinetex XB-C18 100A 2.6 μm 2.1x50 mm column. Mobile phases were water and acetonitrile containing 0.1% formic acid as modifier. The relative drug concentration in each phase was determined by the peak area measurement from LC-UV analysis, UV detection at 254 nm and 210 nm.

Log $D_{7.4}$ is calculated as follows:

$$LogD = Log \left[\frac{\text{peak area of compound in octanol x injection volume of buffer phase}}{\text{peak area of compound in buffer x injection volume of octanol phase}} \right]$$

Three markers were used as controls: caffeine (LogD$_{7.4}$ ~ 0), furosemide (LogD$_{7.4}$ < - 0.5) and reserpine (LogD$_{7.4}$ > 3).

Microsomal stability:

Test compounds (final concentration = 1 μM; final DMSO concentration = 0.1%) were incubated in 0.1 M phosphate buffer pH 7.4 with liver microsomes (human, mouse or rat; 0.5 mg of protein/mL) at 37 °C. Reactions were started by addition of NADPH in 0.1 M phosphate buffer pH 7.4 (final concentration 1 mM). 40 μL aliquots were removed at 2, 5, 10, 15, 20, 30, 40 and 50 min. Reactions were quenched in 80 μL of ice-cold methanol containing internal standard. Samples were subsequently frozen overnight then centrifuged at 3500 rpm for 20 min at 4 °C. The supernatants were removed and transferred into analytical plates and analyzed by LC-MS/MS.

LC-MS/MS method: All samples were analyzed on a Waters Acquity I-Class coupled to a Waters Xevo TQD mass spectrometer. A Waters BEH C18 2.1 x 50 mm 1.7 μm column was used and mobile phases were water and methanol containing 0.1 % formic acid as modifier. Analysis was by multiple reaction monitoring and conditions were optimised for each test compound.

Data analyses: From a plot of ln peak area against time, the gradient of the line is determined. Subsequently, half-life and intrinsic clearance are calculated using the equations below:

Eliminated rate constant (k) = (- gradient)

Half-life ($t_{1/2}$) (min) = $\frac{0.693}{k}$

Intrinsic Clearance (CL$_{int}$)(μL/min/mg protein) = $V x \frac{0.693}{t_{1/2}}$

Where V = Incubation volume (μL)/mg of protein

Four markers were used as controls for each assay:

Human Liver Microsomes	Dextromethorphan	Moderate-high CL$_{int}$
	Quinidine	Low-moderate CL$_{int}$
	Tolbutamide	Low CL$_{int}$
	Verapamil	High CL$_{int}$
Mouse Liver Microsomes	Diphenydramine	High CL$_{int}$
	Metroprolol	Low-moderate CL$_{int}$
	Diclofenac	Moderate CL$_{int}$
	Verapamil	High CL$_{int}$

9. Pharmacokinetic Profiling of Compounds 46 & 47:

Pharmacokinetics of compounds **46** and **47** were evaluated in healthy male CD-1 mice following a single oral, intravenous or intraperitoneal administration. Dosing solutions for intravenous administration were prepared using DMSO: 20% 2-hydroxypropyl β cyclodextrin (2:98) and administered at a dose level of 1 mg/kg. Dosing solutions for oral administration were prepared using 0.5 % methylcellulose and administered at a dose level of 30 mg/kg. Dosing solutions for intraperitoneal administration were prepared using saline and administered at a dose level of 10 mg/kg. Blood samples

were collected up to 24 h post-dose. In-vivo experiments were carried out at Axis BioServices and pharmacokinetic parameters were measured from blood by XenoGesis Ltd.

A total of 27 male CD-1 mice aged 5-8 weeks, weighing approximately 30-35g were used for the study (animals were bred in-house at Axis BioServices). Mice were housed in IVC cages (5 per cage) with individual mice identified by tail mark. The holding room was maintained under standard conditions: 20-24 °C, 40-70 % humidity and a 12 h light/dark cycle. Animals were fed a standard certified commercial laboratory rodent diet except for overnight fasting immediately prior to dosing; animals were allowed access to food 2 hours after test compound administration. Animals were allowed free access to water at all times during the study.

Dosing solutions were freshly prepared as follows:

For IV dosing solutions:

1.0 mg compound **46** was weighed and mixed with DMSO (40 μL). 1960 μL of 20% 2-hydroxypropyl β cyclodextrin was added to give a final concentration of 0.5 mg/mL.

1.3 mg compound **47** was weighed and mixed with DMSO (52 μL). 2548 μL of 20% 2-hydroxypropyl β cyclodextrin was added to give a final concentration of 0.5 mg/mL.

For PO dosing solutions:

12.2 mg compound **46** was weighed and mixed with 1mL 0.5% methylcelluose with a pestle and mortar. A further 1.03 mL 0.5% methylcellulose was used to completely wash any compound into the tube, giving a final concentration of 6 mg/mL.

15.2 mg compound **47** was weighed and mixed with 1mL 0.5% methylcelluose with a pestle and mortar. A further 1.533 mL 0.5% methylcellulose was used to completely wash any compound into the tube, giving a final concentration of 6 mg/mL.

For IP dosing solutions:

4.5 mg compound **46** was weighed and mixed with 1mL sterile saline with a pestle and mortar. A further 3.5 mL saline was added to completely wash any compound into the tube, giving a final concentration of 1 mg/mL.

3.8 mg compound **47** was weighed and mixed with 1mL sterile saline with a pestle and mortar. A further 2.8 mL saline was added to completely wash any compound into the tube, giving a final concentration of 1 mg/mL.

The dosing volumes were 2 mL/kg for IV dosing, 5 mL/kg for PO dosing and 10 mL/kg for IP dosing with individual dose calculated from the bodyweight recorded on the day of dosing. At the required time points 100 uL whole blood was removed from the lateral vein into tubes coated with K_2-EDTA. Blood samples were diluted 1:1 with ultrapure water and stored at -80°C before being transported to XenoGesis Ltd on dry ice for bioanalysis. The time points for blood sampling were 5min, 15min, 30min, 1h, 2h, 4h, 8h and 24h for the IV and IP routes. They were 15min, 30min, 1h, 2h, 4h, 6h, 8h and 24h for the PO route. PK parameters were calculated using Phoenix WinNonlin software.

10. Computational Chemistry:

Docking studies and images were carried out or created using Molecular Operating Environment (MOE, 2015.1001 or 2016.0802; Chemical Computing Group ULC, 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7 (2017)) and/or Maestro (Schrödinger Release 2016-4: Maestro, Schrödinger LLC, New York, NY (2016).

11. Supporting Figure S1: USP7 SPR sensogram of compound **1**:

Name	k_a (M^{-1}s^{-1})	k_d (s^{-1})	K_D (M)
Compound 1	3.82±0.04e3	1.80±0.01	471±5uM
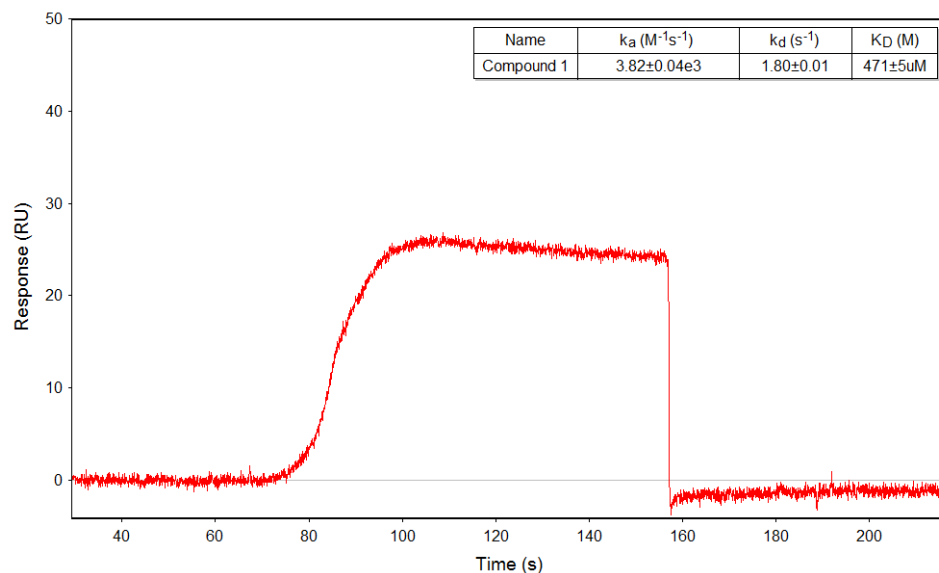
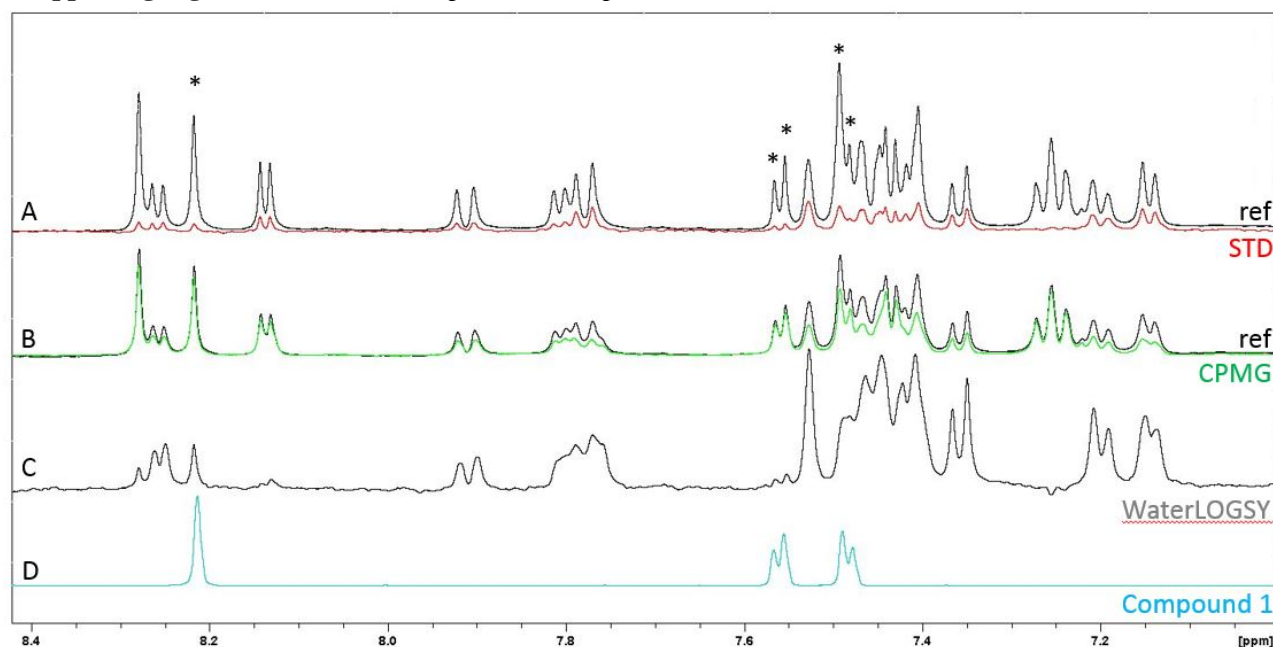**12. Supporting Figure S2:** USP7 NMR spectra of compound **1**:

Figure S2: A-C Aromatic region expansion of 1D ^{1}H NMR spectra illustrating binding of compound **1** to USP7 in cocktail containing 10 μM USP7, 200 μM compound **1** in PBS pH 7.4, collected at 25 °C. **A.** Cocktail reference (black trace) and saturation transfer difference spectra (STD, red trace) illustrating positive STD signal of compound **1**, indicated by asterisks. **B.** Reference (black trace) and T2 Carr-Purcell-Meiboom-Gill (CPMG, green trace) spectra illustrating line broadening of signals of compound **1**. **C.** WaterLOGSY spectrum (black trace) of cocktail illustrating binding of compound **1**. **D.** Aromatic region expansion of reference 1D ^{1}H NMR spectrum (blue trace) of 1 mM compound **1** in PBS pH 7.4.

13. Supporting Figure S3: Structure of trifluromethyl analogue **47**:
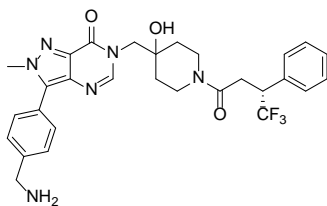
References:

1. Gavory, G.; O'Dowd, C. R.; Helm, M. D.; Flasz, J.; Arkoudis, E.; Dossang, A.; Hughes, C.; Cassidy, E.; McClelland, K.; Odrzywol, E.; Page, N.; Barker, O.; Miel, H.; Harrison, T. Discovery and Characterisation of Highly Potent and Selective Allosteric Inhibitors of USP7. *Nat. Chem. Biol.* **2018**, *14*, 118-125.