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Subtle Modifications to a Thieno[2,3-*d*]pyrimidine Scaffold Yield Negative Allosteric Modulators and Agonists of the Dopamine D_2 Receptor

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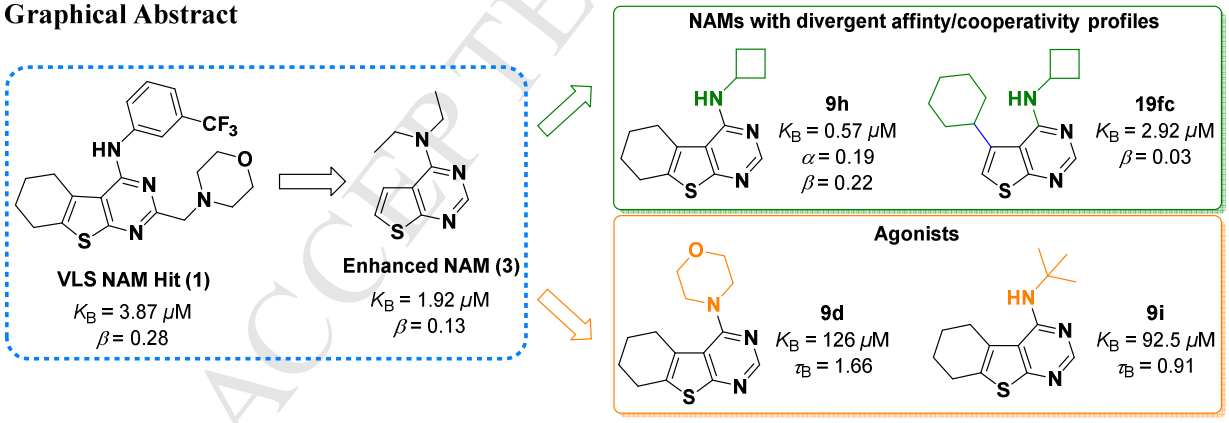
Abstract: We recently described a structurally novel series of negative allosteric modulators (NAMs) of the dopamine D_2 receptor (D_2R) based on thieno[2,3-*d*]pyrimidine **1**, showing it can be structurally simplified to reveal low molecular weight, fragment-like NAMs that retain robust negative cooperativity, such as **3**. Herein, we report the synthesis and functional profiling of analogues of **3**, placing specific emphasis on examining secondary and tertiary amino substituents at the 4-position, combined with a range of substituents at the 5/6-positions (e.g. aromatic/aliphatic carbocycles). We identify analogues with diverse pharmacology at the D_2R including NAMs (**19fc**) with sub-μM affinity (**9h**) and, surprisingly, low efficacy partial agonists (**9d** and **9i**).

Graphical Abstract



Keywords: negative allosteric modulators, dopamine D_2 receptor, thieno[2,3-*d*]pyrimidines, NAMs, agonists.

1. Introduction

The D_2R is a class A G protein-coupled receptor (GPCR) implicated in the pathophysiology and treatment of a number of central nervous system (CNS) disorders, including schizophrenia (SCZ).

As such, drugs targeting this receptor have traditionally targeted the orthosteric site - where the endogenous ligand DA binds. Clinically used antipsychotic drugs (APDs) targeting this site act as D_2R antagonists or low efficacy partial agonists, and are termed typical or atypical APDs based on their propensity to cause extrapyramidal side effects. Unfortunately, the efficacy of these drugs is largely limited to the positive symptoms of this disorder. Typical APDs are associated with extra-pyramidal motor symptoms (EPS) and hyperprolactinaemia, which are mediated by blockade of D_2R signalling in the nigrostriatal and tuberoinfundibular DA pathways, respectively.[1-6] Atypical APDs show a reduced incidence of EPS, but display other off-target side-effects mediated through the interaction with other aminergic receptors, including metabolic disorders and weight gain.[7]

Alternative approaches to target the D_2R have emerged *via* the identification of homo- and heterodimeric complexes.[8] Such complexes in specific tissues may provide novel pharmacological targets for compounds with distinctive functional profiles and improved therapeutic windows.[9-11] Another proposition which entails targeting binding sites topographically distinct to the orthosteric site of GPCRs might also be advantageous. Negative allosteric modulators (NAMs) of the D_2R may represent a safer therapeutic approach for the treatment of SCZ symptoms. A D_2R NAM with limited negative cooperativity with DA may display antipsychotic efficacy but avoid EPS through partial blockade of the D_2R akin to the action of atypical partial agonist APDs such as aripiprazole.[12] Furthermore, as a NAM will allow DA to bind, the spatiotemporal pattern of DA signalling is more likely to be maintained. Finally, NAMs may display greater selectivity for the D_2R over other targets via the targeting of less evolutionarily conserved sites and consequently reduced off target toxicities. Drug-like (NAMs) of DA receptors have recently been identified including the scaffold that is the focus of the present study.[13-19]

Our previous study described the pharmacological validation of a virtual ligand screen hit (**1**, Figure 1), confirming that it binds with low μM functional affinity and exerts its effect via negative allosteric cooperativity, primarily by modulation of dopamine signalling efficacy.[18] Based on the scaffold of **1**, we synthesised a small library of structural analogues to further understand key

molecular features that were responsible for changes in affinity and negative allosteric cooperativity. Of several promising analogues identified, **2** (Figure 1) maintained low μM affinity (K_B = 1.92 μM) and significantly attenuated DA signalling efficacy (β = 0.001). This compound was devoid of the morpholinomethyl moiety present on **1**, and also incorporated replacement of the ((3-trifluoromethyl)phenyl)amino- substituent with an *N*,*N*-diethylamino motif. In addition to this, our previous work focused on assessing various substituents at the 5/6-positions (e.g. phenyl, cyclohexyl) in place of the fused cyclohexane system. Moreover, we discovered that the scaffold could be structurally simplified, to reveal a low molecular weight fragment-like starting point that maintained μM affinity and negative cooperativity (MW = 207, K_B = 4.56 μM, β = 0.13) (**3**, Figure 1). Thus, we identified an appropriate starting point for further structural interrogation and elaboration of the core scaffold using **2** and **3** as our lead compounds with the aim to identify higher affinity NAMs.

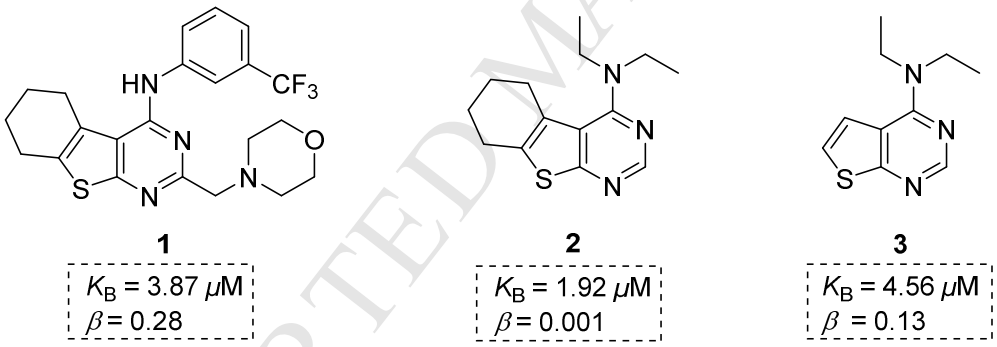


Fig. 1. Promising structural analogues of 1 arising from a preliminary structure-activity relationship (SAR) investigation. Compound **1** was identified from a virtual ligand screen and formed the basis of our previous SAR study. Compound **2** significantly attenuates DA signalling efficacy at a concentration of 10 μM (K_B = 1.92 μM, β = 0.001). Compound **3** retains negative allosteric cooperativity despite being a structurally simplified fragment-like analogue (MW: 207, K_B = 4.56 μM, β = 0.13) of **1**.

To this end, we sought to investigate the influence of varying the nature (both aliphatic and aromatic) of the 4-amino moiety of **2** (series 1, Figure 2). In addition, a second series of compounds was designed to further explore the effect of 5/6-thiophene substitution with respect to altering

functionality at the 4-position (series 2, Figure 2). This would permit further refinement of the structural determinants of D_2R affinity and negative cooperativity. Through the parallel synthesis of an additional thirty-seven structural analogues of **2**, we have identified molecules that exert a range of modulatory behaviour including, surprisingly, derivatives that display agonism. To confirm a D_2R-mediated mechanism of action, all compounds with agonist profiles together with selected NAMs were assessed for their ability to modulate our functional readout in the absence of the D_2R.

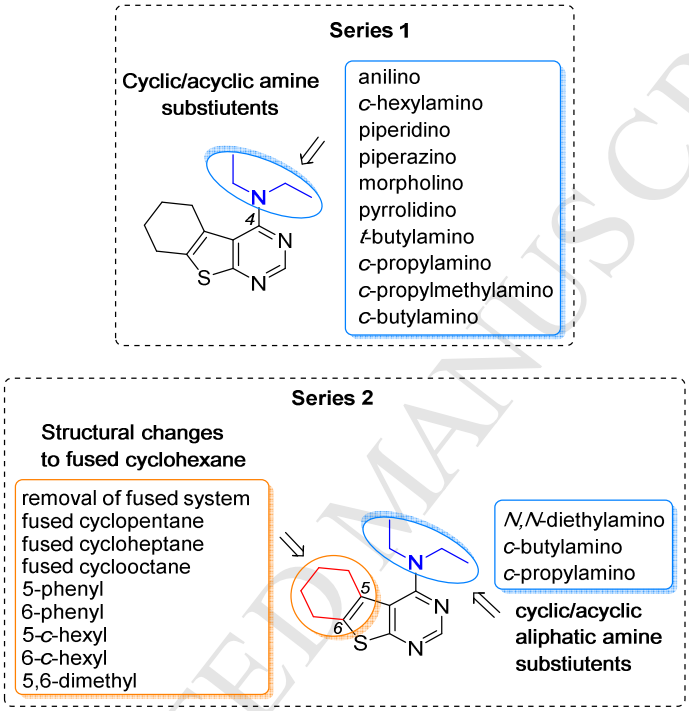


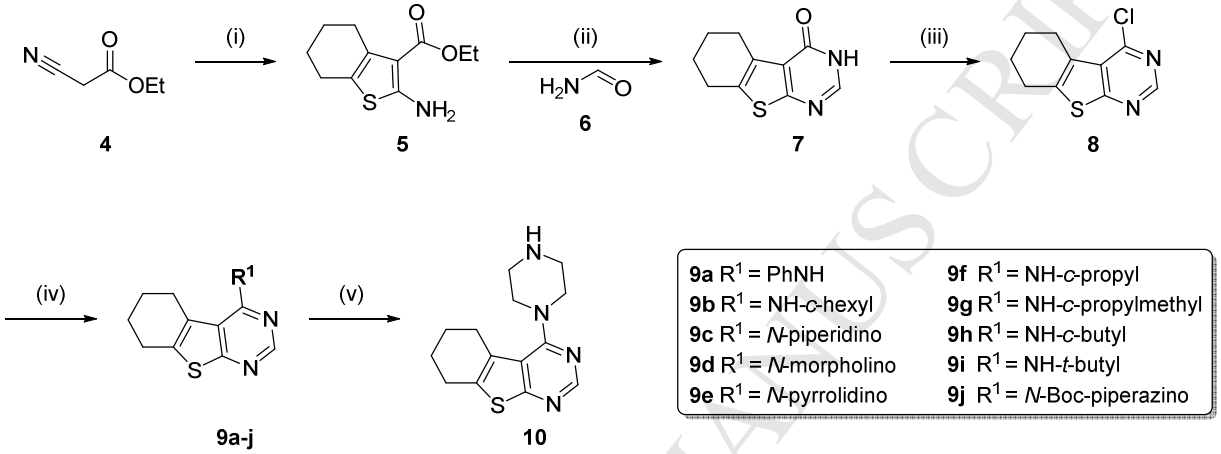
Fig. 2. Areas of structural modification conducted during SAR investigation of 2.

2. Chemistry

The synthesis of all compounds generally followed established methods for the synthesis of **1** and other related compounds previously reported by our research group.[18] Each compound detailed in this study was easily accessed in four steps (schemes 1-3). Briefly, Scheme 1 depicts the synthesis of selected analogues (**9a-j**), beginning with Gewald[20] chemistry to facilitate the one-pot construction of the ethyl 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (**5**),[18] followed by cyclisation with formamide (**6**) to afford the corresponding 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidinone (**7**).[18] $POCl_3$ was employed to convert pyrimidinone

7 to the corresponding key intermediate chloropyrimidine **8**,[18] followed by nucleophilic aromatic substitution (S_NAr) with the desired amine, in the presence of Hünig's base in propan-2-ol. This methodology afforded compounds **9a-j** in high purity after a simple work-up and/or chromatography.

Scheme 1. Synthesis of analogues of **2** modified at the 4-position[a]



[a]Reagents and conditions: (i) S_8, cyclohexanone, morpholine, rt, ~18 h, 81%; (ii) 170 °C, 12 h, 85%; (iii) $POCl_3$, $DMF_{(cat.)}$, toluene, 4 h, 68%; (iv) amine, DIPEA, *i*-PrOH, reflux, 1-3 h, 68-88%; (v) **9j**, TFA, DCM, rt, 4 h, 96%.

*2.1. Analogues of **2** varied at the 4-(N,N-diethylamino) substituent*

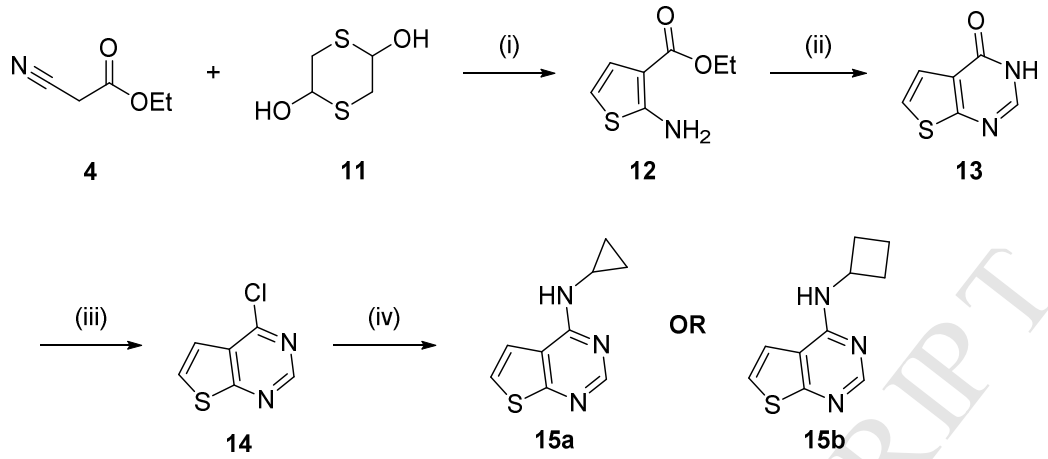
Compound **2** was originally synthesised as an analogue of **1** to observe the effect of simultaneously removing the morpholinomethyl moiety whilst incorporating a 4-(*N*,*N*-diethylamino) group in in the context of retaining the tricyclic structure of the 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidine. As this molecule was found to be a potent NAM of DA efficacy at the D_2R, an additional series of compounds was synthesised to investigate further changes to the 4-position of the pyrimidine ring (Scheme 1). Preliminary modifications included incorporating 6-membered secondary aromatic and aliphatic amino substituents (e.g. anilino (**9a**) and cyclohexylamino (**9b**) together with 6-membered cyclic aliphatic tertiary amine moieties, including piperidino (**9c**), morpholino (**9d**) and piperazino (**10**), followed by the smaller pyrrolidino (**9e**) to assess the influence of a five-membered cyclic aliphatic system. The piperazino analogue **10** was obtained via

the S_NAr reaction between commercially available *N*-Boc piperazine and **8** under microwave conditions as outlined previously (Scheme 1) to afford **9j** as a crystalline solid requiring no further purification. Standard trifluoroacetic acid-mediated *N*-Boc de-protection followed by an alkaline work-up furnished **10**. This molecule is unique relative to its counterparts in that it will impart a positive charge at physiological pH. Moreover, we investigated both monocyclic (cyclopropylamino (**9f**), cyclopropylmethylamino (**9g**), cyclobutylamino (**9h**)) and acylic aliphatic secondary amine substituent (*tert*-butylamino (**9i**)). The *tert*-butylamino substituent was installed to assess the effect of an acylic aliphatic secondary amine as a comparison to *N*,*N*-diethylamino. These syntheses were all achieved using chemistry as previously outlined (Scheme 1), and successfully afforded ten analogues (**9a-i**, and **10**) in yields varying from 68-88% following S_NAr of **8** with the appropriate amine.

*2.2. Fragment analogues of **3***

We previously reported a low molecular weight fragment (*N*,*N*-diethylthieno[2,3-*d*]pyrimidin-4-amine, (**3**)) that retains low μM functional binding affinity and negative allosteric cooperativity at the D_2R (K_B = 4.56 μM, β = 0.13, Figure 1).[18] This molecule was originally synthesised using a deletion strategy to examine the effect of switching from a 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidine to the corresponding thieno[2,3-*d*]pyrimidine scaffold, in conjunction with incorporating a tertiary aliphatic amine at the 4-position. As such, we generated two additional analogues of **3** bearing 4-cyclopropylamino and 4-cyclobutylamino substituents. Their synthesis is depicted in Scheme 2 and begins with the construction of ethyl 2-aminothiophene-3-carboxylate (**12**)[18] using ethyl cyanoacetate (**4**) and 1,4-dithiane-2,5-diol (**11**) under modified Gewald conditions. Refluxing **12** in neat formamide afforded the corresponding pyrimidinone (**13**)[18] in good yield. Subsequent chemistry as outlined previously included deoxychlorination to give **14**,[18] followed by S_NAr with cyclopropylamine or cyclobutylamine to afford the corresponding analogues **15a** and **15b**, respectively.

Scheme 2. Synthesis of Thieno[2,3-*d*]pyrimidine Analogues of **2**[a]



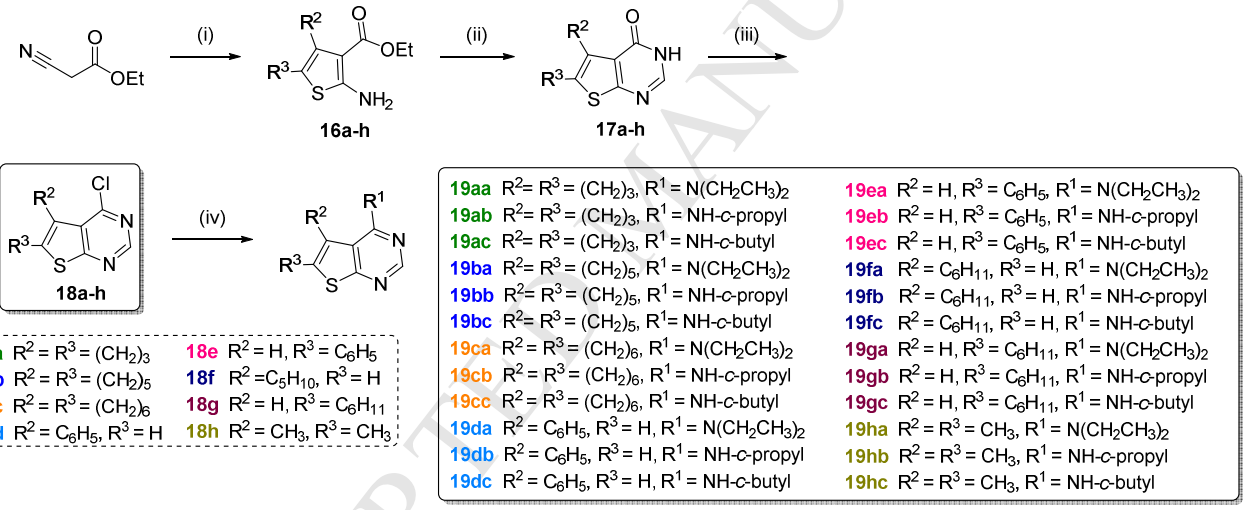
[a]Reagents and conditions: (i) Et_3N, DMF, 45 °C, 0.5 h, 55%; (ii) formamide (neat), 170 °C, 12 h, 76%; (iii) $POCl_3$, $DMF_{(cat.)}$, toluene, 100 °C 4 h, 77%; (iv) amine, DIPEA, *i*-PrOH, 100 °C, 1-3 h, 76% (**15a**), 79% (**15b**).

*2.3. Single modifications to the 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine moiety of **2** whilst varying the nature of the 4-substituent*

Our previously reported SAR study of **1** focused on structural modifications to the fused cyclohexane system, including switching from a 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidine to the corresponding thieno[2,3-*d*]pyrimidine, 5- and 6-aryl substitution, 5- and 6-cyclohexyl substitution, 5,6-dimethyl substitution, as well as incorporation of fused cyclopentane, cycloheptane, and cyclooctane systems.[18] These structural modifications were mostly employed in the presence of a 3-(trifluoromethyl)anilino substituent at the 4-position, and a 2-morpholinomethyl substituent. Only the fused cyclopentane and the 6-cyclohexyl modifications preserved the allosteric pharmacology seen with this scaffold, while the other structural modifications rendered the corresponding molecule functionally inactive. This study, however, had demonstrated the encouraging effects of incorporating *N,N*-diethylamino, cyclopropylamino, or cyclobutylamino to the 4-position of the pyrimidine core, increasing functional affinity as well as maintaining the magnitude of negative allosteric cooperativity whilst bearing the fused cyclohexane system (Series 1, Figure 2). To this end, we wanted to evaluate combining the structural modifications to the

thiophene, whilst concurrently incorporating the aforementioned 4-amino substituents (Series 2, Figure 2). These compounds would further refine the SAR and provide insight into the design of further high affinity NAMs. These modifications are depicted in Figure 2 and their chemical synthesis is outlined in **Scheme 3**. All compounds were accessed using established chemistry as detailed in our previous work, beginning with construction of the appropriate thiophene via Gewald[20] chemistry (**16a-h**),[18] formation of the corresponding pyrmidinones (**17a-h**)[18] and chloropyrimidines (**18a-h**),[18] followed by S_NAr with the appropriate amine to furnish a further twenty-four analogues (**19aa-hc**).

Scheme 3. Synthesis of Functionalised Analogues of **2**[a]



[a]Reagents and conditions: (i) aldehyde or ketone, S_8, morpholine, rt 12-24 h, 38-84%, **16a-h**; (ii) formamide, 170 °C, 12 h, 75-85%, **17a-h**; (iii) $POCl_3$, $DMF_{(cat.)}$, toluene, 100 °C, 3-5 h, 77-91%, **18a-h**; (iv) amine, DIPEA, *i*-PrOH, MWI, 1-3 h, 71-93%, **19aa-hc**.

3. Pharmacology

*3.1. Functional and off-target analyses of analogues of **2** using a BRET biosensor for cAMP*

To measure the effect of these compounds upon the functional response of the D_2R when stimulated by DA we made use of a BRET biosensor of cAMP. This assay measures inhibition of forskolin-stimulated cAMP accumulation through activation of the long isoform of the human D_2R ($hD_{2L}R$)

stably expressed in FlpIn CHO cells and provides a robust measurement of D_2R $G\alpha_{i/o}$ activation to allow estimates of functional binding affinities and quantify the magnitude of modulatory effects.[18] Application of an operational model of allostery to the DA concentration-response data obtained in the presence and absence of increasing concentrations of our test compounds yielded an estimate of their affinity for the unoccupied receptor (K_B), cooperativity with DA affinity (α) and modulation of DA efficacy (β), plus the intrinsic efficacy of the allosteric ligand (τ_B).[21] Values of α or $\beta < 1$ signify negative modulatory effects with DA, and values of α or $\beta > 1$ signify positive modulatory effects. Logarithms of affinity and cooperativity values are normally distributed, whereas the absolute values (antilogarithms) are not.[22] Thus, all interpretation of the SAR described below (Tables 1-3) refer to the logarithmic values. For ease of interpretation, however, the allosteric parameter antilogarithms are also highlighted in the main text for selected key analogues. As reported previously, the lead compound **2** acted as a NAM at the D_2R, with low μM functional affinity (K_B = 1.92 μM, Table 1).[18] The depression in the DA dose-response curve caused by increasing concentrations of **2** is characteristic of the action of a NAM of agonist efficacy and β was fixed to 0.001 when fitting these data to signify high negative cooperativity (Figure 3A, Table 1).

To our surprise, we identified molecules based on this NAM scaffold that now appear to display agonism. It was important, then, to confirm that these effects are D_2R mediated. Luciferase assays may identify false positive 'hits' through a variety of mechanisms, for example, by inhibition of the luciferase enzyme.[23-25] To allow us to discriminate between D_2R-mediated activities and any non-specific effects (Supplementary Figure 1), all compounds observed to display D_2R agonism in our cAMP assay were selected for further analysis (**9c-d**, **9i**, **15a-b**, **19ac**, **19ca**, **19da**, **19db**, **19dc**, **19ec**, and **19ha**). In addition, a number of other compounds were selected, namely the parent NAMs **1** and **2**, together with NAMs **9h**, **19bb**, **19fb**, and **19fc**. We measured their ability to modulate the BRET signal in FlpIn CHO cells transfected with the CAMYEL biosensor,[26] but not expressing the D_2R. All compounds were measured up to a concentration of 100 μM in both the presence and absence of forskolin (10 μM). DA (10 μM) was also used as a control. Two compounds (**9c**, **19ca**)

were found to display effects independent of any action at the D_2R. Importantly, the original VLS hit (**1**), representative NAMs (**2**, **9h**, **19bb**, **19fb**, and **19fc**), as well as all other agonists exert an effect dependent on the presence of the D_2R.

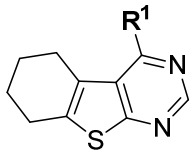
4. Results and discussion

*4.1. Functional analysis of 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine analogues of **2***

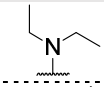
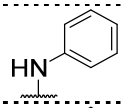
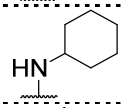
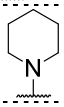
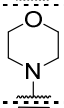
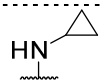
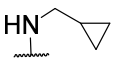
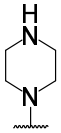
We first examined the effect of introducing various monocyclic/acyclic aliphatic amines to the 4-position of the core scaffold whilst still bearing the fused cyclohexane system. This was based on our finding that **2**, bearing a 4-(*N*,*N*-diethylamino) substituent, displayed high negative cooperativity. As outlined in Table 1, anilino (**9a**) and cyclohexylamino (**9b**) substituents rendered the corresponding analogues functionally inactive. Substitution with the smaller cyclopropylamino substituent (**9f**) resulted in a modest increase in functional affinity (K_B = 0.88 μM) and maintenance of a negative modulatory effect on DA efficacy (β = 0.03, Figure 3C). The cyclopropylmethylamino analogue (**9g)** maintained a functional affinity (K_B = 1.19 μM) similar to that of **2**, but more modest modulatory effects upon dopamine efficacy (β = 0.29). Introduction of a cyclobutylamino substituent (**9h**) not only maintained this negative modulatory action, but acted to increase affinity (K_B = 0.57 μM, α = 0.19, β = 0.22, Figure 3D). Conversely, the *tert*-butylamino analogue (**9i**) lost ~50-fold functional affinity relative to **2** (K_B = 92.5 μM), but now, surprisingly, exerted agonism (τ_B = 0.91). It is interesting to note that introduction of the *tert*-butylamino moiety (**9i**) abolished negative cooperativity, whereas the *N*,*N*-diethylamino substituent (**2**) confers a high degree of negative allosteric modulatory effects upon dopamine efficacy. These data demonstrate that the ring size and nature of the substituent at the 4-position are crucial for both affinity and negative modulatory action. We found that larger 6-membered cyclic substituents bearing secondary amines, both of aromatic and aliphatic nature, are not tolerated, whereas three- and four-membered cyclic substituents (cyclopropylamino and cyclobutylamino, respectively) increase affinity and maintain modulatory properties in conjunction with the fused cyclohexane system. We next examined the

effect of substituents bearing tertiary amines by incorporating various cyclic amines. The piperidino analogue (**9c**) decreased the functional affinity by 10-fold (K_B = 59.3 μM), and was devoid of any modulatory effect on DA but, surprisingly, appeared to exert an intrinsic response in its own right. However, our assay using FlpIn CHO cells that do not express the D_2R revealed that this is effect is most likely an off target effect (Supplementary Figure 1). The morpholino analogue (**9d**) further decreased the functional affinity, some ~65-fold (K_B = 126 μM), but also displayed agonism (τ_B = 1.66) with negligible modulatory action on DA an effect that required the expression of the D_2R (Figure 3B). Interestingly, isosteric replacement of the morpholine O with NH to give the piperazino analogue (**10**), significantly changed the pharmacology as concentrations of **10** caused no decrease in DA maximal response, whilst acting to cause a limitless rightward shift in the DA dose-response curve. Such a pattern could be consistent with either very high negative cooperativity with DA affinity, or conversely, a competitive mode of action. Accordingly, these data could also be fit with a model of competitive antagonism (pA_2 = 4.67 ± 0.09, Schild slope: 1.06 ± 0.12, Figure 3E). If this molecule is indeed competitive with DA we speculate that the presence of the piperazino ionisable nitrogen atom at physiological pH may potentially confer greater affinity for the orthosteric binding pocket, consequently converting the pharmacology from allosteric to competitive. Decreasing the ring size by one carbon relative to **9c** to give pyrrolidine analogue (**9e**), maintained functional affinity (K_B = 4.23 μM), and displayed robust negative allosteric modulatory effects upon DA efficacy (β = 0.17).

Table 1 Functional parameters derived from cAMP BRET assay for analogues of **1** modified with various amines at the 4-position



2, 9a-i, 10

#	R^1	pK_B (K_B, μM)[a]	Logτ_B (τ_B)[b]	Log α (α)[c]	Log β (β)[d]
2		5.72 ± 0.13 (1.92)	= -3.0	-	= -3.0
9a				nd	
9b				nd	
9c		4.23 ± 0.13 (59.3)	NSE	= 0	= 0
9d		3.90 ± 0.27 (126)	-0.22 ± 0.15 (1.66)	= 0	= 0
9e		5.37 ± 0.13 (4.23)		= 0	-0.76 ± 0.06 (0.17)
9f		6.05 ± 0.09 (0.88)		= 0	-1.48 ± 0.08 (0.03)
9g		5.92 ± 0.19 (1.19)		-0.06 ± 0.14 (0.86)	-0.54 ± 0.08 (0.29)
9h		6.25 ± 0.12 (0.57)		-0.72 ± 0.12 (0.19)	-0.65 ± 0.07 (0.22)
9i		4.03 ± 0.31 (92.5)	-0.04 ± 0.15 (0.91)	= 0	= 0
10		4.70 ± 0.09 (20.0)		= -3.0	= 0

[a]Estimate of the negative logarithm of the equilibrium dissociation constant as determined in an cAMP functional assay. [b]Estimate of the logarithm of the net cooperativity factor between the modulator and dopamine as determined in an cAMP functional assay. [c] Estimate of the intrinsic efficacy of the modulator. nd = inactive at concentrations up to 100 μM. NSE, non-specific effect, compound determined to inhibit forskolin-stimulated cAMP accumulation in the absence of the D_2R. Values represent the mean ± S.E.M. from at least three independent experiments performed in duplicate.

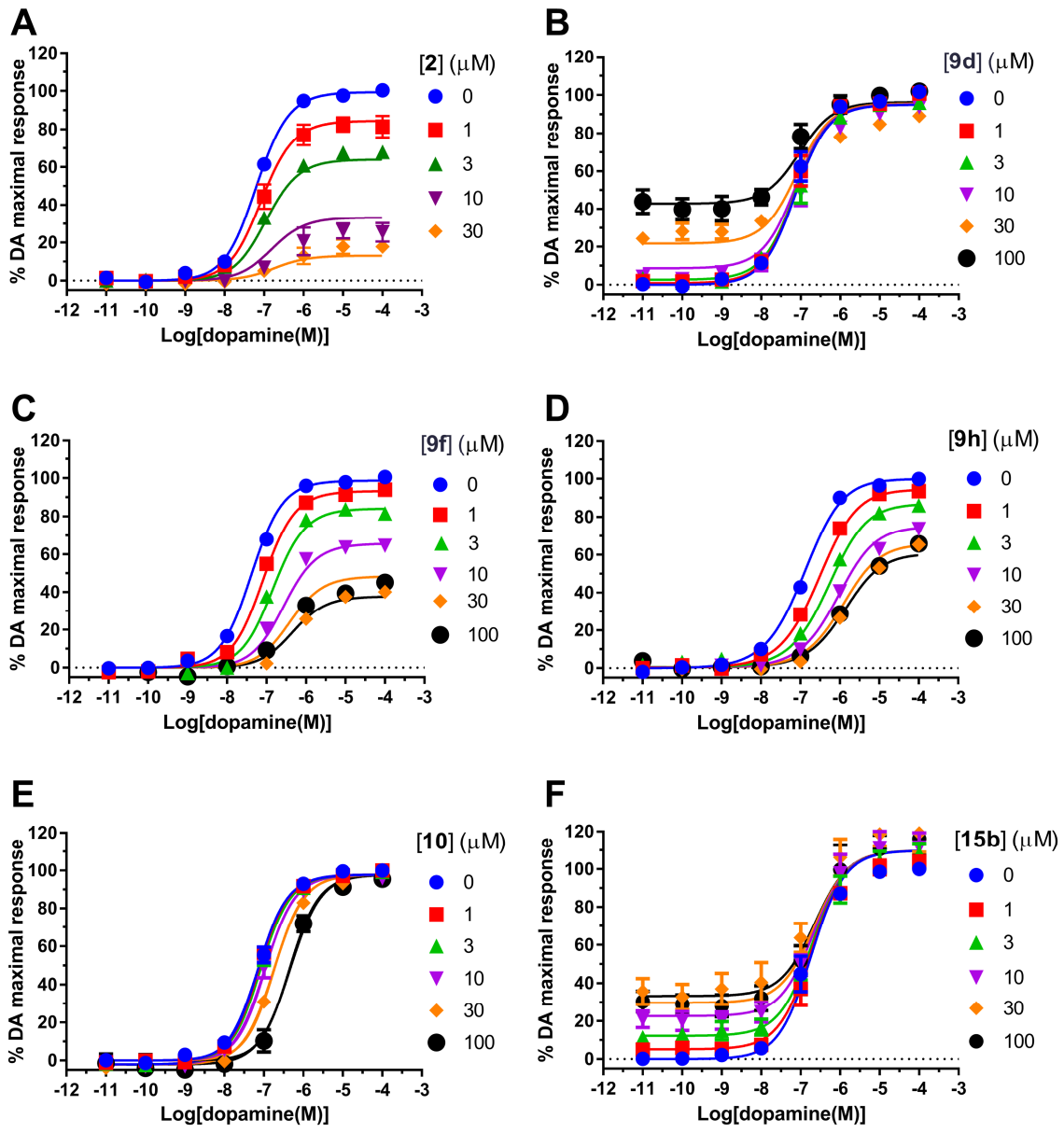
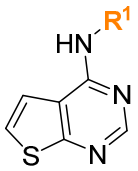


Fig. 3. Modification to the thieno[2,3-*d*]pyrimidine scaffold 4-substituent results in analogues with distinct functional effects at the hD$_{2L}$R. (**A**) *N*,*N*-Diethylamino substitution confers negative allosteric cooperativity on DA signalling efficacy (**2**). (**B**) Introduction of the cyclic tertiary aliphatic morpholino moiety confers allosteric agonism (**9d**). (**C**, **D**) Introduction of cyclopropylamino or cyclobutylamino substituents to the 4-position in conjunction with the fused cyclohexane system results in analogues with sub-μM affinities that maintain negative allosteric cooperativity (**9f**, **9h**). (**E**) Introduction of the piperazino moiety (**10**) converts allosteric to competitive pharmacology. (**F**) Removal of the fused cyclohexane in conjunction with a cyclobutylamino substituent converts allosteric pharmacology to agonism (**15b**). All data used in these graphs are detailed in Tables 1 and 2 and are presented as mean ± SEM from three independent experiments performed in duplicate.

*4.2. Functional analysis of thieno[2,3-d]pyrimidine fragment analogues of **2***

We recently reported that incorporating an *N*,*N*-diethylamino substituent to the 4-position of the thieno[2,3-*d*]pyrimidine scaffold devoid of both the 2-morpholinomethyl moiety and fused cyclohexane systems (**3**), resulted in a low molecular weight fragment-like NAM of the D_2R (Figure 1).[18] As 4-cyclopropylamino and 4-cyclobutylamino substituents on the thieno[2,3-*d*]pyrimidine moiety conferred sub-μM analogues with robust negative cooperativity, we further examined the effect of introducing these substituents to the 4-position of **3** in place of the *N*,*N*-diethylamino functionality (Table 2). We found that introducing the cyclopropylamino substituent (**15a**) did not affect functional affinity, however caused a limitless rightward shift in the DA dose-response curve in addition to stimulating a modest agonist response in its own right. If cooperativity values of α and β were constrained to -3.0 and 0, respectively, we could derive a value of affinity and efficacy (K_B = 21.4 μM, τ_B = 0.22, Table 2). However, this pattern of ligand action is also consistent with that of a competitive low efficacy partial agonist. Furthermore, introduction of a cyclobutylamino substituent (**15b**) maintained affinity but abolished modulatory effects and engendered agonism (K_B = 7.38 μM, τ_B = 0.48, Table 2, Figure 3F). Thus, relatively subtle structural modifications significantly change pharmacology, from compounds which negatively modulate the behaviour of DA from a secondary binding site, to molecules that activate the receptor with no cooperativity. Of note, the D_2R orthosteric agonists pramipexole and sumanirole are both low molecular weight heterocyclic compounds bearing acyclic secondary amines.

Table 2 Functional parameters derived from cAMP BRET assay for fragment analogues of **2** with modifications to the 4-position



#	R^1	pK_B (K_B, μM)[a]	Logτ_B (τ_B)[b]	Log α (α)[c]	Log β (β)[d]
3		5.31 ± 0.07 (4.6)		= 0	-0.89 ± 0.04 (0.13)
15a		4.67 ± 0.08 (21.4)	-0.65 ± 0.07 (0.22)	= -3.0	= 0
15b		5.13 ± 0.19 (7.38)	-0.32 ± 0.06 (0.48)	= 0	= 0

[a]Estimate of the negative logarithm of the equilibrium dissociation constant determined in an cAMP functional assay. [b]Estimate of the logarithm of the net cooperativity factor between the modulator and dopamine determined in an cAMP functional assay. [c] Estimate of the intrinsic efficacy of the modulator. nd = inactive at concentrations up to 100 μM. Values represent the mean ± S.E.M. from at least three independent experiments performed in duplicate.

*4.3. Functional analysis (cAMP) of fused cyclohexane modified analogues of **2***

Our previous study revealed that incorporating various substituents (fused cyclopentane, cycloheptane, cyclooctane, 5- and 6-cyclohexyl, 5- and 6-phenyl, 5,6-dimethyl) to the 5-/6-positions of the thieno[2,3-*d*]pyrimidine generally rendered the corresponding analogues functionally inactive. However, these substituents were all previously examined on molecules also bearing 2-morpholinomethyl and 4-(3-(trifluoromethyl)anilino) substituents. As such, we further extended our study to monitor the effect of incorporating favourable amino substituents (*N*,*N*-diethylamino, cyclopropylamino, or cyclobutylamino) onto the 4-position of the thieno[2,3-*d*]pyrimidine whilst being devoid of the 2-morpholinomethyl moiety present in **1**, and bearing variation in the nature of substituents at the 5- and/or 6-positions (Table 3).

4.3.1. Fused cyclopentane analogues. Our previous work revealed converting the fused cyclohexane system of **1** to the homologous cyclopentane system had no adverse impact on functional affinity, nor the degree of negative cooperativity. Molecular docking studies conducted using the D_2R crystal structure as a template suggest that such fused hydrophobic rings interact with a hydrophobic subpocket located between helices V and VI formed by the residues $I184^{ECL2}$,

V190$^{5.39}$, H394$^{6.55}$ (where superscript numbers refer to Ballesteros-Weinstein numbering system[27]).[18,28] However, these interactions were predicted for compounds bearing a fused hydrophobic ring in conjunction with additional substituents located at the 2-position (morpholinomethyl) and 4-position (3-(trifluoromethyl)anilino). As these groups were now altered, we wanted to re-examine the importance of this ring system for such compounds. The *N*,*N*-diethylamino analogue (**19aa**) displayed an increase in functional affinity (K_B = 0.74 μM) relative to **1** and acted to negatively modulate DA signalling efficacy (β = 0.14, Figure 4A). The cyclopropylamino analogue (**19ab**) maintained low μM affinity (K_B = 6.01 μM) and similarly acted as a NAM of DA efficacy (β = 0.28). Conversely, the cyclobutylamino analogue (**19ac**) displayed a >15-fold decrease in functional affinity (K_B = 102 μM) and acted as an agonist (τ_B = 0.57). These data were surprising as **9h**, the corresponding fused cyclohexane variant of **19ac**, displayed sub-μM affinity at the D_2R and acted as a NAM of DA efficacy.

4.3.2. Larger homologous fused rings (cycloheptane and cyclooctane). Increasing the hydrophobic ring size by one carbon relative to **1** was previously shown to abolish D_2R activity, potentially due to the hydrophobic allosteric pocket failing to accommodate such extended ring sizes.[18] Likewise, further expansion of the hydrophobic ring by one additional carbon in the presence of the 3-(trifluoromethyl)anilino substituent also previously rendered the corresponding analogue inactive.[18] In the presence of a fused cycloheptane system devoid of the 2-morpholinomethyl substituent, incorporation of the 4-(*N*,*N*-diethylamino) group (**19ba**) similarly abolished activity. However, the cyclopropylamino analogue **19bb** (K_B = 2.96 μM, β = 0.05, Figure 4B) and cyclobutylamino analogue **19bc** (K_B = 0.91 μM, β = 0.06, Figure 4C) remained NAMs of DA efficacy at the D_2R, with μM and sub-μM affinities, respectively. Interestingly, all cyclooctane analogues in this study maintained activity at the D_2R. The *N*,*N*-diethylamino analogue **19ca** maintained low μM affinity (K_B = 14.5 μM) and negatively modulated DA signalling efficacy whilst exerting a slight degree of agonism unlike the inactive structural analogue **19ba** bearing the fused cycloheptane system. However, this agonism proved to be a non-specific effect (Supplementary Figure 1). To our surprise

the cyclopropylamino analogue **19cb** displayed the highest improvement in affinity seen for any analogue of **1** to date, some 10-fold compared to **2**, coupled with robust negative modulation of DA signalling efficacy (K_B = 0.53 μM, β = 0.10, Figure 4D). Introduction of a cyclobutylamino substituent (**19cc**) had no effects on affinity compared to **2** and this compound still acted as a NAM of DA efficacy (K_B = 3.98 μM, β = 0.26).

4.3.3. 5-Phenyl substituted analogues. Installing a phenyl substituent at the 5-position of **1** in the presence of the 4-(3-(trifluoromethyl)anilino) substituent had previously been shown to abolish activity.[18] Surprisingly, the *N*,*N*-diethylamino analogue (**19da**) not only lost >10-fold affinity, but caused a limitless rightward shift of the DA concentration response-curve, instead displaying weak agonism. From this limited set of data, however, its mode of action cannot be conclusively determined (i.e. allosteric agonist versus low efficacy competitive partial agonist). In order to derive a value of affinity and efficacy, cooperativity values of α and β were constrained to -3.0 and 0, respectively (K_B = 60.0 μM, τ_B = 0.27). Additionally, both the cyclopropylamino analogue (**19db**) (K_B = 1.93 μM, τ_B = 0.32) and cyclobutylamino analogue (**19dc**) (K_B = 137 μM, τ_B = 0.39) lost their modulatory effect upon DA and instead behaved as agonists.

4.3.4. 6-Phenyl substituted analogues. Converting the fused cyclohexane system of **1** to the corresponding 6-phenyl-substituted thiophene in the presence of 3-(trifluoromethyl)anilino was previously not tolerated.[18] However, in the presence of 4-(*N*,*N*-diethylamino), **19ea** showed improved functional affinity (K_B = 0.90 μM) relative to **1** and was a NAM of DA efficacy. The cyclopropylamino analogue **19eb** also maintained affinity and negative allosteric cooperativity (K_B = 6.35 μM, α = 0.60, β = 0.43). Conversely, the cyclobutylamino analogue **19ec** no longer negatively modulated the action of DA but instead also acted as a weak agonist (K_B = 4.50 μM, τ_B = 0.28). These data further demonstrate that the nature of the amine at the 4-position can dramatically affect the type of functional behaviour exerted by this scaffold at the D_2R.

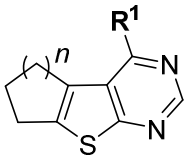
4.3.5. 5-Cyclohexyl substituted analogues. Integration of a 5-cyclohexyl substituent into the scaffold of **1** was previously shown to abolish activity.[18] In line with these data, the *N*,*N*-

diethylamino analogue (**19fa**) was also inactive. However, despite this, analogues **19fb** (K_B = 11.6 μM, β = 0.09) and **19fc** (K_B = 2.92 μM, β = 0.03, Figure 4E) were both NAMs of DA efficacy at the D_2R. These data indicate that incorporation of a 5-cyclohexyl substituent may be favourable depending on the nature of substituents at the 2- and 4-positions.

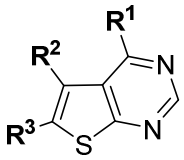
4.3.6. 6-Cyclohexyl substituted analogues. Modifying the thiophene with a cyclohexyl substituent at the 6-position of **1** maintained low μM affinity and negative allosteric cooperativity.[18] This substituent changed observed pharmacology from negative modulation of DA efficacy (β) to negative modulation of DA affinity (α). However, we found that incorporating the cyclohexyl substituent into analogues devoid of the 2-morpholinomethyl moiety (**19ga-c**) abolished activity regardless of the nature of the 4-substituent.

4.3.7. 5,6-Dimethyl substituted analogues. This structural modification in conjunction with the 4-(3-(trifluoromethyl)anilino) group was previously shown to abolish activity at the D_2R.[18] In the presence of an *N*,*N*-diethylamino in the 4-position, however, **19ha** maintained low μM affinity (K_B = 19.5 μM) but failed to have any effects on DA binding and function, instead displaying agonism (τ_B = 0.24). Conversely, the cyclopropylamino analogue (**19hb**) was inactive in our functional assay, while the cyclobutylamino analogue (**19hc**) maintained low μM affinity and was a NAM of DA efficacy (K_B = 2.88 μM, β = 0.13, Figure 4F).

Table 3 Functional parameters derived from cAMP BRET assay for analogues of **2** with modifications to the 5/6-fused system and 4-position

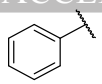
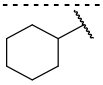


1, 19aa-c, ba-c, ca-c



19da-c, ea-c, fa-c, ga-c, ha-c

#	*n*	R^1	R^2	R^3	pK_B (K_B, μM)[a]	Logτ_B (τ_B)[b]	Log α (α)[c]	Log β (β)[d]
2	2				5.72 ± 0.13 (1.92)	= -3.0	= 0	= -3.0
19aa	1				6.13 ± 0.14 (0.74)		= 0	-0.85 ± 0.07 (0.14)
19ab	1				5.22 ± 0.14 (6.01)		-0.13 ± 0.11 (0.75)	-0.55 ± 0.06 (0.28)
19ac	1				3.99 ± 0.50 (102)	-0.25 ± 0.29 (0.57)	= 0	= 0
19ba	3					nd		
19bb	3				5.53 ± 0.07 (2.96)		= 0	-1.30 ± 0.07 (0.05)
19bc	3				6.04 ± 0.13 (0.91)		= 0	-1.22 ± 0.09 (0.06)
19ca	4				4.84 ± 0.13 (14.5)	NSE	= 0	-3.0
19cb	4				6.27 ± 0.18 (0.53)		= 0	-1.00 ± 0.11 (0.10)
19cc	4				5.40 ± 0.26 (3.98)		= 0	-0.58 ± 0.09 (0.26)
19da	-			H	4.22 ± 0.33 (60.0)	-0.57 ± 0.14 (0.27)	= -3.0	= 0
19db	-			H	5.72 ± 0.22 (1.93)	-0.47 ± 0.09 (0.32)	= -3.0	= 0
19dc	-			H	3.89 ± 0.52 (137)	-0.41 ± 0.31 (0.39)	= -3.0	= 0
19ea	-		H		6.04 ± 0.23 (0.90)		= 0	= -3.0

19eb	-		H		5.92 ± 0.17 (6.35)		-0.22 ± 0.10 (0.60)	-0.37 ± 0.05 (0.43)
19ec	-		H		5.35 ± 0.24 (4.50)	0.55 ± 0.10 (0.28)	= 0	= 0
19fa	-			H		nd		
19fb	-			H	4.93 ± 0.16 (11.6)		= 0	-1.04 ± 0.19 (0.09)
19fc	-			H	5.53 ± 0.13 (2.92)		= 0	-1.55 ± 0.19 (0.03)
19ga	-		H			nd		
19gb	-		H			nd		
19gc	-		H			nd		
19ha	-		CH_3	CH_3	4.71 ± 0.30 (19.5)	-0.61 ± 0.11 (0.24)	= 0	= 0
19hb	-		CH_3	CH_3		nd		
19hc	-		CH_3	CH_3	5.54 ± 0.16 (2.88)			-0.90 ± 0.08 (0.13)

[a]Estimate of the negative logarithm of the equilibrium dissociation constant determined in an cAMP functional assay. [b]Estimate of the logarithm of the net cooperativity factor between the modulator and dopamine determined in an cAMP functional assay. [c]Estimate of the intrinsic efficacy of the modulator determined in an cAMP functional assay. nd = inactive at concentrations up to 100 μM. NSE – non-specific effect, compound determined to inhibit forskolin-stimulated cAMP accumulation in the absence of the D_2R. Values represent the mean ± S.E.M. from at least three independent experiments performed in duplicate.

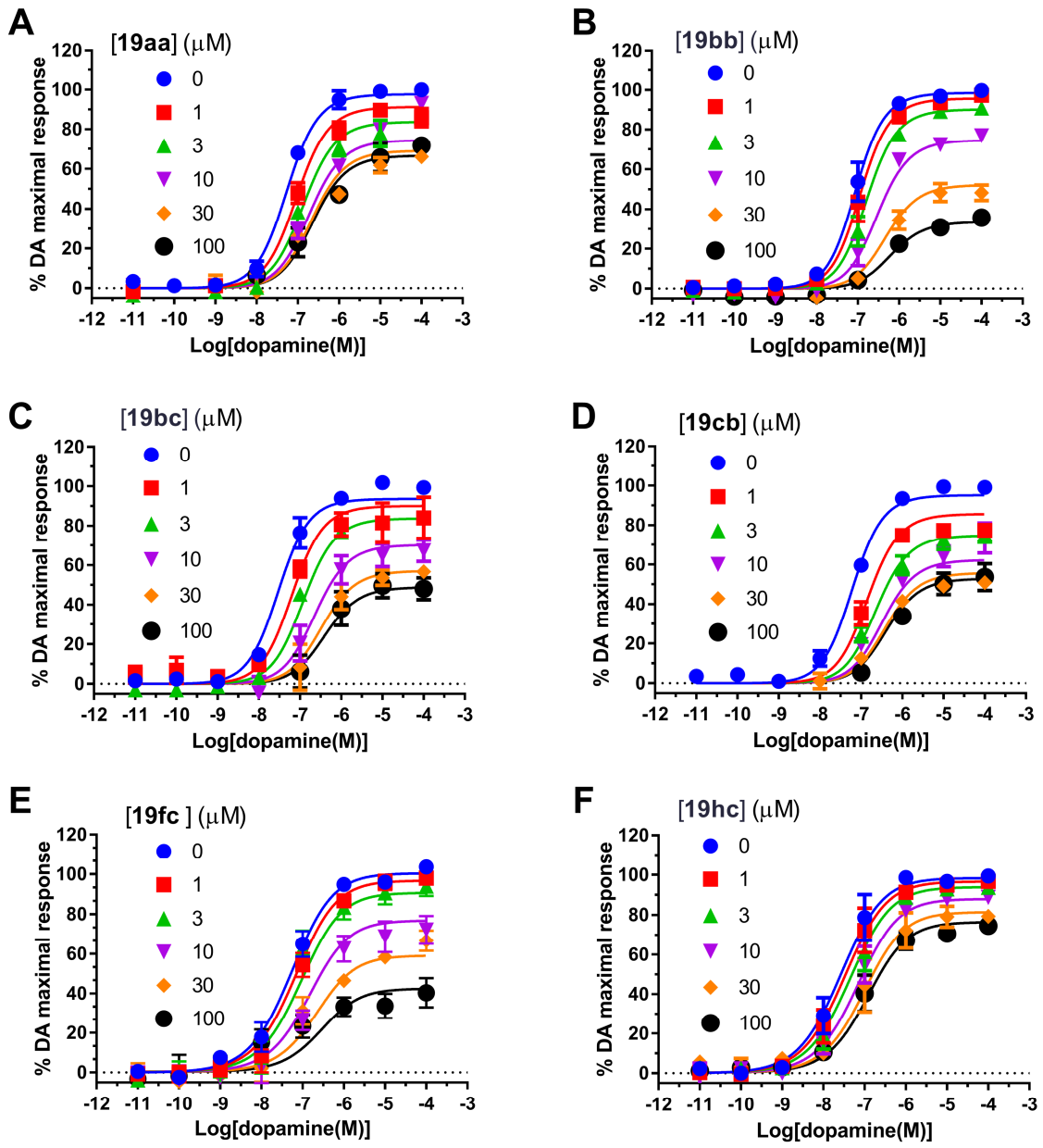


Fig. 4. Various aliphatic amino substituents at the 4-position can recover allosteric pharmacology. Most modifications to the 5- and 6-positions of the thienopyrimidine were previously found to be detrimental to the activity of the target compound. However, these activities can be recovered and even enhanced when using *N*,*N*-diethylamino, cyclopropylamino or cyclobutylamino substituents at the 4-position. (**A**) Fused cyclopentane with 4-(*N*,*N*-diethylamino) substituent (**19aa**, K_B = 0.74 μM, β = 0.14). (**B**) Fused cycloheptane with 4-(cyclopropylamino) substituent (**19bb**, K_B = 2.96 μM, β = 0.05). (**C**) Fused cycloheptane with 4-(cyclobutylamino) substituent (**19bc**, K_B = 0.91 μM, β = 0.06). (**D**). Fused cyclooctane with 4-(cycloproylamino) substituent (**19cb**, K_B = 0.53 μM, β = 0.10). (**E**) 5-Cyclohexyl with 4-(cyclobutylamino) substituent (**19fc**, K_B = 2.92 μM, β = 0.03). (**F**) 5,6-Dimethyl with 4-(cyclobutylamino) substituent (**19hc**, K_B = 2.88 μM, β = 0.13). All data used in these graphs are detailed in Table 3 and are presented as mean ± SEM from three independent experiments performed in duplicate.

Our data reveal that relatively subtle structural changes can cause a change in pharmacology from that of a NAM to that of a weak D_2R agonist. With two exceptions, the actions of the ligands described within this study are mediated through interaction with the D_2R. The phenomenon whereby subtle modifications to a small molecule allosteric scaffold act to modulate modes of pharmacology, presumably via a change in receptor conformation, have been coined as "molecular switches".[29] This phenomenon has been documented for allosteric ligands targeting multiple GPCR and non-GPCR targets, including muscarinic acetylcholine receptors (mAChRs),[30-32] as well as kinase[33] and phospholipase[34,35] allosteric ligands. Molecular switches have been reported to encompass a number of subtle structural changes, for example, stereochemistry, ring size and simple aryl substitution (i.e. fluoro *vs* methyl) to afford compounds with diverse pharmacology (e.g. positive and negative alloseric modulators, partial antagonists, and agonists). Indeed it is not surprising that such changes to allosteric ligands can cause dramatic changes in pharmacology given that similarly subtle changes to orthosteric ligands can convert agonists to antagonists. However, for those compounds that displayed agonism rather than the NAM activity of **1**, the nature of this agonism i.e. whether it is non-competitive (allosteric) or competitive (orthosteric), cannot be conclusively confirmed due to their low D_2R affinity. Thus it is not clear whether such compounds bind to the same allosteric site as **1**, and are examples of molecular switching, or whether the relatively subtle structural changes investigated within this study confer the ability to engage the orthosteric site.[18] Indeed, our recent paper proposed that the allosteric binding site of **1** was in close proximity to the orthosteric site.

In summary, it is clear that the nature of substituents at the thieno[2,3-*d*]pyrimidine 4-, 5- and 6-positions play a crucial role in both binding affinity and functional activity (e.g. anilino (**9a**) and cyclohexylamino (**9b**) at the 4-position are not tolerated). However, other 6-membered substituents (morpholino (**9d**) and piperazino (**10**)) convert NAMs to agonists and competitive antagonists, respectively. Moreover, smaller acyclic and cyclic at the 4-position (*N*,*N*-diethylamino (**2**), cyclopropylamino (**9f**), cyclopropylmethylamino (**9g**) and cyclobutylamino (**9h**)) are tolerated, with

all compounds retaining modulatory activity as well as **9h** showing a 10-fold increase in affinity. Decreasing the fused ring size whilst bearing the cyclobutylamino substituent (**19ac**) abolishes any negative modulatory effects, and instead confers agonism. However the *N*,*N*-diethylamino (**19aa**) and cyclopropylamino (**19ab**) counterparts retain negative modulatory action. Interestingly, increasing the ring size (cycloheptane (**19bc**), cyclooctane (**19cc**)) while bearing the cyclobutylamino substituent maintains negative modulatory action. However, introduction of the *N*,*N*-diethylamino substituent to the cycloheptane analogue (**19ba**) completely abolishes activity. Additionally, introduction of a phenyl ring to the 5-position in the presence of any amine converts all analogues (**19da-c**) to agonists. In most cases, a cyclohexyl substituent at this position appears favourable (**19fb-c**) as these compounds retain affinity and cooperativity, whereas an *N*,*N*-diethylamino substituent (**19fa**) abolishes activity. A phenyl substituent at the 6-posititon, however, is tolerated in the presence of *N*,*N*-diethylamino (**19ea**) and cycloproylamino (**19eb**), yet, with cyclobutylamino (**19ec**), this yields an agonist. Installing a cyclohexyl moiety at the 6-position abolishes activity for **19ga-c**, whereas the activity profile of analogues bearing a 5,6-dimethyl substituent are highly dependent on the amine present at the 4-position (*N*,*N*-diethylamino (**19ha**) = agonist, cyclopropylamino (**19hb**) = inactive, and cyclobutylamino (**19hc**) = NAM). All NAMs identified were modulators of agonist efficacy, whereby structural modifications did not confer modulation of agonist affinity.

5. Conclusions

In this study, we report the functional characterisation of a small library of structural analogues based on a thieno[2,3-*d*]pyrimidine scaffold that we have previously shown to act as D_2R NAMs. The impact of structural modification was initially assessed with respect to the type of amine substituent at the 4-position in the presence of the 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidine moiety (series 1). Having identified favourable amines from series 1, we further extended our study to the analysis of various substituents in place of the 5/6-fused cyclohexane

system in conjunction with one of three selected amines (series 2) at the 4-position. While many of these derivatives maintained NAM activity, some, surprisingly acted as D_2R agonists. It is not clear from the present data if this change in activity relates to a switch from an allosteric to an orthosteric mode of engagement or whether these compounds bind to the same allosteric site yet display distinct pharmacological effects. Future studies are needed to address this but such efforts are complicated by the relatively low affinity of these ligands. In the presence of a fused cyclohexane system, small cyclic aliphatic amines (cyclopropylamino, cyclobutylamino) were found to be vital for negative modulatory action. Two of the highest affinity NAMs to arise from this scaffold were identified, **9h** (K_B = 0.57 μM, fused cyclohexane moiety) and **19cb** (K_B = 0.53 μM, fused cyclopentane moiety), that contain cyclobutylamino and cyclopropylamino substituents, respectively. Conversely, larger heterocyclic substituents (morpholino) and acyclic amines (*tert*-butylamino) conferred agonism and abolished modulatory activity (**9d** and **9i**, respectively). In combination with a range of different substituents at the 5/6-positions, however, small cyclic/acyclic aliphatic amines gave a range of analogues with differential functional pharmacology, ranging from NAMs of DA efficacy, to agonists and compounds with no apparent functional activity. Taken together, this study demonstrates that the pharmacology of analogues functionalised at the 4-, 5-, and 6-positions of the thieno[2,3-*d*]pyrimidine core is difficult to predict, resulting in shallow SAR. Such observations are consistent with SAR studies of allosteric modulators of other GPCR targets, highlighting the challenges associated with GPCR allosteric ligand design.

6. Experimental section

6.1. General methods for chemistry

Chemicals and solvents were purchased from standard suppliers and used without further purification. Davisil silica gel (40−63 μm) for flash column chromatography was supplied by Grace Davison Discovery Sciences (Victoria, Australia), and deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. (USA, distributed by Novachem PTY. Ltd., Victoria,

Australia). Reactions were monitored by thin layer chromatography on commercially available precoated aluminum-backed plates (Merck Kieselgel 60 F_{254}). Visualization was by examination under UV light (254 and 366 nm). A solution of ninhydrin (in ethanol) was used to visualize primary and secondary amines. All organic extracts collected after aqueous workup procedures were dried over anhydrous Na_2SO_4 before gravity/vacuum filtering and evaporation to dryness. Organic solvents were evaporated *in vacuo* at ≤40 °C (water bath temperature). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance Nanobay III 400 MHz Ultrashield Plus spectrometer at 400.13 and 100.62 MHz, respectively. Chemical shifts (δ) are recorded in parts per million (ppm) with reference to the chemical shift of the deuterated solvent. Coupling constants (*J*) are recorded in Hz, and the significant multiplicities described by singlet (s), doublet (d), triplet (t), quadruplet (q), broad (br), multiplet (m), doublet of doublets (dd), and doublet of triplets (dt). Spectra were assigned using appropriate COSY, distortionless enhanced polarization transfer (DEPT), HSQC, and HMBC sequences. All NMR experiments were performed in $CDCl_3$ to permit comparison of the spectra of the various analogues. Experiments were performed in acetone-d_6, DMSO-d_6, or MeOH-d_4 where selected analogues lacked solubility in $CDCl_3$. LCMS experiments were run using one of two systems to verify reaction outcome and purity. System A was the default unless otherwise stated. System A consisted of the following: an Agilent 6100 series single quad coupled to an Agilent 1200 series HPLC instrument using the following buffers: buffer A, 0.1% formic acid in H_2O; buffer B, 0.1% formic acid in MeCN. The following gradient was used with a Phenomenex Luna 3 μm C8(2) 15 mm × 4.6 mm column and a flow rate of 0.5 mL/min and total run time of 12 min: 0−4 min 95% buffer A and 5% buffer B, 4−7 min 0% buffer A and 100% buffer B, 7−12 min 95% buffer A and 5% buffer B. Mass spectra were acquired in positive and negative ion modes with a scan range of 0−1000 *m*/*z* at 5 V. UV detection was carried out at 254 nm. System B consisted of the following: an Agilent 6120 series single quad coupled to an Agilent 1260 series HPLC instrument. The following buffers were used; buffer A, 0.1% formic acid in H_2O; buffer B, 0.1% formic acid in MeCN. The following gradient was used with a Poroshell 120 EC-C18 50 mm

× 3.0 mm, 2.7 μm column and a flow rate of 0.5 mL/min and total run time of 5 min: 0−1 min 95% buffer A and 5% buffer B, from 1 to 2.5 min up to 0% buffer A and 100% buffer B, held at this composition until 3.8 min, 3.8−4 min 95% buffer A and 5% buffer B, held until 5 min at this composition. Mass spectra were acquired in positive and negative ion modes with a scan range of 100−1000 m/z. UV detection was carried out at 214 and 254 nm. All retention times (t_R) are quoted in minutes. System C: Analytical reverse-phase HPLC was performed on a Waters HPLC system coupled directly to a photodiode array detector and fitted with a Phenomenex Luna C8 (2) 100 Å column (150 mm × 4.6 mm, 5 μm) using a binary solvent system: solvent A, 0.1% TFA/H_2O; solvent B, 0.1% TFA/80% CH_3CN/H_2O. Gradient elution was achieved using 100% solvent A to 100% solvent B over 20 min at a flow rate of 1 mL/min. All compounds subjected to biological testing were found to be >95% pure by HPLC at two wavelengths (λ of 254 and 214 nm).

6.2. General synthetic procedures.

*6.3. General procedure A for the Synthesis of **9a-j**, **15a-b**, **19aa-hc***

In a suitable microwave reaction vessel the required chloropyrimidine (1 equiv.) was taken up in *i*-PrOH. To this was added the required amine (1.1 equiv.) and the mixture irradiated under stirring at 120 ˚C for 1-2 h. Upon completion of the reaction, the mixture was directly purified using FCC to afford the compound. Similarly, any precipitate could be collected under vacuum and washed several time with cold *i*-PrOH to afford the desired compound.

*6.3.1. N-Phenyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-amine (**9a**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 105 mg of a white amorphous solid (84%). LCMS (*m/z*): 281.9 $[M+H]^+$. HPLC: t_R 6.244 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{16}H_{15}N_3S$ requires 282.1071 $[M+H]^+$; found 282.1059. ^{1}H NMR ($CDCl_3$) δ 8.48 (s, 1H), 7.64 (dt, *J* = 8.8, 1.7 Hz, 2H), 7.41 – 7.34 (m, 2H), 7.17 – 7.10 (m, 2H), 3.06 (dd, *J* =

8.1, 3.9 Hz, 2H), 2.85 (dd, *J* = 8.0, 3.9 Hz, 2H), 2.03 – 1.89 (m, 4H). ^{13}C NMR ($CDCl_3$) δ 166.3, 155.0, 152.6, 138.5, 134.7, 129.1, 124.8, 124.0, 121.3, 116.6, 26.5, 25.5, 22.5, 22.4.

*6.3.2. N-Cyclohexyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-amine (**9b**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 115 mg of a beige amorphous solid (82%). LCMS (*m/z*): 288.2 $[M+H]^+$. HPLC: t_R 6.383 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{16}H_{21}N_3S$ requires 288.1533 $[M+H]^+$; found 288.1529. ^{1}H NMR ($CDCl_3$) δ 8.35 (s, 1H), 4.23 – 4.12 (m, 1H), 2.91 (dd, *J* = 6.8, 5.1 Hz, 2H), 2.79 (dd, *J* = 8.1, 3.7 Hz, 2H), 2.12 – 2.03 (m, 2H), 1.96 – 1.85 (m, 4H), 1.75 (ddd, *J* = 13.3, 8.8, 5.0 Hz, 2H), 1.69 – 1.61 (m, 1H), 1.55 – 1.42 (m, 2H), 1.32 – 1.21 (m, 3H). ^{13}C NMR ($CDCl_3$) δ 165.3, 156.9, 153.3, 132.9, 125.5, 116.0, 48.9, 33.3, 26.5, 25.8, 25.5, 24.8, 22.7, 22.6.

*6.5.3. 4-(Piperidin-1-yl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine (**9c**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 95.2 mg of a white amorphous solid (68%). LCMS (*m/z*): 274.1 $[M+H]^+$. HPLC: t_R 6.478 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{15}H_{19}N_3S$ requires 224.1374 $[M+H]^+$; found 274.1372. ^{1}H NMR ($CDCl_3$) δ 8.51 (s, 1H), 3.36 – 3.28 (m, 4H), 2.96 – 2.91 (m, 2H), 2.90 – 2.85 (m, *J* = 6.3, 1.6 Hz, 2H), 1.97 – 1.90 (m, 2H), 1.81 (ddd, *J* = 12.1, 5.9, 2.6 Hz, 2H), 1.77 – 1.71 (m, 4H), 1.69 – 1.62 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 168.1, 162.9, 151.6, 134.4, 127.7, 121.5, 51.8, 26.7, 25.8, 25.7, 24.4, 23.1, 22.9.

*6.5.4. 4-(5,6,7,8-Tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-yl)morpholine (**9d**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 161 mg of a white amorphous solid (88%). LCMS (*m/z*): 275.9 $[M+H]^+$. HPLC: t_R 5.179 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{14}H_{17}N_3OS$ requires 275.1065 $[M+H]^+$; found 275.1087.^{1}H NMR (400 MHz, $CDCl_3$) δ 8.46 (s, 1H), 3.82 – 3.77 (m, 4H), 3.36 – 3.31 (m, 4H), 2.87 – 2.77 (m, 4H), 1.91 –

1.84 (m, 2H), 1.79 – 1.71 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 168.39, 162.08, 151.46, 135.46, 126.9, 121.4, 66.6, 51.1, 26.7, 25.8, 22.9, 22.8.

*6.5.5. 4-(Pyrrolidin-1-yl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine (**9e**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 109 mg of a white amorphous solid (82%). LCMS (*m/z*): 260.0 $[M+H]^+$. HPLC: t_R 5.270 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{14}H_{17}N_3S$ requires 259.1112 $[M+H]^+$; found 259.1138. ^{1}H NMR ($CDCl_3$) δ 8.33 (s, 1H), 3.72 – 3.65 (m, 4H), 2.90 – 2.81 (m, 4H), 1.95 – 1.86 (m, 6H), 1.78 – 1.71 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 167.7, 158.8, 150.9, 132.2, 127.5, 117.9, 50.9, 29.2, 25.8, 25.5, 23.3, 22.8.

*6.5.6. N-Cyclopropyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-amine (**9f**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 105 mg of a white amorphous solid (87%). LCMS (*m/z*): 246.0 $[M+H]^+$. HPLC: t_R 4.864 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{13}H_{15}N_3S$ requires 245.0954 $[M+H]^+$; found 245.0981. ^{1}H NMR ($CDCl_3$) δ 8.47 (s, 1H), 5.51 (s, 1H), 2.96 – 2.89 (m, 1H), 2.83 (td, *J* = 5.9, 1.9 Hz, 2H), 2.78 (td, *J* = 6.0, 1.9 Hz, 2H), 1.95 – 1.83 (m, 4H), 0.95 – 0.90 (m, 2H), 0.61 – 0.57 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 165.2, 158.3, 153.1, 133.4, 125.3, 116.2, 26.4, 25.4, 23.9, 22.5, 22.4, 7.6.

*6.5.7. N-(Cyclopropylmethyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-amine (**9g**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 88 mg of a light yellow amorphous solid (85%). LCMS (*m/z*): 260.0 $[M+H]^+$. HPLC: t_R 5.519 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{14}H_{17}N_3S$ requires 260.1215 $[M+H]^+$; found 260.1216. ^{1}H NMR ($CDCl_3$) δ 8.36 (s, 1H), 5.40 (s, 1H), 2.97 – 2.91 (m, *J* = 8.0, 4.1, 1.7 Hz, 1H), 2.82 – 2.78 (m, 2H), 1.98 – 1.85 (m, 4H), 1.20 – 1.08 (m, 1H), 0.62 – 0.55 (m, 2H), 0.33 – 0.28 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 165.27, 157.40, 153.14, 133.1, 125.5, 116.1, 46.1, 26.4, 25.5, 22.7, 22.6, 10.8, 3.5.

*6.5.8. N-Cyclobutyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-amine (**9h**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 101 mg of a white amorphous solid (88%). LCMS (*m/z*): 260.0 $[M+H]^+$. HPLC: t_R 5.612 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{14}H_{17}N_3S$ requires 260.1213 $[M+H]^+$; found 260.1213. ^{1}H NMR ($CDCl_3$) δ 8.37 (s, 1H), 5.41 (d, *J* = 6.2 Hz, 1H), 4.76 – 4.64 (m, 1H), 2.93 (td, *J* = 5.9, 1.6 Hz, 2H), 2.81 (dd, *J* = 9.8, 3.7 Hz, 2H), 2.50 (dddd, *J* = 13.6, 7.7, 3.7, 2.7 Hz, 2H), 1.99 – 1.85 (m, 6H), 1.85 – 1.76 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 165.51, 156.55, 153.20, 133.26, 125.42, 115.9, 46.2, 31.8, 26.5, 25.5, 22.7, 22.6, 15.4.

*6.5.9. N-(tert-Butyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-amine (**9i**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 114 mg of a white amorphous solid (85%). LCMS (*m/z*): 262.0 $[M+H]^+$. HPLC: t_R 4.623 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{14}H_{19}N_3S$ requires 261.1312 $[M+H]^+$; found 261.1294. ^{1}H NMR ($CDCl_3$) δ 8.34 (s, 1H), 5.28 (s, 1H), 2.88 (td, *J* = 5.9, 1.6 Hz, 2H), 2.78 (dd, *J* = 9.8, 3.7 Hz, 2H), 1.95 – 1.82 (m, 4H), 1.53 (s, 9H). ^{13}C NMR ($CDCl_3$) δ 165.1, 157.3, 152.6, 132.7, 125.2, 116.4, 52.3, 29.2, 26.5, 25.5, 22.7, 22.5.

*6.5.10. tert-Butyl-4-(5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-yl)piperazine-1-carboxylate (**9j**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 244 mg of a white amorphous solid (84%). LCMS (*m/z*): 375.2 $[M+H]^+$. HPLC: t_R 7.500 min, >95% purity (214 & 254 nm). ^{1}H NMR ($CDCl_3$) δ 8.45 (s, 1H), 3.53 (dd, *J* = 6.1, 4.1 Hz, 4H), 3.30 – 3.25 (m, 4H), 2.82 (ddd, *J* = 9.2, 7.8, 3.7 Hz, 4H), 1.90 – 1.83 (m, 2H), 1.77 – 1.69 (m, 2H), 1.41 (s, 9H). ^{13}C NMR ($CDCl_3$) δ 168.5, 162.3, 154.9, 151.6, 135.6, 127.0, 121.6, 80.1, 28.5, 26.7, 25.8, 23.1, 22.8.

*6.5.11. 4-(Piperazin-1-yl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine (**10**)*

tert-Butyl-4-(5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-yl)piperazine-1-carboxylate (163 mg, 435 μmol) was dissolved in DCM. To this solution was added trifluoroacetic acid (5.00 mL, 65.3 mmol) and the reaction mixture was stirred at r.t. for 3 h. The solvents were evaporated and the residue the residue was taken up in H_2O (20 mL) and added to a separating funnel. The aqueous solution was washed with Et_2O (3 × 30 mL), and the aqueous phase made alkaline with the addition of 2 M NaOH solution. This phase was then extracted with DCM (3 × 30 mL) and the organic extracts collected and dried over anhydrous Na_2SO_4 to afford 115 mg of the corresponding amine free base as a white amorphous solid (96%). LCMS (*m/z*): 274.9 $[M+H]^+$. HPLC: t_R 4.623 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{14}H_{18}N_3S$ requires 275.1334 $[M+H]^+$; found 275.1334. ^{1}H NMR ($CDCl_3$) δ 8.52 (s, 1H), 3.41 – 3.34 (m, 4H), 3.07 – 3.03 (m, 4H), 2.95 – 2.91 (m, 2H), 2.90 – 2.85 (m, 2H), 2.05 (s, 1H), 1.97 – 1.90 (m, 2H), 1.84 – 1.78 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 168.3, 162.5, 151.5, 135.0, 127.2, 121.4, 51.9, 45.7, 26.8, 25.8, 23.0, 22.8.

*6.5.12. N-Cyclopropylthieno[2,3-d]pyrimidin-4-amine (**15a**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 77.1 mg of a white amorphous solid (76%). LCMS (*m/z*): 191.9 $[M+H]^+$. HPLC: t_R 3.290 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_9H_9N_3S$ requires 192.0587 $[M+H]^+$; found 192.0590. ^{1}H NMR ($CDCl_3$) δ 8.55 (s, 1H), 7.35 (d, *J* = 5.2 Hz, 1H), 7.27 (d, *J* = 6.0 Hz, 1H), 5.92 (s, 1H), 3.03 – 2.97 (m, 1H), 0.99 – 0.93 (m, 2H), 0.74 – 0.68 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 167.3, 158.6, 153.9, 122.8, 118.4, 116.2, 24.6, 8.2.

*6.5.13. N-Cyclobutylthieno[2,3-d]pyrimidin-4-amine (**15b**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 85.1 mg of a white amorphous solid (79%). LCMS (*m/z*): 206.0 $[M+H]^+$. HPLC: t_R 3.952 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{14}H_{17}N_3S$ requires 206.0744 $[M+H]^+$; found 206.0746. ^{1}H NMR ($CDCl_3$) δ

8.50 (s, 1H), 7.27 (d, *J* = 5.9 Hz, 1H), 7.17 (d, *J* = 6.0 Hz, 1H), 5.53 (d, *J* = 5.9 Hz, 1H), 4.81 – 4.71 (m, 1H), 2.57 – 2.46 (m, 2H), 2.07 – 1.92 (m, 2H), 1.85 – 1.75 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 166.7, 156.3, 154.2, 123.0, 117.2, 116.1, 46.4, 31.7, 15.3.

*6.5.14. N,N-Diethyl-6,7-dihydro-5H-cyclopenta[4,5]thieno[2,3-d]pyrimidin-4-amine (**19aa**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 115 mg of a white amorphous solid (78%). LCMS (*m/z*): 248.1 $[M+H]^+$. HPLC: t_R 5.295 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{13}H_{17}N_3S$ requires 248.1217 $[M+H]^+$; found 248.1216. ^{1}H NMR ($CDCl_3$) δ 8.35 (s, 1H), 3.59 (q, *J* = 7.1 Hz, 4H), 3.06 – 2.99 (m, 2H), 2.94 (ddt, *J* = 8.2, 6.6, 1.8 Hz, 2H), 2.44 – 2.34 (m, 2H), 1.19 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR ($CDCl_3$) δ 173.3, 159.4, 151.3, 138.2, 136.0, 116.0, 43.7, 32.4, 29.8, 28.1, 13.1.

*6.5.15. N-Cyclopropyl-6,7-dihydro-5H-cyclopenta[4,5]thieno[2,3-d]pyrimidin-4-amine (**19ab**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 105 mg of a white amorphous solid (77%). LCMS (*m/z*): 232.1 $[M+H]^+$. HPLC: t_R 4.629 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{12}H_{13}N_3S$ requires 232.0903 $[M+H]^+$; found 232.0903. ^{1}H NMR ($CDCl_3$) δ 8.49 (s, 1H), 5.28 (s, 1H), 2.98 (t, *J* = 7.2 Hz, 4H), 2.92 (td, *J* = 6.6, 3.7 Hz, 1H), 2.57 – 2.47 (m, 2H), 0.97 – 0.91 (m, 2H), 0.64 – 0.58 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 170.7, 157.7, 153.2, 138.9, 134.2, 133.1, 29.5, 29.2, 27.9, 23.8, 7.6.

*6.5.16. N-Cyclobutyl-6,7-dihydro-5H-cyclopenta[4,5]thieno[2,3-d]pyrimidin-4-amine (**19ac**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 90.1 mg of a white amorphous solid (83%). LCMS (*m/z*): 246.1 $[M+H]^+$. HPLC: t_R 5.373 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{13}H_{15}N_3S$ requires 246.0466 $[M+H]^+$; found 246.0464. ^{1}H NMR ($CDCl_3$) δ 8.37 (s, 1H), 5.16 (d, *J* = 6.7 Hz, 1H), 4.77 – 4.65 (m, 1H), 3.06 – 2.96 (m, 4H), 2.60 – 2.45 (m,

4H), 1.99 – 1.86 (m, 2H), 1.84 – 1.75 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 155.8, 153.2, 138.6, 134.3, 113.1, 45.9, 31.8, 29.5, 29.2, 27.9, 15.2.

*6.5.17. N,N-Diethyl-6,7,8,9-tetrahydro-5H-cyclohepta[4,5]thieno[2,3-d]pyrimidin-4-amine (**19ba**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 99.4 mg of a white amorphous solid (86%). LCMS (*m/z*): 276.0 $[M+H]^+$. HPLC: t_R 6.323 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{15}H_{21}N_3S$ requires 276.1526 $[M+H]^+$; found 276.1529. ^{1}H NMR ($CDCl_3$) δ 8.40 (s, 1H), 3.36 (q, *J* = 7.1 Hz, 4H), 2.98 – 2.93 (m, 2H), 2.82 – 2.78 (m, 2H), 1.87 – 1.80 (m, 2H), 1.68 – 1.62 (m, 2H), 1.56 (ddd, *J* = 11.4, 6.0, 2.8 Hz, 2H), 1.03 (t, *J* = 7.1 Hz, 7H). ^{13}C NMR ($CDCl_3$) δ 166.3, 161.7, 150.9, 138.2, 132.6, 121.6, 44.5, 32.8, 30.4, 28.7, 27.6, 27.4, 12.3.

*6.5.18. N-Cyclopropyl-6,7,8,9-tetrahydro-5H-cyclohepta[4,5]thieno[2,3-d]pyrimidin-4-amine (**19bb**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 101 mg of a white amorphous solid (88%). LCMS (*m/z*): 274.2 $[M+H]^+$. HPLC: t_R 5.709 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{15}H_{19}N_3S$ requires 274.1374 $[M+H]^+$; found 274.1372. ^{1}H NMR ($CDCl_3$) δ 8.48 (s, 1H), 5.53 (s, 1H), 2.97 – 2.90 (m, *J* = 5.7, 3.4 Hz, 4H), 2.90 – 2.84 (m, 4H), 1.93 – 1.86 (m, 2H), 1.82 (td, *J* = 11.7, 7.2 Hz, 2H), 0.97 – 0.89 (m, 2H), 0.64 – 0.57 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 164.2, 158.4, 152.8, 137.37, 130.2, 117.5, 30.48, 30.32, 29.0, 27.1, 26.4, 24.2, 7.7.

*6.5.19. N-Cyclobutyl-6,7,8,9-tetrahydro-5H-cyclohepta[4,5]thieno[2,3-d]pyrimidin-4-amine (**19bc**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 100 mg of a white amorphous solid (88%). LCMS (*m/z*): 274.0 $[M+H]^+$. HPLC: t_R 5.908 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{15}H_{19}N_3S$ requires 274.1371 $[M+H]^+$; found 274.1372. ^{1}H NMR ($CDCl_3$) δ

8.35 (s, 1H), 5.40 (d, *J* = 6.3 Hz, 1H), 4.74 – 4.63 (m, 1H), 3.04 – 2.99 (m, 2H), 2.87 (dd, *J* = 6.7, 4.3 Hz, 2H), 2.55 – 2.45 (m, 2H), 2.03 – 1.69 (m, 10H). ^{13}C NMR ($CDCl_3$) δ 164.4, 156.5, 152.8, 136.9, 130.2, 117.1, 46.4, 31.8, 30.4, 30.4, 28.9, 27.2, 26.4, 22.5, 15.5.

*6.5.20. N,N-Diethyl-5,6,7,8,9,10-hexahydrocycloocta[4,5]thieno[2,3-d]pyrimidin-4-amine (**19ca**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 113 mg of a white amorphous solid (79%). LCMS (*m/z*): 290.2 $[M+H]^+$. HPLC: t_R 7.076 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{16}H_{23}N_3S$ requires 290.1689 $[M+H]^+$; found 290.1685. ^{1}H NMR ($CDCl_3$) δ 8.57 (s, 1H), 3.43 (q, *J* = 7.1 Hz, 4H), 3.05 – 3.01 (m, 2H), 2.93 – 2.89 (m, 2H), 1.78 – 1.71 (m, 2H), 1.57 (tt, *J* = 8.4, 6.2 Hz, 2H), 1.49 – 1.42 (m, 2H), 1.16 – 1.11 (m, *J* = 8.3, 3.6 Hz, 2H), 1.09 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR ($CDCl_3$) δ 168.0, 162.4, 151.2, 138.5, 129.8, 121.6, 45.5, 31.8, 31.2, 27.9, 26.2, 25.4, 24.6, 12.3.

*6.5.21. N-Cyclopropyl-5,6,7,8,9,10-hexahydrocycloocta[4,5]thieno[2,3-d]pyrimidin-4-amine (**19cb**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 75.2 mg of a white amorphous solid (77%). LCMS (*m/z*): 274.2 $[M+H]^+$. HPLC: t_R 5.709 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{15}H_{19}N_3S$ requires 274.1374 $[M+H]^+$; found 274.1372. ^{1}H NMR ($CDCl_3$) δ 8.52 (d, *J* = 3.2 Hz, 1H), 5.57 (s, 1H), 3.01 – 2.94 (m, 1H), 2.87 (dd, *J* = 12.3, 5.1 Hz, 4H), 1.75 – 1.66 (m, 4H), 1.57 – 1.48 (m, 2H), 1.38 – 1.27 (m, 2H), 0.99 – 0.92 (m, 2H), 0.62 – 0.57 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 164.9, 157.9, 152.9, 136.6, 127.5, 116.3, 31.6, 29.9, 27.8, 26.1, 25.2, 23.9, 7.7.

*6.5.22. N-Cyclobutyl-5,6,7,8,9,10-hexahydrocycloocta[4,5]thieno[2,3-d]pyrimidin-4-amine (**19cc**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 122 mg of a white amorphous solid (86%). LCMS (*m/z*): 288.2 $[M+H]^+$. HPLC: t_R 6.266 min, >95% purity (214 &

254 nm). HRMS (*m/z*): $C_{16}H_{21}N_3S$ requires 288.1531 $[M+H]^+$; found 288.1531. ^1H NMR ($CDCl_3$) δ 8.39 (s, 1H), 5.48 (d, *J* = 6.6 Hz, 1H), 4.80 – 4.67 (m, 1H), 2.95 – 2.90 (m, 2H), 2.90 – 2.85 (m, 2H), 2.57 – 2.47 (m, 2H), 1.98 – 1.86 (m, 2H), 1.86 – 1.74 (m, 4H), 1.74 – 1.65 (m, 2H), 1.57 – 1.49 (m, 2H), 1.35 (dt, *J* = 10.7, 6.1 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 165.1, 156.1, 152.8, 136.3, 127.5, 115.9, 46.1, 31.8, 31.6, 29.9, 27.8, 26.1, 25.2, 15.3.

*6.5.23. N,N-Diethyl-5-phenylthieno[2,3-d]pyrimidin-4-amine (**19da**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 94.1 mg of a white amorphous solid (86%). LCMS (*m/z*): 283.9 $[M+H]^+$. HPLC: t_R 6.1389 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{16}H_{17}N_3S$ requires 284.1213 $[M+H]^+$; found 284.1216. ^1H NMR ($CDCl_3$) δ 8.55 (s, 1H), 7.44 – 7.38 (m, 4H), 7.37 – 7.32 (m, 1H), 7.18 (s, 1H), 3.16 (q, *J* = 7.1 Hz, 4H), 0.89 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR ($CDCl_3$) δ 170.1, 160.9, 152.11, 136.8, 136.4, 128.4, 128.1, 127.6, 120.5, 115.6, 43.7, 12.3.

*6.5.24. N-Cyclopropyl-5-phenylthieno[2,3-d]pyrimidin-4-amine (**19db**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 98.3 mg of a white amorphous solid (86%). LCMS (*m/z*): 267.9 $[M+H]^+$. HPLC: t_R 5.308 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{15}H_{13}N_3S$ requires 268.0830 $[M+H]^+$; found 268.0903. ^1H NMR ($CDCl_3$) δ 8.60 (s, 1H), 7.51 – 7.47 (m, 3H), 7.43 – 7.38 (m, 2H), 7.07 (s, 1H), 5.11 (s, 1H), 2.87 – 2.80 (m, 1H), 0.80 – 0.74 (m, 2H), 0.29 – 0.24 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 166.9, 158.6, 154.4, 136.2, 134.7, 129.3, 129.1, 128.9, 120.5, 114.27, 23.7, 7.3.

*6.5.25. N-Cyclobutyl-5-phenylthieno[2,3-d]pyrimidin-4-amine (**19dc**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 85.1 mg of a white amorphous solid (75%). LCMS (*m/z*): 281.9 $[M+H]^+$. HPLC: t_R 6.183 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{16}H_{15}N_3S$ requires 282.1059 $[M+H]^+$; found 282.1059. ^1H NMR ($CDCl_3$) δ

8.48 (s, 1H), 7.54 – 7.49 (m, 5H), 7.46 (dd, *J* = 6.7, 2.7 Hz, 2H), 7.07 (s, 1H), 5.10 (d, *J* = 5.5 Hz, 1H), 4.62 – 4.50 (m, 1H), 2.37 – 2.27 (m, 2H), 1.73 – 1.59 (m, 2H), 1.57 – 1.44 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 166.9, 156.4, 154.2, 136.2, 134.7, 129.4, 128.89, 128.7, 120.0, 113.9, 45.7, 31.2, 15.2.

*6.5.26. N,N-Diethyl-6-phenylthieno[2,3-d]pyrimidin-4-amine (**19ea**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 96.8 mg of a white amorphous solid (84%). LCMS (*m/z*): 284.0 $[M+H]^+$. HPLC: t_R 6.080 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{16}H_{17}N_3S$ requires 284.1213 $[M+H]^+$; found 284.1216. ^{1}H NMR ($CDCl_3$) δ 8.41 (s, 1H), 7.65 – 7.61 (m, 2H), 7.48 (s, 1H), 7.45 – 7.40 (m, 2H), 7.37 – 7.32 (m, 1H), 3.79 (q, *J* = 7.1 Hz, 4H), 1.36 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR ($CDCl_3$) δ 168.9, 156.9, 153.2, 138.1, 134.1, 129.2, 128.4, 126.3, 116.8, 116.4, 44.2, 13.4.

*6.5.27. N-Cyclopropyl-6-phenylthieno[2,3-d]pyrimidin-4-amine (**19eb**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 99.1 mg of a white amorphous solid (90%). LCMS (*m/z*): 267.9 $[M+H]^+$. HPLC: t_R 5.407 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{15}H_{13}N_3S$ requires 268.0830 $[M+H]^+$; found 268.0903. ^{1}H NMR (401 MHz, $CDCl_3$) δ 8.53 (s, 1H), 7.65 – 7.61 (m, 1H), 7.49 (s, 1H), 7.44 – 7.38 (m, 1H), 7.37 – 7.32 (m, 1H), 5.79 (s, 1H), 3.06 – 2.99 (m, 1H), 1.01 – 0.95 (m, 1H), 0.74 – 0.70 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.8, 158.2, 153.9, 140.7, 133.6, 129.2, 128.7, 126.4, 117.7, 113.3, 24.6, 8.2.

*6.5.28. N-Cyclobutyl-6-phenylthieno[2,3-d]pyrimidin-4-amine (**19ec**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 108 mg of a white amorphous solid (92%). LCMS (*m/z*): 281.9 $[M+H]^+$. HPLC: t_R 5.940 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{16}H_{15}N_3S$ requires 282.1058 $[M+H]^+$; found 282.1059. ^{1}H NMR ($CDCl_3$) δ

8.48 (s, 1H), 7.65 – 7.62 (m, 2H), 7.45 – 7.39 (m, 2H), 7.37 – 7.31 (m, 2H), 5.47 (d, *J* = 7.2 Hz, 1H), 4.83 – 4.72 (m, 1H), 2.63 – 2.41 (m, 2H), 2.07 – 1.95 (m, 2H), 1.87 – 1.77 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 166.1, 155.9, 154.1, 140.9, 133.6, 129.2, 128.7, 126.3, 117.6, 112.5, 46.4, 31.7, 15.3.

*6.5.29. 5-Cyclohexyl-N,N-diethylthieno[2,3-d]pyrimidin-4-amine (**19fa**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 115 mg of a white amorphous solid (81%). LCMS (*m/z*): 290.2 $[M+H]^+$. HPLC: t_R 7.189 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{16}H_{23}N_3S$ requires 290.1689 $[M+H]^+$; found 290.1685. ^{1}H NMR ($CDCl_3$) δ 8.58 (s, 1H), 7.02 (s, 1H), 3.47 (q, *J* = 7.1 Hz, 1H), 3.17 – 3.06 (m, *J* = 2.9 Hz, 1H), 1.98 (dd, *J* = 12.7, 1.3 Hz, 1H), 1.89 – 1.82 (m, 1H), 1.81 – 1.73 (m, 1H), 1.48 – 1.20 (m, 1H), 1.13 (t, *J* = 7.1 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ 169.8, 163.3, 151.6, 141.6, 119.9, 117.3, 45.0, 38.4, 34.8, 26.9, 26.2, 12.4.

*6.5.30. 5-Cyclohexyl-N-cyclopropylthieno[2,3-d]pyrimidin-4-amine (**19fb**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 87.8 mg of a white amorphous solid (88%). LCMS (*m/z*): 274.2 $[M+H]^+$. HPLC: t_R 6.073 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{16}H_{15}N_3S$ requires 274.1375 $[M+H]^+$; found 274.1372. ^{1}H NMR ($CDCl_3$) δ 8.55 (s, 1H), 6.87 (s, 1H), 5.57 (s, 1H), 3.07 – 2.99 (m, 1H), 2.59 (dd, *J* = 13.5, 5.5 Hz, 1H), 2.06 (t, *J* = 11.0 Hz, 2H), 1.95 (t, *J* = 10.5 Hz, 2H), 1.83 (d, *J* = 12.7 Hz, 1H), 1.50 – 1.38 (m, 4H), 1.30 (ddd, *J* = 13.7, 12.5, 4.9 Hz, 1H), 1.01 – 0.95 (m, 2H), 0.63 – 0.56 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 167.7, 158.6, 153.5, 139.7, 115.6, 115.3, 40.9, 33.9, 26.7, 25.9, 24.1, 7.7.

*6.5.31. N-Cyclobutyl-5-cyclohexylthieno[2,3-d]pyrimidin-4-amine (**19fc**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 115 mg of a white amorphous solid (81%). LCMS (*m/z*): 288.2 $[M+H]^+$. HPLC: t_R 6.820 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{16}H_{21}N_3S$ requires 288.1531 $[M+H]^+$; found 288.1529. ^{1}H NMR ($CDCl_3$) δ 8.43 (s, 1H), 6.85 (s, 1H), 5.51 (d, *J* = 6.3 Hz, 1H), 4.79 – 4.69 (m, 1H), 2.74 – 2.66 (m, 1H), 2.60 – 2.50 (m, 2H), 2.15 (d, *J* = 8.2 Hz, 2H), 2.02 – 1.90 (m, 4H), 1.91 – 1.81 (m, 5H), 1.53 – 1.40 (m, 5H), 1.34 (ddd, *J* = 12.5, 8.2, 3.5 Hz, 1H). ^{13}C NMR ($CDCl_3$) δ 167.8, 156.6, 153.5, 139.8, 115.2, 46.2, 41.0, 33.9, 31.7, 26.8, 26.0, 15.4.

*6.5.32. 6-Cyclohexyl-N,N-diethylthieno[2,3-d]pyrimidin-4-amine (**19ga**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 113 mg of a white amorphous solid (91%). LCMS (*m/z*): 290.0 $[M+H]^+$. HPLC: t_R 6.559 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{16}H_{23}N_3S$ requires 290.1683 $[M+H]^+$; found 290.1685. ^{1}H NMR ($CDCl_3$) δ 8.37 (s, 1H), 6.96 (d, *J* = 1.0 Hz, 1H), 3.73 (q, *J* = 7.1 Hz, 4H), 2.83 (tt, *J* = 11.2, 3.5 Hz, 1H), 2.10 – 2.04 (m, 2H), 1.89 – 1.82 (m, 2H), 1.79 – 1.71 (m, 1H), 1.55 – 1.35 (m, 4H), 1.32 (t, *J* = 7.1 Hz, 6H), 1.30 – 1.19 (m, 1H). ^{13}C NMR ($CDCl_3$) δ 167.9, 156.6, 152.5, 146.9, 115.4, 115.3, 44.07, 40.3, 35.1, 26.4, 25.9, 13.6.

*6.5.33. 6-Cyclohexyl-N-cyclopropylthieno[2,3-d]pyrimidin-4-amine (**19gb**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 84.8 mg of a white amorphous solid (87%). LCMS (*m/z*): 274.0 $[M+H]^+$. HPLC: t_R 5.986 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{15}H_{19}N_3S$ requires 274.1369 $[M+H]^+$; found 274.1372. ^{1}H NMR ($CDCl_3$) δ 8.49 (s, 1H), 6.94 (s, 1H), 5.63 (s, 1H), 2.97 (qd, *J* = 6.7, 3.6 Hz, 1H), 2.81 (td, *J* = 10.9, 3.2 Hz, 1H), 2.06 (d, *J* = 10.6 Hz, 2H), 1.87 – 1.81 (m, 2H), 1.74 (d, *J* = 12.7 Hz, 1H), 1.52 – 1.33 (m, 4H), 1.31 – 1.20 (m, 1H), 0.97 – 0.91 (m, 2H), 0.70 – 0.64 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 157.65, 153.3, 149.7, 116.5, 111.8, 40.2, 35.01, 26.3, 25.9, 24.5, 8.15.

*6.5.34. N-Cyclobutyl-6-cyclohexylthieno[2,3-d]pyrimidin-4-amine (**19gc**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 101 mg of a white amorphous solid (88%). LCMS (*m/z*): 288.0 $[M+H]^+$. HPLC: t_R 6.377 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{16}H_{21}N_3S$ requires 288.1528 $[M+H]^+$; found 288.1529. ^1H NMR ($CDCl_3$) δ 8.43 (s, 1H), 6.80 (d, *J* = 1.0 Hz, 1H), 5.31 (d, *J* = 7.2 Hz, 1H), 4.80 – 4.67 (m, 1H), 2.86 – 2.77 (m, 1H), 2.55 – 2.45 (m, 2H), 2.11 – 2.04 (m, 2H), 2.03 – 1.91 (m, 2H), 1.89 – 1.71 (m, 5H), 1.53 – 1.33 (m, 4H), 1.32 – 1.20 (m, 1H). ^{13}C NMR ($CDCl_3$) δ 165.36, 155.53, 153.38, 149.88, 116.4, 111.1, 46.3, 40.2, 35.0, 31.8, 26.4, 25.9, 15.3.

*6.5.35. N,N-Diethyl-5,6-dimethylthieno[2,3-d]pyrimidin-4-amine (**19ha**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 82.3 mg of a white amorphous solid (71%). LCMS (*m/z*): 236.0 $[M+H]^+$. HPLC: t_R 5.322 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{12}H_{17}N_3S$ requires 236.1212 $[M+H]^+$; found 236.1216. ^1H NMR ($CDCl_3$) δ 8.50 (s, 1H), 3.46 (q, *J* = 7.1 Hz, 4H), 2.43 (d, *J* = 0.8 Hz, 3H), 2.41 (d, *J* = 0.7 Hz, 3H), 1.12 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR ($CDCl_3$) δ 167.2, 162.0, 151.1, 131.3, 124.9, 122.3, 44.8, 13.9, 13.7, 12.3.

*6.5.36. N-Cyclopropyl-5,6-dimethylthieno[2,3-d]pyrimidin-4-amine (**19hb**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 101 mg of a white amorphous solid (93%). LCMS (*m/z*): 220.1 $[M+H]^+$. HPLC: t_R 5.322 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{11}H_{13}N_3S$ requires 220.0898 $[M+H]^+$; found 220.0903. ^1H NMR ($CDCl_3$) δ 8.49 (s, 1H), 5.65 (s, 1H), 2.98 – 2.91 (m, 1H), 2.41 (s, 6H), 0.97 – 0.91 (m, 2H), 0.63 – 0.58 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 164.6, 158.4, 153.2, 130.2, 122.9, 117.2, 24.1, 14.5, 13.5, 7.7.

*6.5.37. N-Cyclobutyl-5,6-dimethylthieno[2,3-d]pyrimidin-4-amine (**19hc**)*

General procedure A. Purification by FCC (eluent, 1:1 EtOAc/PE) afforded 85.5 mg of a white amorphous solid (73%). LCMS (*m/z*): 234.0 $[M+H]^+$. HPLC: t_R 5.940 min, >95% purity (214 & 254 nm). HRMS (*m/z*): $C_{16}H_{15}N_3S$ requires 234.1056 $[M+H]^+$; found 234.1059. ^1H NMR ($CDCl_3$) δ

8.37 (s, 1H), 5.55 (d, J = 5.9 Hz, 1H), 4.81 – 4.59 (m, 1H), 2.54 – 2.48 (m, 2H), 2.46 (s, 3H), 2.41 (d, J = 0.6 Hz, 3H), 1.98 – 1.86 (m, 2H), 1.86 – 1.76 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 164.8, 156.5, 153.1, 129.8, 123.0, 116.8, 46.2, 31.8, 15.4, 14.5, 13.5.

6.6. Pharmacological Characterisation

6.6.1. Materials

Dulbecco's modified Eagle's medium, Flp-In CHO cells, and hygromycin B were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from ThermoTrace (Melbourne, VIC, Australia). All other reagents were purchased from Sigma Aldrich (St. Louis, MO).

6.6.2. Cell culture and transfection for cAMP assay

FlpIn CHO cells stably expressing the human dopamine D_{2L} receptor (or WT FlpIn CHO cells for off-target screening assay) were maintained in DMEM supplemented with 5% foetal calf serum (FBS) and 0.2 mg/mL hygromycin at 37 °C in a humidified incubator supplied with 5% CO_2.

6.6.3. cAMP measurement (interaction & off-target assay)

The cellular cAMP levels were measured with the CAMYEL BRET-based biosensor for cAMP.[26] One day after transfection, cells were trypsinised and seeded in white 96-well microplates. The cells were then cultured for an additional day, rinsed twice with Hank's Balanced Salt Solution (HBSS) and were then incubated in fresh HBSS. The *Renilla* luciferase (*RLuc*) substrate coelenterazine-h was added to reach a final concentration of 5 μM.

Interaction assay: FlpIn CHO cells stably expressing the human dopamine D_{2L} receptor were stimulated with dopamine in the presence of 10 μM forskolin (final concentration). For the antagonism assay, the allosteric antagonists were added 30 min prior to stimulation. The BRET signals were measured using a BMG Lumistar counter 30 min after stimulation. The BRET signal (BRET ratio) was determined by calculating the ratio of the light emitted at 535 ± 30 nm (YFP) to the light emitted at 475 ± 30 nm (RLuc).

Off-target assay using non-hD_{2L}R-expressing Flp-In CHO cells: The allosteric antagonists (1-100 μM), and dopamine (10 μM) were added 30 min prior to stimulation in the presence or absence of 10 μM forskolin (final concentration). The BRET signals were measured using a BMG Lumistar counter 30 min after stimulation. The BRET signal (BRET ratio) was determined by calculating the ratio of the light emitted at 535 ± 30 nm (YFP) to the light emitted at 475 ± 30 nm (RLuc).

6.6.4. Data analysis

Computerized nonlinear regression, statistical analyses and simulations were performed using Prism 6.0 (GraphPad Prism 6.0b Software, San Diego, CA).

6.6.5. Analysis of functional data

All concentration-response data were fitted to the following modified four-parameter Hill equation to derive potency estimates.[36]

$$E = Basal + \frac{(E_{max} - Basal).[A]^{nH}}{[A^{nH}] + EC_{50}^{nH}} \quad (1)$$

where E is the effect of the system, nH is the Hill slope and EC_{50} is the concentration of agonist [*A*] that gives the midpoint response between basal and maximal effect of dopamine or other agonists (E_{max}), which are the lower and upper asymptotes of the response, respectively.

To determine the mode of interaction of **1** and analogues of **1** at the D_2R in relation to the agonist dopamine, data were fit to both a competitive and allosteric model and the best fit compared statistically. A logistic equation of competitive agonist-antagonist interaction was globally fitted to data from functional experiments measuring the interaction between dopamine and all analogues of **1**:

$$Response = Bottom + \frac{(E_{max} - Bottom)}{1 + \left(\frac{10^{-pEC_{50}\left[1+\left(\frac{[B]}{10^{-K_B}}\right)\right]^{s}}}{[A]} \right)^{nH}} \quad (2)$$

Where *s* represents the Schild slope for the antagonist and pA_2 represents the negative logarithm of the molar concentration of antagonist that makes it necessary to double the concentration of agonist needed to elicit the original submaximal response obtained in the absence of antagonist. The same data describing the interaction between all analogues of **1** and dopamine were also analyzed using a complete operational model of allosterism and agonism according to equation 5:[37]

$$E = \frac{E_m(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^{nH}}{([A]K_B + K_AK_B + K_A[B] + \alpha[A][B])^{nH} + (\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^{nH}} \quad (3)$$

Where E_m is the maximum possible cellular response, [A] and [B] are the concentrations of orthosteric and allosteric ligands, respectively, K_A and K_B are the equilibrium dissociation constant of the orthosteric and allosteric ligands, respectively, τ_A and τ_B (constrained to -100) are operational measures of orthosteric and allosteric ligand efficacy (which incorporate both signal efficiency and receptor density), respectively, α is the binding cooperativity parameter between the orthosteric and allosteric ligand, and β denotes the magnitude of the allosteric effect of the modulator on the efficacy of the orthosteric agonist. K_A was constrained to 617 nM, and represents a value of functional affinity determined by an operational model of agonism applied to concentration-response data of dopamine in the presence of increasing concentrations of the alkylating agent phenoxybenzamine. For compounds that caused a limited rightward shift of the dopamine dose-response curve but no decrease in E_{max}, data were fit using an operational model of allosterism where Logβ was constrained to 0 to represent neutral cooperativity with dopamine efficacy. For

compounds that produced an unlimited decrease in the maximal response of dopamine Logβ was constrained to -3.

For each of the compounds the two equations (models) were then compared for their fit using an extra-sum-of-squares *F* test. All of the data points and values shown in the figures and tables are the mean ± S.E.M. of at least three separate experiments performed in duplicate unless otherwise stated.

Associated Content

Supporting Information:

^{1}H & ^{13}C NMR spectra are included for all compounds depicted in Figures 3 & 4 (**9d**, **9f**, **10**, **15b**, **19aa**, **19bb**, **19bc**, **19cb**, **19hc**), together with compounds found to non-specifically inhibit cAMP accumulation (**9c** & **19ca**). Reverse-phase analytical HPLC traces and high-resolution mass spectra are included for key compounds highlighted in the TOC graphic (**9d**, **9h**, **9i**, **19fc**).

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Author Contributions

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Abbreviation list

cAMP, cyclic adenosine monophosphate; D_2R, dopamine D_2 receptor; D_3R, dopamine D_3 receptor; dopamine, DA; GPCR, G protein-coupled receptor; EPS, extrapyramidal symptoms; NAM, negative allosteric modulator; PAM, positive allosteric modulator; VLS, virtual ligand screening; SAR, structure-activity relationship; CHO, chinese hamster ovary; BRET, bioluminescence resonance energy transfer; FBS, foetal bovine serum; HBSS, hank's balanced salt solution; SEM, standard error of mean; DMF, *N*,*N*-dimethylformamide, $POCl_3$, phosphorus oxychloride; *i*-PrOH, isopropyl alcohol; EtOAc, ethyl acetate; DCM, dichloromethane; MWI, microwave irradiation.

References

1. Ginovart, N.; Kapur, S. Role of dopamine D(2) receptors for antipsychotic activity. *Handb. Exp. Pharmacol.* **2012,** *212*, 27-52.
2. Miyamoto, S.; Miyake, N.; Jarskog, L. F.; Fleischhacker, W. W.; Lieberman, J. A. Pharmacological treatment of schizophrenia: a critical review of the pharmacology and clinical effects of current and future therapeutic agents. *Mol. Psychiatr.* **2012,** *17*, 1206-1227.
3. Meltzer, H. Y. Update on typical and atypical antipsychotic drugs. *Annu. Rev. Med.* **2013,** *64*, 393-406.
4. Leucht, S.; Corves, C.; Arbter, D.; Engel, R. R.; Li, C.; Davis, J. M. Second-generation versus first-generation antipsychotic drugs for schizophrenia: a meta-analysis. *Lancet* **2009,** *373*, 31-41.
5. Farde, L.; Nordstrom, A. L.; Wiesel, F. A.; Pauli, S.; Halldin, C.; Sedvall, G. Positron emission tomographic analysis of central D1 and D2 dopamine receptor occupancy in patients treated with classical neuroleptics and clozapine. Relation to extrapyramidal side effects. *Arch. Gen. Psychiat.* **1992,** *49*, 538-544.
6. Caron, M. G.; Beaulieu, M.; Raymond, V.; Gagne, B.; Drouin, J.; Lefkowitz, R. J.; Labrie, F. Dopaminergic receptors in the anterior pituitary gland. Correlation of [3H]dihydroergocryptine binding with the dopaminergic control of prolactin release. *J. Biol. Chem.* **1978,** *253*, 2244-2253.
7. Newcomer, J. W. Second-generation (atypical) antipsychotics and metabolic effects: a comprehensive literature review. *CNS Drugs* **2005,** *19*, 1-93.
8. Maggio, R.; Novi, F.; Scarselli, M.; Corsini, G. U. The impact of G-protein-coupled receptor hetero-oligomerization on function and pharmacology. *FEBS J.* **2005,** *272*, 2939-2946.
9. Maggio, R.; Scarselli, M.; Capannolo, M.; Millan, M. J. Novel dimensions of D3 receptor function: Focus on heterodimerisation, transactivation and allosteric modulation. *Eur. Neuropsychopharmacol.* **2015,** *25*, 1470-1479.
10. Maggio, R.; Millan, M. J. Dopamine D2-D3 receptor heteromers: pharmacological properties and therapeutic significance. *Curr. Opin. Pharmacol.* **2010,** *10*, 100-1077.
11. Carli, M.; Kolachalam, S.; Aringhieri, S.; Rossi, M.; Giovannini, L.; Maggio, R.; Scarselli, M. Dopamine D2 receptors dimers: how can we pharmacologically target them? *Curr. Neuropharmacol.* **2018,** *16*, 222-230.
12. Rossi, M.; Fasciani, I.; Marampon, F.; Maggio, R.; Scarselli, M. The first negative allosteric modulator for dopamine D2 and D3 Receptors, SB269652 may lead to a new generation of antipsychotic drugs. *Mol. Pharmacol.* **2017,** *91*, 586-594.

13. Silvano, E.; Millan, M. J.; Mannoury la Cour, C.; Han, Y.; Duan, L.; Griffin, S. A.; Luedtke, R. R.; Aloisi, G.; Rossi, M.; Zazzeroni, F.; Javitch, J. A.; Maggio, R. The tetrahydroisoquinoline derivative SB269,652 is an allosteric antagonist at dopamine D3 and D2 receptors. *Mol. Pharmacol.* **2010,** *78*, 925-934.
14. Shonberg, J.; Draper-Joyce, C.; Mistry, S. N.; Christopoulos, A.; Scammells, P. J.; Lane, J. R.; Capuano, B. Structure-activity study of *N*-((trans)-4-(2-(7-cyano-3,4-dihydroisoquinolin-2(1*H*)-yl)ethyl)cyclohexyl)-1*H*-indole-2-carboxamide (SB269652), a bitopic ligand that acts as a negative allosteric modulator of the dopamine D2 receptor. *J. Med. Chem.* **2015,** *58*, 5287-5307.
15. Lane, J. R.; Donthamsetti, P.; Shonberg, J.; Draper-Joyce, C. J.; Dentry, S.; Michino, M.; Shi, L.; Lopez, L.; Scammells, P. J.; Capuano, B.; Sexton, P. M.; Javitch, J. A.; Christopoulos, A. A new mechanism of allostery in a G protein-coupled receptor dimer. *Nat. Chem. Biol.* **2014,** *10*, 745-752.
16. Kumar, V.; Moritz, A. E.; Keck, T. M.; Bonifazi, A.; Ellenberger, M. P.; Sibley, C. D.; Free, R. B.; Shi, L.; Lane, J. R.; Sibley, D. R.; Newman, A. H. Synthesis and pharmacological characterization of novel trans-cyclopropylmethyl-linked bivalent ligands that exhibit selectivity and allosteric pharmacology at the dopamine D3 receptor (D3R). *J. Med. Chem.* **2017,** *60*, 1478-1494.
17. Lane, J. R.; Chubukov, P.; Liu, W.; Canals, M.; Cherezov, V.; Abagyan, R.; Stevens, R. C.; Katritch, V. Structure-based ligand discovery targeting orthosteric and allosteric pockets of dopamine receptors. *Mol. Pharmacol.* **2013,** *84*, 794-807.
18. Fyfe, T. J.; Zarzycka, B.; Lim, H. D.; Kellam, B.; Mistry, S. N.; Katritch, V.; Scammells, P. J.; Lane, J. R.; Capuano, B. A thieno[2,3-*d*]pyrimidine scaffold is a novel negative allosteric modulator of the dopamine D2 receptor. *J. Med. Chem.* **2018,** *62*, 174-206.
19. Kopinathan, A.; Draper-Joyce, C.; Szabo, M.; Christopoulos, A.; Scammells, P. J.; Lane, J. R.; Capuano, B. Subtle modifications to the indole-2-carboxamide motif of the negative allosteric modulator *N*-((trans)-4-(2-(7-cyano-3,4-dihydroisoquinolin-2(1*H*)-yl)ethyl)cyclohexyl)-1*H*-indole-2-carboxamide (SB269652) yield dramatic changes in pharmacological activity at the dopamine D2 receptor. *J. Med. Chem.* **2018,** *62*, 371-377.
20. Gewald, K.; Schinke, E.; Böttcher, H. Heterocyclen aus CH-aciden nitrilen, VIII. 2-Amino-thiophene aus methylenaktiven nitrilen, carbonylverbindungen und schwefel. *Chem. Ber.* **1966,** *99*, 94-100.
21. Leach, K.; Sexton, P. M.; Christopoulos, A. Allosteric GPCR modulators: taking advantage of permissive receptor pharmacology. *Trends Pharmacol. Sci.* **2007,** *28*, 382-389.
22. Christopoulos, A. Assessing the distribution of parameters in models of ligand-receptor interaction: to log or not to log. *Trends Pharmacol. Sci.* **1998,** *19*, 351-357.
23. Inglese, J.; Johnson, R. L.; Simeonov, A.; Xia, M.; Zheng, W.; Austin, C. P.; Auld, D. S. High-throughput screening assays for the identification of chemical probes. *Nat. Chem. Biol.* **2007,** *3*, 466-479.
24. Auld, D. S.; Southall, N. T.; Jadhav, A.; Johnson, R. L.; Diller, D. J.; Simeonov, A.; Austin, C. P.; Inglese, J. Characterization of chemical libraries for luciferase inhibitory activity. *J. Med. Chem.* **2008,** *51*, 2372-2386.
25. Heitman, L. H.; van Veldhoven, J. P.; Zweemer, A. M.; Ye, K.; Brussee, J.; AP, I. J. False positives in a reporter gene assay: identification and synthesis of substituted *N*-pyridin-2-ylbenzamides as competitive inhibitors of firefly luciferase. *J. Med. Chem.* **2008,** *51*, 4724-4729.
26. Jiang, L. I.; Collins, J.; Davis, R.; Lin, K. M.; DeCamp, D.; Roach, T.; Hsueh, R.; Rebres, R. A.; Ross, E. M.; Taussig, R.; Fraser, I.; Sternweis, P. C. Use of a cAMP BRET sensor to characterize a novel regulation of cAMP by the sphingosine 1-phosphate/G13 pathway. *J. Biol. Chem.* **2007,** *282*, 10576-10584.
27. Ballesteros, J. A.; Weinstein, H. Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. In *Methods Neurosci.*, Sealfon, S. C., Ed. Academic Press: 1995; Vol. *25*, pp 366-428.
28. Wang, S.; Che, T.; Levit, A.; Shoichet, B. K.; Wacker, D.; Roth, B. L. Structure of the D2 dopamine receptor bound to the atypical antipsychotic drug risperidone. *Nature* **2018,** *555*, 269-273.
29. Sharma, S.; Rodriguez, A. L.; Conn, P. J.; Lindsley, C. W. Synthesis and SAR of a mGluR5 allosteric partial antagonist lead: unexpected modulation of pharmacology with slight structural modifications to a 5-(phenylethynyl)pyrimidine scaffold. *Bioorg. Med. Chem. Lett.* **2008,** *18*, 4098-4101.
30. Conn, P. J.; Jones, C. K.; Lindsley, C. W. Subtype-selective allosteric modulators of muscarinic receptors for the treatment of CNS disorders. *Trends Pharmacol. Sci.* **2009,** *30*, 148-155.
31. Bridges, T. M.; Marlo, J. E.; Niswender, C. M.; Jones, C. K.; Jadhav, S. B.; Gentry, P. R.; Plumley, H. C.; Weaver, C. D.; Conn, P. J.; Lindsley, C. W. Discovery of the first highly M5-preferring muscarinic

acetylcholine receptor ligand, an M5 positive allosteric modulator derived from a series of 5-trifluoromethoxy *N*-benzyl isatins. *J. Med. Chem.* **2009,** *52*, 3445-3448.
32. Bridges, T. M.; Phillip Kennedy, J.; Noetzel, M. J.; Breininger, M. L.; Gentry, P. R.; Conn, P. J.; Lindsley, C. W. Chemical lead optimization of a pan Gq mAChR M1, M3, M5 positive allosteric modulator (PAM) lead. Part II: development of a potent and highly selective M1 PAM. *Bioorg. Med. Chem. Lett.* **2010,** *20*, 1972-1975.
33. Lindsley, C. W.; Zhao, Z.; Leister, W. H.; Robinson, R. G.; Barnett, S. F.; Defeo-Jones, D.; Jones, R. E.; Hartman, G. D.; Huff, J. R.; Huber, H. E.; Duggan, M. E. Allosteric Akt (PKB) inhibitors: discovery and SAR of isozyme selective inhibitors. *Bioorg. Med. Chem. Lett.* **2005,** *15*, 761-764.
34. Scott, S. A.; Selvy, P. E.; Buck, J. R.; Cho, H. P.; Criswell, T. L.; Thomas, A. L.; Armstrong, M. D.; Arteaga, C. L.; Lindsley, C. W.; Brown, H. A. Design of isoform-selective phospholipase D inhibitors that modulate cancer cell invasiveness. *Nat. Chem. Biol.* **2009,** *5*, 108-117.
35. Lavieri, R. R.; Scott, S. A.; Selvy, P. E.; Kim, K.; Jadhav, S.; Morrison, R. D.; Daniels, J. S.; Brown, H. A.; Lindsley, C. W. Design, synthesis, and biological evaluation of halogenated *N*-(2-(4-oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-8-yl)ethyl)benzamides: discovery of an isoform-selective small molecule phospholipase D2 inhibitor. *J. Med. Chem.* **2010,** *53*, 6706-6719.
36. Motulsky, H. J. C. Fitting models to biological data using linear and nonlinear regression: a practical guide to curve fitting. In Graphpad Software Inc., San Diego CA.: 2003.
37. Christopoulos, A.; Kenakin, T. G protein-coupled receptor allosterism and complexing. *Pharmacol. Rev.* **2002,** *54*, 323-374.

Subtle Modifications to a Thieno[2,3-*d*]pyrimidine Scaffold Yield Negative Allosteric Modulators and Agonists of the Dopamine D_2 Receptor

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Highlights

- Structural changes furnished allosteric fragment-like cores with improved ligand efficiency
- Substituents at the thieno[2,3-*d*]pyrimidine core are crucial for binding and function at the D_2R
- Subtle variations cause a change in pharmacology from that of a D_2R NAM to that of a weak D_2R agonist