

Discovery of a novel class of D-amino acid oxidase (DAO) inhibitors with the Schrödinger computational platform

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INTRODUCTION

D-serine is a physiological co-agonist of the N-methyl D-aspartate (NMDA) type of glutamate receptor, a key excitatory neurotransmitter receptor in the brain. D-Serine in the brain is synthesized from its L-isomer by serine racemase and is metabolized by the D-amino acid oxidase (DAO, DAAO), a flavoenzyme that catalyzes the oxidative degradation of D-amino acids including D-serine to the corresponding α -keto acids. The function of the NMDA receptor requires the presence of both the agonist (glutamate) and the co-agonist (D-serine, glycine, and/or D-alanine). Importantly, D-serine has been reported to be the predominant NMDA co-agonist in the forebrain and linked directly to schizophrenia.¹ D-serine concentrations in serum and cerebrospinal fluid have been reported to be decreased in schizophrenia patients,² and oral administration of D-serine improved symptoms of schizophrenia when used as an adjuvant to typical and atypical antipsychotics.³ Thus, it is plausible to explore pharmaceutical inhibition of DAO function as putative novel therapeutics to treat the positive (psychotic), negative and cognitive symptoms in schizophrenia.

The simplest DAO inhibitor benzoic acid (**1**) was reported in 1956.⁴ Since the early 2000s, many small molecule DAO inhibitors have been reported in the literature (Figure 1).⁵ They all mimic the substrate D-serine and bind to the catalytic site of DAO. The early inhibitors (**1-6**)⁶ can all be characterized as aryl carboxylic acids or corresponding acid-bioisosteres with low molecular weight. Although they are potent and highly ligand efficient, they lack the vectors that are needed for optimization of potency and physicochemical properties. To that end in 2013, Astellas reported a new class of DAO inhibitors (**7**) which contain a tail group reaching into a hydrophobic pocket perpendicular to the head group.⁷ Takeda also worked on a similar chemical series which culminated in the discovery of their clinical candidate TAK-831 (**8**). The kojic acid derivatives (**9**) were also explored by a Johns Hopkins research group.⁸ In addition, Sunovion reported a new class of DAO inhibitors (**10**) that stabilize an active-site lid-open conformation, although the lead compounds suffer from poor pharmacokinetic and brain penetration properties.⁹

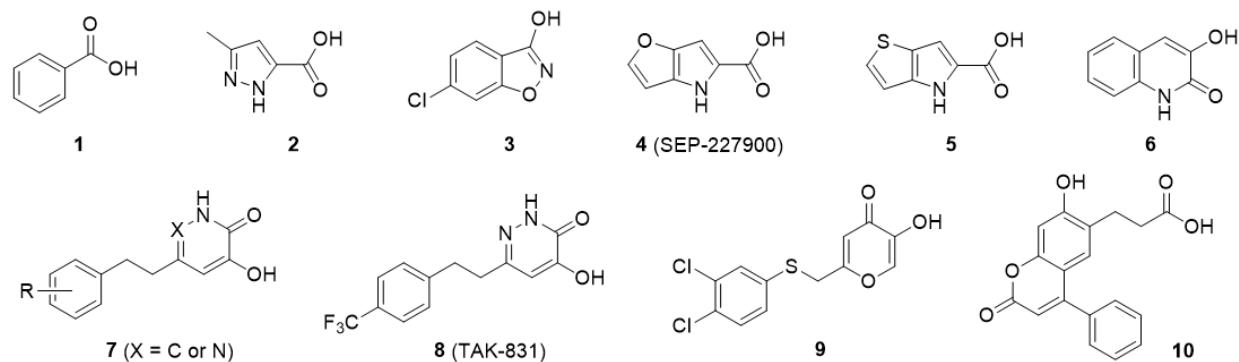


Figure 1. Previously reported inhibitors of DAO

A few DAO inhibitors have entered into clinical development. Currently, only SyneuRx is actively developing NaBen® (sodium salt of **1**) in a phase II/III clinical trial for refractory schizophrenia in adults.¹⁰ Sepracor was developing SEP-227900 for neuropathic pain around 2010. Takeda was developing TAK-831 (luvadaxistat, **8**) in phase 2 clinical trials¹¹ for the treatment of schizophrenia, which was the subject of a license agreement with Neurocrine in 2020.¹² In March 2021, Neurocrine reported topline data from the Phase II INTERACT study in adults with negative symptoms of schizophrenia treated with luvadaxistat (NBI-1065844/TAK-831). Although luvadaxistat did not meet its primary endpoint in the study, as measured by the change from baseline on the PANSS NSFS at Day 84, Luvadaxistat met secondary endpoints of cognitive assessment, which merit further clinical evaluation.¹³ The improvement of cognitive function for TAK-831 in schizophrenic patients is consistent with improvement of cognitive performance in rodent models. For example, another DAO inhibitor SEP-227900 increased D-serine in the cerebellum of rats in a dose dependent manner, and pretreatment of rats with this DAO inhibitor increased memory of the test object in the novel object recognition model in rats, suggesting improved cognitive function.¹⁴

There are many published DAO co-crystal structures in the literature. Figure 2 shows the co-crystal structure of human DAO enzyme with a hydroxy pyridazinone ligand (**11**), which was one of the most potent DAO inhibitors reported by both Takeda and Astellas.⁷ Overall, the ligand adopts an L-shaped conformation in the binding site. The hydroxy pyridazinone head group is stacked between the flavin ring of FAD (flavin adenine dinucleotide) and Tyr224. The hydroxy-carbonyl moiety forms a salt bridge with Arg283, and the N-H forms an H-bond with Gly313. On the other side, the phenyl ring sticks into a relatively hydrophobic pocket and stacks with Tyr224 to form a π -edge interaction.

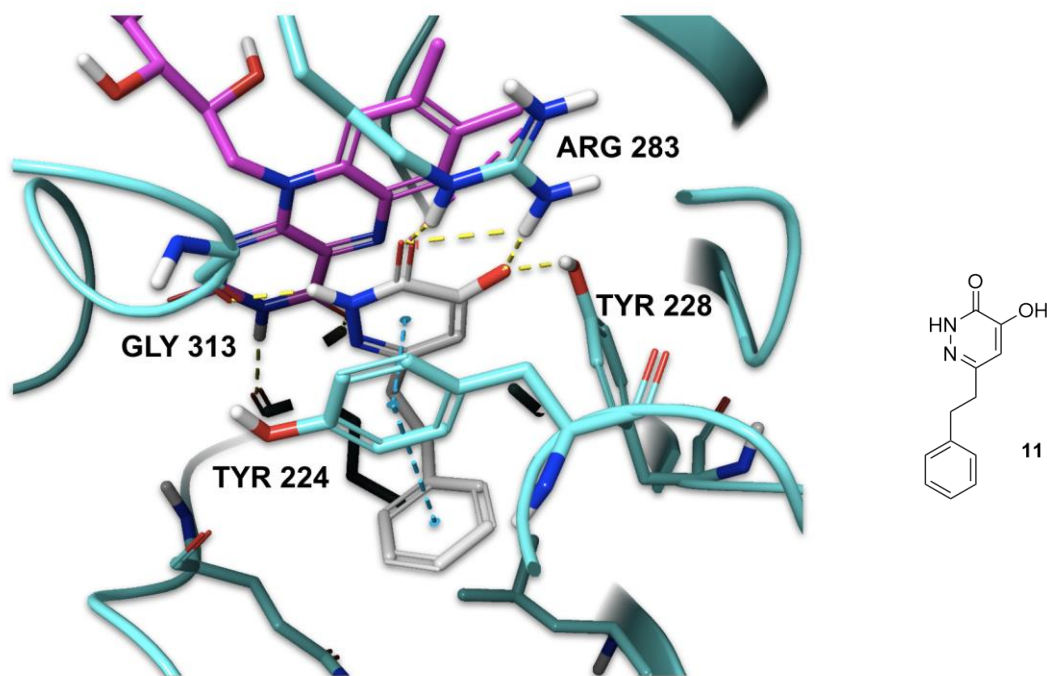
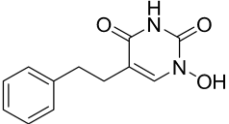
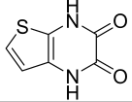
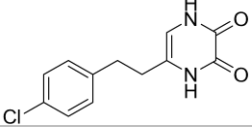


Figure 2. Co-crystal structure of human DAO with compound **11** (PDB: 3W4K) in grey and coenzyme FAD in magenta.

Intrigued by the target biology and therapeutic potential for treatment of cognitive impairment in schizophrenia or other neurological disorders, we initiated a program to identify novel DAO inhibitors with best-in-class properties. The program leveraged the Schrödinger physics-based modeling technology, specifically, a human DAO Free Energy Perturbation (FEP+) model which was developed on the basis of published SAR data.¹⁵ Key protein-ligand interactions presented in the co-crystal structures were taken into account as novel ligands were designed by multiple internal medicinal and computational chemists. The designs were further evaluated with the hDAO FEP+ model, and the top ideas were prioritized for synthesis. Among them (Table 1), **12**, **13** and **14**¹⁶ showed good hDAO biochemical potency, which is consistent with the FEP+ model prediction.¹⁷ Thanks to their low molecular weights, all three compounds have good ligand efficiency (LE) and lipophilic ligand efficiency (LLE). Initially SAR work indicated that various substituents can be tolerated on the phenyl ring of both dihydropyrazine dione (**12**, DHP dione) and N-hydroxyl pyrimidine dione (**13**, NHP dione) hit classes. For instance, the CF₃ group of **12** can be replaced with a chlorine to yield **15** which shares similar DAO potency. Similarly, a chlorine can be incorporated at the para-position of **13** to afford **16**, which is slightly more potent than **13**. In order to understand the in vivo pharmacokinetic (PK) properties of the hits, especially their ability to cross the blood-brain barrier (BBB), compounds **14**, **15**, and **16** were dosed as a cassette in mice along with **17**¹⁸ (Table 3) as a reference compound.

Table 1. Novel DAO inhibitors identified by FEP+ enabled lead hopping

Compound ID	Chemical Structure	FEP+ Pred. IC ₅₀ (μM)	hDAO IC ₅₀ (μM)	LE	LLE	Papp A→B (10 ⁻⁶ cm/s), ER
12		0.011	0.34	0.46	3.6	11, 0.7

13		0.034	0.028	0.62	7.1	15, 0.4
14		0.12	0.12	0.88	6.5	8.8, 0.8
15		0.22	0.32	0.53	4.4	12, 0.4
16		0.016	0.011	0.62	6.9	24, 0.5

Overall, compound **17** showed good plasma exposure following oral dosing but poor CNS exposure with a measured $K_{p,uu}$ (brain/plasma) of 0.01.¹⁹ Compound **15** also has good plasma exposure with relatively low clearance in the study. The brain exposure, however, is also lower than the plasma exposure with a K_p of 0.17. Due to lower free brain fraction than free plasma fraction, the $K_{p,uu}$ was further reduced to 0.04. Nevertheless, the PK and brain penetration data of **15** suggests that the DHP dione hit class is suitable for hit-to-lead development. The NHP dione series suffers from high clearance across all analogs we profiled, as exemplified by compound **16**. Compound **14** exhibits a low clearance and an acceptable oral bioavailability in the plasma. However, the level of brain exposure was very low. In addition, the thiophene pyrazine hit class had limited opportunity for potency improvement and narrow existing SAR. Based on the data, the NHP dione and thiophene pyrazine hit classes were deprioritized, and further work was focused on the development of the DHP dione hit class.

Table 2. Mouse PK and brain distribution data

Study		17	15	16	14
PK study with C57/BL6 mice: Cassette dosing with 2 mg/kg po and 0.5 mg/kg IV; n = 3	CL (mL/min/kg)	3.6	5.9	108	1.8
	Vdss (L/kg)	0.5	1.0	1.9	0.2
	$t_{1/2}$, IV (h)	2.5	2.4	NR	1.2
	AUCN, po (ng*h/mL)	2407	2175	13.6	6266
	F%	51	77	9	34
	K_p (brain/plasma)	0.02*	0.17*	BLQ	0.004**
	Fu, plasma (brain)	0.17 (0.1)	0.20 (0.05)	BLQ	0.22 (0.26)
	$K_{p,uu}$ (brain/plasma)	0.01*	0.04*	BLQ	0.005**

* Calculated from PO AUC; **Calculated from IV AUC

To understand the binding interactions of the DHP dione chemical series, a co-crystal structure of **12** was obtained *via* a soaking experiment with the hDAO *apo* crystal. As shown in Figure 3, compound **12** binds to the hDAO enzyme in a fashion very similar to ligand **11**. The dihydropyridazinone head group is stacked between the flavin ring and Tyr224. The hydroxy-carbonyl moiety forms a salt bridge with Arg283, and the NH forms H-bond with Gly313. On the tail side, the 4-trifluoromethyl phenyl sticks into the hydrophobic pocket. Unlike the acidic hydroxy pyridazinone head group in compound **11**, the pKa of **12** was measured at 9.7,²⁰ which would imply a pKa penalty in binding to DAO, as only the anionic form can actively bind to DAO. In addition, the head group of **12** is *pseudo*-symmetric with two possible anionic tautomers, and substitution on the head group can impact the tautomer

distribution. Fortunately, quantum mechanics (QM) calculations suggested that the active tautomer is strongly favored for **12**, by 0.8 kcal/mol. Although the DHP dione chemical series is relatively weaker than the hydroxy pyridazinone²¹ chemical series due to the higher pKa of the head group, it may benefit from other properties such as pharmacokinetics and brain penetration.

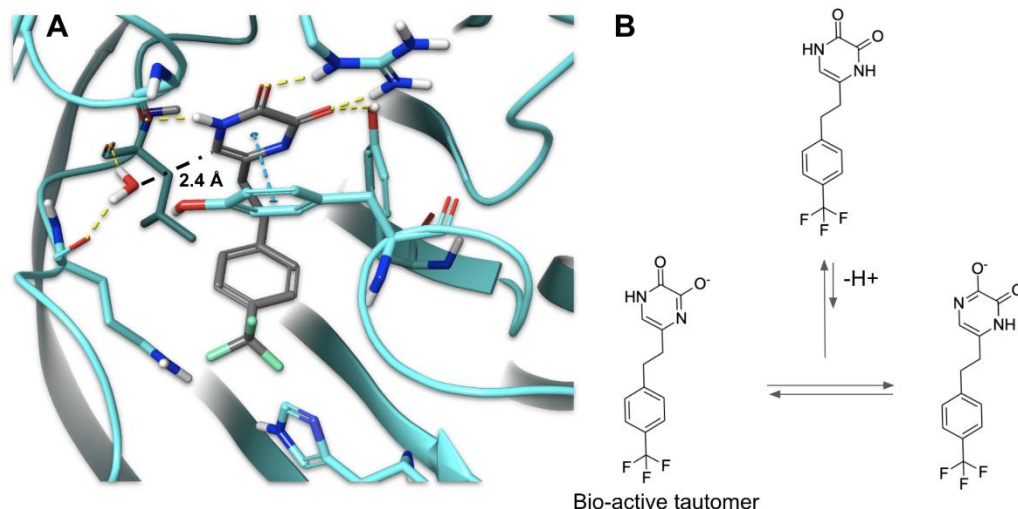


Figure 3. (a) Co-crystal structure of human DAO with compound **12**; (b) Equilibrium of **12** (approx. 80% QM predicted preference for the bio-active tautomer (left) vs the alternative tautomer (right))

SAR DEVELOPMENT

Initial SAR exploration was focused on the aromatic tail region of compound **12**. Both rational design by medicinal chemists and computational enumeration by Schrödinger's AutoDesigner algorithm were applied to generate a diverse set of design ideas. The large number of designs were filtered by molecular properties, a CNS MPO, a drug-likeness MPO and synthetic tractability, etc. The top scoring designs were progressed into FEP+ calculations to predict hDAO inhibitory potency. The compounds with favorable predicted hDAO potency were selected for synthesis at Charles River Laboratories (CRL). Additionally, active compounds were tested in the MDCK-MDR1 assays to assess cell permeability and efflux ratio (ER).

Compound **17** was also included in Table 3 as a reference compound, which was measured 17 nM in the hDAO biochemical assay. It is slightly right shifted in the hDAO cell assay, but about 4-fold left shifted in the mouse DAO cell assay. In the DHP dione chemical series, *para*-substitution on the phenyl ring is beneficial to potency, as the unsubstituted analog **18** is much less active. At the *para*-position, CN substitution (**19**) can also be tolerated in addition to Cl, while the methoxy analog **20** is less potent. From the mono Cl-substituted analogs **15**, **21**, and **22**, *para*-substitution is the most preferred, while *ortho*-substitution is not tolerated. Compound **19** can be substituted with a fluorine *ortho* to the cyano group as in analog **23**, while 3,5-dichloro substituted analog **24** is less active when compared to the mono-substituted analog **21**. The tail region tolerates other hetero aromatic rings such as pyridine (**25**) and bicyclic aromatic rings such as quinoline (**26**) with some loss of potency. Polar groups can also be tolerated in this region as exemplified by compound **27** and **28**. It is worth noting that both compound **27** and **28** were designed by the AutoDesigner algorithm featuring uncommon yet drug-like functionalities. In terms of hDAO FEP+ model performance, the majority of the prediction is within 1 log unit of the experimental IC₅₀ value. Compared to **17**, most analogs showed lower but moderate cell permeability and low efflux ratio in the MDCK-MDR1 assay, which may partially account for the near 10-fold shift in the hDAO cell assay. To ensure that the compound activity is not an artifact from their redox potential, the horseradish peroxidase assay (HRP) was developed as a counter screen. All compounds in Table 3 were shown to be clean up to 10 μM in the HRP assay.

Table 3. Tail SAR of the DHP dione chemical series

Compound ID	Chemical Structure	FEP+ Pred. IC ₅₀ (μM)	hDAO IC ₅₀ (μM)	hDAO Cell EC ₅₀ (μM)	mDAO Cell EC ₅₀ (μM)	Papp A→B (10 ⁻⁶ cm/s), ER
17		ND	0.017	0.029	0.0042	53, 0.8
12		0.011	0.34	3.1	1.6	11, 0.7
18		2.2	5.8	ND	ND	4, 0.4
15		0.22	0.32	3.3	0.85	12, 0.4
19		0.16	0.19	1.8	1.5	3.0, 0.6
20		0.71	1.6	ND	ND	3, 0.4
21		0.49	0.47	3.5	ND	6.8, 0.9
22		1.5	6.2	ND	ND	ND
23		0.097	0.18	1.9	ND	3.3, 0.5
24		0.25	2.5	ND	ND	ND
25		0.060	0.67	8.2	ND	ND

26		0.081	0.58	8.4	ND	3.1, 0.7
27		0.001	0.32	2.9	1.8	21, 0.1
28		0.047	0.64	ND	4.3	3.4, 0.4

ND: No data

In the hDAO co-crystal structure of **12**, there is a water molecule 2.4 Å away from the 6-H of the dihydropyrazine dione head group (Figure 3a). It is possible to substitute at the 6-position to interact with or even replace the water molecule. In addition, the substituents may also modify the pKa of the head group. Table 4 includes a subset of analogs that were explored in this area. The Cl-substituted analog **29** was predicted to be quite potent by FEP+. However, it was found to be much less active in the hDAO assay. A QM calculation later suggested that the Cl substitution would strongly disfavor the bio-active tautomer to about 1% due to electronic effects. Attempts were also made to synthesize the F-substituted analog **30**, as fluorine is much less electron donating than chlorine, and it was also predicted to have much lower pKa (Macro-pKa ~ 5.7) than **12**. Unfortunately, compound **30** proved to be unstable during synthesis. The methyl substituted analog **31** was also synthesized. However, it was much less active in the hDAO biochemical assay than predicted by the FEP+ model, suggesting hydrophobic groups are not tolerated at this position. Also interesting is the cyano analog **32**, which was measured to have much lower pKa (6.4) than **12**. Compound **32** showed similar potency to **12** as predicted by FEP+, and it showed lower cell shift in both the human and mouse DAO cell assays.

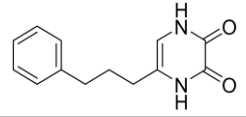
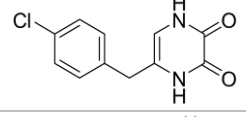
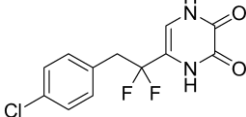
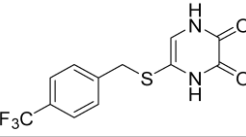
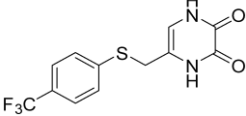
Table 4. C-6 SAR of the DHP dione chemical series

Compound ID	Chemical Structure	FEP Pred. IC ₅₀ (μM)	hDAO IC ₅₀ (μM)	hDAO Cell EC ₅₀ (μM)	mDAO Cell EC ₅₀ (μM)	Papp A→B (10 ⁻⁶ cm/s), ER
29		0.0028	1.5	0.50	ND	8.1, 0.7
30		0.047	NA	NA	NA	NA
31		0.050	3.9	ND	ND	4.8, 2.4

32		0.19	0.25	0.54	0.34	5.5, 0.35
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SAR of the linker region was also explored (Table 5). In terms of the linker length, the 2-carbon linker (**18**) is superior to the 3-carbon linker (**33**) according to the FEP+ predictions.²² It is also better than the 1-carbon linker based on the matched pair of **15** and **34**. Analogs with fluorinated linker (i.e. **35**) are also interesting, as fluorine-substituted linkers were predicted to lower the pKa of the head group. However, compound **35** failed in the synthesis due to chemical stability issues. Linkers with hetero atoms were also explored. Although analogs with an oxygen linker failed in synthesis, the sulfur-linked analogs are stable enough for further SAR development. Encouragingly, both **36** and **37** are about 3-fold more potent than **12** in the hDAO biochemical assay. The enhancement in biochemical potency may partly be attributed to lower pKa's of the head groups in **36** and **37**. Compound **36** has a measured pKa of 8.5, and compound **37** 9.2. Compared to **12** with a pKa of 9.7, **36** and **37** are more favored to form the bio-active anionic structures. In addition, they both show a lower cell shift when compared to **12**, possibly due to moderately higher cell permeability as measured in the MDCK-MDR1 assay.

Table 5. Linker SAR in the DHP dione chemical series

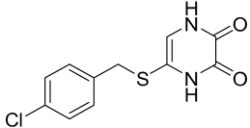
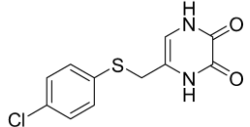
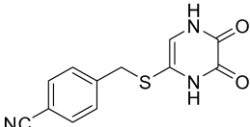
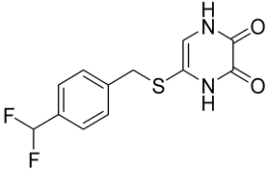
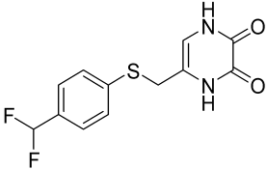
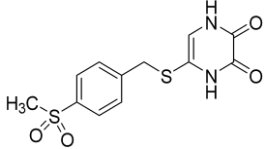
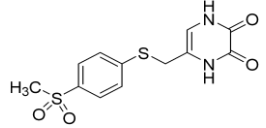
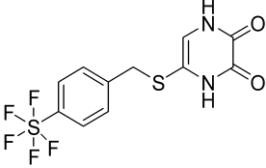
Compound ID	Chemical Structure	FEP Pred. IC ₅₀ (μM)	hDAO IC ₅₀ (μM)	hDAO Cell EC ₅₀ (μM)	mDAO Cell EC ₅₀ (μM)	Papp A→B (10 ⁻⁶ cm/s), ER
33		61	NA	NA	NA	NA
34		13	3.3	ND	ND	ND
35		0.084	NA	NA	NA	NA
36		0.034	0.091	0.42	0.24	18, 0.3
37		0.040	0.092	0.60	0.51	14, 0.4

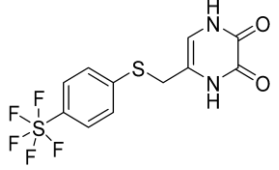
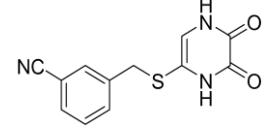
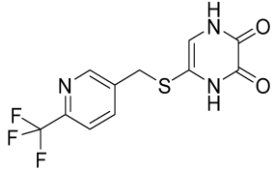
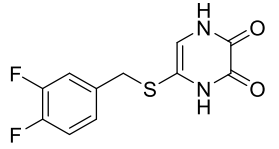
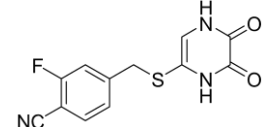
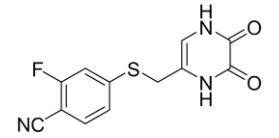
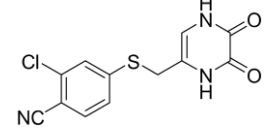
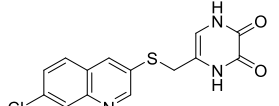
NA: Not available because the compound was not synthesized.

The tail SAR of the sulfur-linked analogs **36** and **37** largely resembles that of **12** (Table 6). Further exploration of the tail SAR of sulfur-linked analogs led to significant potency improvement in the hDAO biochemical and cell assays. In addition to Cl (**38** & **39**) and CN (**40**), difluoro methyl (**41** & **42**), methyl sulfone (**43** & **44**) and pentafluoro sulfanyl (**45** & **46**) groups can be tolerated at the *para*-position. In general, *para*-substitution is still the most preferred, while *meta*-substitution clearly reduces potency (**40** vs. **47**). The *ortho*-CN substituted analog (not shown) was predicted to be 26 μM by the FEP+ model. As demonstrated by **48**, the phenyl ring can be replaced with

a pyridine ring with some loss of potency. Di-substitutions can be tolerated at the *para* and *meta*-positions as exemplified by compounds **49**, **50**, **51**, and **52**. The tail region can also tolerate bicyclic aromatic rings as exemplified by **53**, a compound designed by our AutoDesigner algorithm. In terms of cell potency, the sulfur-linked compounds tend to be less right shifted in the DAO cell assays compared to the carbon linked analogs, which is desirable. However, analogs with polar substituents tend to have larger cell shifts possibly due to decreased cell permeability (e.g. **42**, **44**, and **51**).

Table 6. SAR of sulfur-linked analogs

Compound ID	Chemical Structure	FEP Pred. IC ₅₀ (μM)	hDAO IC ₅₀ (μM)	hDAO Cell EC ₅₀ (μM)	mDAO Cell EC ₅₀ (μM)	Papp A→B (10 ⁻⁶ cm/s), ER
38		0.36	0.15	0.40	0.51	19, 0.6
39		0.25	0.13	0.68	0.39	11, 0.4
40		0.24	0.043	0.25	0.31	4.4, 0.4
41		0.26	0.070	0.45	0.59	8.9, 0.4
42		0.27	0.072	0.62	0.62	5.6, 0.4
43		0.097	0.090	0.44	0.54	0.9, 1.1
44		0.60	0.082	0.83	2.3	1.7, 0.4
45		0.078	0.097	0.38	0.50	15, 0.7

46		0.029	0.080	0.71	0.79	10, 0.7
47		1.6	0.44	3.8	ND	ND
48		1.7	0.25	1.1	0.59	2.4, 0.4
49		1.3	0.30	0.81	ND	9.8, 0.4
50		0.080	0.054	0.20	0.68	3.1, 0.4
51		0.092	0.046	0.44	0.97	4, 0.5
52		0.12	0.098	0.88	0.56	4.6, 0.6
53		0.016	0.087	1.1	1.4	4.3, 0.9

In order to further enhance compound inhibitory potency against hDAO, fused ring designs in the linker region were also assessed by the FEP+ model. One of the ideas that stood out was **54**, which was designed by cyclizing the linker of **37** to form a fused 1,4-oxathiane ring. It was predicted that cyclization would lead to a gain in potency resulting at least in part from stabilization of the linker and tail piece. FEP+ predicted this compound to be a 3 nM inhibitor in the hDAO assay (Figure 4a). To our delight, the compound showed an IC₅₀ of 25 nM in the assay, a 3-fold improvement from **37**. Thus, cyclized analogs with the best substituents from the chemical series were prepared. Most of these analogs showed significant improvement when compared to their acyclic counterparts in the hDAO biochemical assay. However, there was less improvement in the human and mouse cell DAO assays due to larger cell shift for the cyclized analogs. The binding mode of the cyclized analogs was confirmed by X-ray co-crystal structure of **59** (Figure 4b), which very much resembles the FEP+ snapshot of compound **54** binding to hDAO. The biggest changes are in the tail region due to different substituents at the *para*-position.

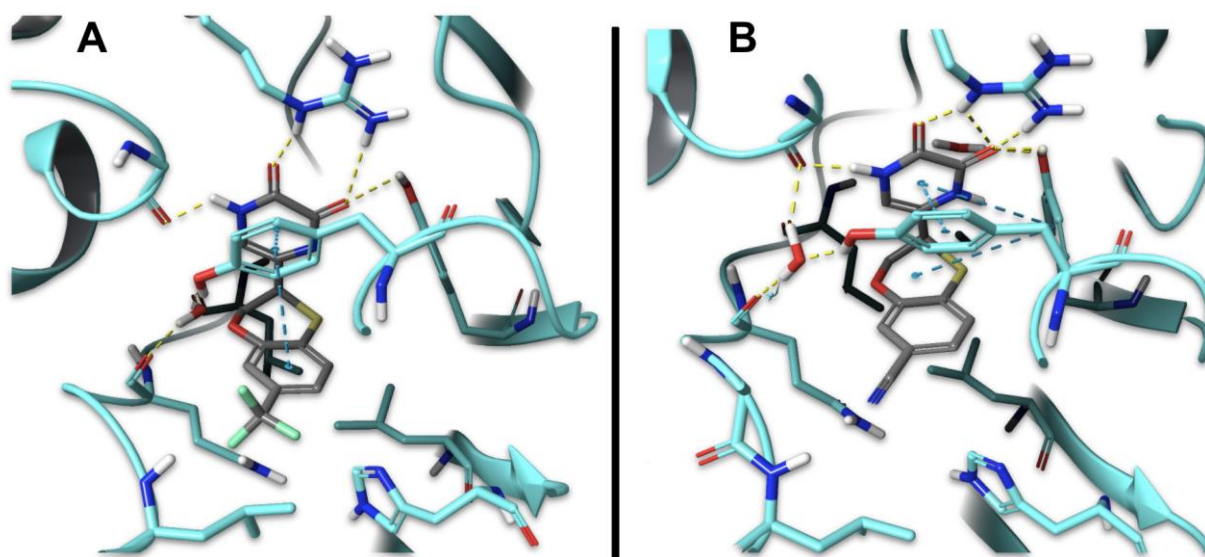


Figure 4. (A) FEP+ snapshot of compound **54** binding to hDAO. (B) Crystal structure of compound **59**.

Table 7. SAR of the fused 1,4-oxathiane analogs

Compound ID	Chemical Structure	FEP Pred. IC ₅₀ (μM)	hDAO IC ₅₀ (μM)	hDAO Cell EC ₅₀ (μM)	mDAO Cell EC ₅₀ (μM)	Papp A→B (10 ⁻⁶ cm/s), ER
54		0.003	0.025	0.29	0.43	ND
55-rac		0.049#	0.406	1.76	0.72	ND
56		0.039	0.094	0.56	0.54	7.6, 0.3
57		0.033	0.021	0.14	0.22	15, 0.5
58		0.062	0.013	0.13	0.29	ND
59		0.0017	0.020	0.63	0.78	3.1, 0.5

60		0.072	0.025	0.27	1.2	ND
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FEP+ model prediction was for the eutomer.

Another interesting design on the cyclized analogs is quaternary methyl adduct **61**. While this methyl addition was not initially predicted by our FEP+ model to lead to any gain in potency when compared to the des-methyl analog **55**, the racemic quaternary methyl **61-rac**²³ was tested to be about 5-fold more potent than **55-rac**. That is because we did not have more closely related starting references for our FEP+ model at the time of the original prediction for **61**. Subsequently we troubleshooted the FEP+ model by using the more closely related des-methyl analog **55** as the starting reference and observed that the methyl group displaces a high-energy water molecule concurrent with a predicted gain of potency (Figure 5). The quaternary methyl group was incorporated into other analogs and resulted in roughly 2-fold improvement in DAO biochemical and cellular assays. Notably, compound **63** showed inhibition potency near 100 nM in both human and mouse DAO cell assays.

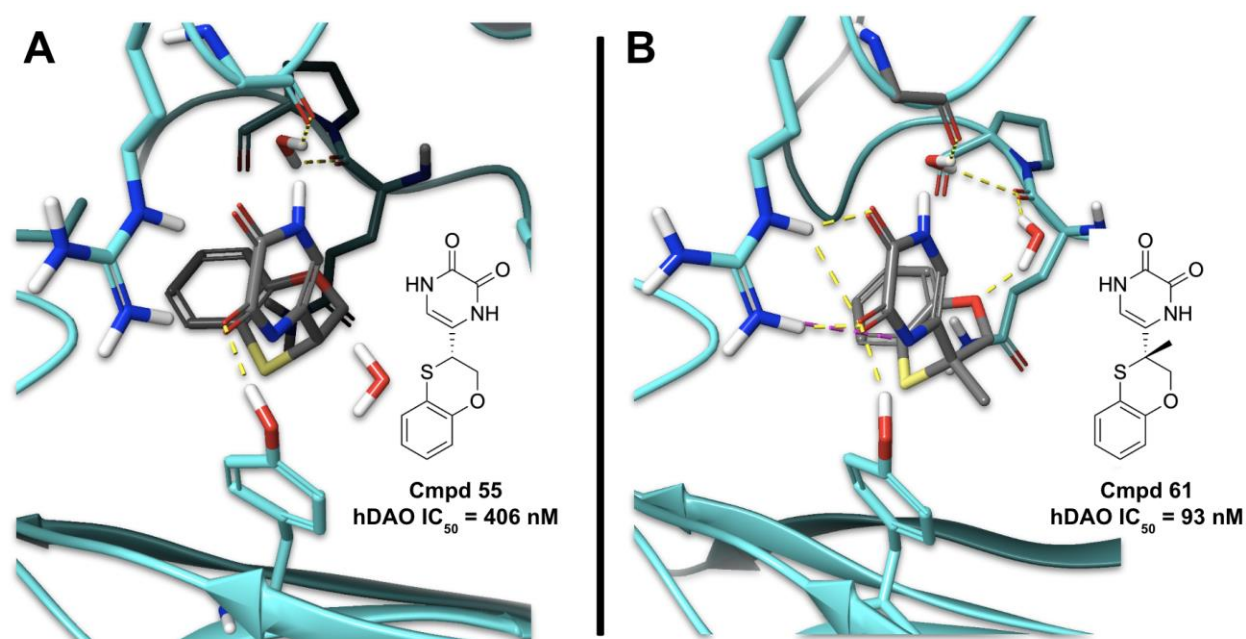
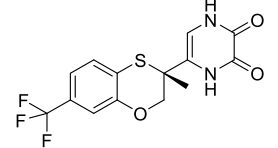
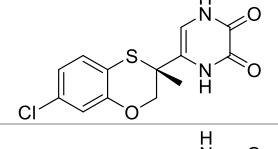
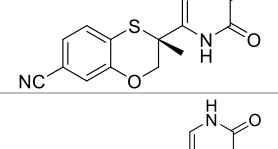
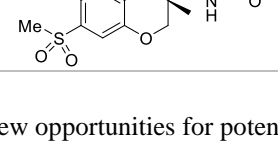


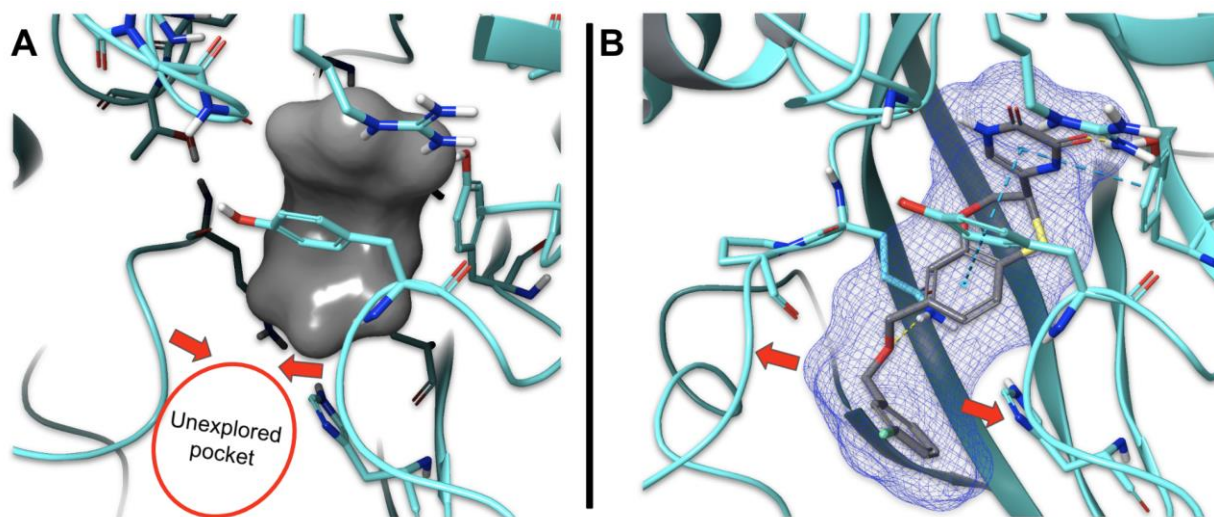
Figure 5. (a) FEP+ snapshot of compound **55** binding to hDAO. (b) **61** binding to hDAO.

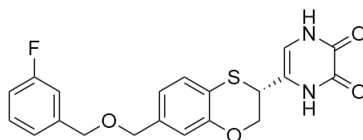
Table 8. Key analogs with the quaternary methyl group

Compound ID	Chemical Structure	FEP Pred. IC ₅₀ (μM)	hDAO IC ₅₀ (μM)	hDAO Cell EC ₅₀ (μM)	mDAO Cell EC ₅₀ (μM)
61-rac		0.150*	0.093	0.46	0.25

62		0.010	0.009	0.14	0.26
63		0.013	0.009	0.095	0.10
64		0.005	0.020	0.24	0.12
65		0.022	0.051	0.39	1.5

In an effort to explore new opportunities for potency enhancement, careful examination of the DAO catalytic site revealed a subpocket just beyond the tail region, which was not explored by other groups (Figure 6a). In order to design into this subpocket, we employed our AutoDesigner algorithm to enumerate novel design ideas using compound **54** as a template. Initially over 198 million design ideas were generated by the algorithm, which were filtered by an array of criteria such as molecular properties, CNS and drug-like MPO's, and synthetic complexity. After GLIDE docking into the hDAO crystal structure, the surviving compounds were evaluated by the hDAO FEP+ model for potency. Only three top compounds were selected for synthesis, among which compound **66** stood out as a single digit nM hDAO inhibitor on the project. With just one round of synthesis, we were able to confirm that the subpocket is a viable design space to further enhance compound binding potency to the hDAO enzyme, which opens up much needed new SAR space for this target.





66

FEP+ DAO IC₅₀ = 37 nM

Exp hDAO IC₅₀ = 8 nM

Figure 6. (a) Overlay of publicly disclosed crystallized inhibitors with surface representation (grey). (b) Binding model of compound **66** with novel subpocket outlined in blue.

ADME/PK PROPERTIES

In order to demonstrate the therapeutic potential of the DHP dione chemical class, the team next tried to identify a suitable candidate to probe PK/PD relationship *in vivo*. As mentioned earlier in the SAR, most analogs showed moderate permeability and low efflux ratio in the MDCK-MDR1 cell line. They showed excellent stability in the human and mouse liver microsome assay. The compounds have also shown good stability in human and mouse hepatocytes, as no significant turnover was observed for most compounds under the assay conditions employed.

Table 9. *In vitro* ADME properties of selected compounds.

Compound ID	LM Clint h(m) (μ L/min/mg)	Hepatocyte Clint h(m) (μ L/min/mil cells)
36	<14 (<14)	<5 (<5)
37	<14 (<14)	<5 (<5)
42	<14 (<14)	<5 (<5)
46	<14 (<14)	8 (10)
52	<14 (<14)	49 (57)
53	<14 (35)	ND
63	ND	6 (9)
66	ND	16 (11)

The *in vivo* drug metabolism and pharmacokinetic (DMPK) properties were assessed in cassettes of five compounds each, including **17** as the reference. Cassette administration is an extremely useful approach to generate *in vivo* PK data quickly in a cost effective and animal sparing fashion. A cassette dosing strategy also enabled direct comparison of drug brain penetrability among a set of compounds within the same set of animals. In practice, cassette doses were prepared for both intravenous (IV) and oral (PO) administration utilizing a standard dose formulation for each route throughout the project.

Table 10 shows mouse plasma PK of a few compounds in the chemical series. Most analogs showed low to moderate clearance and normal volume of distribution in mice, which resulted in good half-life values. They are also well absorbed when dosed orally with oral bioavailability generally over 40%. Not surprisingly, compound **66** showed reduced and less favorable oral bioavailability, possibly due to multiple rotatable bonds in the structure.

Table 10. Mouse plasma PK of representative compounds

Compound ID	Cl (mL/min/kg)	Vdss (L/kg)	AUCN (iv) (ng*hr/mL*mg)	T1/2 (h)	AUCN (po) (ng*hr/mL*mg)	%F
36	2.7	0.5	5800	2.2	2400	46
37	3.0	0.6	4800	2.9	3700	73

42	14	1.6	1140	2.3	1300	110
46	17	3.6	860	2.9	490	57
52	37	1.3	440	1.3	180	42
63	11	0.9	1390	1.5	400	30
66	74	4.3	180	1.7	26	15

The brain PK parameters of the same set of compounds are presented in Table 11. Compared to **17**, the DHP dione analogs are generally more brain penetrant, which can be a key advantage. The best analogs achieved K_{p,uu} around 0.5. Overall, compounds **37**, **42** and **46** gave the highest brain drug exposure. Surprisingly, compound **36**, a close analog to **37**, showed very low brain exposure and K_{p,uu} despite very nice plasma PK. Disappointingly, both **63** and **66** showed little to no brain exposure in mice, clearly suggesting that the more potent analogs still require much improvement in PK/brain penetration.

Table 11. Mouse brain PK of representative compounds

Compound ID	AUCN (iv) (ng*hr/mL*mg)	AUCN (po) (ng*hr/mL*mg)	%Fu (plasma)	%Fu (brain)	K _p (po)	K _{p,uu} (po)
36	160	52	8	6	0.022	0.016
37	820	460	9	5.2	0.12	0.07
42	240	240	17	20	0.18	0.22
46	200	480	4	2	0.98	0.54
52	67	38	5	9	0.23	0.43
63	108	23	7.8	2.2	0.06	0.02
66	40	BLQ	1.1	0.4	0	0

IN VIVO PK/PD MODEL

Based on compound potency and brain exposure, both **37** and **42** were considered as potential PK/PD candidates. Compound **46** was deprioritized due to lower free drug fraction in the brain. To enable selection of a PK/PD candidate, high dose oral PK studies were carried out at 10 and 100 mg/kg for both compounds. Compound **37** demonstrated good dose linearity in brain, while **42** showed sub dose proportionality at 100 mg/kg (Figure 7).

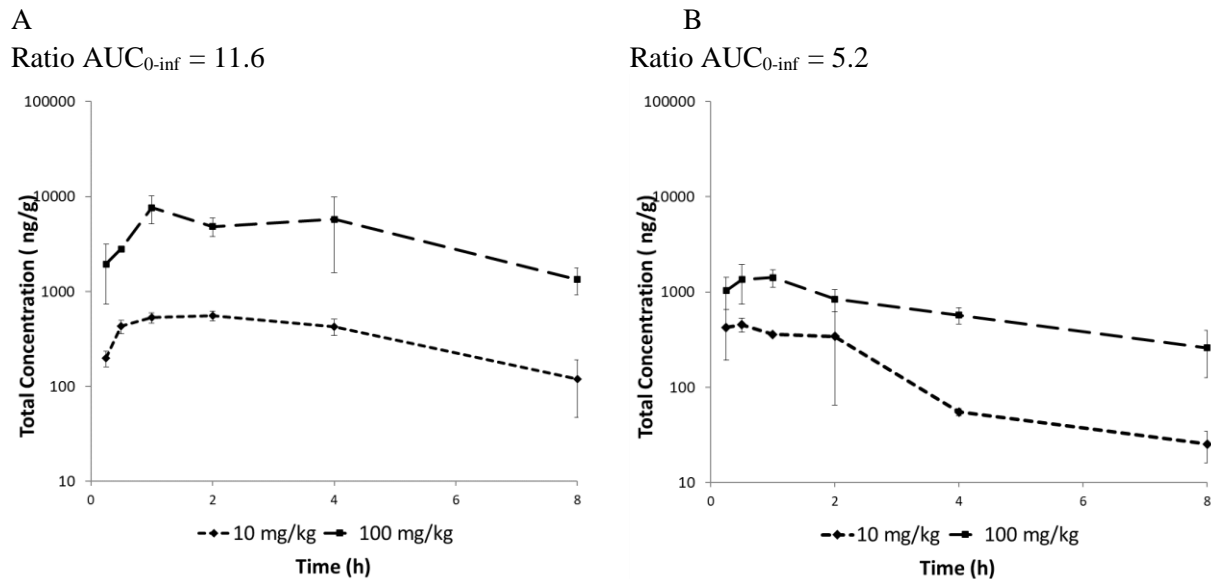


Figure 7. Oral pharmacokinetic profile of compound **37** (A) and compound **42** (B) in mouse cerebella following administration at 10 and 100 mg/kg

Modeling of the PK and theoretical enzyme occupancy (Equation 1) after a single 100 mg/kg dose identified **37** to be the optimal compound to progress into a PK/PD study with a 150 mg/kg BID, Q4hr dosing regimen. This study design, in conjunction with the measured mouse cell IC₅₀, the concentration of **37** in the cerebellum and the corresponding free fraction in this tissue was predicted to provide enzyme occupancy and coverage commensurate with an *in vivo* biomarker response (see Figure 7). Projected tissue concentrations at 15, 50 and 150 mg/kg were calculated following a linear extrapolation of the measured values obtained from the 100 mg/kg dosing cohort illustrated in Figure 7. These data were used in Equation 1 to generate the %tEO profiles in Figure 8.

$$tEO (\%) = \frac{[D_{tissue}]_{unbound}}{IC_{50} + [D_{tissue}]_{unbound}} \times 100$$

Equation 1. Calculation of theoretical enzyme occupancy (%tEO)

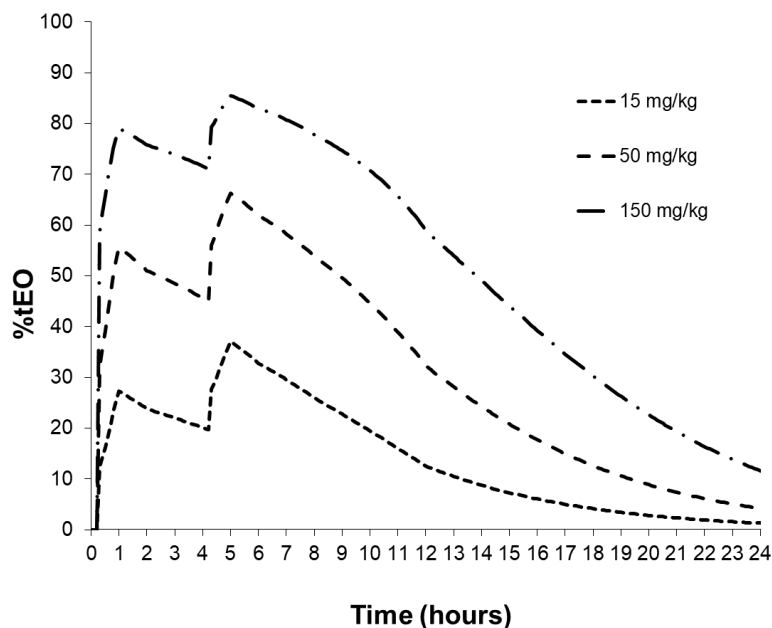


Figure 8. Projected enzyme occupancy levels following BID Q4hr dosing with compound **37**.

Both **37** and **42** have been extensively screened *in vitro* for potential off-targets. In the Eurofins Safety/Diversity panel (Table 12), COX2 is the only off-target for both **37** and **42**, representing about 93-fold *in vitro* selectivity for **37** and 132-fold for **42**. In addition, the compounds have also been screened against six additional CNS targets at Eurofins, and none of them showed significant activity at 10 μ M on the six off-targets. No significant inhibition of the major human CYP enzymes (<40%, 3A4, 2D6, 2C9, 2C19, 2C8, 1A2) was observed for either compound at 10 μ M. In addition, there was a complete absence of any cytotoxicity signal for either compound when they were tested at 100 μ M in a HepG2 assay that measured 72-hour ATP production and 24-hour Glu/Gal mitotoxicity.

Table 12. Eurofins selectivity data summary

Compound ID	37	42
Eurofins Safety/Diversity Panel	COX2 IC ₅₀ 9.0 μ M (93x)	COX2 IC ₅₀ 9.5 μ M (132x)
5-LOX Human Lipoxygenase Enzymatic Assay	> 10 μ M	> 10 μ M
A3 Human Adenosine GPCR Binding Assay	> 10 μ M	> 10 μ M
GR Human Glucocorticoid NHR Binding Assay	> 10 μ M	> 10 μ M
PPAR gamma Human NHR Binding Assay	> 10 μ M	> 10 μ M
Sigma (Non-Selective) Guinea Pig Binding Assay	> 10 μ M	> 10 μ M
Sodium Channel, Site 2	> 10 μ M	> 10 μ M
CYP Inhibition @ 10 μ M	< 40%	< 40%
HepG2 Glu/Gal	> 100 μ M	> 100 μ M
HepG2 72-hr ATP	> 100 μ M	> 100 μ M

Following ethical review and approval of the study protocol, the PK/PD assessment was undertaken to measure the modulation of D-serine levels in the cerebella of mice following administration of the test compound at one dose using the regimen described above. Two cohorts of animals were tested (compound and vehicle) using 33 animals in total (n=8/group for **37** and n=3/timepoint for vehicle). In both cases plasma and cerebella samples were collected

following animal “take-down” at 4-hour, prior to 2nd dosing, 6-hour, and 10-hour after the initial dosing. The levels of D-serine in plasma and brain tissue were quantitatively determined using a chiral LC-MS/MS method, ensuring both adequate sensitivity and selectivity. In addition, CSF was sampled from the animals at the 10-hour timepoint to determine the free, unbound levels of **37**.

The bioanalytical results obtained from the PK/PD study are shown in Figure 9a. As can be seen based on a mouse cell EC_{50} of ~150 ng/mL and brain tissue binding of 94.8%, free drug exposures exceeding the mouse cell EC_{50} were observed at 10 hours in the plasma, cerebellum and CSF. A significant increase of D-serine levels compared to vehicle was also observed in both the plasma and cerebellum at all three time points measured (4, 6 and 10-hour) (Figure 9b).

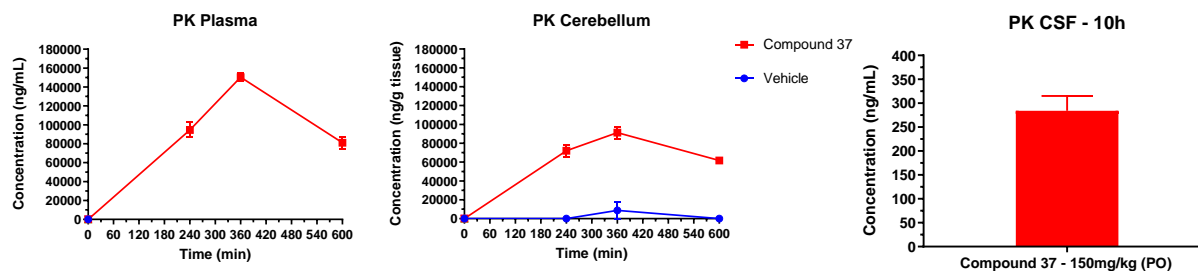


Figure 9a. Bioanalytical data from the PK/PD study

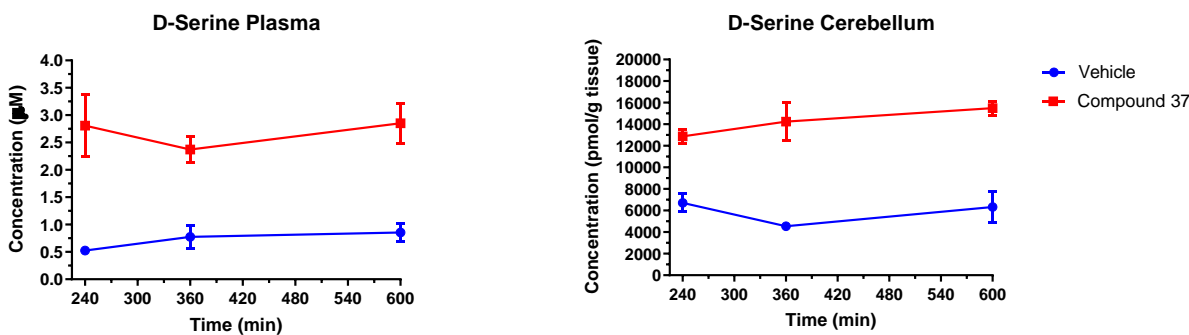


Figure 9b. D-Serine levels in plasma and cerebellum

In addition, a parallel study was run to assess the receptor occupancy (RO) in the cerebellum with the Takeda tracer compound PGM019260 following the protocol published in *Neurochemistry Research* 2017 (Ref. 18). As shown in Figure 10, the study confirmed significant RO of compound **37** in the PK/PD study, as projected by PK modeling (Figure 8).

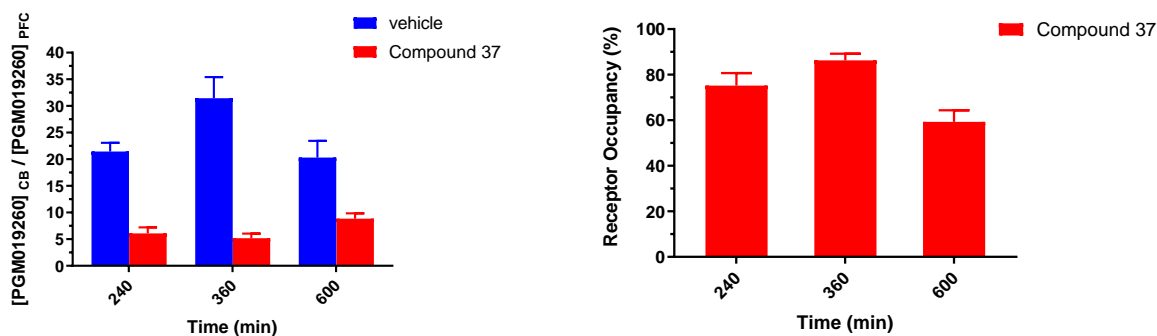


Figure 10. Receptor occupancy of **37** dose at orally at 150 mg/kg in the PK/PD study

The results of the PK/PD study are summarized in Table 13. Based upon the data, the PK/PD study with compound **37** has successfully demonstrated the pharmacological potential of hDAO inhibitors from the DHP dione chemical series.

Table 13. Summary of the PK/PD study with compound **37**.

Time Points	37 free drug		Fold increase in D-serine levels		%RO
	Plasma (ng/mL)	Cerebellum (ng/g)	Plasma	Cerebellum	Cerebellum
4 hr	8505	3746	5.4	1.9	75
6 hr	13534	4750	3.1	3.1	86
10 hr	7300	3206	3.3	2.4	59

In parallel with the PK/PD study, compound **37** was also assessed in a catalepsy model using the same dosing regimen (150 mg/kg p.o. BID, Q4hr) that had generated the positive response in the PK/PD study. During this study, plasma samples were taken and used to assess the prolactin levels at 6 hours post the first dose, which was predicted to be around C_{max}. As shown in Figure 11, no catalepsy or increase in prolactin levels was observed in this study. Plasma and brain concentrations of **37** were determined indicating that levels were similar to those achieved in the PK/PD study (data not shown). This study confirms that **37** is well tolerated *in vivo* at exposure levels required to evoke the desired PD responses.

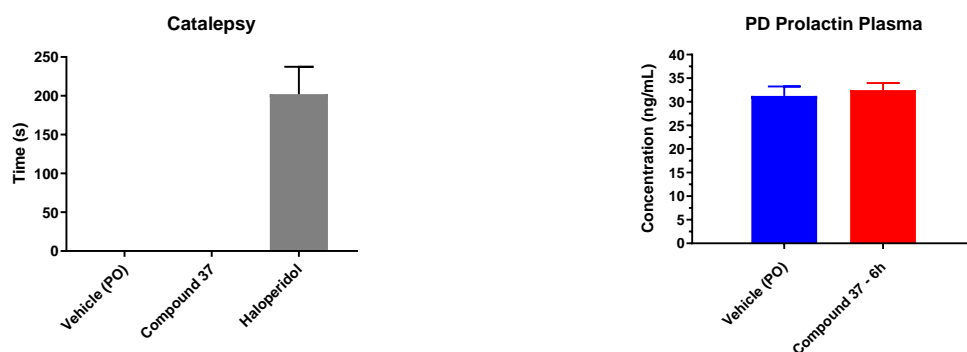
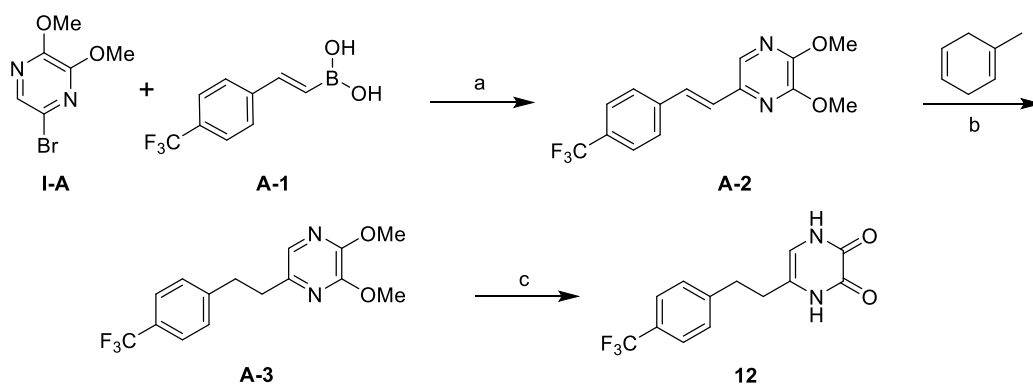


Figure 11. (a) Catalepsy model of **37** dosed orally at 150 mg/kg BID, Q4hr, (b) plasma prolactin level with compound **37** dosed orally at 150 mg/kg BID, Q4hr.

CHEMICAL SYNTHESIS

Due to the diversity of the SAR, a wide variety of chemistry was attempted to synthesize the compounds on this project. A few typical procedures applied in the syntheses were described below. Please refer to the experimental section for detailed synthesis of the individual analogs.

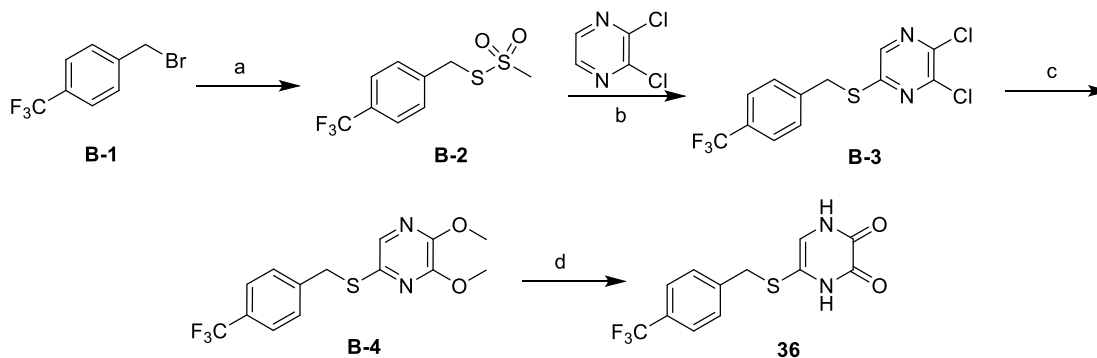
The synthesis of compound **12** is illustrated in Scheme A. Treatment of commercial material **I-A** with 4-(trifluoromethyl)styryl boronic acid **A-1** under Suzuki-Miyaura cross coupling conditions afforded **A-2**, which was subsequently reduced to the corresponding alkane **A-3** by catalytic phase-transfer hydrogenation. Finally, compound **12** was obtained by refluxing **A-3** in a 1:1 mixture of dioxane and 2N aqueous HCl.



Scheme A. Synthesis of compound **12**.

Reaction conditions: (a) Pd(PPh₃)₄, Na₂CO₃, dioxane/H₂O, 95 °C; (b) Pd(OH)₂/C, EtOH reflux; (c) 2N HCl, dioxane 100 °C.

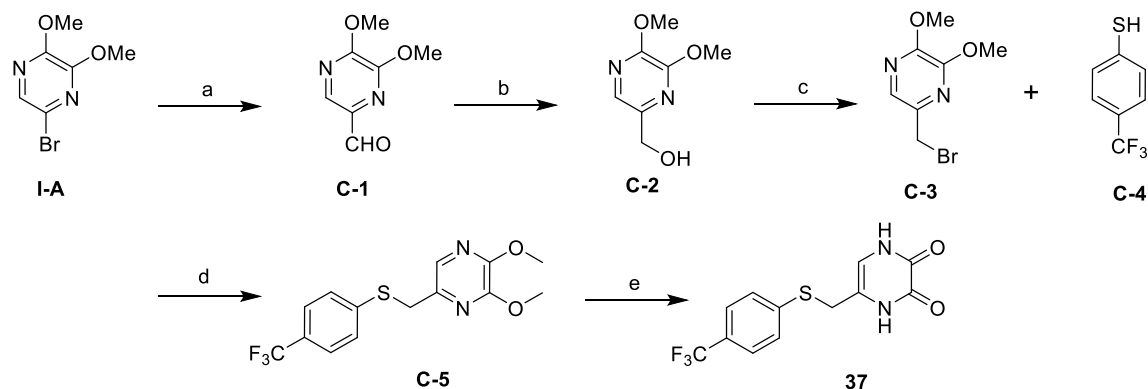
Synthesis of the α -thioether analogs follows the general procedure in Scheme B, unless otherwise noted. Treatment of **B-1** with sodium methanesulfonate gave rise to **B-2**, which was reacted with the lithium salt of 2,3-dichloropyridazine to afford **B-3**. Subsequently **B-3** was converted to **B-4** by reacting with methanol under basic conditions. Finally, hydrolysis of **B-4** with 2N HCl produced compound **36**.



Scheme B. General Procedure for the synthesis of the α -thioether compounds.

Reaction conditions: (a) NaSSO₂, DMF, RT; (b) TMPMgCl*LiCl, 0 °C; (c) NaH, MeOH, RT; (d) 2N HCl, dioxane, 100 °C.

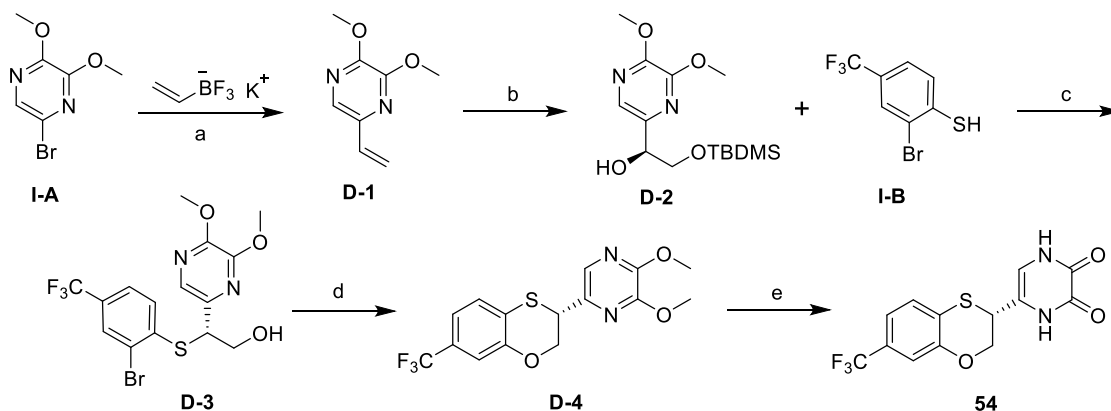
Scheme C describes the synthesis of compound **37**. Treatment of **I-A** with *n*-butyllithium and subsequent quenching with *N,N*-dimethylformamide yielded 5,6-dimethoxypyrazine-2-carbaldehyde **C-1**. Reduction of **C-1** with sodium borohydride and bromination of the resulting alcohol **C-2** using Appel conditions generated key bromide intermediate **C-3** which has been prepared using this route on multi-gram scale. Treatment of **C-3** with 4-(trifluoromethyl)benzenethiol **C-4** in *N,N*-dimethylformamide with cesium carbonate and cesium fluoride afforded 2,3-dimethoxy-5-(((4-(trifluoromethyl)phenyl)thio)methyl)pyrazine **C-5**. Finally refluxing **C-5** with 2N HCl in dioxane afforded compound **37**.



Scheme C. Synthesis of compound **37**.

Reaction conditions: (a) *n*-BuLi, THF, -78 °C, then DMF; (b) NaBH₄, MeOH, 0 °C; (c) CBr₄, PPh₃; (d) Cs₂CO₃, CsF, DMF, 50 °C; (e) 2N HCl, dioxane, 100 °C.

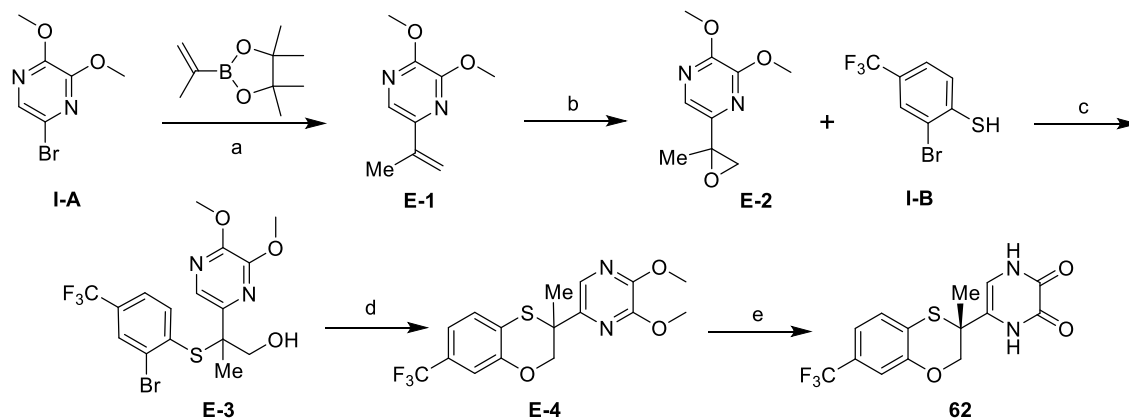
Synthesis of fused-ring analogs follows the general procedure in Scheme D, unless otherwise noted. Starting from commercial **I-A**, coupling to potassium vinyltrifluoroborate produced alkene **D-1**. Treatment of **D-1** with AD mix- α gave rise to the corresponding chiral diol asymmetrically, which was converted to the silyl ether **D-2** subsequently. Reacting **D-2** with thiol **I-B** furnished thiol ether **D-3**, which was treated under palladium catalyzed conditions to afford the cyclized ether **D-4**. Final hydrolysis of the methoxy groups under acidic conditions yielded fuse-ring analog **D-5**.



Scheme D. General procedure for the fused-ring analogs.

Reaction conditions: (a) Pd(dppf)Cl₂ DCM complex; (b) (1) AD Mix- α ; (2) TBDMS-Cl; (c) (1) DIAD, PPh₃; (2) TBAF; (d) Pd(OAc)₂, TrixiePhos, Cs₂CO₃; (2) 2N HCl, dioxane, 100 °C.

Palladium catalyzed coupling of **I-A** to 2-Isopropenyl-4,4,5,5-tetramethyl-1,3,2-dioxaborolane gave rise to **E-1**, which was treated with NBS and NaOH to produce epoxide **E-2**. Reacting **E-2** to thiol **I-B** under activation by InCl₃ furnished thiol ether **E-3**, which was subsequently converted to the cyclized ether **E-4** with palladium catalysis. Final hydrolysis of **E-4** under acidic conditions followed by chiral resolution yielded the quaternary methyl compound **62**.



Scheme E. Synthesis of the quaternary methyl analogs.

Reaction conditions: (a) Pd(PPh₃)₄; (b) NBS, NaOH; (c) I-B, InCl₃; (d) Pd(OAc)₂, TrixiePhos, Cs₂CO₃; (e) 2N HCl, dioxane, 100 °C.

CONCLUSIONS

In summary, we have discovered a novel class of small molecule inhibitors against the human D-amino Acid Oxidase (DAO). Different from the earlier lead compounds, this chemical class features a non-acidic dihydropyrazine dione head moiety. Starting from hit compound **12**, SAR work in the linker region led to the discovery of thioether linker analogs which showed enhanced DAO potency with desirable PK and brain penetration properties. With tool compound **37**, we were able to demonstrate PK/PD in an *in vivo* mouse model at drug exposure levels devoid of any adverse events. Continued SAR work has led to compounds with significant improvement in both DAO biochemical and cellular potency.

We have leveraged Schrödinger's computational modeling technology extensively to accelerate the program execution. Free energy perturbation (FEP+) technology was applied to prioritize compounds based on prospective binding potency predictions. Overall, the FEP+ models have performed well in predicting compounds binding potency to the hDAO enzyme. As shown in Figure 12, compound experimental hDAO inhibitory potency correlates well with prospectively predicted potency across the three chemical series.²⁴ Of the ~11000 ideas designed and profiled *in silico*, we synthesized 208 compounds and only 20 of these were unexpectedly inactive (>10 uM), demonstrating that the physics-based methods allowed us to quickly prioritize compounds of interest and deprioritize compounds that did not meet project objectives. In addition to structure-based design by seasoned medicinal chemists and modelers, we have also applied computational enumeration with our AutoDesigner algorithm to generate novel design ideas. Most notably, this effort has helped to identify a novel subpocket for further SAR development on the project. As is common for CNS programs, the challenge is to balance compound potency with desirable PK/brain penetration properties. While a working model to predict PK/brain penetration has been elusive on this project, we will continue to apply the Schrödinger computational modeling technology along with drug-likeness and CNS MPO filters to prioritize compounds for synthesis. Further optimization work toward a development candidate will be reported in due course.

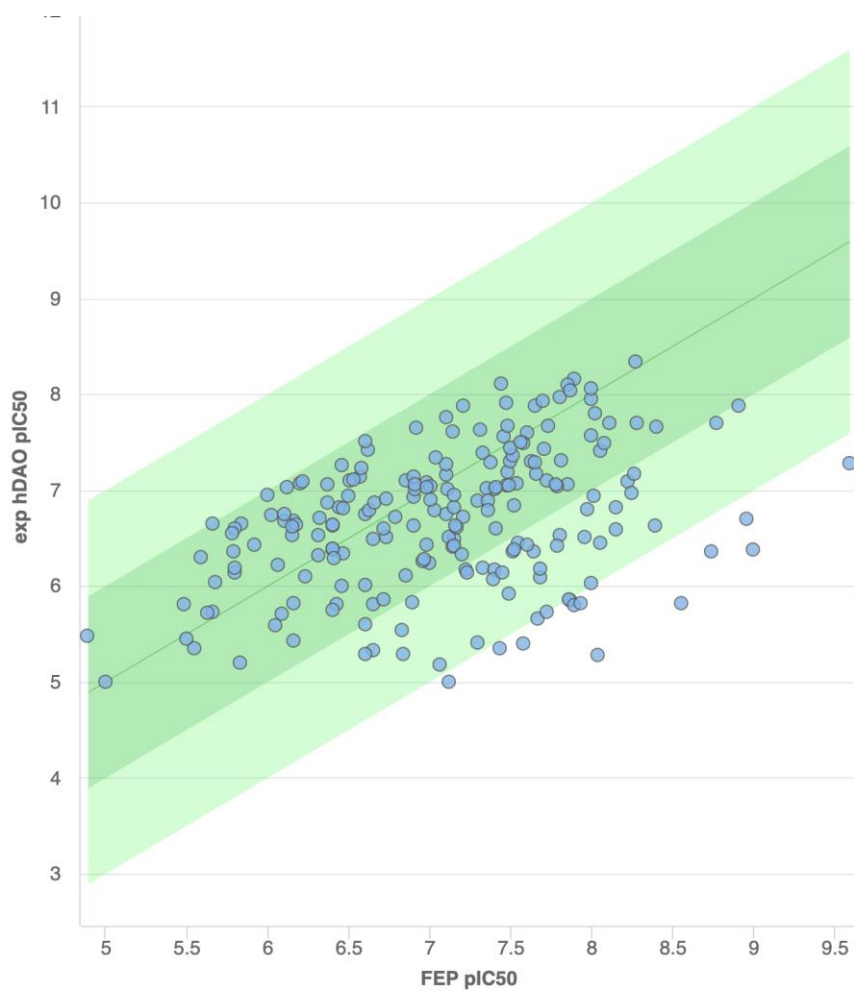


Figure 12. FEP+ prospective performance. Mean Unsigned Error = 0.71

EXPERIMENTAL SECTION

In vitro assay protocols

The D-amino acid oxidase (DAO) assays are fluorescence-based assays, in which the hydrogen peroxide (H_2O_2) generated from the reaction of D-serine with DAO and Flavin Adenine Dinucleotide (FAD), is linked to oxidation of Amplex Red in the presence of horseradish peroxidase (HRP). The Amplex Red reagent reacts with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin, which is measured fluorometrically.

Preparation of Assay Ready Plates

100-fold concentrated, 12-point serial dilutions of test compounds (250 nL) in duplicate were prepared using DMSO in 384 well black plates (262260, Nunc) using the Echo555 Acoustic Dispenser (Labcyte). These assay ready plates were employed in the biochemical and cellular screening assays described below.

Human DAO Biochemical Assay

The human DAO biochemical assay was performed using reagents at the following final assay concentrations: 1 nM recombinant full-length human DAO protein, D-Serine at Km concentration (10 mM), 50 µM FAD (excess), 50 µM Amplex Red and 0.1 U/mL HRP in the presence of compound or DMSO vehicle (1%). All reagents were made up in assay buffer containing 20 mM Tris, pH 7.4 + 0.1% BSA. The final assay volume was 25 µL/well.

Briefly, 10 µL of a working solution containing 2.5 nM hDAO (TECC-1280-14AA, Takeda) and 125 µM FAD (F6625, Sigma) in assay buffer was added to all the wells in the assay ready plate (containing 250 nL compound / DMSO vehicle per well) except for the negative control wells. 10 µL of 125 µM FAD (working solution) was added to the negative control wells containing 250 nL of DMSO vehicle. The plates were incubated at 25°C for 20 minutes (pre-incubation of compound with human DAO).

10 µL of a working solution containing 125 µM Amplex Red and 0.25 U/mL HRP (A22188, ThermoFisher Scientific) in assay buffer was then added to all the wells. The reaction was initiated by the addition of 5 µL of 50 mM D-Serine (S4250, Sigma-Aldrich) to all the wells. The plate was incubated for 4 hours in the dark at 25°C before measuring fluorescence in each well using the Envision plate reader with excitation at 530 nm and emission at 595 nm. Concentration response curves were generated using ActivityBase (IDBS). IC₅₀ values were determined by plotting % Inhibition vs Log₁₀ compound concentration using a sigmoidal fit with a variable slope (four parameter fit).

Horseradish Peroxidase (HRP) Counter Screen

The HRP counter screen was routinely performed using reagents at the following final assay concentrations: D-Serine (10 mM), FAD (50 µM), Amplex Red (50 µM), HRP (0.1 U/mL) and hydrogen peroxide (H₂O₂) (2 µM) in the presence of compound or DMSO vehicle (1%). All reagents were made up in assay buffer containing 20 mM Tris, pH7.4 + 0.1% BSA. The final assay volume was 25 µL/well.

Briefly, 5 µL of 250 µM FAD and 5 µL of 50mM D-Serine were added to all the wells in the assay ready plate (containing 250 nL compound / DMSO vehicle per well). 5 µL of a working solution containing 250 µM Amplex Red and 0.5 U/mL HRP was added to all the wells except for the negative control wells. 5 µL of 250 µM Amplex Red was added to the negative control wells containing 250 nL of DMSO vehicle. 10 µL of 5 µM H₂O₂ was added to all the wells. The plate was incubated for 10 minutes in the dark at 25°C before measuring fluorescence in each well using the Envision plate reader with excitation at 530 nm and emission at 595 nm.

Dose response curves were generated using ActivityBase (IDBS). IC₅₀ values were determined by plotting % Inhibition vs Log₁₀ compound concentration using a sigmoidal fit with a variable slope (four parameter fit).

Human DAO Cell Assay

The human DAO cell assay routinely employed a CHO-K1 clone, which was stably transfected with a mammalian expression plasmid containing the human DAO nucleotide encoding the full-length human DAO protein. This cell line was originally generated as described in Howley *et al*, 2017 (Supplementary Information). The human DAO CHO-K1 stable cell line was routinely cultured in Gibco Ham's F-12 Nutrient Mix (31765-027, ThermoFisher Scientific) containing 10% FBS (10082-147, ThermoFisher Scientific) and 500 µg/mL Geneticin™ Selective Antibiotic (10131-027, ThermoFisher Scientific).

The human DAO cell assay was performed using the following final assay conditions: 25,000 human DAO CHO-K1 stable cells/well, 50 mM D-Serine, 50 µM Amplex Red and 0.125 U/mL HRP. All cells and reagents were made up in 10 mM HEPES buffer (15630-056, ThermoFisher Scientific). The final assay volume was 25 µL/well.

The human DAO CHO-K1 stable cells were trypsinised, resuspended in complete medium and centrifuged at 1200 rpm for 4 minutes at room temperature. The cell pellet was then washed in 10 mM HEPES buffer and centrifuged at

1200 rpm for 4 minutes at room temperature. The resulting cell pellet was resuspended in 10 mM HEPES buffer at 1.25×10^6 cells/mL.

25,000 human DAO CHO-K1 stable cells (20 μ L in 10 mM HEPES buffer) were added to all the wells in the assay ready plate (containing 250 nL compound / DMSO vehicle per well). 5 μ L of a working solution containing 250 mM D-Serine, 250 μ M Amplex Red and 0.625 U/mL HRP in assay buffer was added to all the wells except for the negative control wells. 5 μ L of a working solution of 250 μ M Amplex Red and 0.625 U/mL HRP in assay buffer was added to the negative control wells. The plate was incubated for 30 minutes in the dark at 25°C before measuring fluorescence in each well using the Envision plate reader with excitation at 530 nm and emission at 595 nm.

Dose response curves were generated using ActivityBase (IDBS). IC₅₀ (Point of Inflection) values were determined by plotting % Inhibition vs Log₁₀ compound concentration using a sigmoidal fit with a variable slope (four parameter fit).

Mouse DAO Cell Assay

The mouse DAO cell assay routinely employed CHO-K1 cells, which were transiently transfected with an expression plasmid containing the mouse DAO nucleotide encoding the full-length mouse DAO protein. The CHO-K1 cell line was routinely cultured in Gibco Ham's F-12 Nutrient Mix containing 10% FBS.

The mouse DAO cell assay was performed using the following final assay conditions: 35,000 CHO-K1 cells transiently transfected with mouse DAO/ pcDNA3.1+C_(K)-DYK expression plasmid, 50 mM D-Serine, 50 μ M Amplex Red and 0.125 U/mL HRP. All cells and reagents were made up in 10 mM HEPES buffer. The final assay volume was 25 μ L/well.

Briefly, 5×10^6 CHO-K1 cells were seeded into two T175 flasks, in Gibco Ham's F-12 Nutrient Mix containing 10% FBS and incubated for 24 hours at 37 °C/5% CO₂.

The T175 flasks, which were seeded with CHO-K1 cells were transfected with mouse DAO/ pcDNA3.1+C_(K)-DYK expression plasmid as follows. A transfection mix for each T175 flask was made up containing 43.75 μ L mouse DAO/ pcDNA3.1+C_(K)-DYK expression plasmid (OMu05394D_pcDNA3.1+ C_(K)-DYK endotoxin free (maxi-prep, 1 mg/mL, Genscript), 8575 μ L of Optimem (31985-062, ThermoFisher Scientific) and 131.25 μ L of Lipofectamine LTX (15338-100, ThermoFisher Scientific). The transfection mix was incubated at room temperature for 25 minutes. During this incubation period the complete growth medium was removed by aspiration from the CHO-K1 cells, which were seeded 24 hours previously and replaced with 35ml of fresh Gibco Ham's F-12 Nutrient Mix containing 10% FBS. The transfection mix was then added to each flask containing CHO-K1 cells and incubated for a further 24 hours at 37 °C/5% CO₂.

The mouse DAO transiently transfected CHO-K1 cells were trypsinised, resuspended in complete medium and centrifuged at 1200 rpm for 4 minutes at room temperature. The cell pellet was then washed in 10mM HEPES buffer and centrifuged at 1200 rpm for 4 minutes at room temperature. The resulting cell pellet was resuspended in 10 mM HEPES buffer at 1.75×10^6 cells/mL.

35,000 mouse DAO CHO-K1 transiently transfected cells (20 μ L in 10 mM HEPES buffer) were added to all the wells in the assay ready plate (containing 250 nL compound / DMSO vehicle per well). 5 μ L of a working solution containing 250 mM D-Serine, 250 μ M Amplex Red and 0.625 U/mL HRP in assay buffer was added to all the wells except for the negative control wells. 5 μ L of a working solution of 250 μ M Amplex Red and 0.625 U/mL HRP in assay buffer was added to the negative control wells. The plate was incubated for 30 minutes in the dark at 25°C before measuring fluorescence in each well using the Envision plate reader with excitation at 530 nm and emission at 595 nm.

Dose response curves were generated using ActivityBase (IDBS). IC_{50} (Point of Inflection) values were determined by plotting % Inhibition vs Log₁₀ compound concentration using a sigmoidal fit with a variable slope (four parameter fit).

Mouse pharmacokinetics

Male C57Bl/6NCrl mice, Inbred, SPF-Quality, Charles River, Germany between 8 and 10 weeks of age, ranging from 20 to 40 grams were used to study the pharmacokinetics of test compounds. On arrival and following randomization animals were housed individually in polycarbonate cages equipped with water bottles, unless contraindicated by study procedures (such as pharmacokinetic blood sampling) or clinical signs. Pelleted rodent diet (SM R/M-Z from SSNIFF® Spezialdiäten GmbH, Soest, Germany) was provided ad libitum throughout the study, except during designated procedures. The compounds were administered to the mice via a single intravenous (slow bolus) injection to the tail vein using a vehicle comprising DMSO, PEG200 and Water. Terminal blood samples were collected via aorta puncture following inhalation anaesthesia into K2EDTA tubes and stored on wet ice. Oral cohorts were dosed by gavage using a vehicle of 0.5% (w/v) methylcellulose and 0.1% (v/v) Tween80 in water with bloods collected using a similar procedure. Whole blood was processed to plasma by centrifugation (3000g for 10 minutes at 5°C) within 30 minutes of collection. Plasma samples were transferred into 96 well plates (matrix tubes) and stored at < -75°C. Following termination, brains were collected from the animals and the cerebella separated. Both tissues were rinsed with saline, weighed and stored at ≤ -75 °C prior to analysis using LC-MS-MS.

Plasma and brain samples were extracted by protein precipitation using acetonitrile containing an appropriate internal standard. Specific reaction monitoring transitions were identified using automated instrumental optimization procedures for each compound studied, to ensure adequate linearity of response and define the upper and lower limits of quantitation. Samples were injected (SIL-30AC Autosampler, Shimadzu, Kyoto, Japan) onto a reverse phase chromatography system (A: 0.1% formic acid in ultrapure water; B: 0.1% formic acid in acetonitrile, Waters Corporation Acquity® UPLC column HSS T3 1.8µ). Analysis was performed using an API 5000 triple quadrupole mass spectrometer fitted with an electrospray ionisation source (AB Sciex, Ontario, ON, Canada). Pharmacokinetic analysis was performed with IDBS E-WorkBook v10 using mean data, non-compartmental analysis and the nominal dose of test item administered to the study animals.

Hepatic microsomal stability

The stability of the test compounds (1 µM) was measured following incubation at 37 °C with hepatic microsomes (0.5 mg protein/mL for all species) in the presence of the cofactor, NADPH. Incubates were prepared in duplicate, with aliquots removed at 0, 5, 10, 20 and 40 minutes and reactions terminated and compound extracted by the addition of acetonitrile containing an analytical internal standard. The disappearance of parent compound was monitored by LC-MS/MS and the half-life determined over the time-course of incubation. The half-life values were used to calculate their *in vitro* intrinsic clearance expressed as µL/min/mg protein.

Cryopreserved hepatocyte stability

The stability of test compounds (1 µM) were measured following incubation at 37 °C with cryopreserved hepatocytes in suspension at a cell density of 0.5 million cells per mL. Incubates were prepared in duplicate with aliquots removed at seven time points over a period of 120 minutes and reactions terminated and compound extracted by the addition of acetonitrile containing an analytical internal standard. The disappearance of the parent compounds were monitored by LC-MS/MS and half-life values determined over the course of the incubation. The half-life values obtained were used to calculate their *in vitro* intrinsic clearance expressed as µL/min/million cells.

MDCK assay protocol

MDR1-MDCK cells were seeded into 24 well Transwell plates and cultured for 3 days to form monolayers. The test compounds were prepared at 10 μ M in Hanks' Balanced Salt Solution containing 25 mM HEPES and loaded into the donor compartments of Transwell plates bearing the cell monolayers (pH 7.4 for both donor and receiver compartments). Lucifer Yellow was added to the apical buffer in all wells to assess integrity of the cell monolayer. Duplicate wells were prepared and incubated at 37°C in a CO₂ incubator. Samples were removed at time zero and 60 minutes and test compound analysed by LC-MS/MS. Concentrations of Lucifer Yellow in the samples were measured using a fluorescence plate reader. The apparent permeability (P_{app}) values of test compound were determined for both the apical to basal (A>B) and basal to apical (B>A) permeation and the efflux ratio (B>A: A>B) determined.

Animal Models

In vivo studies were performed at Charles River Laboratories (South San Francisco, CA, USA) under animal welfare protocols approved by the Institutional Animal Care and Use Committee of Charles River Laboratories, South San Francisco, and they adhere to the ACS Ethical Guidelines for animal studies.

In the PK/PD study, adult male C57Bl/6 mice with 7-8 weeks of age were dosed orally with compound **37** as a suspension in 1% Tween 80 in 0.5% methylcellulose at 150 mg/kg BID q4h. Terminal tissue collection was conducted at 4, 6, and 10 hours after treatment (11 mice/timepoint). Mice being euthanized for the 4-hour group were euthanized before 2nd dosing. At each collection timepoint, mice were euthanized by CO₂ asphyxiation and blood was collected via cardiac puncture into vials containing K⁺EDTA anticoagulant. Then, brains were extracted, and cerebellum dissected, separated into 2 equal parts then placed into pre-weighed 1.5ml tubes. Terminal CSF was collected for 10h treatment group only. Upon collection, all tissue samples and CSF were weighed, snap frozen in liquid nitrogen and stored at -80 °C for analysis.

The receptor occupancy study followed very similar protocol as the PK/PD study. In addition to treatment group with compound **37** and the vehicle group, a third group of C57Bl/6 mice (n = 12, 4 at each timepoint) were dosed IV with tracer compound PGM019260 at 60 μ g/kg in 10% DMSO in 0.5% 90% HP- β -CD, 20 minutes prior to the defined takedown time. Terminal tissue collection was conducted at 4, 6, and 10 hours after treatment (14 mice/timepoint). At each collection timepoint, mice were euthanized by CO₂ asphyxiation and brains were extracted and dissected in cerebellum and prefrontal cortex tissue samples and placed into pre-weighed 2 ml tubes. Upon collection, all tissue samples were weighed, snap frozen in liquid nitrogen and stored at -80 °C for analysis.

Compound Synthesis

Materials and Methods

All chemicals were purchased from commercial suppliers and used as received.

Liquid Chromatography-Mass Spectrometry

UPLC-MS was performed on a Waters Acquity I-Class with Waters Diode Array Detector coupled to a Waters SQD2 single quadrupole mass spectrometer using an Waters HSS C18 column (1.8 μ m, 100 \times 2.1 mm) or on a Waters DAD + Waters SQD2, single quadrupole UPLC-MS spectrometer using an Acquity UPLC BEH Shield RP18 1.7 μ m 100 \times 2.1mm (plus guard cartridge), maintained at 40 °C. The columns were initially held at 5% acetonitrile/water (with 0.1% formic acid or 10 mM ammonium bicarbonate in each mobile phase), followed by a linear gradient of 5-100% and then held at 100%.

NMR

¹H Nuclear magnetic resonance (NMR) spectroscopy was carried out using a Bruker instrument operating at 400 MHz using the stated solvent at around room temperature unless otherwise stated. In all cases, NMR data were consistent with the proposed structures. Characteristic chemical shifts (δ) are given in parts-per-million using conventional abbreviations for designation of major peaks: e.g. s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; dt, doublet of triplets; m, multiplet; br, broad.

Preparative reverse-phase HPLC conditions

Preparative HPLC purification was performed by reverse phase HPLC using a Waters Fractionlynx preparative HPLC system (2525 pump, 2996/2998 UV/VIS detector, 2767 liquid handler) or an equivalent HPLC system such as a Gilson Trilution UV directed system. The Waters 2767 liquid handler acted as both auto-sampler and fraction collector. The columns used for the preparative purification of the compounds were a Waters Sunfire OBD Phenomenex Luna Phenyl Hexyl or Waters Xbridge Phenyl at 10 μ m 19 \times 150 mm or Waters CSH Phenyl Hexyl, 19 \times 150, 5 μ m column. Appropriate focused gradients were selected based on acetonitrile and methanol solvent systems under either acidic or basic conditions. The modifiers used under acidic/basic conditions were formic acid or trifluoroacetic acid (0.1% V/V) and ammonium bicarbonate (10 mM) respectively. The purification was controlled by Waters Fractionlynx software through monitoring at 210-400 nm, and triggered a threshold collection value at 260 nm and, when using the Fractionlynx, the presence of target molecular ion as observed under API conditions. Collected fractions were analyzed by LCMS (Waters Acquity systems with Waters SQD).

The diastereomeric separation of compounds was achieved by Supercritical Fluid Chromatography (SFC) using a Waters Thar Prep100 preparative SFC system (P200 CO₂ pump, 2545 modifier pump, 2998 UV/VIS detector, 2767 liquid handler with Stacked Injection Module). The Waters 2767 liquid handler acted as both auto-sampler and fraction collector. Appropriate isocratic methods were selected based on methanol, ethanol or isopropanol solvent systems under un-modified or basic conditions. The standard SFC method used was modifier, CO₂, 100 mL/min, 120 Bar backpressure, 40 °C column temperature. The modifier used under basic conditions was diethylamine (0.1% V/V). The modifier used under acidic conditions was either formic acid (0.1% V/V) or trifluoroacetic acid (0.1% V/V). The SFC purification was controlled by Waters Fractionlynx software through monitoring at 210-400 nm and triggered at a threshold collection value, typically 260 nm. Collected fractions were analyzed by SFC (Waters/Thar SFC systems with Waters SQD). The fractions that contained the desired product were concentrated by vacuum centrifugation.

Supercritical Fluid Chromatography – Mass Spectrometry analytical conditions

Method 1

SFC-MS was performed on a Waters/Thar SFC systems with Waters SQD using a Lux Cellulose-3 column with a 15% methyl alcohol/CO₂ (with 0.5% diethylamine) isocratic run at 5 mL/min, 120 Bar backpressure, 40 °C column temperature.

Method 2

SFC-MS was performed on a Waters/Thar SFC systems with Waters SQD using a YMC Amylose-C column with a 30% (methyl alcohol /MeCN)/CO₂ isocratic run at 5 mL/min, 120 Bar backpressure, 40 °C column temperature.

Method 3

SFC-MS was performed on a Waters/Thar SFC systems with Waters SQD using a YMC Cellulose-C column with a 5-60% IPA (with 0.1% diethylamine)/ heptane isocratic run at 1 mL/min, 120 Bar backpressure, 40 °C column temperature.

All final compounds except **54** have demonstrated HPLC purity >95%. Compound **54** has a purity of 93.1% by LCMS analysis.

Synthesis of 5-(4-(Trifluoromethyl)phenethyl)-1,4-dihydropyrazine-2,3-dione (**12**)

Step A: (E)-2,3-Dimethoxy-5-(4-(trifluoromethyl)styryl)pyrazine

To a degassed mixture of 5-bromo-2,3-dimethoxypyrazine (1.10 g, 5.00 mmol), (E)-4-(trifluoromethyl)styrylboronic acid (2.16 g, 10.0 mmol) and sodium carbonate (1.59 g, 15.0 mmol) in 1,4-dioxane (30 mL) and water (10 mL) was added tetrakis(triphenylphosphine)Pd(0) (0.58 g, 0.50 mmol) and the reaction was heated to 95 °C for 4 hrs. The reaction was cooled to room temperature and diluted with ethyl acetate (10 mL). The organics were separated and further extracted with ethyl acetate (2 x 10 mL). The combined organics were washed with brine (10 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude material was purified by flash column chromatography (0 – 100% dichloromethane in *iso*-hexane) to yield the title compound as a yellow solid (1.45 g, 94%). ¹H NMR (CDCl₃, 400 MHz) δ 7.65-7.60 (m, 5 H), 7.52 (d, *J* = 15.5 Hz, 1 H), 7.09 (d, *J* = 15.5 Hz, 1 H), 4.13 (s, 3H), 4.05 (s, 3 H).

Step B: 2,3-Dimethoxy-5-(4-(trifluoromethyl)phenethyl)pyrazine

To a degassed solution of (E)-2,3-dimethoxy-5-(4-(trifluoromethyl)styryl)pyrazine (1.45 g, 4.67 mmol) and 1-methyl-1,4-cyclohexadiene (10.5 mL, 93.5 mmol) in dry ethanol (80 mL) was added Pdhydroxide (20% on carbon, 1.47 g, 2.09 mmol) and the reaction was heated to reflux overnight. The reaction was cooled to room temperature and filtered through Celite™ under a flow of nitrogen. The filtrate was concentrated under reduced pressure and the crude material was purified by flash column chromatography (0 – 15% ethyl acetate in *iso*-hexane) yielding the title compound as a pale yellow oil (1.40 g, 96%). ¹H NMR (CDCl₃, 400 MHz) δ 7.51 (d, *J* = 8.0 Hz, 2 H), 7.36 (s, 1 H), 7.28 (d, *J* = 8.0 Hz, 2 H), 4.00 (s, 3 H), 3.99 (s, 3 H), 3.08 (t, *J* = 7.5 Hz, 2 H), 2.93 (t, *J* = 7.5 Hz, 2 H).

Step C: 5-(4-(Trifluoromethyl)phenethyl)-1,4-dihydropyrazine-2,3-dione

A solution of 2,3-dimethoxy-5-(4-(trifluoromethyl)phenethyl)pyrazine (1.40 g, 4.48 mmol) in 2 M aqueous hydrochloric acid (23 mL) and 1,4-dioxane (23 mL) was heated to reflux for 16 hrs. The reaction was cooled to room temperature and concentrated under reduced pressure. The crude material was purified by flash column chromatography (0 – 20% methanol in DCM) to afford the title compound a white solid (720 mg, 57%). ¹H NMR (DMSO-*d*₆, 400 MHz) δ 11.40 (s, 1 H), 11.01 (s, 1 H), 7.67 (d, *J* = 8.0 Hz, 2 H), 7.45 (d, *J* = 8.0 Hz, 2 H), 6.01 (s, 1 H), 2.90 (t, *J* = 8.0 Hz, 2 H), 2.51 (t, *J* = 8.0 Hz, 2 H). MS (ESI+) *m/z* 285.1 (M+H)⁺.

The following compounds were prepared following the same procedure as **12**.

5-(4-Chlorophenethyl)-1,4-dihydropyrazine-2,3-dione (**15**): ¹H NMR (400 MHz, DMSO) δ 11.33 (s, 1 H), 10.98 (s, 1 H), 7.35 (dt, *J*=9.0, 2.3 Hz, 2H), 7.24 (dt, *J*=9.0, 2.3 Hz, 2H), 5.98 (s, 1H), 2.80 (t, *J*=7.8 Hz, 2H), 2.47 (t, *J*=7.8 Hz, 2H). MS (ESI+) *m/z* 251.1, (M+H)⁺.

5-(4-Methoxyphenethyl)-1,4-dihydropyrazine-2,3-dione (**20**): ¹H NMR (400 MHz, DMSO): δ 11.33-11.28 (m, 1 H), 11.02-10.97 (m, 1 H), 7.13 (d, J = 8.7 Hz, 2 H), 6.85 (d, J = 8.7 Hz, 2 H), 5.97 (s, 1 H), 3.72 (s, 3 H), 2.77-2.71 (m, 2 H), 2.48-2.41 (m, 2 H). MS (ESI+) m/z 247 (M+H)⁺.

5-(3-Chlorophenethyl)-1,4-dihydropyrazine-2,3-dione (**21**): ¹H NMR (400 MHz, DMSO): δ 11.33-11.32 (m, 1 H), 10.98-10.98 (m, 1 H), 7.36-7.24 (m, 3 H), 7.18 (d, J = 7.6 Hz, 1 H), 6.01 (s, 1 H), 2.85-2.78 (m, 2 H), 2.50-2.47 (m, 2 H). MS (ES+) m/z 251 (M+H)⁺.

5-(2-Chlorophenethyl)-1,4-dihydropyrazine-2,3-dione (**22**): ¹H NMR (400 MHz, DMSO): δ 11.36 (s, 1 H), 10.99 (s, 1 H), 7.42 (dd, J = 1.4, 7.7 Hz, 1 H), 7.36-7.22 (m, 3 H), 5.93 (s, 1 H), 2.98-2.91 (m, 2 H), 2.51-2.48 (m, 2 H). MS (ES+) m/z 251 (M+H)⁺.

5-Phenethyl-1,4-dihydropyrazine-2,3-dione (**18**): 2,3-Dimethoxy-5-phenethylpyrazine was isolated as a by-product from the reduction of (E)-5-(4-chlorostyryl)-2,3-dimethoxypyrazine and deprotection of 2,3-dimethoxy-5-phenethylpyrazine following the general procedure used to prepare 5-(4-(trifluoromethyl)phenethyl)-1,4-dihydropyrazine-2,3-dione. ¹H NMR (400 MHz, DMSO) δ 11.35 (s, 1 H), 10.98 (s, 1 H), 7.32 - 7.27 (m, 2 H), 7.23 - 7.19 (m, 3 H), 5.99 (s, 1 H), 2.81 (t, J=8.0 Hz, 2 H), 2.52 - 2.45 (m, 2 H). MS (ESI-) m/z 215.1 (M-H)⁻.

5-(3,5-Dichlorophenethyl)-1,4-dihydropyrazine-2,3-dione (**24**)

Step A: 2,3-Dimethoxy-5-((trimethylsilyl)ethynyl)pyrazine

Anhydrous THF (50 mL) was added to 5-bromo-2,3-dimethoxypyrazine (4.6 g, 20 mmol), bis(triphenylphosphine)Pd(II) dichloride (0.98 g, 1.4 mmol) and copper (I) iodide (0.11 g, 0.6 mmol) and degassed with a stream of nitrogen whilst being stirred at room temperature. Triethylamine (6.0 g, 60 mmol) was added followed by ethynyltrimethylsilane (2.45 g, 25 mmol) and the mixture was stirred at room temperature for 18 hrs. The mixture was partitioned between water (100 mL) and ethyl acetate (200 mL) and the aqueous layer was extracted with further portions of ethyl acetate (3 x 100 mL). The combined organic extracts were washed with brine (100 mL), dried over sodium sulfate, filtered and evaporated. The crude material was purified by flash column chromatography (0-10% ethyl acetate in cyclohexane) to elute the desired product as a yellow oil (4.7 g, 99%). ¹H NMR (400 MHz, CDCl₃): δ 7.81 (s, 1 H), 4.05-4.03 (m, 6 H), 0.28-0.27 (m, 9 H).

Step B: 5-Ethynyl-2,3-dimethoxypyrazine

2,3-Dimethoxy-5-((trimethylsilyl)ethynyl)pyrazine (4.7 g, 20 mmol), was dissolved in methanol (60 mL) and stirred at room temperature. Potassium carbonate (2.8 g, 20 mmol) was added and the mixture was stirred at room temperature for 5.5 hrs. The solution was partitioned between dichloromethane (200 mL) and water (100 mL) and the aqueous layer was extracted with further portions of dichloromethane (3 x 100 mL). The combined organic extracts were washed with brine (100 mL), dried over sodium sulfate, filtered and evaporated. The crude material was purified by flash column chromatography (0-10% ethyl acetate in cyclohexane) to elute the desired product as an off-white solid (2.85 g, 87%). ¹H NMR (400 MHz, CDCl₃): δ 7.84 (s, 1 H), 4.04 (d, J = 3.0 Hz, 6 H), 3.16 (s, 1 H).

Step C: 5-((3,5-Dichlorophenyl)ethynyl)-2,3-dimethoxypyrazine

A solution of 5-ethynyl-2,3-dimethoxypyrazine (0.16 g, 1 mmol), 1-bromo-3,5-dichlorobenzene (0.24 g, 1.1 mmol) and triethylamine (0.3 g, 0.42 mL, 3 mmol) in DMF (12 mL) was degassed with a stream of nitrogen for 1 minute. Copper iodide (0.019 g, 0.1 mmol) and bis(triphenylphosphine)Pd(II) dichloride (0.1 g, 0.15 mmol) were added and the mixture was stirred at room temperature for 18 hrs. The mixture was diluted with ethyl acetate (30 mL) and filtered through a plug of Celite™. Water (50 mL) was added to the filtrate and the organic layer extracted. The aqueous layer was extracted with further portions of ethyl acetate (2 x 30 mL). The organic layers were combined, dried over magnesium sulfate, filtered and concentrated. The crude material was purified by flash column chromatography (5% ethyl acetate in cyclohexane isocratic) to afford the title compound as a yellow solid (0.29 g, 94%). ¹H NMR (400 MHz, DMSO): δ 7.79 (s, 1 H), 7.65 (m, 1 H), 7.48-7.42 (m, 1 H), 7.33-7.20 (m, 1 H), 4.02 (m, 3 H), 3.94 (s, 3 H).

Step D: 5-(3,5-Dichlorophenethyl)-2,3-dimethoxypyrazine

10% Palladium hydroxide on carbon (0.1 g) was added to a solution of 5-((3,5-dichlorophenyl)ethynyl)-2,3-dimethoxypyrazine (0.28 g, 0.9 mmol) and 1-methyl-1,4-cyclohexadiene (1.7 g, 18 mmol) and the mixture stirred at 65 °C for 3 h. A further portion of 1-methyl-1,4-cyclohexadiene (1.7 g, 18 mmol) was added and the mixture was stirred at 65 °C for a further 3 hrs. The solution was then cooled and filtered through a plug of Celite™. The Celite™ was washed with a portion of ethyl acetate (10 mL), dichloromethane (10 mL) and methanol (10 mL). The filtrate was concentrated in vacuo and the crude residue partitioned between water (10 mL) and ethyl acetate (10 mL). The organic layer was washed with brine (10 mL) and the combined aqueous layers washed with ethyl acetate (2 x 10 mL). The organic layers were combined, dried over magnesium sulfate, filtered and concentrated. The crude material was purified by flash column chromatography (0-10% ethyl acetate in cyclohexane) to afford the title compound as a pale yellow solid (0.08 g, 28%). ¹H NMR (400 MHz, DMSO): δ 7.45-7.32 (m, 2 H), 7.23 (d, J = 1.9 Hz, 2 H), 3.82-3.79 (m, 6 H), 2.94-2.81 (m, 4 H).

Step E: 5-(3,5-Dichlorophenethyl)-1,4-dihydropyrazine-2,3-dione

A solution of 5-(3,5-dichlorophenethyl)-2,3-dimethoxypyrazine (0.08 g, 0.25 mmol) in 2 M aqueous hydrochloric acid (2.6 mL) and 1,4-dioxane (3 mL) was heated to reflux for 16 hrs. The reaction was cooled to room temperature and concentrated under reduced pressure. The crude material was purified by reverse phase preparative HPLC to afford the title compound as a white solid (23 mg, 32%). ¹H NMR (400 MHz, DMSO): δ 11.03 (s, 2 H), 7.45 (t, J = 1.9 Hz, 1 H), 7.32 (d, J = 1.9 Hz, 2 H), 6.02 (s, 1 H), 2.82 (t, J = 8.0 Hz, 2 H), 2.49-2.47 (m, 2 H). MS (ES+) m/z 285 (M+H)⁺.

The following compounds were prepared following the same procedures as compound **24**.

5-(2-(Quinolin-6-yl)ethyl)-1,4-dihydropyrazine-2,3-dione (**26**): ¹H NMR (400 MHz, DMSO): δ 11.40 (s, 1 H), 10.97-10.92 (m, 1 H), 8.84 (dd, J = 1.8, 4.3 Hz, 1 H), 8.30-8.27 (m, 1 H), 7.95 (d, J = 8.7 Hz, 1 H), 7.75-7.74 (m, 1 H), 7.65 (dd, J = 1.9, 8.7 Hz, 1 H), 7.50 (dd, J = 4.1, 8.3 Hz, 1 H), 6.00-5.97 (m, 1 H), 3.01 (t, J = 7.8 Hz, 2 H), 2.59 (t, J = 7.8 Hz, 2 H). MS (ES+) m/z 268 (M+H)⁺.

5-(2-(3-(Trifluoromethyl)-1H-indazol-6-yl)ethyl)-1,4-dihydropyrazine-2,3-dione (**28**): ¹H NMR (400 MHz, DMSO): δ 14.08-13.65 (m, 1 H), 11.51-11.22 (m, 1 H), 11.09-10.83 (m, 1 H), 7.73 (d, J = 8.4 Hz, 1 H), 7.49 (s, 1 H), 7.23 (d, J = 9.2 Hz, 1 H), 6.00 (s, 1 H), 2.98 (dd, J = 7.8, 7.8 Hz, 2 H), 2.58-2.53 (m, 2 H). MS (ESI+) m/z 325 (M+H)⁺.

4-(2-(5,6-Dioxo-1,4,5,6-tetrahydropyrazin-2-yl)ethyl)benzotrile (**19**)

Sodium iodide (0.14 g, 0.91 mmol) and TMSCl (0.099, 0.12 mL, 0.91 mmol) were added to a solution of 4-(2-(5,6-dimethoxypyrazin-2-yl)ethyl)benzotrile (synthesised following step A-D of **24**, used to prepare 5-(3,5-dichlorophenethyl)-1,4-dihydropyrazine-2,3-dione) (0.049 g, 0.18 mmol), in acetonitrile (1 mL) and the solution stirred at room temperature for 2 hrs. The mixture was evaporated to dryness and then dissolved in a 1:1:1 mixture of DMSO/ MeCN and water. The crude compound was purified by reverse phase preparative HPLC to afford the title compound as an off-white solid (6 mg, 14%). ¹H NMR (400 MHz, DMSO): δ 11.38-11.37 (m, 1 H), 11.01 (s, 1 H), 7.84-7.79 (m, 2 H), 7.49-7.45 (m, 2 H), 6.05-6.01 (m, 1 H), 3.34 (m, 2 H), 2.98-2.91 (m, 2 H). MS (ES+) m/z 242 (M+H)⁺.

4-(2-(5,6-dioxo-1,4,5,6-tetrahydropyrazin-2-yl)ethyl)-2-fluorobenzotrile (**23**): prepared using an analogous method to that used to prepare compound **19**.

¹H NMR (400 MHz, DMSO) δ 11.32 (s, 1 H), 10.98 (s, 1 H), 7.86 (dd, J=7.2, 7.9 Hz, 1 H), 7.43 (dd, J=1.4, 10.8 Hz, 1 H), 7.27 (dd, J=1.4, 7.9 Hz, 1 H), 6.01 (s, 1 H), 2.95 - 2.90 (m, 2 H), 2 H obscured by DMSO peak. MS (ESI+) m/z 260 (M+H)⁺.

5-(2-(6-(Difluoromethoxy)pyridin-3-yl)ethyl)-1,4-dihydropyrazine-2,3-dione (**25**)

Step A: 2,3-Bis(benzyloxy)-5-bromopyrazine

To a solution of 2,3-bis(benzyloxy)pyrazine (2.5 g, 8.6 mmol) in DMF (15 mL) was added *N*-bromosuccinimide (1.6 g, 9 mmol) in one portion and the reaction mixture was stirred at room temperature for 48 hrs. A further portion of *N*-bromosuccinimide (1.6 g, 9 mmol) was added and the solution was stirred at room temperature for a further 48 hrs. The reaction mixture was concentrated under reduced pressure and the resulting crude material purified by flash column chromatography (0-30% diethyl ether in cyclohexane) to elute the desired product as a white solid (0.65 g, 21%). ¹H NMR (400 MHz, CDCl₃): δ 7.70 (s, 1 H), 7.50-7.29 (m, 10 H), 5.45-5.40 (m, 4 H).

Step B: 2,3-Bis(benzyloxy)-5-((trimethylsilyl)ethynyl)pyrazine

Anhydrous THF (6 mL) was added to 2,3-bis(benzyloxy)-5-bromopyrazine (0.65 g, 1.75 mmol), bis(triphenylphosphine)Pd(II) dichloride (0.086 g, 0.123 mmol) and copper (I) iodide (0.01 g, 0.0525 mmol) and degassed with a stream of nitrogen whilst being stirred room temperature. Triethylamine (0.53 g, 0.73 mL, 5.25 mmol) was added followed by ethynyltrimethylsilane (0.21 g, 2.2 mmol) and the mixture was stirred at 55 °C for 18 hrs. The mixture was filtered through a plug of silica and washed with ethyl acetate (2 x 10 mL). The filtrate was concentrated under reduced pressure and the resulting crude material purified by flash column chromatography (0-10% ethyl acetate in cyclohexane) to elute the desired product as a pale brown solid (0.68 g, quant.). ¹H NMR (400 MHz, CDCl₃): δ 7.81 (s, 1 H), 7.49-7.28 (m, 10 H), 5.47-5.45 (m, 4 H), 0.28-0.28 (m, 9 H).

Step C: 2,3-Bis(benzyloxy)-5-ethynylpyrazine

2,3-bis(benzyloxy)-5-((trimethylsilyl)ethynyl)pyrazine (0.68 g, 1.75 mmol), was dissolved in methanol (18 mL) and stirred at room temperature. Potassium carbonate (0.24 g, 1.75 mmol) was added and the mixture was stirred at room temperature for 2.5 hrs. The solution was partitioned between dichloromethane (40 mL) and water (4 mL) and the aqueous layer was extracted with further portions of dichloromethane (2 x 10 mL). The combined organic extracts were washed with brine (100 mL), passed through a phase separator and evaporated. The crude material was

purified by flash column chromatography (0-25% diethyl ether in cyclohexane) to elute the desired product as a light brown solid (0.49 g, 88%). ¹H NMR (400 MHz, CDCl₃): δ 7.84-7.83 (m, 1 H), 7.50-7.42 (m, 4 H), 7.40-7.30 (m, 6 H), 5.47-5.45 (m, 4 H), 3.16-3.14 (m, 1 H).

Step D: 2,3-Bis(benzyloxy)-5-((6-(difluoromethoxy)pyridin-3-yl)ethynyl)pyrazine

A solution of 2,3-bis(benzyloxy)-5-ethynylpyrazine (0.26 g, 0.82 mmol), 5-bromo-2-(difluoromethoxy)pyridine (0.23 g, 1.0 mmol) and triethylamine (0.25 g, 0.34 mL, 2.5 mmol) were dissolved in acetonitrile (10 mL) and degassed with a stream of nitrogen for 1 minute. Copper iodide (0.005 g, 0.024 mmol) and bis(triphenylphosphine)Pd(II) dichloride (0.04 g, 0.06 mmol) were added and the mixture was stirred at 70 °C for 18 hrs. The mixture was diluted with ethyl acetate (10 mL) and filtered through a plug of Celite™. Water (10 mL) was added to the filtrate and the organic layer extracted. The aqueous layer was washed with further portions of ethyl acetate (2 x 10 mL). The organic layers were combined, dried over magnesium sulfate, filtered and concentrated. The crude material was purified by flash column chromatography (0-50% dichloromethane in cyclohexane) to afford the title compound as a yellow oil (0.08 g, 21%). ¹H NMR (400 MHz, CDCl₃): δ 7.72 (dd, J = 7.1, 13.4 Hz, 5 H), 7.53-7.30 (m, 10 H), 5.48 (d, J = 10.6 Hz, 4 H).

Step E: 5-(2-(6-(Difluoromethoxy)pyridin-3-yl)ethyl)-1,4-dihydropyrazine-2,3-dione

Pd hydroxide on carbon (0.02 g) was added to a solution of 2,3-bis(benzyloxy)-5-((6-(difluoromethoxy)pyridin-3-yl)ethynyl)pyrazine (0.015 g, 0.033 mmol) and 1-methyl-1,4-cyclohexadiene (0.061 g, 0.653 mmol) and the mixture stirred at 85 °C for 18 hrs. The solution was cooled and filtered through a plug of Celite™. The Celite™ was washed with ethyl acetate (5 mL), dichloromethane (5 mL) and methanol (5 mL). The filtrate was concentrated in vacuo and the crude residue purified by reverse preparative HPLC to afford the title compound as a white solid (1 mg, 7%). ¹H NMR (400 MHz, DMSO): δ 11.34-11.33 (m, 1 H), 11.00-10.99 (m, 1 H), 8.08-8.07 (m, 1 H), 7.78-7.74 (m, 1 H), 7.67 (t, J = 73.1 Hz, 1 H), 7.04-7.01 (m, 1 H), 5.98 (s, 1 H), 2.84-2.78 (m, 2 H), 2.48-2.44 (m, 2 H). MS (ES+) m/z 284 (M+H)⁺.

5-(3-Fluoro-4-(3-hydroxy-1H-pyrazol-5-yl)phenethyl)-1,4-dihydropyrazine-2,3-dione (**27**)

Step A: 4-(2-(5,6-Dimethoxypyrazin-2-yl)ethyl)-2-fluorobenzoic acid

Tert-butyl 4-(2-(5,6-dimethoxypyrazin-2-yl)ethyl)-2-fluorobenzoate (synthesized following general procedures A-D of compound **24**) (0.42 g, 1.17 mmol) was stirred at room temperature in dichloromethane (15 mL) with trifluoroacetic acid (4.0 g, 2.7 mL, 35 mmol) for 3 hrs. The mixture was concentrated under reduced pressure to afford the title compound as a white solid (0.35 g, 98%). ¹H NMR (400 MHz, CDCl₃): δ 7.92 (dd, J = 7.8, 7.8 Hz, 1 H), 7.38 (s, 1 H), 7.06-6.98 (m, 2 H), 4.01 (s, 3 H), 3.99 (s, 3 H), 3.11-3.06 (m, 2 H), 2.97-2.91 (m, 2 H). Acidic OH not observed.

Step B: Ethyl 3-(4-(2-(5,6-dimethoxypyrazin-2-yl)ethyl)-2-fluorophenyl)-3-oxopropanoate

Magnesium chloride (0.1 g, 1.1 mmol) and ethyl potassium malonate (0.26 g, 1.5 mmol) were combined in dry THF (3 mL) and heated to reflux for 4 hrs. 4-(2-(5,6-Dimethoxypyrazin-2-yl)ethyl)-2-fluorobenzoic acid (0.35 g, 1.14 mmol) and CDI (0.22 g, 1.4 mmol) were combined in dry THF (3 mL) and heated to reflux for 30 min. Both solutions were cooled down to room temperature and then the mixture of 4-(2-(5,6-dimethoxypyrazin-2-yl)ethyl)-2-fluorobenzoic acid and CDI was added portion-wise to the mixture of magnesium chloride and ethyl potassium

malonate. The resulting suspension was stirred at room temperature for 18 hrs. The mixture was cooled to 0 °C, quenched with 1 M hydrochloric acid solution (aq) (10 mL), diluted with ethyl acetate (10 mL) and then stirred for 1 hour. The mixture was further diluted with water (10 mL) and ethyl acetate (10 mL) and the aqueous layer extracted with ethyl acetate (3 x 40 mL). The combined organic layers were then washed with brine, dried over magnesium sulfate, filtered and concentrated. The crude material was purified by flash column chromatography (10-30% ethyl acetate in cyclohexane) to afford the title compound as a pale-yellow oil (0.35 g, 83%). ¹H NMR (400 MHz, CDCl₃): δ 7.88-7.76 (m, 1 H), 7.38-7.37 (m, 1 H), 7.08-6.92 (m, 2 H), 4.24 (ddd, J = 6.5, 13.7, 20.7 Hz, 2 H), 4.01-3.98 (m, 6 H), 3.10-3.02 (m, 2 H), 2.96-2.91 (m, 2 H), 1.44-1.42 (m, 1 H), 1.34-1.24 (m, 4 H).

Step C: 5-(3-Fluoro-4-(3-hydroxy-1H-pyrazol-5-yl)phenethyl)-1,4-dihydropyrazine-2,3-dione

Ethyl 3-(4-(2-(5,6-dimethoxypyrazin-2-yl)ethyl)-2-fluorophenyl)-3-oxopropanoate (0.35 g, 0.84 mmol) was dissolved in dry methanol (3 mL) under a nitrogen atmosphere. Glacial acetic acid (5.7 mg, 0.0054 mL, 0.094 mmol) was added followed by hydrazine hydrate (0.23 g, 0.23 mL, 4.7 mmol) and the reaction mixture was heated to reflux for 3 hrs. The mixture was cooled to room temperature and then evaporated to dryness. The crude material was purified by flash column chromatography (10-30% ethyl acetate in cyclohexane) to afford 5-(4-(2-(5,6-Dimethoxypyrazin-2-yl)ethyl)-2-fluorophenyl)-1H-pyrazol-3-ol as a white solid (0.23 g, 78%). 5-(4-(2-(5,6-Dimethoxypyrazin-2-yl)ethyl)-2-fluorophenyl)-1H-pyrazol-3-ol (0.23 g, 0.66 mmol) was treated with 2N HCl to afford the title compound (0.014 g, 18%). ¹H NMR (400 MHz, DMSO) δ 12.04 (s, 1 H), 11.35 (s, 1 H), 11.00 (s, 1 H), 9.72 (s, 1 H), 7.74 - 7.65 (m, 1 H), 7.15 (d, J=12.4 Hz, 1 H), 7.09 (d, J=8.0 Hz, 1 H), 6.01 (s, 1 H), 5.82 (s, 1 H), 2.87 - 2.81 (m, 2 H), (2 H obscured by DMSO peak). MS (ESI+) m/z 317 (M+H)⁺.

5-Chloro-6-(4-(trifluoromethyl)phenethyl)-1,4-dihydropyrazine-2,3-dione (**29**)

Step A: 2-Chloro-5,6-dimethoxy-3-(4-(trifluoromethyl)phenethyl)pyrazine

2,3-Dimethoxy-5-(4-(trifluoromethyl)phenethyl)pyrazine (0.1 g, 0.32 mmol) was dissolved in DMF (3.2 mL) and *N*-chlorosuccinimide (0.051 g, 0.384 mmol) was added. The reaction mixture was stirred at 50 °C for 2hrs and then for 18 hrs at room temperature. The mixture was concentrated in vacuo and dissolved in ethyl acetate (5 mL) and washed with water (2 x 5 mL) and brine (5 mL). The organic phase was dried over sodium sulfate, filtered and concentrated. The crude material was purified by flash column chromatography (0-10% ethyl acetate in cyclohexane) to elute the title product as a white solid (0.084 g, 76%). ¹H NMR (400 MHz, CDCl₃): δ 7.55-7.51 (m, 2 H), 7.35-7.30 (m, 2 H), 4.01 (s, 3 H), 3.96 (s, 3 H), 3.09 (s, 4 H).

Step B: 5-Chloro-6-(4-(trifluoromethyl)phenethyl)-1,4-dihydropyrazine-2,3-dione

5-Chloro-6-(4-(trifluoromethyl)phenethyl)-1,4-dihydropyrazine-2,3-dione was prepared following step E of compound **24** (0.055 g, 41%). ¹H NMR (400 MHz, DMSO): δ 12.01 (s, 1 H), 11.64 (s, 1 H), 7.67 (d, J=8.0 Hz, 2 H), 7.45 (d, J=8.0 Hz, 2 H), 2.90 (dd, J=7.7, 7.7 Hz, 2 H), 2.62 (dd, J=7.5, 7.5 Hz, 2 H). MS (ESI+) m/z 319.2 (M+H)⁺.

5-Methyl-6-(4-(trifluoromethyl)phenethyl)-1,4-dihydropyrazine-2,3-dione (**31**)

Step A: 2,3-Dimethoxy-5-methyl-6-(4-(trifluoromethyl)phenethyl)pyrazine

To a stirred solution of 2-chloro-5,6-dimethoxy-3-(4-(trifluoromethyl)phenethyl)pyrazine (0.042 g, 0.121 mmol) and Pd(dppf)₂Cl₂ (0.0089 g, 0.012 mmol) in toluene (1 mL) was added dimethyl zinc (0.24 mL, 2 M solution in toluene,

0.48 mmol) and the reaction mixture was heated at 80 °C for 27 hrs before a further portion of Pd(dppf)₂Cl₂ (0.0089 g, 0.0121 mmol) was added and the reaction was stirred at 80 °C a further 24 hrs. The mixture was cooled and water (2 mL) added. Ethyl acetate (5 mL) was added and the organic layer extracted. The aqueous layer was washed with further ethyl acetate (2 x 5 mL). The organic layers were combined, dried over magnesium sulfate, filtered and concentrated. The crude material was purified by flash column chromatography (0-5% ethyl acetate in cyclohexane) to afford the title compound as a pale-yellow solid (0.019 g, 48%). ¹H NMR (400 MHz, CDCl₃): δ 7.53-7.50 (m, 2 H), 7.26 (s, 2 H), 3.98-3.95 (m, 6 H), 3.09-3.04 (m, 2 H), 2.96-2.92 (m, 2 H), 2.24 (s, 3 H).

Step B: 5-Methyl-6-(4-(trifluoromethyl)phenethyl)-1,4-dihydropyrazine-2,3-dione

A solution of 2,3-dimethoxy-5-methyl-6-(4-(trifluoromethyl)phenethyl)pyrazine (0.019 g, 0.0582 mmol) in 2 M aqueous hydrochloric acid (2 mL) and 1,4-dioxane (0.6 mL) was heated to reflux for 2 hrs. The reaction was cooled to room temperature and concentrated under reduced pressure. The crude material was purified by flash column chromatography (0 – 20% methanol in DCM; dry load on Celite™; 80 g column) to afford the title compound a white solid (9 mg, 53%). ¹H NMR (400 MHz, DMSO): δ 11.20 (s, 1 H), 11.01 (s, 1 H), 7.68 - 7.64 (m, 2 H), 7.47 - 7.43 (m, 2 H), 2.87 - 2.81 (m, 2 H), 1.68 (s, 3 H) 2 H obscured by DMSO peak. MS (ESI+) m/z 299 (M+H)⁺.

5,6-Dioxo-3-(4-(trifluoromethyl)phenethyl)-1,4,5,6-tetrahydropyrazine-2-carbonitrile (32)

Step A: 5,6-Dimethoxy-3-(4-(trifluoromethyl)phenethyl)pyrazine-2-carbonitrile

A solution of 2-chloro-5,6-dimethoxy-3-(4-(trifluoromethyl)phenethyl)pyrazine (0.05 g, 0.144 mmol), Pd(pi-cinnamyl) chloride dimer (0.0075 g, 0.0144 mmol), Xphos (0.021 g, 0.0433 mmol) and N,N-diisopropylethylamine (0.037 g, 0.288 mmol) in previously degassed 1-butanol (0.7 mL) was heated at 80 °C under a nitrogen atmosphere. Acetone cyanohydrin (0.015 g, 0.173 mmol) solubilized in 0.3 mL of 1-butanol was then added drop-wise over 4 hrs. After the addition was completed the reaction was stirred at room temperature for 18 hrs. Saturated NaHCO₃ (2 mL) was then added and the reaction mixture was stirred for 10 min. The aqueous solution was extracted with ethyl acetate (2 x 5 mL). The organic layers were combined, dried over magnesium sulfate, filtered and concentrated. The crude material was purified by flash column chromatography (0-20% ethyl acetate in cyclohexane) to afford the title compound as a white solid (0.039 g, 80%). ¹H NMR (400 MHz, CDCl₃): δ 7.56-7.52 (m, 2 H), 7.34-7.31 (m, 2 H), 4.04-4.01 (m, 6 H), 3.22-3.09 (m, 4 H).

Step B: 5,6-Dioxo-3-(4-(trifluoromethyl)phenethyl)-1,4,5,6-tetrahydropyrazine-2-carbonitrile

Sodium iodide (0.033 g, 0.22 mmol) and TMSCl (0.024, 0.028 mL, 0.22 mmol) were added to a solution of 5,6-dimethoxy-3-(4-(trifluoromethyl)phenethyl)pyrazine-2-carbonitrile (0.025 g, 0.074 mmol), in acetonitrile (0.4 mL) and the solution stirred at 60 °C for 2 hrs. The reaction was diluted with ethyl acetate (2 mL) and washed with water (2 mL) and brine (2 mL). The organic layer was dried over magnesium sulfate, filtered and concentrated. Crude compound was purified by reverse phase preparative HPLC to afford the title compound as an off-white solid (6 mg, 26%). ¹H NMR (400 MHz, DMSO): δ 11.97 (s, 2 H), 7.71 - 7.67 (m, 2 H), 7.47 - 7.44 (m, 2 H), 2.98 (t, J=7.6 Hz, 2 H), 2.71 (t, J=7.6 Hz, 2 H). MS (ESI-) m/z 308 (M-H)⁻.

5-(4-Chlorobenzyl)-1,4-dihydropyrazine-2,3-dione (34)

Step A: 5-(4-Chlorobenzyl)-2,3-dimethoxypyrazine

To a solution of 4-chlorobenzyl magnesium chloride (2.4 mL, 0.5 M soln. in THF, 1.2 mmol) in THF (1 mL) was added a zinc chloride (0.7 mL, 2 M solution in THF, 1.3 mmol) and the mixture was stirred at room temperature for 45 min. A suspension of 5-bromo-2,3-dimethoxypyrazine (0.13 g, 0.6 mmol) and bis(triphenylphosphine)Pd(II) dichloride in THF (1 mL) was added and the mixture was stirred at room temperature for 64 hrs. The mixture was filtered through Celite™ and washed with ethyl acetate (2 x 5 mL). The filtrate was concentrated under reduced pressure and the resulting yellow residue purified by flash column chromatography (0-20% ethyl acetate in cyclohexane) to afford the title compound as a colorless solid (0.09 g, 57%). ¹H NMR (400 MHz, CDCl₃): δ 7.45-7.43 (m, 1 H), 7.25-7.19 (m, 3 H), 7.12-7.06 (m, 1 H), 3.99-3.97 (m, 6 H), 3.91-3.90 (m, 2 H).

Step B: 5-(4-Chlorobenzyl)-1,4-dihydropyrazine-2,3-dione

A solution of 5-(4-chlorobenzyl)-2,3-dimethoxypyrazine (0.09 g, 0.34 mmol) in 2 M aqueous hydrochloric acid (3.4 mL) and 1,4-dioxane (3.4 mL) was heated to reflux for 2 hrs. The reaction was cooled to room temperature and concentrated under reduced pressure. The crude material was purified by preparative HPLC to afford the title compound a white solid (17 mg, 22%). ¹H NMR (400 MHz, DMSO): δ 11.16 (s, 2 H), 7.37 (d, J = 8.4 Hz, 2 H), 7.31 (d, J = 8.4 Hz, 2 H), 6.09 (s, 1 H), 3.52 (s, 2 H), MS (ESI+) m/z 237.2 (M+H)⁺.

5-((4-(Trifluoromethyl)benzyl)thio)-1,4-dihydropyrazine-2,3-dione (**36**)

Step A: S-(4-(Trifluoromethyl)benzyl) methanesulfonothioate

To a solution of 4-(trifluoromethyl)benzyl bromide (0.78 mL, 5.00 mmol) in dry DMF (12 mL) under nitrogen was added sodium methanethiosulfonate (838 mg, 6.25 mmol) and the reaction mixture was stirred at room temperature for 18 hrs. The mixture was diluted with water (30 mL) and ethyl acetate (100 mL). The aqueous layer was extracted with ethyl acetate (2 x 70 mL) and the combined organic layers were washed with 10% lithium chloride solution (30 mL) and brine (40 mL). The organic layer was dried over magnesium sulfate, filtered and concentrated under reduced pressure to yield the title compound as a colorless oil (1.33 g, 98%). ¹H NMR (CDCl₃, 400 MHz): δ 7.65 (d, J = 8.2 Hz, 2 H), 7.54 (d, J = 8.0 Hz, 2 H), 4.42 (s, 2 H), 3.00 (s, 3 H).

Step B: 2,3-Dichloro-5-((4-(trifluoromethyl)benzyl)thio)pyrazine

To a solution of 2,3-dichloropyrazine (305 mg, 2.05 mmol) in dry THF (2 mL) under nitrogen at room temperature was added dropwise over 10 min. a solution of 2,2,6,6-tetramethylpiperidinylmagnesium chloride lithium chloride complex in THF (1.0 M, 2.3 mL, 2.26 mmol). The reaction mixture was stirred at room temperature for 30 min. before being cooled to 0 °C. A solution of S-(4-(trifluoromethyl)benzyl) methanesulfonothioate (665 mg, 2.46 mmol) in dry THF (2 mL) was added dropwise over 10 min. The reaction mixture was stirred at 0 °C for 30 minutes, allowed to warm to room temperature and stirred for 3 hrs. Saturated aqueous ammonium chloride solution (15 mL) was added followed by water (10 mL). The mixture was extracted with ethyl acetate (100 mL, 20 mL, 20 mL) and the combined organic layers were washed with brine (20 mL), dried over magnesium sulfate, filtered and concentrated under reduced pressure. The crude material was purified by flash column chromatography (10 – 20% dichloromethane in cyclohexane) to yield the title compound as a yellow oil (269 mg, 39%). ¹H NMR (400 MHz, CDCl₃): δ 8.15 (s, 1 H), 7.60-7.53 (m, 4 H), 4.41 (s, 2 H).

Step C: 2,3-Dimethoxy-5-((4-(trifluoromethyl)benzyl)thio)pyrazine

To a suspension of sodium hydride (60% in mineral oil, 188 mg, 7.81 mmol) in dry dioxane (3 mL) under nitrogen at room temperature was added dry methanol (0.32 mL, 7.81 mmol) dropwise over 10 min. The reaction mixture was stirred at room temperature for 1 hour. A solution of 2,3-dichloro-5-((4-(trifluoromethyl)benzyl)thio)pyrazine (265 mg, 0.781 mmol) in dry dioxane (2 mL) was added over 5 min. and the mixture was stirred at room temperature for 18 hrs. Saturated aqueous ammonium chloride solution (15 mL) was added followed by water (10 mL). The mixture was extracted with ethyl acetate and the combined organic layers were washed with brine (20 mL), dried over magnesium sulfate, filtered and concentrated under reduced pressure. The crude material was purified by flash column chromatography (10 – 20% dichloromethane in cyclohexane) to yield the title compound as a pale-yellow oil (138 mg, 53%). ¹H NMR (400 MHz, CDCl₃): δ 7.55-7.51 (m, 3 H), 7.43 (d, J = 8.0 Hz, 2 H), 4.31 (s, 2 H), 4.01 (s, 3 H), 3.98 (s, 3 H).

Step D: 5-((4-(Trifluoromethyl)benzyl)thio)-1,4-dihydropyrazine-2,3-dione

To a solution of 2,3-dimethoxy-5-((4-(trifluoromethyl)benzyl)thio)pyrazine (138 mg, 0.418 mmol) in dioxane (10 mL) was added 2 M hydrochloric acid (10.4 mL) and the mixture was heated at 100 °C for 4 hrs. The mixture was cooled to room temperature and the solvent removed under reduced pressure. The crude material was purified by preparative HPLC to yield the title compound as an off-white (73 mg, 58%). ¹H NMR (400 MHz, DMSO): δ 11.43 (s, 2 H), 7.71-7.67 (m, 2 H), 7.45-7.41 (m, 2 H), 6.12 (s, 1 H), 4.10 (s, 2 H). MS (ESI+) m/z 303 (M+H)⁺.

The following compounds were synthesized following the same procedure as **36**.

5-((4-Chlorobenzyl)thio)-1,4-dihydropyrazine-2,3-dione (**38**): ¹H NMR (400 MHz, DMSO): δ 11.94 - 11.01 (m, 2 H), 7.37 (d, J = 8.4 Hz, 2 H), 7.23 (d, J = 8.4 Hz, 2 H), 6.12 (s, 1 H), 4.01 (s, 2 H). MS (ESI+) m/z 269 (M+H)⁺

4-(((5,6-Dioxo-1,4,5,6-tetrahydropyrazin-2-yl)thio)methyl)-benzonitrile (**40**): ¹H NMR (400 MHz, DMSO) δ 11.72-11.03 (m, 2 H), 7.79 (d, J = 8.3 Hz, 2 H), 7.40 (d, J = 8.4 Hz, 2 H), 6.09 (s, 1 H), 4.09 (s, 2 H). MS (ESI+) m/z 260 (M+H)⁺.

5-((4-(Difluoromethyl)-benzyl)thio)-1,4-dihydropyrazine-2,3-dione (**41**): ¹H NMR (400 MHz, DMSO): δ 11.62 (s, 1 H), 11.21 (s, 1 H), 7.50 (d, J = 7.9 Hz, 2 H), 7.34 (d, J = 7.9 Hz, 2 H), 7.00 (t, J = 55.8 Hz, 1 H), 6.10 (s, 1 H), 4.06 (s, 2 H). MS (ESI+) m/z 285 (M+H)⁺.

5-((4-(Methylsulfonyl)-benzyl)thio)-1,4-dihydropyrazine-2,3-dione (**43**): ¹H NMR (400 MHz, DMSO): δ 11.42-11.25 (m, 2 H), 7.87 (d, J = 8.5 Hz, 2 H), 7.48 (d, J = 8.4 Hz, 2 H), 6.14 (s, 1 H), 4.12 (s, 2 H), 3.20 (s, 3 H). MS (ESI+) m/z 313 (M+H)⁺.

5-(((6-(Trifluoromethyl)-pyridin-3-yl)methyl)thio)-1,4-dihydropyrazine-2,3-dione (**48**): ¹H NMR (400 MHz, DMSO): δ 11.60 (s, 1 H), 11.29 (s, 1 H), 8.65 (s, 1 H), 7.93-7.89 (m, 2 H), 6.21 (s, 1 H), 4.18-4.17 (m, 2 H). MS (ESI+) m/z 304 (M+H)⁺.

3-(((5,6-Dioxo-1,4,5,6-tetrahydro pyrazin-2-yl)thio) methyl)-benzonitrile (**47**): The hydrolysis step was carried out with sodium iodide and TMSCl conditions. ¹H NMR (400 MHz, DMSO): δ 11.41 (s, 2 H), 7.75-7.70 (m, 2 H), 7.54-7.52 (m, 2 H), 6.14 (s, 1 H), 4.07-4.05 (m, 2 H). MS (ESI+) m/z 260 (M+H)⁺.

5-((3,4-Difluorobenzyl)thio)-1,4-dihydropyrazine-2,3-dione (**49**)

Step A: 2-((3,4-Difluorobenzyl)thio)-6-methoxypyrazine

To a solution of 3,4-difluorobenzyl mercaptan (665 mg, 4.15 mmol) in dry acetonitrile (8 mL) under nitrogen was added sodium *tert*-butoxide (997 mg, 10.38 mmol). The mixture was stirred at room temperature for 5 min. A solution of 2-chloro-6-methoxypyrazine (500 mg, 3.46 mmol) was added and the mixture was heated at 85 °C for 90 min. The reaction mixture was cooled to room temperature, diluted with water (20 mL) and extracted with ethyl acetate (2 x 30 mL). The combined organic layers were washed with brine (20 mL), passed through a phase separation and concentrated under reduced pressure. The crude material was purified by flash column chromatography (15% ethyl acetate in cyclohexane) to yield the title compound as a red oil (708 mg, 76%). ¹H NMR (400 MHz, CDCl₃): δ 8.02 (s, 1 H), 7.90 (s, 1 H), 7.26-7.20 (m, 1 H), 7.12-7.06 (m, 2 H), 4.35 (s, 2 H), 3.95 (s, 3 H).

Step B: 2-Bromo-5-((3,4-difluorobenzyl)thio)-3-methoxypyrazine

To a solution of 2-((3,4-difluorobenzyl)thio)-6-methoxypyrazine (400 mg, 1.50 mmol) in dry dichloromethane (8 mL) under nitrogen cooled to 0 °C was added *N*-bromosuccinimide (290 mg, 1.65 mmol). The reaction mixture was stirred at 0 °C for 2 hours, warmed to room temperature and stirred for 18 hrs. The reaction mixture was diluted with saturated sodium hydrogencarbonate solution (20 mL) and extracted with dichloromethane (2 x 50 mL). The combined organic layers were dried over magnesium sulfate, filtered and concentrated under reduced pressure. The crude material was purified by flash column chromatography (0-80% dichloromethane in cyclohexane) to yield the title compound as a yellow oil (350 mg, 67%). ¹H NMR (400 MHz, CDCl₃): δ 7.83 (s, 1 H), 7.25-7.18 (m, 1 H), 7.15-7.06 (m, 2 H), 4.32 (s, 2 H), 4.02 (s, 3 H).

Step C: 5-((3,4-Difluorobenzyl)thio)-1,4-dihydropyrazine-2,3-dione

Following steps C-D from compound **36** used to prepare 5-((4-(trifluoromethyl)benzyl)thio)-1,4-dihydropyrazine-2,3-dione yielded the title compound as an off-white solid (37 mg, 13% over two steps). ¹H NMR (400 MHz, DMSO): δ 11.32 (d, J = 115.6 Hz, 2 H), 7.33-7.21 (m, 2 H), 6.97-6.94 (m, 1 H), 6.10-6.08 (m, 1 H), 3.91 (s, 2 H). MS (ESI+) m/z 271(M+H)⁺.

5-((4-(Pentafluoro-λ⁶-sulfaneyl)benzyl)thio)-1,4-dihydropyrazine-2,3-dione (**45**) was prepared following the procedure used to prepare **49** yielded after purification by preparative HPLC the title compound as an off-white solid (4.6 mg, 2% over four steps). ¹H NMR (400 MHz, DMSO): δ 11.40-11.33 (m, 2 H), 7.91-7.88 (m, 2 H), 7.50-7.45 (m, 2 H), 6.19 (s, 1 H), 4.14-4.13 (m, 2 H). MS (ESI+) m/z 361 (M+H)⁺.

4-(((5,6-Dioxo-1,4,5,6-tetrahydropyrazin-2-yl)thio)methyl)-2-fluorobenzonitrile (**50**)

Step A: 5-((3-Bromobenzyl)thio)-2,3-dimethoxypyrazine

Following steps A-C from compound **36** used to prepare 5-((4-(trifluoromethyl)benzyl)thio)-1,4-dihydropyrazine-2,3-dione yielded the title compound as a brown oil (32% over three steps). ¹H NMR (400 MHz, CDCl₃): δ, 7.55 (s, 1 H), 7.45-7.41 (m, 1 H), 7.14-6.97 (m, 2 H), 4.22-4.21 (m, 2 H), 4.01 (s, 3 H), 3.98 (s, 3 H).

Step B: 4-(((5,6-Dimethoxypyrazin-2-yl)thio)methyl)-2-fluorobenzonitrile

To a degassed solution of 5-((3-bromobenzyl)thio)-2,3-dimethoxypyrazine (300 mg, 0.835 mmol) in dry butanol (4 mL) was added DIPEA (0.29 mL, 1.67 mmol), Pd(pi-cinnamyl) chloride dimer (43 mg, 0.0835 mmol) and X-Phos (119 mg, 0.251 mmol). The mixture was heated at 80 °C under nitrogen before a solution of acetone cyanohydrin

(0.092 mL, 1.0 mmol) in dry butanol (1 mL) was added dropwise over 90 min. The mixture was cooled to room temperature and saturated sodium hydrogen carbonate solution (10 mL) was added. The mixture was extracted with ethyl acetate (3 x 20 mL) and the combined organic layers were washed with brine (10 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude material was purified by flash column chromatography (0 – 30% ethyl acetate in cyclohexane) to yield the title compound as a pale-yellow oil (140 mg, 55%). ¹H NMR (400 MHz, CDCl₃): δ 7.55-7.43 (m, 2 H), 7.39-7.29 (m, 2 H), 5.56 (s, 2 H), 5.51 (s, 3 H), 5.46 (s, 3 H).

Step C: 4-(((5,6-Dioxo-1,4,5,6-tetrahydropyrazin-2-yl)thio)methyl)-2-fluorobenzonitrile

4-(((5,6-Dimethoxypyrazin-2-yl)thio)methyl)-2-fluorobenzonitrile was hydrolyzed with NaI and TMSCl to afford the title compound after purification by preparative HPLC as an off-white solid (61 mg, 48%). ¹H NMR (400 MHz, DMSO): δ 11.80-11.05 (m, 2 H), 7.87 (dd, J = 7.5, 7.5 Hz, 1 H), 7.42 (dd, J = 1.2, 10.5 Hz, 1 H), 7.23 (dd, J = 1.4, 8.0 Hz, 1 H), 6.17 (s, 1 H), 4.08 (s, 2 H). MS (ESI+) m/z 278 (M+H)⁺.

5-(((4-(Trifluoromethyl)phenyl)thio)methyl)-1,4-dihydropyrazine-2,3-dione (**37**)

Step A: (5,6-Dimethoxypyrazin-2-yl)methanol

To 5-bromo-2,3-dimethoxypyrazine (2.0 g, 9.1 mmol) in THF (25 mL) at -70 °C was added *n*-butyl lithium (4.0 mL, 2.5 M solution in THF, 10.0 mmol) over 15 min. The reaction was stirred at -70 °C for 1 hour and DMF (1.4 mL, 18.3 mmol) was then added over 5 min. at -70 °C. The reaction was stirred at -70 °C for 1.5 hrs. Methanol (10 mL) and sodium borohydride (690 mg, 18.3 mmol) were added and the reaction was stirred for 0.5 hrs. The reaction was brought to -20 °C and quenched with saturated ammonium chloride solution (10 mL). The reaction was diluted with ethyl acetate (20 mL). The organics were separated and the aqueous further extracted with ethyl acetate (2 x 20 mL). The combined organics were washed with brine, dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude material was purified by flash column chromatography (0 – 50% ethyl acetate in ether) to yield the title compound as a pale-yellow solid (1.31 g, 84%). ¹H NMR (400 MHz, CDCl₃): δ 7.62 (d, J = 0.8 Hz, 1 H), 4.63 (d, J = 0.8 Hz, 2 H), 4.04 (s, 3 H), 4.02 (s, 3 H), 2.41 (m, 1 H).

Step B: 5-(Bromomethyl)-2,3-dimethoxypyrazine

To (5,6-dimethoxypyrazin-2-yl)methanol (6.2 g, 36.7 mmol) was added triphenylphosphine (9.6 g, 36.7 mmol) in dichloromethane at 0 °C. Carbon tetrabromide (12.2 g, 36.7 mmol) was added. The reaction was stirred at room temperature for 48 hrs. The reaction was concentrated under reduced pressure. The crude material was purified by flash column chromatography (0 – 20% ethyl acetate in cyclohexane) to yield the title compound as a colorless solid (7.06 g, 82%). ¹H NMR (400 MHz, CDCl₃): δ 7.69 (s, 1 H), 4.45 (s, 2 H), 4.04 (s, 3 H), 4.02 (s, 3 H).

Step C: 2,3-Dimethoxy-5-(((4-(trifluoromethyl)phenyl)thio)methyl)pyrazine

To 4-(trifluoromethyl)thiophenol (3.36 g, 18.8 mmol) was added cesium carbonate (6.15 g, 18.8 mmol) and tetrabutylammonium iodide (6.97 g, 18.8 mmol) in DMF (85 mL), the reaction was stirred at room temperature for 1 hour. 5-(Bromomethyl)-2,3-dimethoxypyrazine (4.00 g, 17.1 mmol) was added and the reaction was stirred at room temperature for 24 hrs. The reaction was diluted with ethyl acetate (40 mL) and water (40 mL). The organics were separated and the aqueous further extracted with ethyl acetate (2 x 40 mL). The combined organics were washed with brine (20 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude material

was purified by flash column chromatography (0 – 25% ethyl acetate in cyclohexane) to yield the title compound as an off-white solid (5.62 g, 99%). ¹H NMR (400 MHz, CDCl₃): δ 7.59-7.58 (m, 1 H), 7.52-7.44 (m, 4 H), 4.15 (s, 2 H), 3.97 (d, J = 13.9 Hz, 6 H).

Step D: 5-(((4-(Trifluoromethyl)phenyl)thio)methyl)-1,4-dihydropyrazine-2,3-dione

2,3-Dimethoxy-5-(((4-(trifluoromethyl)phenyl)thio)methyl)pyrazine (5.60 g, 16.9 mmol) was dissolved in 1,4-dioxane (170 mL) and 2 M HCl (169.0 mL, 339.0 mmol) was added. The reaction was heated at 100 °C for 24 hrs under nitrogen. The reaction was cooled to room temperature and concentrated under reduced pressure. The crude material was purified by flash column chromatography (0 – 20% MeOH in dichloromethane), yielding the title compound as a white solid (2.82 g, 55%). ¹H NMR (400 MHz, DMSO): δ 11.38 (s, 1 H), 11.07 (d, J = 4.5 Hz, 1 H), 7.69-7.65 (m, 2 H), 7.60-7.56 (m, 2 H), 6.25 (d, J = 4.0 Hz, 1 H), 3.96 (s, 2 H). MS (ESI+) m/z 303 (M+H)⁺.

The following compounds were prepared following the same procedure as compound **37**.

5-(((4-Chlorophenyl)thio)methyl)-1,4-dihydropyrazine-2,3-dione (**39**): ¹H NMR (400 MHz, DMSO): δ 11.27-11.04 (m, 2 H), 7.38 (s, 4 H), 6.05 (s, 1 H), 3.80 (s, 2 H). MS (ES+) m/z 269 (M+H)⁺

5-(((4-(Methylsulfonyl)phenyl)thio)methyl)-1,4-dihydropyrazine-2,3-dione (**44**): ¹H NMR (400 MHz, DMSO): δ 11.21-11.20 (m, 2 H), 7.83-7.80 (m, 2 H), 7.61-7.58 (m, 2 H), 6.30 (s, 1 H), 3.98 (s, 2 H), 3.20 (s, 3 H). MS (ES+) m/z 313 (M+H)⁺.

5-(((4-(Pentafluoro-λ6-sulfaneyl)phenyl)thio)methyl)-1,4-dihydropyrazine-2,3-dione (**46**)

Step A: Triisopropyl((4-(pentafluoro-λ6-sulfaneyl)phenyl)thio)silane

To 1-bromo-4-(pentafluorosulfanyl)benzene (132 mg, 0.4 mmol) was added triisopropylsilanethiol (0.10 mL, 0.5 mmol) and lithium bis(trimethylsilyl)amide (0.51 mL, 0.5 mmol) in toluene (2.5 mL). The reaction was degassed for 5 min. and [1,1'-bis(diphenylphosphino)ferrocene]dichloroPd(II) complex with dichloromethane (38 mg, 0.05 mmol) was added and the reaction was stirred at 110 °C for 2 hrs. The mixture was cooled to room temperature and concentrated under reduced pressure. The crude material was purified by flash column chromatography (0 – 10% dichloromethane in cyclohexane) to yield the title compound as an orange oil (180 mg, 98%). ¹H NMR (400 MHz, CDCl₃): δ 7.57-7.55 (m, 4 H), 1.31-1.23 (m, 3 H), 1.10-1.08 (m, 18 H).

Step B: 2,3-Dimethoxy-5-(((4-(pentafluoro-λ6-sulfaneyl)phenyl)thio)methyl)pyrazine

To triisopropyl((4-(pentafluoro-λ6-sulfaneyl)phenyl)thio)silane (180 mg, 0.5 mmol) was added 5-(bromomethyl)-2,3-dimethoxypyrazine (128 mg, 0.6 mmol) (prepared following steps A-B, compound **37**), cesium fluoride (139 mg, 0.9 mmol) and cesium carbonate (74 mg, 0.23 mmol) in DMF (2.5 mL) and the reaction was stirred at 50 °C for 1.5 hrs. The reaction was diluted with ethyl acetate (5 mL) and water (5 mL). The organics were separated and the aqueous further extracted with ethyl acetate (2 x 5 mL). The combined organics were washed with brine, dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude material was purified by flash column chromatography (0 – 20% ethyl acetate in cyclohexane) to yield the title compound as a pale-yellow oil (160 mg, 92%). ¹H NMR (400 MHz, CDCl₃): δ 7.64 (s, 1 H), 7.61 (d, J = 6.2 Hz, 2 H), 7.45-7.41 (m, 2 H), 4.16-4.15 (s, 2 H), 4.00 (s, 3 H), 3.95 (s, 3 H).

Step C: 5-(((4-(Pentafluoro-λ6-sulfaneyl)phenyl)thio)methyl)-1,4-dihydropyrazine-2,3-dione

To 2,3-dimethoxy-5-(((4-(pentafluoro- λ 6-sulfanyl)phenyl)thio)methyl)pyrazine (155 mg, 0.4 mmol) was added sodium iodide (299 mg, 2.0 mmol) and chlorotrimethylsilane (0.25 mL, 2.0 mmol) in acetonitrile (4 mL) and the reaction was heated at 60 °C for 1 hour. The reaction was cooled to room temperature and concentrated under reduced pressure. The crude material was purified by reverse phase preparative HPLC to yield the title compound as a white solid (21 mg, 15%). ¹H NMR (400 MHz, DMSO): δ 11.29-11.28 (m, 1 H), 11.00-10.96 (m, 1 H), 7.76-7.71 (m, 2 H), 7.52-7.46 (m, 2 H), 6.21 (s, 1 H), 3.25 (s, 2 H). MS (ES+) m/z 361 (M+H)⁺.

The following compounds were prepared following the same procedure as compound **46**.

5-(((4-(Difluoromethyl)phenyl)thio)methyl)-1,4-dihydropyrazine-2,3-dione (**42**): ¹H NMR (400 MHz, DMSO): δ 11.26 (s, 2 H), 7.56-7.54 (m, 4 H), 7.0 (t, 1 H), 6.27-6.25 (m, 1 H), 3.95-3.94 (m, 2 H). MS (ES+) m/z 285 (M+H)⁺.

5-(((7-chloroquinolin-3-yl)thio)methyl)-1,4-dihydropyrazine-2,3-dione (**53**): ¹H NMR (400 MHz, DMSO): δ 11.41-11.40 (m, 1 H), 10.98-10.93 (m, 1 H), 8.87 (d, J = 2.3 Hz, 1 H), 8.46 (d, J = 2.1 Hz, 1 H), 8.08-8.00 (m, 2 H), 7.69 (dd, J = 2.1, 8.8 Hz, 1 H), 6.10 (dd, J = 2.3, 5.5 Hz, 1 H), 3.95-3.94 (m, 2 H). MS (ES+) m/z 320 (M+H)⁺.

4-(((5,6-Dioxo-1,4,5,6-tetrahydropyrazin-2-yl)methyl)thio)-2-fluorobenzonitrile (**51**)

Step A: O-(4-Cyano-3-fluorophenyl) dimethylcarbamothioate

To 2-fluoro-4-hydroxybenzonitrile (1.00 g, 7.2 mmol) was added dimethylthiocarbonyl (1.08 g, 8.7 mmol), 4-dimethylaminopyridine (0.09 g, 0.7 mmol) and trimethylamine (3.0 mL, 21.8 mmol) in dichloromethane (20 mL) and the reaction was stirred at 40 °C for 24 hrs. The reaction was cooled to room temperature and diluted with dichloromethane (20 mL) and water (20 mL). The aqueous layer was separated and further extracted with dichloromethane (2 x 20 mL). The combined organics were passed through a phase separator and concentrated under reduced pressure. The crude solid was triturated with cyclohexane to give a white solid (1.64 g, 100%). ¹H NMR (400 MHz, CDCl₃): δ 7.68-7.60 (m, 1 H), 7.02-6.98 (m, 2 H), 3.45 (s, 3 H), 3.35 (s, 3 H).

Step B: S-(4-Cyano-3-fluorophenyl) dimethylcarbamothioate

O-(4-Cyano-3-fluorophenyl) dimethylcarbamothioate (1.64 g, 7.3 mmol) was melted and stirred at 210 °C for 6 hrs. The reaction was cooled to room temperature. The crude material was purified by flash column chromatography (0 – 50% ethyl acetate in cyclohexane) to yield the title compound (0.92 g, 56%). ¹H NMR (400 MHz, CDCl₃): δ 7.64-7.56 (m, 1 H), 7.47-7.36 (m, 2 H), 3.10 (s, 6 H).

Step C: 2-Fluoro-4-mercaptobenzonitrile

S-(4-Cyano-3-fluorophenyl) dimethylcarbamothioate (0.50 g, 2.2 mmol) was dissolved in THF (5.7 mL) and potassium hydroxide (0.23 g, 4.1 mmol) and methanol (7.7 mL) were added. The reaction was stirred at room temperature for 24 hrs. The mixture was concentrated under reduced pressure then diluted with ethyl acetate (10 mL) and 2 M hydrogen chloride (10 mL). The organics were separated and the aqueous further extracted with ethyl acetate (2 x 10 mL). The combined organics were dried over magnesium sulfate, filtered and concentrated under reduced pressure to yield the title compound (0.34 g, 100%). ¹H NMR (400 MHz, CDCl₃): δ 7.49-7.41 (m, 2 H), 7.12-7.07 (m, 1 H), 2.93-2.88 (m, 1 H).

Step D: 4-(((5,6-Dimethoxypyrazin-2-yl)methyl)thio)-2-fluorobenzonitrile

To 2-fluoro-4-mercaptobenzonitrile (0.10 g, 0.7 mmol) was added cesium carbonate (0.23 g, 0.7 mmol) and tetrabutylammonium iodide (0.261 g, 0.7 mmol) in DMF (3.5 mL) and the reaction was stirred at room temperature for 1 hour. 5-(Bromomethyl)-2-3-dimethoxypyrazine (0.15 g, 0.6 mmol) was added at 0 °C and stirred at room temperature for 1 hour. The reaction was diluted with ethyl acetate (5 mL) and water (5 mL). The aqueous was separated and further extracted with ethyl acetate (2 x 5 mL). The combined organics were passed through a phase separator cartridge and concentrated under reduced pressure. The crude material was purified by flash column chromatography (0 – 20% ethyl acetate in cyclohexane, 12 g column) to yield the title compound as a white solid (0.135 g, 69%). ¹H NMR (400 MHz, CDCl₃): δ 7.64 (s, 1 H), 7.49-7.44 (m, 1 H), 7.38-7.33 (m, 1 H), 7.19 (dd, J = 1.3, 8.3 Hz, 1 H), 4.18 (s, 2 H), 4.01-3.98 (m, 6 H).

Step E: 4-(((5,6-Dioxo-1,4,5,6-tetrahydropyrazin-2-yl)methyl)thio)-2-fluorobenzonitrile

To 4-(((5,6-dimethoxypyrazin-2-yl)methyl)thio)-2-fluorobenzonitrile (130 mg, 0.4 mmol) was added sodium iodide (320 g, 2.1 mmol) and chlorotrimethylsilane (0.27 mL, 2.1 mmol) in acetonitrile (4.5 mL) and the reaction was heated at 60 °C for 2 hrs. The mixture was cooled to room temperature and concentrated under reduced pressure onto silica. The crude material was purified by flash column chromatography (0 – 20% MeOH in dichloromethane) and then further purified by preparative HPLC to yield the title compound as a white solid (81 mg, 67%). ¹H NMR (400 MHz, DMSO): δ 11.27 - 11.27 (m, 2 H), 7.87 (t, J=7.7 Hz, 1 H), 7.65 (dd, J=1.3, 10.7 Hz, 1 H), 7.41 (dd, J=1.3, 8.3 Hz, 1 H), 6.43 (s, 1 H), 4.07 (s, 2 H). MS (ESI+) m/z 278.1 (M+H)⁺.

2-Chloro-4-(((5,6-dioxo-1,4,5,6-tetrahydropyrazin-2-yl)methyl)thio)benzonitrile (**52**)

Step A: 2-Chloro-4-mercaptobenzonitrile

To 2-chloro-4-fluorobenzonitrile (0.50 g, 3.2 mmol) was added sodium sulphide (0.27 g, 3.5 mmol) and in DMF (2.5 mL) and the reaction was stirred at room temperature for 2 hrs. 1 M Sodium hydroxide was added and the mixture washed with dichloromethane (5 mL). The aqueous layer was acidified to pH 1-2 with 1 M hydrogen chloride solution (2 mL) and extracted with dichloromethane (2 x 5 mL). The combined organic layers were washed with brine (5 mL), dried over magnesium sulfate, filtered and concentrated under reduced pressure to provide a crude residue. To the residue was added 10 % hydrogen chloride solution (2 mL) and the mixture was cooled with an ice-water bath. Zinc dust (2.00 g, 30.6 mmol) was added and the mixture was stirred for 1 hour. Ethyl acetate (5 mL) was added and the mixture was stirred for an additional 30 min. The organic layer was separated and washed with water (10 mL) and brine (10 mL), dried over magnesium sulfate, filtered and concentrated under reduced pressure to provide the desired product as an orange oil (0.48 g, 88%). ¹H NMR (400 MHz, CDCl₃): δ 7.52-7.46 (m, 1 H), 7.40-7.36 (m, 1 H), 7.26 (s, 1 H), 3.74 (s, 1 H).

Step B: 4-(((5,6-Dimethoxypyrazin-2-yl)methyl)thio)phthalonitrile

To 2-chloro-4-mercaptobenzonitrile (0.08 g, 0.4 mmol) was added cesium carbonate (0.15 g, 0.4 mmol) and tetrabutylammonium iodide (0.17 g, 0.4 mmol) in DMF (2.5 mL) and the reaction was stirred at room temperature for 1 hour. 5-(Bromomethyl)-2-3-dimethoxypyrazine (0.10 g, 0.4 mmol) was added at 0 °C and the mixture was stirred at room temperature for 1 hour. The reaction was diluted with dichloromethane (5 mL) and water (5 mL). The organics were separated and the aqueous was extracted with dichloromethane (2 x 10 mL). The combined organics were passed through a phase separator and concentrated under reduced pressure. The crude material was

purified by flash column chromatography (0 – 30% ethyl acetate in cyclohexane) to yield the title compound as an off-white solid (0.14 g, 98%). ¹H NMR (400 MHz, CDCl₃): δ 7.63 (d, J = 6.1 Hz, 2 H), 7.53-7.48 (m, 1 H), 7.29-7.27 (m, 1 H), 4.18 (s, 2 H), 4.01-3.98 (m, 6 H).

Step C: 2-Chloro-4-(((5,6-dioxo-1,4,5,6-tetrahydropyrazin-2-yl)methyl)thio)benzotrile

To 4-(((5,6-dimethoxy-pyrazin-2-yl)methyl)thio)phthalonitrile (0.13 g, 0.4 mmol) was added sodium iodide (0.30 g, 2.0 mmol) and chlorotrimethylsilane (0.22 g, 2.0 mmol) in acetonitrile (4.0 mL) and the reaction was heated at 60 °C for 1 hour. The mixture was cooled to room temperature and concentrated under reduced pressure onto silica gel. The crude material was purified by flash column chromatography (0 – 20% MeOH in dichloromethane) to yield the title compound (53 mg, 45%). ¹H NMR (400 MHz, DMSO): δ 11.43-11.38 (m, 1 H), 11.14 (d, J = 5.1 Hz, 1 H), 7.93-7.89 (m, 1 H), 7.82-7.80 (m, 1 H), 7.55 (dd, J = 1.5, 8.3 Hz, 1 H), 6.40 (d, J = 3.3 Hz, 1 H), 4.10-4.07 (m, 2 H). MS (ESI+) m/z 294 (M+H)⁺.

(R)-5-(7-(Trifluoromethyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione (**54**)

Step A: 2,3-Dimethoxy-5-vinylpyrazine

5-Bromo-2,3-dimethoxy-pyrazine (500 mg, 2.28 mmol), potassium vinyl trifluoroborate (612 mg, 4.57 mmol), Pd(dppf)Cl₂ DCM complex (186 mg, 0.23 mmol) and triethylamine (0.95 mL, 6.85 mmol) were dissolved in 2-propanol (10 mL), degassed with nitrogen and heated to reflux for 16 hrs. The reaction mixture was allowed to cool, diluted with ethyl acetate (10 mL) and then filtered through Celite™. The filtrate was concentrated onto silica and purified by flash column chromatography (0 – 50% ethyl acetate in cyclohexane) to provide the desired product as a colourless oil (310 mg, 82%). ¹H NMR (400 MHz, CDCl₃): δ 7.53 (s, 1 H), 6.66 (dd, J = 10.7, 17.0 Hz, 1 H), 6.15 (dd, J = 1.7, 17.1 Hz, 1 H), 5.35 (dd, J = 1.8, 10.7 Hz, 1 H), 4.06 (s, 3 H), 4.02 (s, 3 H).

Step B: (S)-1-(5,6-Dimethoxy-pyrazin-2-yl)ethane-1,2-diol

To a solution of 2,3-dimethoxy-5-vinylpyrazine (150 mg, 0.90 mmol) in a 3:1 mixture of tert-butanol (6 mL)/water (2 mL) cooled to 0 °C, was added AD mix alpha (1.40 g) and the reaction mixture was stirred for 72 hours, while being allowed to warm to room temperature. The reaction mixture was then filtered through Celite™, washing with methanol. The filtrate was concentrated directly onto silica and purified by flash column chromatography (0 – 20% methanol in DCM) to provide the desired product as a colorless oil (80 mg, 44% yield). ¹H NMR (400 MHz, CDCl₃): δ 7.73-7.71 (m, 1 H), 4.75 (dd, J = 3.7, 6.8 Hz, 1 H), 4.26 (s, 1 H), 4.00 (s, 3 H), 3.99 (s, 3 H), 3.90 (dd, J = 3.7, 11.4 Hz, 1 H), 3.80 (dd, J = 6.8, 11.4 Hz, 1 H), 3.73 (s, 1 H).

Step C: (S)-2-((tert-Butyldimethylsilyl)oxy)-1-(5,6-dimethoxy-pyrazin-2-yl)ethan-1-ol

To a solution of (S)-1-(5,6-dimethoxy-pyrazin-2-yl)ethane-1,2-diol (90 mg, 0.45 mmol) and tert-butyldimethylsilyl chloride (81 mg, 0.54 mmol) in DCM (5 mL) cooled to 0 °C, was added imidazole (61 mg, 0.90 mmol) and the reaction mixture stirred for 4 hrs. The reaction mixture was concentrated directly onto silica and purified by flash column chromatography (0 – 40% ethyl acetate in cyclohexane) to provide the desired product as a colourless oil (90 mg, 64%). ¹H NMR (400 MHz, CDCl₃): δ 7.73 (s, 1 H), 4.70-4.64 (m 1 H), 4.01 (s, 3 H), 4.00 (s, 3 H), 3.85 (dd, J = 4.7, 10.0 Hz, 1 H), 3.80 (dd, J = 6.3, 9.9 Hz, 1 H), 3.11 (d, J = 5.5 Hz, 1 H), 0.86 (s, 9 H), 0.02 (s, 3H), 0.00 (s, 3 H).

Step D: (R)-5-(1-((2-Bromo-4-(trifluoromethyl)phenyl)thio)-2-((tert-butyldimethylsilyl)oxy)ethyl)-2,3-dimethoxypyrazine

Tributylphosphine (0.10 mL, 0.38 mmol) was dissolved in anhydrous THF (2.50 mL) and cooled to 0 °C. DIAD (0.06 mL, 0.38 mmol) was added dropwise and reaction stirred at 0 °C for 15 min. 2-bromo-4-trifluoromethylthiophenol (98 mg, 0.38 mmol) and (S)-2-((tert-butyldimethylsilyl)oxy)-1-(5,6-dimethoxypyrazin-2-yl)ethan-1-ol (90 mg, 0.32 mmol) were dissolved in THF (2.50 mL) and added slowly to the solution of DIAD and tributyl phosphine. The reaction was allowed to warm up slowly to room temperature and left stirring for 16 hrs. The reaction mixture was concentrated directly onto silica and purified by flash column chromatography (0 – 20% ethyl acetate in cyclohexane), to give the title compound as a colorless oil (103 mg, 59%). ¹H NMR (400 MHz, CDCl₃): δ 7.77 (s, 1 H), 7.70 (s, 1 H), 7.46 (s, 2 H), 4.44 (dd, J = 5.4, 7.7 Hz, 1 H), 4.19 (dd, J = 7.7, 10.2 Hz, 1 H), 4.07-4.03 (m, 4 H), 4.00 (s, 3 H), 0.81 (s, 9 H), -0.03 (s, 3 H), -0.06 (s, 3 H).

Step E: (R)-2-((2-Bromo-4-(trifluoromethyl)phenyl)thio)-2-(5,6-dimethoxypyrazin-2-yl)ethan-1-ol

To a solution of (R)-5-(1-((2-bromo-4-(trifluoromethyl)phenyl)thio)-2-((tert-butyldimethylsilyl)oxy)ethyl)-2,3-dimethoxypyrazine (90 mg, 0.19 mmol) in THF (3 mL) cooled to 0 °C, was added tetrabutylammonium fluoride (1 M, 0.28 mL, 0.28 mmol) and stirred at 0 °C for 1 hour. The reaction was then diluted with ethyl acetate (5 mL) and washed with water (10 mL). The organics were then concentrated onto silica and purified by flash column chromatography (0 – 50% ethyl acetate in cyclohexane) to provide the desired product as a colorless oil (72 mg, 89%). ¹H NMR (400 MHz, CDCl₃): δ 7.82 (s, 1 H), 7.64 (s, 1 H), 7.49-7.47 (m, 2 H), 4.50 (dd, J = 5.4, 6.4 Hz, 1 H), 4.23-4.07 (m, 2 H), 4.02 (s, 3 H), 4.00 (s, 3 H), 2.92-2.77 (m, 1 H).

Step F: (R)-2,3-Dimethoxy-5-(7-(trifluoromethyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)pyrazine

A suspension of (R)-2-((2-bromo-4-(trifluoromethyl)phenyl)thio)-2-(5,6-dimethoxypyrazin-2-yl)ethan-1-ol (72 mg, 0.16 mmol), Pd(II) acetate (11 mg, 0.05 mmol), TrixiePhos (20 mg, 0.05 mmol) and cesium carbonate (80 mg, 0.25 mmol) in toluene (2 mL) in a microwave vial was thoroughly degassed. The reaction mixture was then heated to 150 °C for two hrs under microwave irradiation. The reaction mixture was then concentrated directly onto silica and purified by flash column chromatography (0 – 40% ethyl acetate in cyclohexane) to provide the desired product as an orange oil (22 mg, 38%). ¹H NMR (400 MHz, CDCl₃): δ 7.72 (s, 1 H), 7.20 (d, J = 8.8 Hz, 1 H), 7.12 (d, J = 6.8 Hz, 2 H), 4.65 (dd, J = 2.4, 10.8 Hz, 1 H), 4.58-4.49 (m, 2 H), 4.02 (s, 3 H), 3.98 (s, 3 H).

Step G: (R)-5-(7-(Trifluoromethyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione

To a solution of (R)-2,3-dimethoxy-5-(7-(trifluoromethyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)pyrazine (16 mg, 0.04 mmol) in 1,4-dioxane (0.5 mL) was added hydrochloric acid (2 M, 0.45 mL) and the reaction mixture was heated to reflux for 2 hrs. The reaction mixture was then allowed to cool to room temperature and concentrated before purification via reverse phase chromatography to provide the desired product as a colorless solid (9 mg, 50%). ¹H NMR (400 MHz, DMSO): δ 11.51 (s, 1 H), 11.22 (d, J = 4.8 Hz, 1 H), 7.44 (d, J = 7.8 Hz, 1 H), 7.29 (d, J = 8.6 Hz, 2 H), 6.35 (d, J = 4.8 Hz, 1 H), 4.64 (dd, J = 5.9, 12.0 Hz, 1 H), 4.56 (dd, J = 2.8, 11.9 Hz, 1 H), 4.48 (dd, J = 2.4, 5.8 Hz, 1 H), MS (ES⁺) m/z 331 (M+H)⁺.

The following compounds were synthesized using the same procedure described for compound **54**.

(R)-5-(7-Fluoro-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione (**56**): ¹H NMR (400 MHz, DMSO): δ 11.44 (s, 1 H), 11.18 (s, 1 H), 7.20-7.15 (m, 1 H), 6.87-6.79 (m, 2 H), 6.26 (d, J = 4.8 Hz, 1 H), 4.59-4.45 (m, 2 H), 4.33 (dd, J = 2.4, 5.7 Hz, 1 H). MS (ES+) m/z 281 (M+H)⁺.

(R)-5-(7-Chloro-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione (**57**): ¹H NMR (400 MHz, DMSO): δ 11.21 (s, 2 H), 7.18 (d, J = 8.4 Hz, 1 H), 7.03-6.97 (m, 2 H), 6.27 (s, 1 H), 4.55 (dd, J = 5.8, 11.7 Hz, 1 H), 4.47 (dd, J = 2.6, 11.7 Hz, 1 H), 4.36 (dd, J = 2.6, 5.8 Hz, 1 H). MS (ES+) m/z 297 (M+H)⁺.

5-(2,3-Dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione (**55**)

Step A: 2-((tert-Butyldimethylsilyloxy)-1-(5,6-dimethoxy-pyrazin-2-yl)ethan-1-ol (rac)

5-Bromo-2,3-dimethoxy-pyrazine (2.00 g, 9.13 mmol) was dissolved in dry THF (40 mL) and cooled to -78 °C. n-Butyl lithium (2.5 M in THF, 4.00 mL, 10.04 mmol) was added dropwise and the mixture stirred for 30 min. (tert-Butyldimethylsilyloxy)acetaldehyde (2.10 mL, 10.96 mmol) was dissolved in THF (2.5 mL) and added slowly. The reaction was stirred at -78 °C for 75 min. before warming to room temperature and quenching with saturated ammonium chloride solution (10 mL). The organics were separated and the aqueous extracted with ethyl acetate (3 x 20 mL). The organics were combined and dried over sodium sulfate before concentrating and purifying by flash column chromatography (0 – 30% ethyl acetate in cyclohexane) to give 2-((tert-butyl dimethylsilyloxy)-1-(5,6-dimethoxy-pyrazin-2-yl)ethan-1-ol as a colorless oil (1.22 g, 43%). ¹H NMR (400 MHz, CDCl₃): δ 7.74 (s, 1 H), 4.69 (q, J = 5.4 Hz, 1 H), 4.02 (s, 3 H), 4.02 (s, 3 H), 3.89-3.78 (m, 2 H), 3.12 (d, J = 5.5 Hz, 1 H), 0.87 (s, 9 H), 0.03 (s, 3 H), 0.01 (s, 3 H).

Step B: 5-(2,3-Dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione

Following the same steps for compound **54** yielded the title compound as a colorless solid. ¹H NMR (400 MHz, DMSO): δ 11.19 (s, 2 H), 7.12 (dd, J = 1.4, 7.7 Hz, 1 H), 7.05 (ddd, J = 7.7, 7.7, 1.9 Hz, 1 H), 6.94 - 6.88 (m, 2 H), 6.29 (s, 1 H), 4.53 - 4.42 (m, 2 H), 4.35 - 4.31 (m, 1 H). MS (ES+) m/z 263 (M+H)⁺.

(R)-5-(7-(Difluoromethyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione (**58**)

Step A: Methyl 3-bromo-4-mercaptobenzoate

Sodium sulfide (0.50 g, 6.44 mmol) was dissolved in DMF (10 mL) and stirred, methyl 3-bromo-4-fluorobenzoate (1.00 g, 4.29 mmol) was added in three portions and reaction stirred at room temperature for 2 hrs. The reaction was diluted with water (20 mL) and washed with ethyl acetate (10 mL). The aqueous layer was then acidified to pH 2 with 2 M hydrochloric acid and extracted with ethyl acetate (3 x 10 mL). The combined organics were dried over magnesium sulfate, concentrated *in vacuo* and purified by flash column chromatography (0 – 100% ethyl acetate in cyclohexane) to provide the desired product. (215 mg, 20%). ¹H NMR (400 MHz, CDCl₃): δ 8.16 (d, J = 1.9 Hz, 1 H), 7.79 (dd, J = 1.9, 8.1 Hz, 1 H), 7.37 (d, J = 8.1 Hz, 1 H), 4.21 (s, 1 H), 3.90 (s, 3 H).

Step B: Methyl (R)-3-(5,6-dimethoxy-pyrazin-2-yl)-2,3-dihydrobenzo[b][1,4]oxathiine-7-carboxylate

Using intermediate (S)-2-((tert-butyl dimethylsilyloxy)-1-(5,6-dimethoxy-pyrazin-2-yl)ethan-1-ol following the same procedure as compound **54** provided the desired product. ¹H NMR (400 MHz, CDCl₃): δ 7.72 (s, 1 H), 7.57-7.53 (m, 2 H), 7.15 (d, J = 8.6 Hz, 1 H), 4.67-4.61 (m, 1 H), 4.56-4.51 (m, 2 H), 4.01 (s, 3 H), 3.98 (s, 3 H), 3.89 (s, 3 H).

Step C: (R)-(3-(5,6-Dimethoxy pyrazin-2-yl)-2,3-dihydrobenzo[b][1,4]oxathiin-7-yl)methanol

To an ice cooled solution of methyl (R)-3-(5,6-dimethoxy pyrazin-2-yl)-2,3-dihydrobenzo[b][1,4]oxathiin-7-carboxylate (273 mg, 0.78 mmol) in THF (8 mL) under nitrogen was added lithium borohydride solution (2 M in THF, 0.78 mL) dropwise and the reaction stirred for 16 hrs. Additional lithium borohydride solution was then added (2 M, 0.78 mL) and reaction stirred for further 48 hrs. The reaction was quenched by addition of water followed by extraction of the organics with ethyl acetate (3 × 10 mL). The organics were combined, washed with brine (1 × 10 mL) and dried over magnesium sulfate before concentrating *in vacuo*. The crude material was then purified by flash column chromatography (0 – 100% ethyl acetate in cyclohexane) to give the desired product as a off-white solid, (152 mg, 61%). ¹H NMR (400 MHz, CDCl₃): δ 7.74 (s, 1 H), 7.09 (d, J = 8.4 Hz, 1 H), 6.91-6.89 (m, 2 H), 4.64-4.59 (m, 2 H), 4.56-4.47 (m, 2 H), 4.01 (s, 3 H), 4.00 (s, 3 H), 1.26 (t, J = 7.2 Hz, 1 H).

Step D: (R)-3-(5,6-Dimethoxy pyrazin-2-yl)-2,3-dihydrobenzo[b][1,4]oxathiin-7-carbaldehyde

(R)-3-(5,6-dimethoxy pyrazin-2-yl)-2,3-dihydrobenzo[b][1,4]oxathiin-7-yl)methanol (70 mg, 0.22 mmol) was dissolved in DCM (2 mL) under nitrogen and Dess-Martin periodinane (102 mg, 0.24 mmol) was added. The reaction was stirred at room temperature for 2 hrs. The reaction mixture was then concentrated and purified via flash column chromatography (0 – 100% ethyl acetate in cyclohexane) to give the desired product (55 mg, 79%). ¹H NMR (400 MHz, CDCl₃): δ 9.87 (s, 1 H), 7.73 (s, 1 H), 7.41 (dd, J = 1.8, 8.0 Hz, 1 H), 7.37 (d, J = 1.6 Hz, 1 H), 7.26-7.23 (m, 1 H), 4.69-4.63 (m, 1 H), 4.58-4.54 (m, 2 H), 4.02 (s, 3 H), 3.98 (s, 3 H).

Step E: (R)-5-(7-(Difluoromethyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-2,3-dimethoxy pyrazine

(R)-3-(5,6-Dimethoxy pyrazin-2-yl)-2,3-dihydrobenzo[b][1,4]oxathiin-7-carbaldehyde (55 mg, 0.17 mmol) was dissolved in DCM (2 mL). (Diethylamino)sulfur trifluoride (0.14 mL, 1.04 mmol) was then added and the reaction stirred for 16 hrs. The reaction mixture was then diluted with 2 M potassium hydroxide (15 mL) and DCM (10 mL) and the organics were separated and dried through an hydrophobic frit. The organics were then concentrated and purified by flash column chromatography (0 – 100% ethyl acetate in cyclohexane) to give the desired product (32 mg, 54%). ¹H NMR (400 MHz, CDCl₃): δ 7.73 (s, 1 H), 7.17 (d, J = 8.6 Hz, 1 H), 7.03 (d, J = 5.3 Hz, 2 H), 6.55 (t, J = 56.6 Hz, 1 H), 4.64 (dd, J = 2.3, 10.9 Hz, 1 H), 4.57-4.48 (m, 2 H), 4.01 (s, 3 H), 3.98 (s, 3 H).

Step F: (R)-5-(7-(Difluoromethyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione

Hydrolysis of (R)-5-(7-(Difluoromethyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-2,3-dimethoxy pyrazine with NaI and TMSCl yielded the title compound as a white solid (19 mg, 0.06 mmol). ¹H NMR (400 MHz, DMSO): δ 11.46-11.45 (m, 1 H), 11.18-11.18 (m, 1 H), 7.30 (d, J = 8.0 Hz, 1 H), 7.13-7.09 (m, 2 H), 6.94 (t, J = 56.1 Hz, 1 H), 6.29-6.29 (m, 1 H), 4.57 (dd, J = 5.8, 11.7 Hz, 1 H), 4.49 (dd, J = 2.7, 11.8 Hz, 1 H), 4.40 (dd, J = 2.5, 5.8 Hz, 1 H), MS (ES+) m/z 313 (M+H)⁺.

(R)-5-(7-(Methylsulfonyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione (**60**)

Step A: 2-Bromo-1-fluoro-4-(methylsulfonyl)benzene

A solution of 1-fluoro-4-(methylsulfonyl)benzene (1.00 g, 5.74 mmol) and N-bromosuccinimide (1.12 g, 6.31 mmol) in sulfuric acid (6 mL) was heated to 50 °C, with stirring, for 16 hrs. The reaction mixture was poured over ice and the solid collected by filtration to provide the desired product as a colourless solid (1.45 g, quant.) ¹H NMR

(400 MHz, CDCl₃): δ 8.19 (dd, J = 2.3, 6.3 Hz, 1 H), 7.91 (ddd, J = 2.3, 4.4, 8.6 Hz, 1 H), 7.34-7.29 (m, 1 H), 3.08 (s, 3 H).

Step B: 2-Bromo-4-(methylsulfonyl)benzenethiol

2-Bromo-1-fluoro-4-(methylsulfonyl)benzene (1.00 g, 3.95 mmol), triisopropylsilanethiol (0.85 mL, 3.95 mmol) and potassium carbonate (0.82 g, 5.93 mmol) were dissolved in DMF (50.00 mL) and stirred at 50 °C for 2 hrs. The reaction mixture was then allowed to cool to room temperature and was then washed with ethyl acetate (50 mL).

The aqueous layer was then acidified to pH <3 with 2 M hydrochloric acid and extracted with ethyl acetate (3 × 30 mL). The organics were combined and concentrated in vacuo, then triturated with DCM (1 × 10 mL) to give the desired product as white solid (260 mg, 25 %). ¹H NMR (400 MHz, CDCl₃): δ 8.08 (d, J = 2.0 Hz, 1 H), 7.71 (dd, J = 2.0, 8.3 Hz, 1 H), 7.52 (d, J = 8.3 Hz, 1 H), 4.31 (s, 1 H), 3.05 (s, 3 H), 2.96 (s, 6 H), 2.88 (s, 6 H), 1.05 (s, 1 H).

Step C: (R)-5-(7-(Methylsulfonyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione

Using intermediate (S)-2-((*tert*-butyldimethylsilyloxy)-1-(5,6-dimethoxypyrazin-2-yl)ethan-1-ol (prepared following the procedure of compound **54**) provided the desired product as an off white solid (13.3 mg, 45%). ¹H NMR (400 MHz, DMSO): δ 11.48 (s, 1 H), 11.23-11.23 (m, 1 H), 7.45-7.43 (m, 2 H), 7.39 (dd, J = 0.6, 1.6 Hz, 1 H), 6.31 (s, 1 H), 4.62 (dd, J = 5.8, 12.0 Hz, 1 H), 4.52 (dd, J = 2.7, 11.7 Hz, 1 H), 4.46 (dd, J = 2.4, 5.8 Hz, 1 H), 3.22 (s, 3 H). MS (ES⁺) m/z 341 (M+H)⁺.

(R)-3-(5,6-Dioxo-1,4,5,6-tetrahydropyrazin-2-yl)-2,3-dihydrobenzo[b][1,4]oxathiine-7-carbonitrile (**59**)

Step A: 5-bromo-2-chloro-4-hydroxybenzonitrile

To a suspension of 2-chloro-4-hydroxybenzonitrile (1.00 g, 6.51 mmol) in acetonitrile (20 mL) cooled to -30 °C was added trifluoromethanesulfonic acid (0.63 mL, 7.16 mmol) as drops and the reaction stirred for 10 minutes. *N*-Bromosuccinimide (1.39 g, 7.81 mmol) was added, and the reaction stirred overnight being allowed to warm to room temperature. Saturated aqueous sodium bicarbonate was added, and the organics extracted with EtOAc (3×). The aqueous phase was acidified to pH3 with 1M HCl(aq) and the organics were further extracted with EtOAc (2×). The combined organics were dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude material was purified by flash column chromatography (0 to 100% EtOAc in cyclohexane) to give 5-bromo-2-chloro-4-hydroxybenzonitrile as a white solid (626 mg, 41% yield). ¹H NMR (400 MHz, CDCl₃) δ 7.79 (s, 1H), 7.17 (s, 1H), 6.11 (s, 1H).

Step B: *S*-(2-bromo-5-chloro-4-cyanophenyl) dimethylcarbamothioate

A solution of 5-bromo-2-chloro-4-hydroxybenzonitrile (625 mg, 2.69 mmol), dimethylthiocarbamoyl chloride (498 mg, 4.03 mmol) and 1,4-diazabicyclo[2.2.2]octane (754 mg, 6.72 mmol) in DMF (13 mL) was stirred overnight. The reaction was diluted with diethyl ether and filtered and the solid washed with diether ether (2×). The solid was dried under vacuum yielding *O*-(2-bromo-5-chloro-4-cyanophenyl) dimethylcarbamothioate as a white solid (604 mg, 70% yield). Neat *O*-(2-bromo-5-chloro-4-cyanophenyl) dimethylcarbamothioate (600 mg, 1.88 mmol) was heated to 200 °C for 2.5 hours. The reaction was allowed to cool to room temperature and the sublimed material rinsed back into the reaction flask with DCM. The solution was concentrated under reduced pressure yielding *S*-(2-bromo-5-chloro-4-cyanophenyl) dimethylcarbamothioate as a pale yellow solid (595 mg, 99% yield). ¹H NMR (400 MHz, CDCl₃) δ 7.92 (s, 1H), 7.84 (s, 1H), 3.13 (s, 3H), 3.06 (s, 3H).

Step C: 5-bromo-2-chloro-4-mercaptobenzonitrile

A mixture of *S*-(2-bromo-5-chloro-4-cyanophenyl) dimethylcarbamothioate (595, 1.86 mmol) and potassium hydroxide (313 mg, 5.59 mmol) in methanol (2 mL) and THF (5 mL) was stirred at room temperature for 90 minutes. Water was added and the organics separated. The aqueous phase was acidified to pH2 with 2M HCl(aq) and the organics further extracted with EtOAc (2×). The combined organics were washed with brine, dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude material was purified by flash column chromatography (5 to 95% DCM in cyclohexane yielding 5-bromo-2-chloro-4-mercaptobenzonitrile as a pale yellow solid (403 mg, 87% yield). ¹H NMR (400 MHz, CDCl₃) δ 7.75 (s, 1H), 7.48 (s, 1H), 4.30 (s, 1H).

Step D: (R)-6-chloro-3-(5,6-dimethoxy-pyrazin-2-yl)-2,3-dihydrobenzo[b][1,4]oxathiine-7-carbonitrile

Following steps D-F from Example 54 yielded the title compound as a yellow solid (136 mg, 25% over 3 steps). ¹H NMR (400 MHz, CDCl₃): ¹H NMR (400 MHz, CDCl₃) δ 7.69 (s, 1H), 7.22 (s, 1H), 7.14 (s, 1H), 4.66 - 4.60 (m, 1H), 4.55 - 4.49 (m, 2H), 4.02 (s, 3H), 3.97 (s, 3H).

Step E: (R)-3-(5,6-Dimethoxy-pyrazin-2-yl)-2,3-dihydrobenzo[b][1,4]oxathiine-7-carbonitrile

To a degassed suspension of (R)-6-chloro-3-(5,6-dimethoxy-pyrazin-2-yl)-2,3-dihydrobenzo[b][1,4]oxathiine-7-carbonitrile (100 mg, 0.29 mmol) and S-Phos (47 mg, 0.11 mmol) in toluene (3 mL) was added 4,4,5,5-tetramethyl-1,3,2-dioxaborolane (0.06 mL, 0.43 mmol) and heated to 100 °C for 16 hrs. The reaction was then allowed to cool and filtered. The solid was washed with ethyl acetate (3 × 10 mL) and the organic phases combined and concentrated *in vacuo* and purified by flash column chromatography (0 – 100% ethyl acetate in cyclohexane) to give the desired product as an off white solid (23 mg, 26%). ¹H NMR (400 MHz, DMSO): δ 11.49 (s, 1 H), 11.23 (d, J = 5.1 Hz, 1 H), 7.46 - 7.38 (m, 3 H), 6.34 (d, J = 5.1 Hz, 1 H), 4.64 - 4.47 (m, 3 H).

Step F: (R)-3-(5,6-Dioxo-1,4,5,6-tetrahydropyrazin-2-yl)-2,3-dihydrobenzo[b][1,4]oxathiine-7-carbonitrile

(R)-3-(5,6-Dimethoxy-pyrazin-2-yl)-2,3-dihydrobenzo[b][1,4]oxathiine-7-carbonitrile (20 mg, 0.06 mmol) was treated with NaI (48 mg, 0.32 mmol) and TMSCl (34 mg, 0.32 mmol) at 60 °C for 45 min. The mixture was concentrated under reduced pressure and purified by reverse phase HPLC to yield the title compound as a white solid (9 mg, 50%). ¹H NMR (400 MHz, DMSO): δ 11.49 (s, 1 H), 11.23 (d, J=5.1 Hz, 1H), 7.46 - 7.38 (m, 3 H), 6.34 (d, J = 5.1 Hz, 1 H), 4.64 - 4.47 (m, 3 H), MS (ES+) m/z 288 (M+H)⁺.

(R)-5-(3-Methyl-7-(trifluoromethyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione (62)

Step A: 2,3-Dimethoxy-5-(prop-1-en-2-yl)pyrazine

5-Bromo-2,3-dimethoxy-pyrazine (3.00 g, 13.70 mmol), isopropenylboronic acid pinacol ester (2.76 g, 16.44 mmol), Pd(0) tetrakis(triphenyl)phosphine (0.47 g, 0.41 mmol) and sodium carbonate (4.35 g, 41.09 mmol) were dissolved in 1,4-dioxane (30.00 mL) and water (20.00 mL) and degassed. The reaction was heated to reflux for 16 hrs. The reaction mixture was allowed to cool and diluted with ethyl acetate (30 mL) and water (30 mL). The organics were separated and the aqueous layer extracted with further ethyl acetate (2 × 30 mL). The combined organics were washed with water (4 × 20 mL) and dried over magnesium sulfate before concentrating onto silica and purification by flash column chromatography (0 to 10% ethyl acetate in hexane) to give the desired compound as a colorless oil (1.89 g, 77%). ¹H NMR (400 MHz, CDCl₃): δ 7.73 (s, 1 H), 5.91 (d, J = 1.4 Hz, 1 H), 5.16 (dd, J = 1.6, 1.6 Hz, 1 H), 4.05 (s, 3 H), 4.03 (s, 3 H), 2.15 (s, 3 H).

Step B: 2,3-Dimethoxy-5-(2-methyloxiran-2-yl)pyrazine

To a solution of 2,3-dimethoxy-5-(prop-1-en-2-yl)pyrazine (1.80 g, 9.99 mmol) in tert-butanol (40 mL) and water (40 mL) was added N-bromosuccinimide (2.13 g, 11.99 mmol). The reaction was heated to 60 °C for 2 hrs and then cooled to 0 °C before addition of 2 M sodium hydroxide (12.5 mL). The reaction mixture was stirred for a further 30 min. before dilution with ethyl acetate (40 mL). The organics were separated and the aqueous further extracted with ethyl acetate (3 × 40 mL) before combining and washing with brine (30 mL). The organics were then concentrated to a pink oil, which solidified on cooling, to provide the desired product (1.84 g, 94%). ¹H NMR (400 MHz, CDCl₃): δ 7.71 (s, 1 H), 4.03 (s, 3 H), 4.01 (s, 3 H), 3.13 (d, J=5.9 Hz, 1 H), 2.97 (d, J=5.5 Hz, 1 H), 1.75 (s, 3 H).

Step C: 2-((2-Bromo-4-(trifluoromethyl)phenyl)thio)-2-(5,6-dimethoxypyrazin-2-yl)propan-1-ol

To a solution of 2,3-dimethoxy-5-(2-methyloxiran-2-yl)pyrazine (500 mg, 2.55 mmol) and 2-bromo-4-(trifluoromethyl)benzenethiol (655 mg, 2.55 mmol) in DCM (20 mL) cooled to 0 °C was added indium (III) chloride (56 mg, 0.26 mmol) and the reaction stirred for 30 min. The reaction mixture was then washed with water (2 × 15 mL) and passed through a phase separator before concentrating *in vacuo* and purifying by flash column chromatography (0 – 30% ethyl acetate in cyclohexane) to provide the desired product as a colourless oil (608 mg, 53%). ¹H NMR (400 MHz, CDCl₃): δ 7.85 (d, J=1.3 Hz, 1 H), 7.68 (s, 1 H), 7.39 (dd, J=1.4, 8.2 Hz, 1 H), 7.32 (d, J=8.1 Hz, 1 H), 4.02 (s, 5 H), 3.86 (s, 3 H), 3.15 (dd, J=5.8, 7.8 Hz, 1 H), 1.72 (s, 3 H).

Step D: 2,3-Dimethoxy-5-(3-methyl-7-(trifluoromethyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)pyrazine

A suspension of 2-((2-bromo-4-(trifluoromethyl)phenyl)thio)-2-(5,6-dimethoxypyrazin-2-yl)propan-1-ol (600 mg, 1.32 mmol), Pd(II) acetate (89 mg, 0.40 mmol), TrixiePhos (158 mg, 0.40 mmol) and caesium carbonate (647 mg, 1.99 mmol) in toluene (6.00 mL) in a microwave vial was thoroughly degassed. The reaction mixture was then heated to 150 °C for two hrs under microwave irradiation. The reaction mixture was then concentrated onto silica and purified by flash column chromatography (0 – 40% ethyl acetate in cyclohexane) to provide the desired product as an orange oil (440 mg, 90%). ¹H NMR (400 MHz, CDCl₃): δ 7.88 (s, 1 H), 7.18 (d, J = 7.9 Hz, 1 H), 7.13 - 7.08 (m, 2 H), 4.76 (d, J = 11.2 Hz, 1 H), 4.23 (d, J=11.2 Hz, 1 H), 4.01 (s, 3 H), 3.99 (s, 3 H), 1.75 (s, 3 H).

Racemic 2,3-Dimethoxy-5-(3-methyl-7-(trifluoromethyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)pyrazine was resolved by chiral SFC to afford single enantiomers.

2,3-Dimethoxy-5-(3-methyl-7-(trifluoromethyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)pyrazine (Isomer 1), 63 mg, chiral analysis (Method 3) at 1.80 min.

2,3-Dimethoxy-5-(3-methyl-7-(trifluoromethyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)pyrazine (Isomer 2), 60 mg, chiral analysis (Method 3) at 2.30 min.

Step E: (R)-5-(3-Methyl-7-(trifluoromethyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione

2,3-Dimethoxy-5-(3-methyl-7-(trifluoromethyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)pyrazine (Isomer 2) was treated with 2N HCl followed by reverse phase HPLC to yield the title compound as a white solid. (32 mg, 58%) ¹H NMR (400 MHz, DMSO): δ 11.42 (s, 1 H), 11.16-11.15 (m, 1 H), 7.39-7.37 (m, 1 H), 7.26-7.23 (m, 2 H), 6.27 (d, J = 4.5 Hz, 1 H), 4.88 (d, J = 12.2 Hz, 1 H), 4.17 (d, J = 12.0 Hz, 1 H), 1.64 (s, 3 H). MS (ES⁺) m/z 345.1 (M+H)⁺

5-(3-Methyl-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione (**61**-rac): prepared following the same procedure as **62** without the SFC resolution. ¹H NMR (400 MHz, DMSO): δ 11.36-11.36 (m, 1 H), 11.14-

11.14 (m, 1 H), 7.10 (dd, J = 1.5, 7.7 Hz, 1 H), 7.07-7.02 (m, 1 H), 6.94-6.88 (m, 2 H), 6.28 (s, 1 H), 4.80 (d, J = 11.9 Hz, 1 H), 4.08 (d, J = 12.0 Hz, 1 H), 1.60 (s, 3 H). MS (ES+) m/z 277 (M+H)⁺.

(R)-5-(7-Chloro-3-methyl-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione (**63**):

Racemic 5-(7-Chloro-3-methyl-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione was prepared following the same procedure as compound **62**.

Racemic 5-(7-Chloro-3-methyl-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione was resolved by chiral SFC to afford single enantiomers.

(R)-5-(7-Chloro-3-methyl-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione (Isomer 1) (**63**):

¹H NMR (400 MHz, DMSO): δ 11.39 (s, 1 H), 11.16 (s, 1 H), 7.16 (d, J = 8.3 Hz, 1 H), 7.02-6.97 (m, 2 H), 6.26-6.23 (m, 1 H), 4.83 (d, J = 12.0 Hz, 1 H), 4.12 (d, J = 12.0 Hz, 1 H), 1.60 (s, 3 H). MS (ES+) m/z 311 (M+H)⁺.

HPLC method 1, Rt 3.50 min.

(R)-3-(5,6-Dioxo-1,4,5,6-tetrahydropyrazin-2-yl)-3-methyl-2,3-dihydrobenzo[b][1,4]oxathiine-7-carbonitrile (**64**)

Step A: 3-Bromo-4-mercaptobenzonitrile

3-Bromo-4-fluorobenzonitrile (0.75 g, 3.75 mmol) triisopropylsilanethiol (0.81 mL, 3.75 mmol) and potassium carbonate (0.77 g, 5.62 mmol) were dissolved in DMF (30 mL) and stirred at 50 °C for 3 hrs. The reaction mixture was then allowed to cool to room temperature and was then washed with ethyl acetate (30 mL). The aqueous layer was then acidified to pH <3 with 2 M hydrochloric acid and extracted with ethyl acetate (3 × 20 mL). The organics were combined and concentrated in vacuo, then triturated with DCM (10 mL) to give the desired product as a yellow solid (528 mg, 66%). ¹H NMR (400 MHz, CDCl₃): δ 7.80 (t, J = 1.0 Hz, 1 H), 7.44-7.43 (m, 2 H), 4.27 (s, 1 H).

Step B: 3-(5,6-Dimethoxypyrazin-2-yl)-3-methyl-2,3-dihydrobenzo[b][1,4]oxathiine-7-carbonitrile

The title compound was prepared following the same procedure as compound **62** as an orange oil (172 mg, 37% over two steps). ¹H NMR (400 MHz, CDCl₃) δ 7.85 (s, 1 H), 7.16 - 7.14 (m, 2 H), 7.10 (d, J = 1.5 Hz, 1 H), 4.75 (d, J = 11.2 Hz, 1 H), 4.23 (d, J = 11.2 Hz, 1 H), 4.01 (s, 3 H), 4.00 (s, 3 H), 1.76 (s, 3 H).

Step C: 3-(5,6-Dimethoxypyrazin-2-yl)-3-methyl-2,3-dihydrobenzo[b][1,4]oxathiine-7-carbonitrile (Isomers 1 and 2)

Racemic 3-(5,6-dimethoxypyrazin-2-yl)-3-methyl-2,3-dihydrobenzo[b][1,4]oxathiine-7-carbonitrile was resolved by chiral SFC to afford single enantiomers.

3-(5,6-dimethoxypyrazin-2-yl)-3-methyl-2,3-dihydrobenzo[b][1,4]oxathiine-7-carbonitrile (Isomer 1), white solid, (38 mg, 0.21 mmol), chiral analysis (Method 2) at 6.00 min.

3-(5,6-dimethoxypyrazin-2-yl)-3-methyl-2,3-dihydrobenzo[b][1,4]oxathiine-7-carbonitrile (Isomer 2), white solid, (39 mg, 0.21 mmol), chiral analysis (Method 2) at 5.00 min.

Step D: (R)-3-(5,6-Dioxo-1,4,5,6-tetrahydropyrazin-2-yl)-3-methyl-2,3-dihydrobenzo[b][1,4]oxathiine-7-carbonitrile

Treating 3-(5,6-dimethoxypyrazin-2-yl)-3-methyl-2,3-dihydrobenzo[b][1,4]oxathiine-7-carbonitrile (Isomer 1) with 2N HCl provided the product as a colorless solid (3 mg, 0.01 mmol, 6%). ¹H NMR (400 MHz, DMSO): δ 11.38 (s,

1 H), 11.16 (s, 1 H), 7.16 (d, J = 8.4 Hz, 1 H), 7.02-6.97 (m, 2 H), 6.27-6.25 (m, 1 H), 4.83 (d, J = 12.2 Hz, 1 H), 4.12 (d, J = 12.0 Hz, 1 H), 1.60 (s, 3 H). MS (ES+) m/z 295 (M+H)⁺.

(R)-5-(3-Methyl-7-(methylsulfonyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione (**65**)
Racemic 5-(3-Methyl-7-(methylsulfonyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione was prepared following the same procedure as **62**. ¹H NMR (400 MHz, DMSO): δ 11.41 (s, 1 H), 11.18 (s, 1 H), 7.43-7.38 (m, 3 H), 6.30-6.28 (m, 1 H), 4.92-4.87 (m, 1 H), 4.21-4.16 (m, 1 H), 3.21 (s, 3 H), 1.64 (s, 3 H). MS (ES+) m/z 355.0 (M+H)⁺.

Racemic 5-(3-Methyl-7-(methylsulfonyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione was resolved by chiral SFC to afford single enantiomers.

(R)-5-(3-Methyl-7-(methylsulfonyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione (Isomer 1) (**65**): ¹H NMR (400 MHz, DMSO): δ 11.06 (s, 2 H), 7.47 (s, 2 H), 7.43 (s, 1 H), 6.33 (s, 1 H), 4.93 (d, J = 12.1 Hz, 1 H), 4.22 (d, J = 12.1 Hz, 1 H), 3.25 (s, 3 H), 1.68 (s, 3 H), MS (ES+) m/z 355 (M+H)⁺. Chiral analysis (Method 1) at 3.26 min.

(R)-5-(7-(((3-Fluorobenzyl)oxy)methyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione (**66**)
Step A: (R)-5-(7-(((3-Fluorobenzyl)oxy)methyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-2,3-dimethoxypyrazine (R)-(3-(5,6-dimethoxypyrazin-2-yl)-2,3-dihydrobenzo[b][1,4]oxathiin-7-yl)methanol (prepared following steps A-C of compound **58**) (125 mg, 0.39 mmol) was dissolved in THF (3 mL) and cooled to 0 °C. Sodium hydride (60% in mineral oil, 62 mg, 1.56 mmol) was then added and the solution stirred at 0 °C for 15 min. before addition of 3-fluorobenzyl bromide (0.06 mL, 0.47 mmol). The reaction was stirred at 0 °C for 3 hrs before allowing to rise to room temperature and quenching with dropwise addition of water (3 mL). The mixture was extracted with ethyl acetate (3 × 10 mL), combined and washed with brine (1 × 10 mL) and dried over magnesium sulfate before concentrating *in vacuo* and purifying via flash column chromatography (0 – 30% ethyl acetate in cyclohexane) to give the desired product as a yellow oil, (74 mg, 55%). ¹H NMR (400 MHz, CDCl₃): δ 7.74 (s, 1 H), 7.34-7.27 (m, 1 H), 7.13-7.06 (m, 3 H), 7.01-6.94 (m, 1 H), 6.91-6.88 (m, 2 H), 4.62 (dd, J = 2.5, 10.9 Hz, 1 H), 4.57-4.47 (m, 6 H), 4.01 (s, 3 H), 3.99 (s, 3 H).

Step B: (R)-5-(7-(((3-Fluorobenzyl)oxy)methyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-1,4-dihydropyrazine-2,3-dione

(R)-5-(7-(((3-Fluorobenzyl)oxy)methyl)-2,3-dihydrobenzo[b][1,4]oxathiin-3-yl)-2,3-dimethoxypyrazine (60 mg, 0.14 mmol) was treated with NaI (76 mg, 0.7 mmol) and TMSCl (105 mg, 0.7 mmol) at 60 °C for 45 min. The mixture was concentrated under reduced pressure and purified by reverse phase HPLC to yield the title compound as a white solid (18 mg, 32%). ¹H NMR (400 MHz, DMSO): δ 11.35-11.18 (m, 2 H), 7.45-7.39 (m, 1 H), 7.22-7.10 (m, 4 H), 6.95-6.89 (m, 2 H), 6.28 (s, 1 H), 4.55-4.45 (m, 6 H), 4.33 (dd, J = 2.3, 5.9 Hz, 1 H), MS (ES+) m/z 401 (M+H)⁺.

1-Hydroxy-5-phenethylpyrimidine-2,4(1H,3H)-dione (**13**)

Step A: 1-(benzyloxy)pyrimidine-2,4(1H,3H)-dione

To a solution of benzyloxy urea (3.00 g, 18.05 mmol) and methyl 3,3-demethoxy propionate (2.60 ml, 18.05 mmol) in DMSO (15 mL) was added sodium hydride 60% (0.65 g, 18.05 mmol). The reaction was heated to 65 °C for 2 hours before allowing to cool and neutralising with 2M HCl, the resultant precipitate was collected via filtration and further drying to afford 1-(benzyloxy)pyrimidine-2,4(1H,3H)-dione (1.50 g, 38%).

¹H NMR (400 MHz, DMSO) δ 11.55 (s, 1H), 7.85 (d, J=8.2 Hz, 1H), 7.57 - 7.47 (m, 5H), 5.47 (d, J=8.6 Hz, 1H), 5.17 (s, 2H).

Step B: 3-Benzyl-1-(benzyloxy)pyrimidine-2,4(1H,3H)-dione

To a solution of 1-(benzyloxy)pyrimidine-2,4(1H,3H)-dione (2.00 g, 9.17 mmol) and potassium carbonate (1.52 g, 11.00 mmol) in DMF (20 mL) was added benzyl bromide (1.1 mL, 9.17 mmol) and the reaction heated to 80 ° for 1 hour. The reaction was then allowed to cool to room temperature, followed by addition of water (40 mL) resulting in precipitation of a colourless solid. The solid was isolated via filtration to afford 3-benzyl-1-(benzyloxy)pyrimidine-2,4(1H,3H)-dione (2.51 g, 89%) as a colourless solid.

¹H NMR (400 MHz, CDCl₃) δ 7.49 (d, J=6.7 Hz, 2H), 7.42 - 7.26 (m, 8H), 6.99 (d, J=8.4 Hz, 1H), 5.15 (s, 2H), 5.12 (s, 2H).

Step C: 3-Benzyl-1-(benzyloxy)-2,4-dioxo-1,2,3,4-tetrahydropyrimidine-5-carbaldehyde

To a solution of 3-benzyl-1-(benzyloxy)pyrimidine-2,4(1H,3H)-dione (1.00 g, 3.24 mmol) in TFA (10.00 mL) was added Hexamethylenetetramine (0.50 g, 3.57 mmol) and the reaction mixture heated to reflux for 6 hours. The reaction was then allowed to cool and poured of 2N HCl and stirred for a further 30 mins. The organics were then extracted with DCM (3 × 15 mL) before combining and washing with 2N HCl (10 mL) and drying over MgSO₄, concentrated *in vacuo* and purified by flash column chromatography (10 – 60% ethyl acetate in cyclohexane) to afford 3-benzyl-1-(benzyloxy)-2,4-dioxo-1,2,3,4-tetrahydropyrimidine-5-carbaldehyde (0.37 g, 36%) as a colorless solid.

¹H NMR (400 MHz, CDCl₃) δ 9.90 (s, 1H), 7.86 (s, 1H), 7.49 (d, J=6.6 Hz, 2H), 7.42 - 7.31 (m, 8H), 5.22 (s, 2H), 5.16 (s, 2H).

Step D: (E)-3-Benzyl-1-(benzyloxy)-5-styrylpyrimidine-2,4(1H,3H)-dione

To a solution of benzyltriphenylphosphonium bromide (322 mg, 0.74 mmol) in THF (5 mL) was added sodium hydride 60% (45 mg, 1.11 mmol) and the solution stirred for 20 minutes. A solution of 3-benzyl-1-(benzyloxy)-2,4-dioxo-1,2,3,4-tetrahydropyrimidine-5-carbaldehyde (250 mg, 0.74 mmol) in THF (5 mL) was then added the reaction refluxed. The reaction was cooled, and the precipitate removed via filtration. The filtrate was concentrated *in vacuo* and purified by flash column chromatography (10 – 60% ethyl acetate in cyclohexane) to afford (E)-3-benzyl-1-(benzyloxy)-5-styrylpyrimidine-2,4(1H,3H)-dione (55 mg, 17%) as a pale yellow solid.

¹H NMR (400 MHz, CDCl₃) δ 7.50 (d, J=7.2 Hz, 2H), 7.36 - 7.18 (m, 15H), 7.04 (s, 1H), 6.65 (d, J=13.3 Hz, 1H), 6.32 (d, J=11.7 Hz, 1H), 5.16 (s, 2H), 4.92 (s, 2H).

Step E: 1-Hydroxy-5-phenethylpyrimidine-2,4(1H,3H)-dione (**13**)

(E)-3-Benzyl-1-(benzyloxy)-5-styrylpyrimidine-2,4(1H,3H)-dione was dissolved in methanol (conc. < 0.05 M), and Hydrogenated over Pd/C cat cart using a H-Cube (flow 1 mLmin⁻¹). The resulting solution was then concentrated

under reduced pressure and suspended in *m*-Xylene and transferred to a pressure tube. Boron tribromide (1M DCM, 0.5 mL) was then added and the reaction heated to reflux for 1 hour. The reaction mixture was allowed to cool before careful quenching with methanol and concentrating *in vacuo* and purified by preparative HPLC to afford the title compound (5.6 mg, 21%)

¹H NMR (400 MHz, DMSO) δ 9.23 - 9.23 (m, 2H), 7.59 (s, 1H), 7.30 - 7.25 (m, 2H), 7.20 - 7.15 (m, 3H).

MS (ES+) *m/z* 233.1 (M+H)⁺.

5-(4-Chlorophenethyl)-1-hydroxypyrimidine-2,4(1H,3H)-dione (**16**)

Step A: (E)-3-Benzyl-1-(benzyloxy)-5-(4-chlorostyryl)pyrimidine-2,4(1H,3H)-dione

To a solution of (4-chlorobenzyl)triphenylphosphonium bromide (287 mg, 0.60 mmol) in THF (5 mL) was added sodium hydride 60% (26 mg, 0.89 mmol) and the solution stirred for 20 minutes. A solution of 3-benzyl-1-(benzyloxy)-2,4-dioxo-1,2,3,4-tetrahydropyrimidine-5-carbaldehyde (250 mg, 0.60 mmol) in THF (5 mL) was then added the reaction refluxed. The reaction was cooled and precipitate removed via filtration. The filtrate was concentrated *in vacuo* and purified by flash column chromatography (10 – 60% ethyl acetate in cyclohexane) to afford (E)-3-benzyl-1-(benzyloxy)-5-(4-chlorostyryl)pyrimidine-2,4(1H,3H)-dione (143 mg, 54%) as a pale yellow solid.

¹H NMR (400 MHz, CDCl₃) δ 7.50 (dd, *J*=1.3, 8.0 Hz, 2H), 7.38 - 7.29 (m, 7H), 7.26 - 7.24 (m, 5H), 7.04 (d, *J*=0.4 Hz, 1H), 6.56 (d, *J*=12.4 Hz, 1H), 6.34 (dd, *J*=0.8, 12.2 Hz, 1H), 5.16 (s, 2H), 4.97 (s, 2H).

Step B: 5-(4-Chlorophenethyl)-1-hydroxypyrimidine-2,4(1H,3H)-dione

(E)-3-Benzyl-1-(benzyloxy)-5-(4-chlorostyryl)pyrimidine-2,4(1H,3H)-dione (143 mg, 0.31 mmol) was dissolved in methanol (conc. < 0.05 M), and Hydrogenated over Pd/C cat cart using a H-Cube (flow 1 mLmin⁻¹). The resulting solution was then concentrated under reduced pressure and suspended in *m*-xylene and transferred to a pressure tube. Boron tribromide (1M DCM, 0.5 mL) was then added and the reaction heated to reflux for 1 hour. The reaction mixture was allowed to cool before careful quenching with methanol and concentrating *in vacuo* and purifying by preparative HPLC to afford the title compound (11 mg, 6%) as an off-white amorphous solid.

¹H NMR (400 MHz, DMSO) δ , 7.62 (s, 1H), 7.34 (d, *J*=8.7 Hz, 2H), 7.22 (d, *J*=8.5 Hz, 2H), 2.75 (t, *J*=8.0 Hz, 2H), 2.46 (t, *J*=8.3 Hz, 2H). MS (ES+) *m/z* 267.2 (M+H)⁺.

ASSOCIATED CONTENT

Supporting Information

- 1) Macro-PK protocol
- 2) Computation of tautomer distributions
- 3) hDAO co-crystallization conditions with data collection and refinement statistics with **12** and **59**
- 4) H-NMR and LC-MS spectra of key compounds **37** and **42**.
- 5) H-NMR of other final compounds
- 6) All compounds with prospective FEP+ hDAO potency and experimental biochemical potency
- 7) Molecular formula strings

Access Codes

Coordinates and structure factors for hDAO complexes with compounds **12** and **59** will be submitted to the PDB.

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ABBREVIATIONS USED

DAO D-amino Acid Oxidase; FAD flavin adenine dinucleotide; NMDA N-methyl D-aspartate; FEP free energy perturbation; Papp apparent permeability; ER efflux ratio; QM quantum mechanics; SAR structure-activity relationship; HRP horseradish peroxidase; PK pharmacokinetics; PD pharmacodynamics; MPO multiparameter optimization; CNS MPO central nervous system multiparameter optimization; LE ligand efficiency; LLE lipophilic ligand efficiency; MDR1 multidrug resistance protein 1; MDCK Madin-Darby canine kidney; CSF cerebrospinal fluid

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¹⁶ Compound **14** was previously disclosed in WO1993008197A1 as an antagonist at the glycine-binding site on the NMDA receptor complex.

¹⁷ To track performance of the hDAO FEP+ model, the predicted hDAO IC₅₀'s are also included in the table. Predictions with over 1 log difference from the experimental value are colored in yellow, and those with 2 logs difference are colored in red.

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¹⁹ The PK data of **17** is consistent with that disclosed in *Neurochemical Research*, **2017**, *42*(11), 3279-3288.

²⁰ The pKa value was prospectively predicted to be 9.5 by the Schrödinger Macro-pKa protocol based on quantum mechanics energy calculations. See supplementary information for detailed description of the protocol and validation data. The protocol has been used on the program to predict pKa of design ideas.

²¹ Compound **11** has a pKa of 6.4 as predicted by the Schrödinger Macro-pKa protocol.

²² No synthesis was attempted on **33** based on predicted FEP+ potency.

²³ The compound could not be resolved due to technical difficulties, so it was tested as a racemate.

²⁴ FEP+ performance was sufficient to drive synthesis decisions. The mean unsigned error (MUE) of all predictions to date is 0.7 log.