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Novel Fused Arylpyrimidinone Based Allosteric Modulators of the M_1 Muscarinic Acetylcholine Receptor

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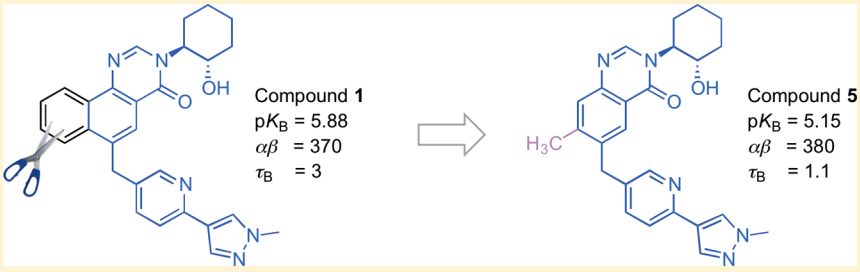

ABSTRACT: Benzoquinazolinone **1** is a positive allosteric modulator (PAM) of the M_1 muscarinic acetylcholine receptor (mAChR), which is significantly more potent than the prototypical PAM, 1-(4-methoxybenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (BQCA). In this study, we explored the structural determinants that underlie the activity of **1** as a PAM of the M_1 mAChR. We paid particular attention to the importance of the tricyclic scaffold of compound **1**, for the activity of the molecule. Complete deletion of the peripheral fused benzene ring caused a significant decrease in affinity and binding cooperativity with acetylcholine (ACh). This loss of affinity was rescued with the addition of either one or two methyl groups in the 7- and/or 8-position of the quinazolin-4(3*H*)-one core. These results demonstrate that the tricyclic benzo[*h*]quinazolin-4(3*H*)-one core could be replaced with a quinazolin-4(3*H*)-one core and maintain functional affinity. As such, the quinazolin-4(3*H*)-one core represents a novel scaffold to further explore M_1 mAChR PAMs with improved physicochemical properties.

KEYWORDS: *M_1 muscarinic acetylcholine receptor, positive allosteric modulator*

Alzheimer's disease is a progressive and irreversible neurodegenerative disorder affecting around 25 million people worldwide.[1] The disorder is primarily observed in the aging population, and characteristic symptoms of the disease include memory loss, confusion, and dementia.[2,3] Currently, pharmacological interventions for Alzheimer's disease remain limited, and provide only symptomatic relief to patients.[4,5]

The reduction of cholinergic neurons in the basal nuclear complex is associated with the cognitive deficits observed in patients with Alzheimer's disease.[6,7] A link between mAChR function and disease pathology has been suggested, with the M_1 mAChR subtype particularly highlighted for a role in cognition.[7,8]

Acetylcholinesterase inhibitors are currently used to treat the cognitive deficits associated with Alzheimer's disease, but this approach is limited by the moderate improvement in the cognitive function of patients, as well as debilitating side effects including nausea, diarrhea, hypotension and vomiting.[9] However, they exert a nonselective effect at all muscarinic receptor subtypes, because acetylcholine esterase inhibitors act to inhibit acetylcholine breakdown. It is likely that such side-effects are due activation of M_2 and M_3 mAChRs expressed in the periphery.[7]

Accordingly, there has been considerable focus upon the design of ligands that selectively activate the M_1 mAChR. However, the design of selective orthosteric agonists for the M_1 mAChR has proven difficult, due to the highly conserved orthosteric pocket of all the mAChRs (M_1–M_5).[10] However, efforts to target the less-conserved, and topographically distinct allosteric site of the receptor have proven more fruitful.[11−14] Ligands that target such allosteric sites may act to potentiate the binding and signaling activity of an orthosteric receptor agonist (positive allosteric modulators, PAM) and/or activate the receptor themselves (allosteric agonists). Allosteric ligands of the M_1 mAChR have been recognized as a potentially promising novel drug class for the treatment of Alzheimer's disease.[14]

BQCA was reported as the first highly selective PAM for the M_1 mAChR.[15] Structure−activity relationship (SAR) studies around BQCA have revealed structurally related compounds with higher affinity and potency.[16−18] Furthermore, our group has reported an enriched SAR study that used analytical modeling of pharmacological data, to relate structural modification to BQCA analogues, with variations in binding affinity (pK_B), binding (α) and functional ($\alpha\beta$) cooperativity,

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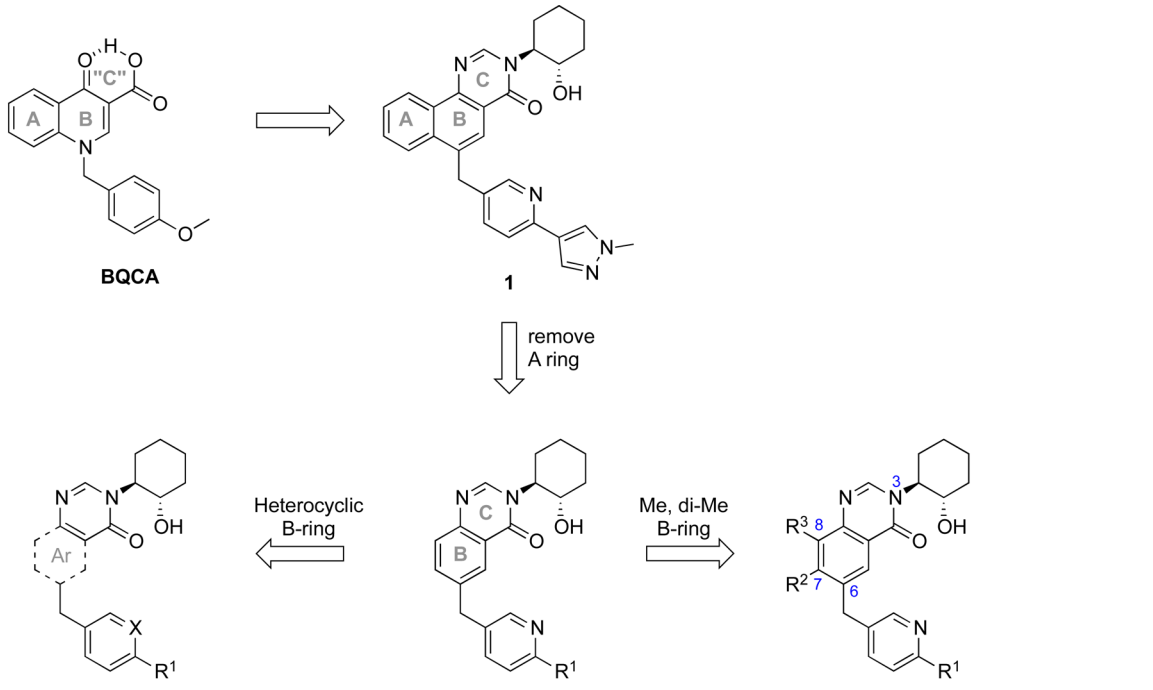
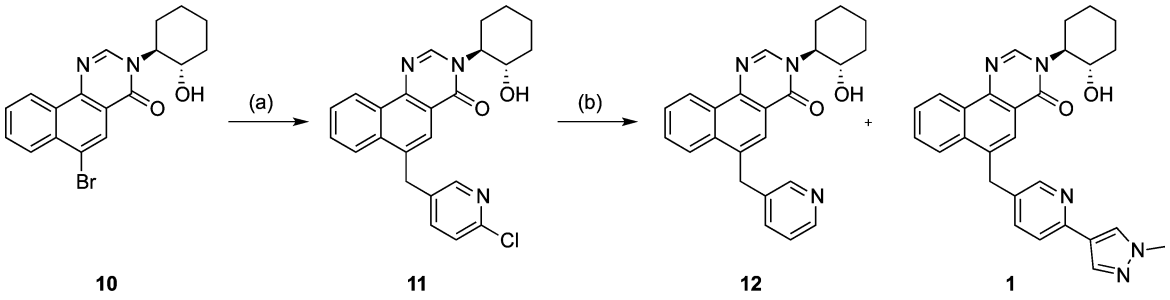


Figure 1. Top: Chemical structure of lead compounds 1-(4-methoxybenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (BQCA) and 3-((1*S*,2*S*)-2-hydroxycyclohexyl)-6-((6-(1-methyl-1*H*-pyrazol-4-yl)pyridin-3-yl)methyl)benzo[*h*]quinazolin-4(3*H*)-one (**1**). Bottom: Overview of the structures and design strategy behind the novel analogues derived from compound **1**. The numbering of the atoms in the quinazolinone ring bearing substituents is shown in blue.

Scheme 1. Synthesis of Compound 1[a]



[a]Reagents and conditions: (a) (i) cat. $Pd(P(^tBu)_3)_2$, degassed anhydrous THF, 0 °C; (ii) 0.5 M (2-chloro-5-pyridyl)methylzinc chloride/THF, 0 °C to rt, 79%; (b) 1-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole, cat. $PdCl_2(PPh_3)_2$, 1 M $Na_2CO_{3(aq)}$/THF 1:3 degassed, 100 °C, 64% (59% **1**: 5% **12**).

and intrinsic efficacy (τ_B).[19] 3-((1*S*,2*S*)-2-Hydroxycyclohexyl)-6-((6-(1-methyl-1*H*-pyrazol-4-yl)pyridin-3-yl)methyl)benzo-[*h*]quinazolin-4(3*H*)-one (**1**), is a significantly more potent M_1 mAChR PAM with a structural ancestry originating from BQCA and related compounds (Figure 1).[17,20] We recently used a combination of site-directed mutagenesis, modeling of pharmacological data and molecular dynamics simulations to propose a binding mode for **1** at the M_1 mAChR, similar to that predicted for BQCA. In particular, residues $Y85^{2.64}$ and $Y82^{2.61}$ in transmembrane (TM) bundle 2, Y179 in extracellular loop (ECL) 2, and $W400^{7.35}$ in TM 7 were shown to be important for the binding and function of both BQCA and compound **1**.[21] This approach also revealed that the higher potency of **1** was predominantly driven by an increase in affinity, rather than cooperativity with ACh, for the M_1 mAChR allosteric site. The current study aimed to explore the structural determinants that underlie the activity of **1** as a PAM of the M_1 mAChR and, in particular, those that are responsible for its superior potency. Furthermore, we aimed to move away from the tricyclic scaffold of compound **1** and determine the importance of this moiety for the activity of the molecule (Figure 1), using approaches such as core trimming (compounds **2**–**9**). An enriched SAR profile was compiled to explore the important features of these novel compounds to maintain high affinity, cooperativity, and intrinsic efficacy.

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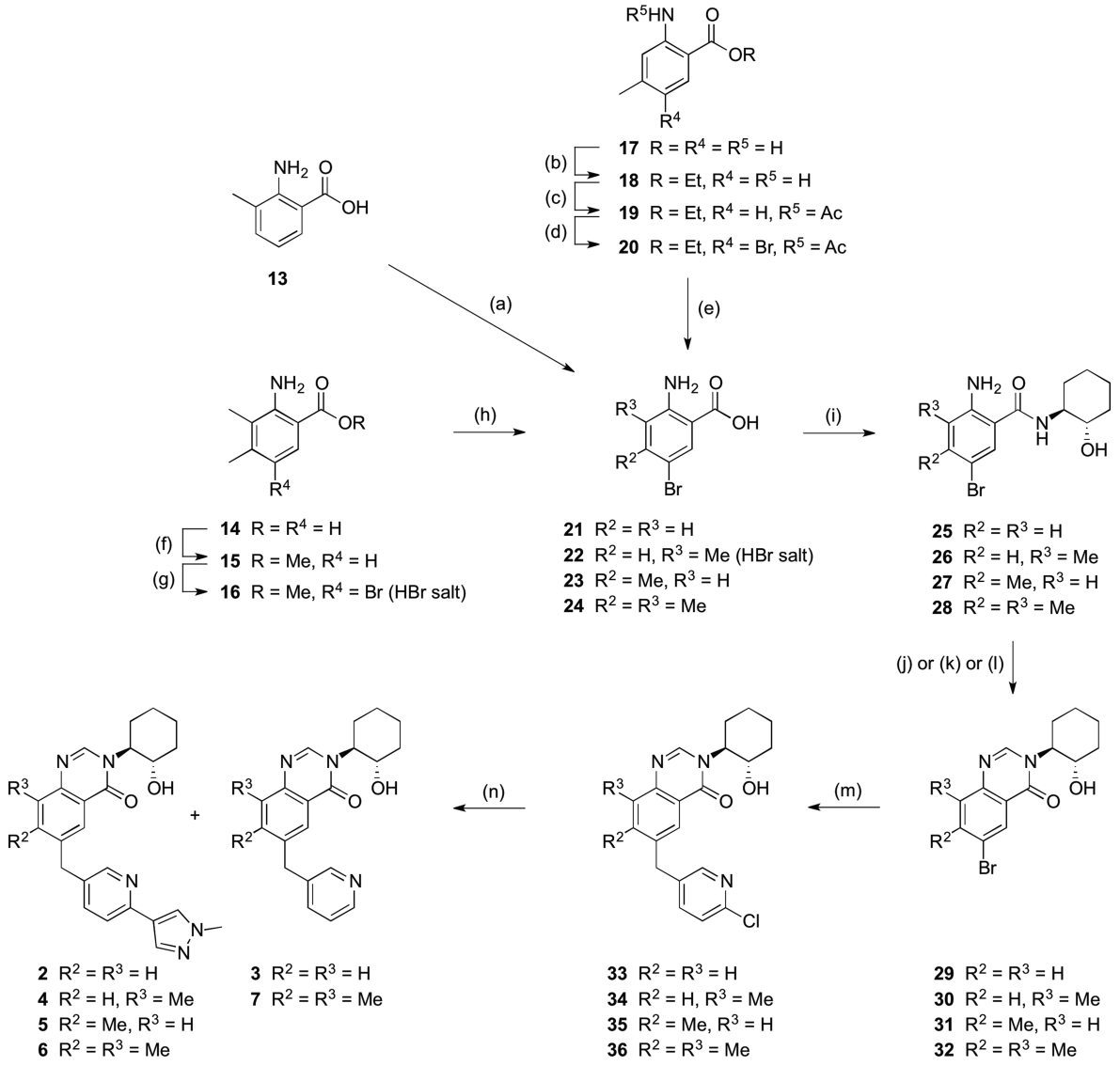
Scheme 2. Synthesis of Analogues 2−7[a]



[a]Reagents and conditions: (a) Br_2, AcOH, DCM, rt, 95%; (b) cat. conc. $H_2SO_{4(aq)}$, EtOH, reflux, 75%; (c) acetyl chloride, TEA, DCM, 0 °C to rt, 100%; (d) Br_2, AcOH, rt, 23% (brsm); (e) 5 M $NaOH_{(aq)}$, EtOH, 90 °C, 81%; (f) cat. $H_2SO_{4(aq)}$, MeOH, reflux, 83%; (g) (i) 1,4-dioxane/CCl_4 1:1, 0 °C; (ii) Br_2, 1,4-dioxane/CCl_4 1:1, dropwise, 0 °C, 93%; (h) LiOH·H_2O, THF, water, rt, 94%; (i) HCTU, (1*S*,2*S*)-2-aminocyclohexanol hydrochloride, DIPEA, DMF, rt, 77−95%; (j) DMF-DMA, 85 or 115 °C, 63−95%; (k) formamide, 150 or 180 °C, 44−69%; (l) triethylorthoformate, 100−150 °C, 65%; (m) (i) cat. $Pd(P(^tBu)_3)_2$, dry THF, 0 °C; (ii) 0.5 M (2-chloro-5-pyridylmethyl)zinc chloride/THF, 0 °C to RT or 55 °C, 14−90%; (n) 1-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-*1H*-pyrazole, cat. $PdCl_2(PPh_3)_2$, 1 M $Na_2CO_{3(aq)}$/THF 1:3 degassed, 100 °C, Suzuki product 15−96%, dehalogenation product 0−14%.

RESULTS AND DISCUSSION

Chemistry. The synthesis of lead compound **1**, though previously reported in patent literature,[20] has only recently been fully optimized and characterized in our hands.[21] Seeking to understand the basis for the PAM activity of compound **1**, we initially decided to pharmacologically characterize key synthetic intermediates. Compound **10** was synthesized as previously described, with subsequent Negishi coupling carried out according to established methodology,[20,21] affording intermediate **11** in good yield. After the final Suzuki coupling of **11** with commercially available 1-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-*1H*-pyrazole, we were able to isolate and characterize both the desired Suzuki product **1**, and corresponding dehalogenated product **12** (Scheme 1).[21]

Aside from pharmacological characterization of compounds **10**, **12**, and **1**, which represent a stepwise buildup of the (pyridin-3-yl)methyl pendant group, we also sought to investigate the nature of the polyaromatic core. Given the precedence for polyaromatic heterocycles potentially imparting toxic DNA-chelation behavior to a scaffold,[22] investigation of related heteroaromatics seemed a prudent avenue of investigation. Initially we envisaged generating comparable analogues of **1**, incorporating gradual deletion of the benzo[*h*]-quinazolin-4(3*H*)-one core toward a quinazolin-4(3*H*)-one core. The deletion of the fused benzene ring was also anticipated to make these analogues more "druglike", through reductions in lipophilicity (assessed through calculated log *P*), topological polar surface area (tPSA), and molecular weight.[23] With this strategy in mind, we synthesized analogues with

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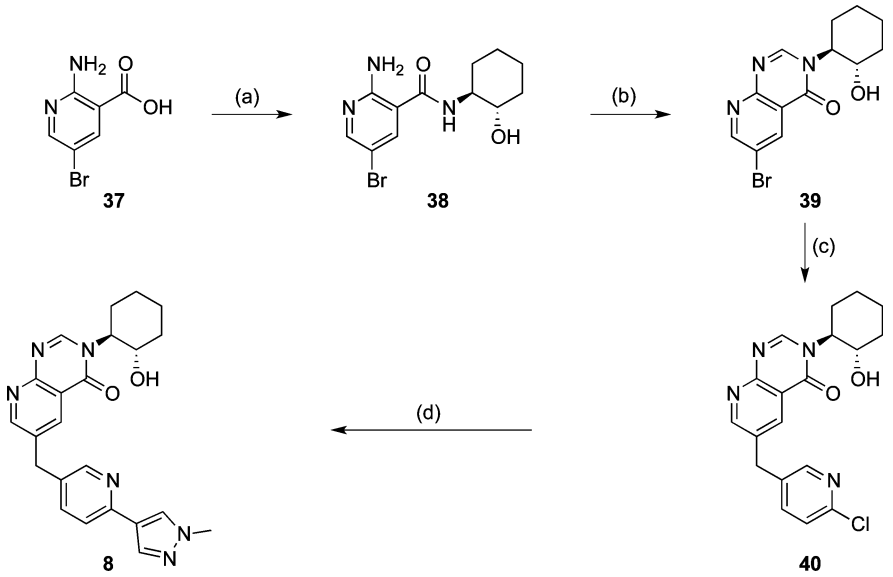
Scheme 3. Synthesis of Compound 8 Containing a Pyrido[2,3-*d*]pyrimidinone Core[a]



[a]Reagents and conditions: (a) HCTU, (1*S*,2*S*)-2-aminocyclohexanol hydrochloride, DIPEA, DMF, rt, 94%; (b) formamide, 150 or 180 °C, 69%; (c) (i) cat. $Pd(P(^tBu)_3)_2$, dry THF, 0 °C; (ii) 0.5 M (2-chloro-5-pyridylmethyl)zinc chloride/THF, 0 °C to RT or 55 °C, 90%; (d) 1-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-*1H*-pyrazole, cat. $PdCl_2(PPh_3)_2$, 1 M $Na_2CO_{3(aq)}$/THF 1:3 degassed, 100 °C, 24%.

either complete deletion of the peripheral fused benzene ring (**2**), or the presence of one or two methyl groups in the 7/8 positions of the quinazolin-4(3*H*)-one core, to give **4**, **5** and **6**. Evaluation of these compounds was anticipated to address whether the peripheral fused benzene ring of literature compound **1**, could be replaced by the steric presence of either one or two methyl groups.

Our recent work determining the structural nature of the interaction of compound **1** with the allosteric binding site at the M_1 mAChR, highlighted the importance of Y179 in ECL2, making aromatic edge-face interactions with both the benzylic pendant group and benzo[*h*]quinazolin-4(3*H*)-one core.[21] In addition, the proximity of the phenol moiety of Y179 could facilitate additional polar interactions with an appropriately positioned heteroatom incorporated into the ligand. With this in mind, we also synthesized **8**, the pyrido[2,3-*d*]pyrimidin-4(3*H*)-one analogue of **2**.

Synthesis of these analogues was carried out in a similar manner to that of lead compound **1**. In the case of **2**, commercially available 2-amino-5-bromobenzoic acid (**21**) was employed, while the remaining substituted 2-amino-5-bromobenzoic acid intermediates (**22**−**24**), required synthesis from unbrominated starting materials.

2-Amino-3,4-dimethylbenzoic acid (**14**) underwent initial Fischer esterification to give the corresponding methyl ester **15**, followed by selective bromination of the 5-position to give intermediate **16** as the hydrobromide salt, in excellent yield. Subsequent basic hydrolysis afforded the desired 2-amino-5-bromo-3,4-dimethylbenzoic acid (**24**). In the case of 2-amino-3-methylbenzoic acid (**13**), we were able to directly brominate in acetic acid at room temperature, to give **22**, without the need for esterification of the carboxylic acid moiety (Scheme 2). Finally, in the case of 2-amino-4-methylbenzoic acid (**23**), our attempts at direct bromination in the same manner as for **22**, gave a mixture of mono- and dibrominated products, in addition to unreacted starting material, which proved difficult to separate. However, selective bromination in the 5-postion was achieved through esterification of the carboxylic acid moiety, and acetylation of the aniline group. The acetanilide derivative **19** facilitated selective monobromination of the 5-position, allowing the isolation of **23** following the saponification of the ester in the modest yield of 19% over these two steps.

Subsequent synthetic steps proceeded in accordance with our reported synthesis of lead compound **1**.[21] Briefly, the 2-amino-5-bromobenzoic acid (**21**−**24**) intermediates first underwent *O*-(1*H*-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU)-mediated coupling with (1*S*,2*S*)-2-aminocyclohexanol hydrochloride in the presence of DIPEA, in DMF at room temperature, furnishing the corresponding amides (**25**−**28**) in good to excellent yield. Cyclisation of these 2-amino arylcarboxamide derivatives was achieved through heating in *N*,*N*-dimethylformamide-dimethylacetal (DMF-DMA), formamide or triethylorthoformate, affording **29**-**32**. The more forcing conditions (150 or 180 °C in formamide) were found to prevent formation of stable 2-(dimethylamino)-2,3-dihydroquinazolin-4(1*H*)-one-type intermediates, which occurred in the presence of DMF-DMA. In some cases, these intermediates failed to undergo elimination of *N*,*N*-dimethylamine to furnish the desired product, necessitating the use of more forcing conditions.

Installation of the (pyridin-3-yl)methyl pendant group was achieved as described for lead compound **1**, through a sequence of Negishi and Suzuki reactions, to give the desired compounds **2** and **4**-**6**, bearing the (6-(1-methyl-1*H*-pyrazol-4-yl)pyridin-3-yl)methyl group. During Suzuki coupling of final compounds **2** and **6**, appreciable amounts of the dehalogenation side-product, **3** and **7** were also isolated, and deemed of interest for pharmacological evaluation.

As part of the strategy to develop fused heteroaromatic analogues of lead compound **1**, the investigation of alternative cores was also of interest. The pyrido[2,3-*d*]pyrimidinone analogue of **2** (compound **8**) was prepared using an analogous

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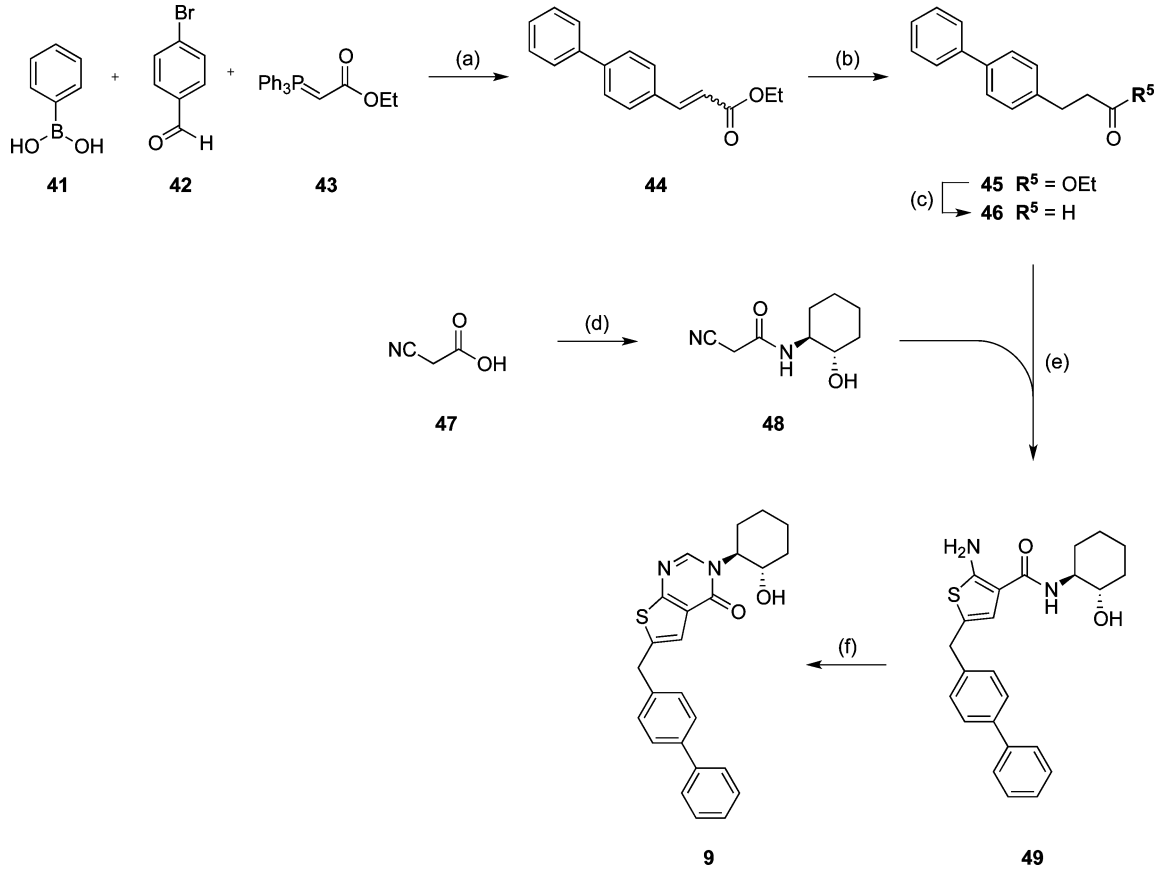
Scheme 4. Synthesis of Analogue 9 Containing a Thienopyrimidinone Core[a]



[a]Reagents and conditions: (a) (i) cat. PPh_3, cat. $PdCl_2(PPh_3)_2$, degassed DME/2 M $Na_2CO_{3(aq)}$, 70 °C; (ii) 100 °C; (iii) 85 °C, 84% (*E*/*Z* 4:1); (b) H_2, wet 10% Pd/C, EtOAc, rt, 99%; (c) (i) anhydrous toluene, −78 °C; (ii) 1 M DIBAL-H in toluene, dropwise, −78 °C; (iii) MeOH quench, −78 °C; 88%; (d) (1*S*,2*S*)-2-aminocyclohexanol hydrochloride, HCTU, DIPEA, DMF, rt, 71%; (e) sulfur, TEA, EtOH, 60 °C, 57%; (f) formamide, 180 °C, 56%.

approach starting from 2-amino-5-bromonicotinic acid (**37**) (Scheme 3).

Having synthesized the pyrido[2,3-*d*]pyrimidin-4(3*H*)-one analogue **8**, we turned our attention to 5−6 fused heterocyclic scaffolds. The thieno[2,3-*d*]pyrimidin-4(3*H*)-one core was of interest, since spatially isosteric replacements for the quinazolin-4(3*H*)-one core have been reported in a number of medicinal chemistry lead optimization campaigns, possessing "drug-like" properties and biological activity.[24−26]

The thieno[2,3-*d*]pyrimidin-4(3*H*)-one core was accessible through synthesis of the appropriately substituted 2-amino-thiophene-3-carboxamide, which in turn was assembled in a one-pot Gewald synthesis based on literature precedent.[5] Components for the Gewald reaction were readily synthesized from commercially available reagents (Scheme 4). Cyanoacetamide **48**, was obtained through HCTU-mediated coupling of cyanoacetic acid and (1*S*,2*S*)-2-aminocyclohexanol hydrochloride in the presence of DIPEA and DMF at room temperature. A one-pot Wittig-Suzuki reaction was employed to construct ester **44**, from phenylboronic acid (**41**), 4-bromobenzaldehyde (**42**), and (ethoxycarbonylmethylene)-triphenylphosphorane (**43**), according to previously described methodology.[6] Though intermediate **44** was isolated as both the *E*- and *Z*-isomers, these were combined before hydrogenation of the double bond, to give saturated ester **45**. Reduction of **45** in the presence of DIBAL-H at −78 °C, afforded the desired aldehyde **46** in excellent yield.

The combination of **46**, **48**, and sulfur in the presence of TEA and ethanol at 60 °C (one-pot Gewald conditions) gave the substituted 2-aminothiophene-3-carboxamide **49** in moderate yield. Finally cyclization of **49** was achieved by heating in formamide at 180 °C, affording the desired thieno[2,3-*d*]pyrimidin-4(3*H*)-one derivative **9**. We elected to synthesize **9** bearing the biphenylmethyl pendant group, for initial ease of access and to establish synthetic methodology. Furthermore, this moiety has been shown to impart good affinity and cooperativity on the BQCA scaffold (an early precursor of lead compound **1** and related structures).[7]

PHARMACOLOGY

We recently published an SAR study of the M_1 mAChR PAM, BQCA.[19] By incorporating modeling into our pharmacological analysis, we were able to relate modifications of the structural features of BQCA, to changes in parameters that describe allosteric ligand action. These comprise the affinity of the modulator for the free receptor (K_B), its modulatory effects on the binding and efficacy of acetylcholine (α and β, respectively), and its intrinsic efficacy (direct allosteric agonism) in the system (τ_B). In particular, alternative substitution of the quinolone ring in the 5- and 8-positions modulated intrinsic efficacy; isosteric replacement of the carboxylic acid moiety or

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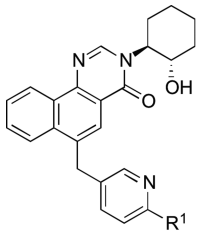
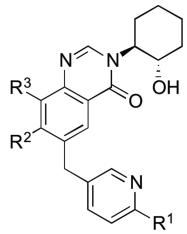
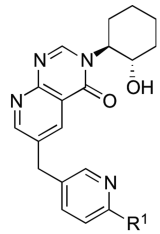
Table 1. Binding and Functional Parameters of 4-Phenylpyridin-2-one Analogues 2−12 at the M_1 mAChR



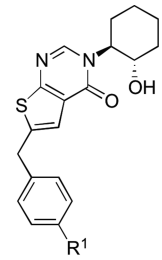
1, 11, 12



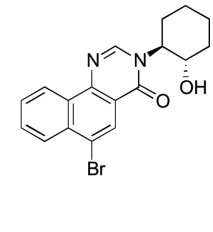
2-7



8



9



10

				Radioligand binding ([^{3}H]NMS)			IP_1 accumulation	
	R^1	R^2	R^3	pK_B (K_B, μM)	Log α' [a]	Log α (α) [b]	Log $\alpha\beta$ ($\alpha\beta$) [c]	Log τ_B (τ_B) [d]
BQCA				4.78 ± 0.06 (17)*	-3	1.77 ± 0.13 (58)*	1.84 ± 0.13 (69)*	-0.60 ± 0.10 (0.2)
1		–	–	5.88 ± 0.02 (1.3)	-3	2.84 ± 0.13 (692)	2.57 ± 0.15 (370)	0.44 ± 0.07 (3)
2		H	H	4.28 ± 0.13 (52)*	-3	1.96 ± 0.17 (91)*	2.29 ± 0.07 (195)	0.60 ± 0.02 (4)
3	H	H	H	4.21 ± 0.06 (61)*	-3	1.01 ± 0.26 (10)*	1.24 ± 0.07 (17)*	-3
4		Me	H	4.98 ± 0.11 (10)*	-3	2.32 ± 0.09 (209)	2.42 ± 0.10 (263)	0.44 ± 0.04 (2.8)
5		H	Me	5.15 ± 0.18 (7.1)	-3	2.58 ± 0.16 (380)	2.66 ± 0.05 (457)	0.05 ± 0.03 (1.1)*
6		Me	Me	5.14 ± 0.13 (7.2)*	-3	2.41 ± 0.21 (257)	2.69 ± 0.11 (490)	0.81 ± 0.05 (6.5)*
7	H	Me	Me	4.76 ± 0.14 (17)*	-3	2.34 ± 0.17 (219)	2.86 ± 0.09 (776)	1.06 ± 0.02 (11)*
8		–	–	4.10 ± 0.07 (79)*	-3	1.84 ± 0.16 (69)*	1.82 ± 0.04 (66)*	-3
9	Ph	–	–	4.66 ± 0.20 (22)	-0.32 ± 0.12	-0.04 ± 0.09 (0.91)	n/a	n/a
10	–	–	–	4.99 ± 0.22 (10)*	0.08 ± 0.05	0.60 ± 0.08 (4)*	0.67 ± 0.09 (5)*	-3
11	Cl	–	–	4.06 ± 0.08 (87)*	-0.55 ± 0.18	0.67 ± 0.08 (5)*	1.12 ± 0.11 (13)*	-0.36 ± 0.11 (0.4)*
12	H	–	–	5.29 ± 0.09 (5)	-3	2.35 ± 0.18 (223)	2.64 ± 0.13 (436)	1.14 ± 0.02 (14)*

[a]Binding cooperativity with [^{3}H]NMS; for instances where a complete inhibition of [^{3}H]NMS binding by the allosteric modulator was observed (consistent with a high level of negative cooperativity), log α' was fixed to −3. [b]Binding cooperativity with ACh. [c]Functional cooperativity with ACh. [d]Intrinsic efficacy of the modulator; for instances where no intrinsic efficacy was observed, log τ_B was fixed to −3. *Significant difference ($p < 0.05$) relative to same parameter determined for **1**, one-way ANOVA with Tukeys's post-test. Values represent the mean ± SEM from at least three experiments performed in duplicate.

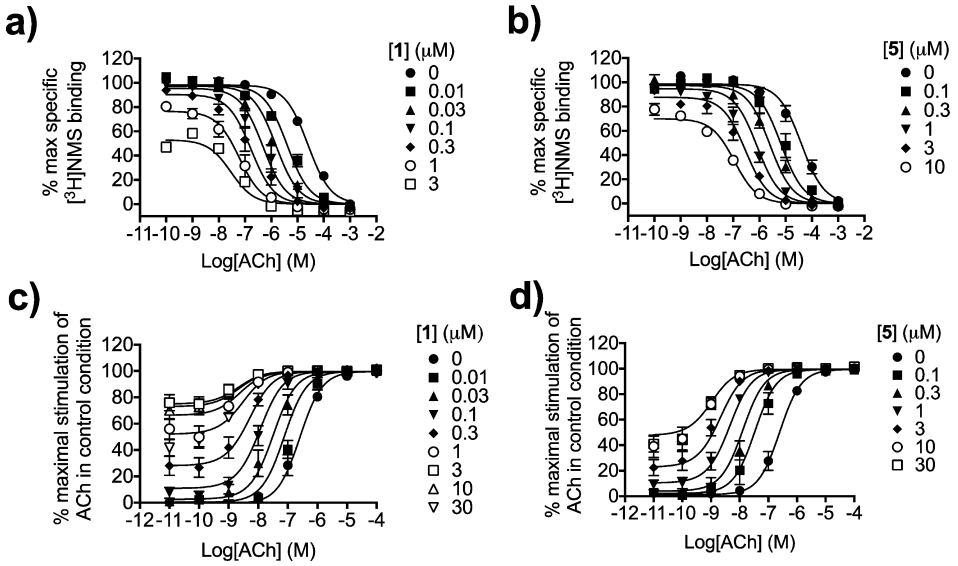


Figure 2. (a−d) Pharmacological characterization of **1** and **5** in binding and function at the M_1 mAChR. (a,b) Radioligand binding experiments were performed using FlpIn-CHO cells expressing the M_1 mAChR, 0.1 nM of the radiolabeled antagonist [^{3}H]NMS, increasing concentrations of ACh, with or without increasing concentrations of either **1** (a) or **5** (b). (c,d) IP_1 accumulation experiments were performed using FlpIn-CHO cells expressing the M_1 mAChR and increasing concentrations of ACh with or without increasing concentrations of either compound **1** (c) or **5** (d). 100% represents the maximal stimulation of ACh in the absence of test compound.

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amide derivatives of the acid function was important in determining cooperativity, and replacement of the *N*-alkyl group modulated ligand affinity.[19] More recently, we focused on the binding mode of 3-((1*S*,2*S*)-2-hydroxycyclohexyl)-6-((6-(1-methyl-1*H*-pyrazol-4-yl)pyridin-3-yl)methyl)benzo[*h*]-quinazolin-4(3*H*)-one (**1**), a significantly more potent M_1 mAChR PAM with a structural ancestry originating from BQCA.[21] To understand the structural determinants that underlie the superior PAM activity of **1**, we applied the same approach as described above for BQCA for all ligands described in this study. Competition binding studies between ACh and the radiolabeled antagonist [^{3}H]NMS at the M_1 mAChR expressed in FlpIN CHO cells were performed in the absence and presence of increasing concentrations of each test compound. Data were analyzed with an allosteric ternary complex,[27] to determine the K_B of the test compound for the allosteric site on the unoccupied M_1 mAChR, and its binding cooperativity (α) with ACh. To assess the ability of our test compounds to modulate ACh function, we used myoinositol-1-phosphate (IP_1) accumulation as a measure of M_1 mAChR activation. Concentration curves of ACh were generated in the presence of increasing concentrations of test compound, and an operational model of allostery was applied to the data with the K_B fixed to that determined in the binding studies, thus allowing an overall estimate of both functional cooperativity with ACh ($\alpha\beta$) and the intrinsic efficacy of the allosteric ligand. Values of α or $\beta > 1$ describe a positive modulatory effect upon ACh, whereas values between 0 and 1 describe a negative modulatory effect. It should be noted that because the logarithms of affinity and cooperativity values are normally distributed, whereas the corresponding absolute (antilogarithms) are not,[28] all statistical comparisons were performed on the logarithmic values (Table 1).

As described before, **1** displays a significant 13-fold higher affinity (K_B = 1.3 μM) for the M_1 mAChR as compared to BQCA. In addition, in comparison to BQCA, **1** displays a 12-fold increase in binding cooperativity (α = 692) and a 5-fold increase in functional cooperativity ($\alpha\beta$ = 370) with ACh (Figure 2, Table 1). Finally, **1** displays superior intrinsic efficacy compared to BQCA with a 15-fold increase in τ_B (τ_B = 3). Complete deletion of the peripheral fused benzene ring, as in **2**, caused a 40-fold decrease in affinity (K_B = 52) and an 8-fold decrease in binding cooperativity with ACh (α = 91). However, no change in intrinsic efficacy was observed (τ_B = 4). Further deletion of the 4-(1-methylpyrazole-4-yl) substitution of the (pyridin-3-yl)methyl pendant group to give **3**, caused no change in affinity relative to **2**, but caused a significant 9-fold loss of binding cooperativity, a significant 11-fold loss of functional cooperativity, and a complete loss of observed intrinsic efficacy. The addition of a methyl group at the 8- or 7-positions of the quinazolin-4(3*H*)-one core (compounds **4** and **5**, respectively) caused a significant 5- to 7-fold increase in affinity compared to **2**, but with no significant change in cooperativity with ACh. While methyl substitution at the 8-position (**4**) resulted in similar intrinsic activity relative to **2**, the addition of methyl at the 7-position (**5**) caused a significant 4-fold decrease in intrinsic efficacy (Figure 2). The addition of a methyl group at both the 7- and 8-positions in compound **6** caused a 7-fold increase in affinity, and no significant change in cooperativity with ACh or intrinsic efficacy relative to **2**. Indeed this analogue displayed a 2-fold higher intrinsic efficacy than **1**. Together these data reveal that the benzo[*h*]quinazolin-4(3*H*)-one core is an important determinant of the affinity of **1**. Deletion of the fused benzene ring of this core (**2**) was associated with a significant loss of affinity that was partly rescued with the addition of methyl groups in the 7 and/or 8 position of a quinazolin-4(3*H*)-one core (**5** and **6**). Indeed, compound **5** displayed no significant difference in affinity compared to **1** ($p > 0.05$, one-way ANOVA with Tukey's post-test). In our recent SAR study,[19] we observed that the replacement of the *N*-alkyl group of the quinolone core of BQCA modulated ligand affinity. In particular, changing the *N*-(4-methoxy)benzyl group to *N*-(4-phenyl)benzyl tended to improve affinity for the receptor without improving cooperative binding with ACh. In contrast, the absence of a 4-(1-methylpyrazole-4-yl) substituent from the (pyridin-3-yl)methyl pendant group of **2** (with a quinazolin-4(3*H*)-one core) had no effect upon affinity but instead caused a decrease in intrinsic activity (compare compounds **2** and **3**). However, when two methyl groups were present on the quinazolin-4(3*H*)-one core, the absence of the 4-(1-methylpyrazole-4-yl) moiety caused no change in affinity, cooperativity or intrinsic activity (**6** compared to **7**, Table 1).

To further explore the role of the (pyridin-3-yl)methyl pendant group in the determination of the activity of **1**, we characterized the synthetic intermediates **10** and **11** and synthetic byproduct **12**. Replacement of the (6-(1-methyl-1*H*-pyrazol-4-yl)pyridin-3-yl)methyl group with a bromo-substituent (**10**) caused an 8-fold loss of affinity, a 170-fold and 70-fold loss in binding and functional cooperativity with ACh, respectively. In addition, a complete loss of intrinsic efficacy was observed. Compound **12**, which possessed an unsubstituted (pyridin-3-yl)methyl group, displayed an affinity for the M_1 mAChR that was not significantly different from **1** and had similar binding and functional cooperativity with ACh (α = 223, $\alpha\beta$ = 436). Furthermore, **12** displayed 5-fold higher intrinsic efficacy than **1** (τ_B = 14). In contrast, the (6-chloropyridin-3-yl)methyl derivative **11** displayed 66-fold lower affinity than **1**, 140-fold and 30-fold lower binding and functional cooperativity with ACh and 8-fold lower intrinsic efficacy. Together these data illustrate the importance of the benzylic pendant group for the activity of **1**. In contrast to our findings with BQCA, we found that this moiety was not only important for affinity, but also for the cooperativity with ACh and intrinsic efficacy displayed by **1**. However, removal of the 4-(1-methylpyrazole-4-yl) substitution of the (pyridin-3-yl)methyl pendant group of **1** was tolerated both in terms of affinity, cooperativity with ACh and intrinsic efficacy.

The pyrido[2,3-*d*]pyrimidin-4(3*H*)-one analogue (**8**: α = 69, $\alpha\beta$ = 66) displayed attenuated binding and functional cooperativity with ACh relative to compound **2** (**2**: α = 91, $\alpha\beta$ = 195). Finally, the thieno[2,3-*d*]pyrimidin-4(3*H*)-one **9** showed negligible binding cooperativity with ACh.

CONCLUSIONS

We have recently reported that 3-((1*S*,2*S*)-2-hydroxycyclohexyl)-6-((6-(1-methyl-1*H*-pyrazol-4-yl)pyridin-3yl)methyl)benzo-[*h*]quinazolin-4(3*H*)-one (**1**), while structurally related to BQCA, is a significantly more potent PAM of the M_1 mAChR, driven both by an increased affinity for the M_1 mAChR and an increased level of positive cooperativity with ACh.[21] In addition, **1** displays higher intrinsic efficacy than BQCA. Furthermore, we recently proposed a binding mode of **1** within the M_1 mAChR that is similar to that predicted for BQCA.[21] In this study, we wanted to explore the structural determinants that underlie the activity of **1** as a PAM of the M_1

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mAChR. We have previously demonstrated that changing the *N*-alkyl group of the quinolone core of BQCA modulated ligand affinity but not cooperativity with ACh.[19] In this study, we demonstrate that the (pyrid-3-yl)methyl pendant group of **1** is not only important for affinity, but has an additional role in determining cooperativity with ACh. In addition, we found that removal of the 1-methyl-1*H*-pyrazol-4-yl moiety of **1** was well tolerated in terms of both affinity and cooperativity with ACh and generated a PAM (**7**) with superior intrinsic efficacy. We paid particular attention to the importance of the tricyclic scaffold of compound **1** for the activity of the molecule. Complete deletion of the peripheral fused benzene ring caused a significant decrease in affinity and binding cooperativity with ACh, but no change in intrinsic activity. However, this loss of affinity was partially rescued with the addition of methyl groups in the 7- and/or 8-position of the quinazolin-4(3*H*)-one core (compounds **4**, **5** and **6**). Indeed, compound **5** with the addition of a methyl group in the 8-position displayed no significant difference in affinity for the M_1 mAChR compared to **1**, but lower intrinsic activity (Figure 2). The addition of methyl groups at the 7- and 8-positions (**6**) maintained this affinity and rescued intrinsic efficacy. These results demonstrate that the tricyclic benzo[*h*]quinazolin-4(3*H*)-one core could be replaced with a quinazolin-4(3*H*)-one core. This may be important, given the precedence of polyaromatic heterocycles as DNA chelators. In addition, the quinazolin-4(3*H*)-one core represents a novel scaffold to explore further M_1 mAChR PAMs with improved physicochemical properties.

METHODS

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

Unless otherwise stated, reactions were carried out at ambient temperature. 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). General staining was carried out with $KMnO_4$ or phosphomolybdic acid. 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 $MgSO_4$ or Na_2SO_4 before gravity filtering and evaporation to dryness. Organic solvents were evaporated in vacuo at ≤40 °C (water bath temperature). Purification using preparative layer chromatography (PLC) was carried out on Analtech preparative TLC plates (200 mm × 200 mm × 2 mm).

^{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) were recorded in parts per million (ppm) with reference to the chemical shift of the deuterated solvent. Coupling constants (J) and carbon−fluorine coupling constants (J_{CF}) were 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. Specific optical rotation was determined using a Jasco P-2000 polarimeter.

LC-MS were run to verify reaction outcome and purity using following system: Agilent 6120 Series Single Quad coupled to an Agilent 1260 Series HPLC. 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 × 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 mode 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. High resolution mass spectra (HRMS) were obtained from a Waters LCT Premier XE (TOF) mass spectrometer fitted with an ESI ion source, coupled to a 2795 Alliance Separations Module.

Preparative HPLC was performed using an Agilent 1260 infinity coupled with a binary preparative pump and Agilent 1260 FC-PS fraction collector, using Agilent OpenLAB CDS software (Rev C.01.04), and an Altima 5 μM C8 22 × 250 mm column. The following buffers were used: buffer A, H_2O; buffer B, MeCN, with sample being run at a gradient of 5% buffer B to 100% buffer B over 20 min, at a flow rate of 20 mL/min All screening compounds were of >95% purity unless stated otherwise.

General Procedure A: HCTU-Mediated Amide Bond Formation. Carboxylic acid (1 equiv), HCTU (1.1 equiv), and amine or amine salt (1.1 equiv) were dispersed or dissolved in DMF (∼2 mL/mmol) at RT. To this was added DIPEA (2.5 eq, for amine salts, an additional 1.0 equiv per salt form was also added), and the mixture allowed to stir at RT overnight. LC-MS analysis was used to confirm reaction completion. The mixture was diluted with water/sat. $NaHCO_{3(aq)}$ (1:1, ∼20 mL/mmol) and stirred for 30 min at RT. Where a solid precipitate formed, this was collected by filtration (vacuum) and washed with water. Where no solid could be isolated in this manner, the aqueous slurry was extracted with EtOAc (3 times) and the combined organic layers washed with brine, then concentrated under reduced pressure. Where necessary, further purification was carried out by FCC.

General Procedure B: Negishi Coupling of Aryl Bromides with (2-Chloro-5-pyridylmethyl)zinc Chloride. Aryl bromide (1.0 equiv) was dissolved in dry THF (2 mL/mmol), under an atmosphere of nitrogen, and degassed for 5 min under a stream of nitrogen. $Pd(P(^tBu)_3)_2$ (0.03 equiv) was added and then vessel was evacuated and refilled with nitrogen, before cooling to 0 °C over an ice bath. A solution of 2-chloro-5-pyridylmethyl)zinc chloride (0.5 M in THF, 1.25 equiv) was added in a dropwise fashion, and stirring continued over the ice bath for a further 5 min, before allowing the mixture to warm to RT. Reactions were monitored by LC-MS analysis and generally left to stir overnight. To quench, the mixture was cooled to 0 °C over an ice bath and a small amount of water added with care. The quenched mixture was diluted with water, then washed three times with equal volumes of EtOAc. The combined organic layers were washed with brine, before drying over $MgSO_4$, and concentrating under reduced pressure. The crude product was further purified by FCC (eluent EtOAc/PE 50:50 to 100:0).

General Procedure C: Suzuki Coupling of Substituted 2-Chloropyridines with 1-Methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole. Substituted 2-chloropyridine (1.0 equiv) and 1-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole (1.5 equiv) were dispersed in 1 M $Na_2CO_{3(aq)}$/THF (1:3, ∼ 10 mL/mmol) in a 10 mL microwave tube. The mixture was sonicated for 5 min, then degassed under a stream of nitrogen. $PdCl_2(PPh_3)_2$ (0.1 equiv) was added, and the tube sealed, before heating (hot plate) at 100 °C for 2 h. The mixture was cooled to RT, then diluted with water (20 mL), before extracting with EtOAc (3 × 20 mL). The combined organic extracts were washed with brine (20 mL), then concentrated under reduce pressure. The crude product was purified by normal phase silica chromatography as specified under each monologue.

*3-((1S,2S)-2-Hydroxycyclohexyl)-6-((6-(1-methyl-1H-pyrazol-4-yl)pyridin-3-yl)methyl)quinazolin-4(3H)-one (**2**) and 3-((1S,2S)-2-Hydroxycyclohexyl)-6-(pyridin-3-ylmethyl)quinazolin-4(3H)-one (**3**).* 6-((6-Chloropyridin-3-yl)methyl)-3-((1*S*,2*S*)-2-hydroxycyclohexyl)-quinazolin-4(3*H*)-one (**33**) (70 mg, 0.19 mmol) underwent Suzuki coupling according to General Procedure C. The crude product was purified by PLC (MeOH/EtOAc 6:94, plate run three times). The

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higher running band was found to be dehalogenated starting material: 3-((1*S*,2*S*)-2-Hydroxycyclohexyl)-6-(pyridin-3-ylmethyl)quinazolin-4(3*H*)-one (**3**), isolated as 9 mg (14%) of a white solid. ^{1}H NMR ($CDCl_3$) δ 8.62−8.41 (m, 2H), 8.09 (s, 1H), 8.04 (d, J = 1.8 Hz, 1H), 7.68 (d, J = 7.9 Hz, 1H), 7.59 (d, J = 8.3 Hz, 1H), 7.49 (dd, J = 8.4/2.0 Hz, 1H), 7.45−7.32 (m, 1H), 4.74−4.40 (m, 1H), 4.13 (s, 2H), 4.08−3.85 (m, 1H), 2.30−2.17 (m, 1H), 2.03−1.77 (m, 4H), 1.58−1.35 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 161.8, 147.2, 146.4, 145.2, 144.9, 139.4, 138.2, 137.6, 135.1, 128.1, 126.7, 124.7, 122.2, 70.4, 38.8, 35.7, 31.0, 25.4, 24.5; m/z MS (TOF ES$^+$) 336.2 [MH]$^+$; HRMS - $C_{20}H_{22}N_3O_2$ [MH]$^+$ calcd 336.1712; found 336.1716; LC-MS t_R: 2.76 min; $[\alpha]_D^{27}$ = +5.10° (0.11, DMSO).

The lower running band was found to be 3-((1*S*,2*S*)-2-hydroxycyclohexyl)-6-((6-(1-methyl-1*H*-pyrazol-4-yl)pyridin-3-yl)-methyl)quinazolin-4(3*H*)-one (**2**, isolated as 42 mg (53%) of a glassy solid. ^{1}H NMR ($CDCl_3$) δ 8.39 (d, J = 1.7 Hz, 1H), 8.08 (s, 1H), 8.05 (s, 1H), 8.00 (d, J = 1.6 Hz, 1H), 7.88 (s, 1H), 7.53 (d, J = 8.3 Hz, 1H), 7.51−7.42 (m, 2H), 7.39 (d, J = 8.2 Hz, 1H), 4.51 (s, 1H), 4.06−3.85 (m, 6H), 2.29−2.15 (m, 1H), 2.01−1.73 (m, 4H), 1.58−1.33 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 161.8, 149.5, 148.1, 146.2, 144.6, 139.0, 138.6, 137.7, 135.1, 133.6, 129.6, 127.8, 126.5, 122.1, 120.0, 113.6, 71.6, 39.4, 38.5, 35.6, 31.0, 25.4, 24.6; m/z MS (TOF ES$^+$) 416.3 [MH]$^+$; HRMS - $C_{24}H_{26}N_5O_2$ [M-Na]$^+$ calcd 438.1906; found 438.1872; LC-MS t_R: 2.85 min; $[\alpha]_D^{27}$ = −1.04° (0.26, DMSO).

*3-((1S,2S)-2-Hydroxycyclohexyl)-8-methyl-6-((6-(1-methyl-1H-pyrazol-4-yl)pyridin-3-yl)methyl)quinazolin-4(3H)-one (**4**).* 6-((6-Chloropyridin-3-yl)methyl)-3-((1*S*,2*S*)-2-hydroxycyclohexyl)-8-methylquinazolin-4(3*H*)-one (**34**) (200 mg, 0.52 mmol) underwent Suzuki coupling according to General Procedure C. The crude product was purified by FCC (eluent MeOH/DCM 0:100 to 10:90) to give 214 mg (96%) of pale yellow glassy solid. ^{1}H NMR (DMSO-d_6) δ 8.46 (d, J = 1.6 Hz, 1H), 8.38 (s, 1H), 8.22 (s, 1H), 7.93 (d, J = 0.6 Hz, 1H), 7.84 (d, J = 1.6 Hz, 1H), 7.61 (dd, J = 8.1/2.3 Hz, 1H), 7.58 (dd, J = 1.9/0.7 Hz, 1H), 7.55 (dd, J = 8.1/0.7 Hz, 1H), 4.90 (d, J = 5.4 Hz, 1H), 4.39 (s, 1H), 4.05 (s, 2H), 3.93 (s, 1H), 3.86 (s, 3H), 2.50 (s, 3H), 2.14−1.50 (m, 5H), 1.50−1.21 (m, 3H); ^{13}C NMR (DMSO-d_6) δ 160.6, 149.8, 149.4, 144.9, 144.4, 139.1, 137.0, 136.9, 135.4, 135.2, 133.5, 129.6, 123.2, 122.7, 121.6, 119.1, 69.0, 38.7, 37.4, 35.2, 30.3, 25.0, 24.0, 17.0; m/z MS (TOF ES$^+$) 430.3 [MH]$^+$; HRMS - $C_{25}H_{28}N_5O_2$ [MH]$^+$ calcd 430.2243; found 430.2248; LC-MS t_R: 3.29 min; $[\alpha]_D^{27}$ = +6.93° (0.29, DMSO).

*3-((1S,2S)-2-Hydroxycyclohexyl)-7-methyl-6-((6-(1-methyl-1H-pyrazol-4-yl)pyridin-3-yl)methyl)quinazolin-4(3H)-one (**5**).* 6-((6-Chloropyridin-3-yl)methyl)-3-((1*S*,2*S*)-2-hydroxycyclohexyl)-7-methylquinazolin-4(3*H*)-one (**35**) (328 mg, 0.97 mmol) underwent Suzuki coupling according to General Procedure C. The crude product was purified by FCC (eluent EtOAc/PE 50:50 to 100:0) to give 334 mg (90%) of an off-white solid. ^{1}H NMR (DMSO-d_6) δ 8.38 (d, J = 1.6 Hz, 1H), 8.34 (s, 1H), 8.23 (s, 1H), 7.94 (d, J = 0.7 Hz, 1H), 7.84 (s, 1H), 7.56 (dd, J = 8.1/0.6 Hz, 1H), 7.53−7.42 (m, 2H), 4.90 (d, J = 5.4 Hz, 1H), 4.37 (s, 1H), 4.11 (s, 2H), 3.94 (s, 1H), 3.87 (s, 3H), 2.39 (s, 3H), 2.11−1.49 (m, 5H), 1.46−1.13 (m, 3H); ^{13}C NMR (DMSO-d_6) δ 160.2, 149.8, 149.6, 146.2, 146.0, 143.7, 138.3, 137.0, 136.9, 132.3, 129.3, 127.8, 126.2, 122.6, 119.1, 119.0, 69.0, 38.7, 35.2, 35.0, 29.9, 25.1, 24.0, 19.7; m/z MS (TOF ES$^+$) 430.3 [MH]$^+$; HRMS - $C_{25}H_{28}N_5O_2$ [MH]$^+$ calcd 430.2243; found 430.2244; LC-MS t_R: 3.24 min; $[\alpha]_D^{27}$ = +6.11° (0.67, DMSO).

*3-((1S,2S)-2-Hydroxycyclohexyl)-7,8-dimethyl-6-((6-(1-methyl-1H-pyrazol-4-yl)pyridin-3-yl)methyl)quinazolin-4(3H)-one (**6**) and 3-((1S,2S)-2-Hydroxycyclohexyl)-7,8-dimethyl-6-(pyridin-3-ylmethyl)quinazolin-4(3H)-one (**7**).* 6-((6-Chloropyridin-3-yl)-methyl)-3-((1*S*,2*S*)-2-hydroxycyclohexyl)-7,8-dimethylquinazolin-4(3*H*)-one (**36**) (61 mg, 0.15 mmol) underwent Suzuki coupling according to General Procedure C. The crude product was purified by PLC (MeOH/EtOAc 5:95, plate run three times). The higher running band was found to be dehalogenated starting material: 3-((1*S*,2*S*)-2-Hydroxycyclohexyl)-7,8-dimethyl-6-(pyridin-3-ylmethyl)quinazolin-4(3*H*)-one (**7**), isolated as 5 mg (9%) of an off-white solid. ^{1}H NMR ($CDCl_3$) δ 8.45 (s, 2H), 8.10 (s, 1H), 7.90 (s, 1H), 7.46 (d, J = 7.9 Hz, 1H), 7.33−7.19 (m, 1H), 4.53 (s, 1H), 4.13 (s, J = 7.5 Hz, 2H), 3.97 (s, 1H), 3.82−2.86 (m, 1H), 2.52 (s, 3H), 2.33−2.13 (m, 4H), 2.03−1.69 (m, 4H), 1.61−1.33 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 162.3, 148.3, 146.1, 145.1, 143.3, 142.1, 137.7, 136.6, 134.9, 129.0, 125.3, 124.2, 119.7, 72.0, 37.7, 35.7, 31.0, 25.5, 24.6, 17.0, 13.7; m/z MS (TOF ES$^+$) 364.2 [MH]$^+$; HRMS - $C_{22}H_{26}N_3O_2$ [MH]$^+$ calcd 364.2025; found 364.2031; LC-MS t_R: 2.90 min; $[\alpha]_D^{27}$ = +13.87° (0.18, DMSO).

The lower running band was found to be 3-((1*S*,2*S*)-2-hydroxycyclohexyl)-7,8-dimethyl-6-((6-(1-methyl-1*H*-pyrazol-4-yl)-pyridin-3-yl)methyl)quinazolin-4(3*H*)-one (**6**), isolated as 10 mg (15%) of white solid. ^{1}H NMR ($CDCl_3$) δ 8.20 (s, 1H), 8.08−7.93 (m, 2H), 7.83 (s, 1H), 7.81 (s, 1H), 7.48−7.32 (m, 2H), 4.35 (s, 1H), 4.05 (s, 2H), 4.01−3.73 (m, 4H), 2.43 (s, 3H), 2.17 (s, 3H), 2.07 (d, J = 10.1 Hz, 1H), 1.97−1.58 (m, 4H), 1.48−1.21 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 162.1, 151.6, 146.8, 144.9, 143.5, 142.2, 139.5, 137.6, 136.6, 134.6, 133.9, 129.9, 124.9, 120.6, 119.5, 112.1, 70.6, 38.9, 37.1, 35.1, 31.0, 25.2, 24.3, 16.7, 13.4; m/z MS (TOF ES$^+$) 444.3 [MH]$^+$; HRMS - $C_{26}H_{30}N_5O_2$ [MH]$^+$ calcd 444.2400; found 444.2411; LC-MS t_R: 3.01 min; $[\alpha]_D^{27}$ = +6.44° (0.16, DMSO).

*3-((1S,2S)-2-Hydroxycyclohexyl)-6-((6-(1-methyl-1H-pyrazol-4-yl)pyridin-3-yl)methyl)pyrido[2,3-d]pyrimidin-4(3H)-one (**8**).* 6-((6-Chloropyridin-3-yl)methyl)-3-((1*S*,2*S*)-2-hydroxycyclohexyl)pyrido-[2,3-*d*]pyrimidin-4(3*H*)-one (**40**) (33 mg, 0.09 mmol) underwent Suzuki coupling according to General Procedure C. The crude product was purified by PLC (MeOH/EtOAc 7:93, plate run six times) to give 9 mg (24%) of white solid. ^{1}H NMR (CD_3OD) δ 8.88 (d, J = 2.4 Hz, 1H), 8.52 (s, 1H), 8.49−8.40 (m, 2H), 8.12 (s, 1H), 7.99 (d, J = 0.5 Hz, 1H), 7.70 (dd, J = 8.2/2.3 Hz, 1H), 7.62 (d, J = 8.2 Hz, 1H), 4.44 (s, 1H), 4.23 (s, 2H), 4.18−3.98 (m, 1H), 3.94 (s, 3H), 2.29−1.69 (m, 5H), 1.56−1.37 (m, 3H); ^{13}C NMR (CD_3OD) δ 163.1, 157.4, 157.2, 151.6, 150.4, 150.3, 139.3, 138.5, 137.3, 137.1, 134.6, 130.9, 123.9, 121.5, 118.0, 71.1, 39.1, 36.2, 36.0, 30.8, 26.4, 25.4; m/z MS (TOF ES$^+$) 417.3 [MH]$^+$; HRMS- $C_{23}H_{25}N_6O_2$ [MH]$^+$ calcd 417.2039; found 417.2025; LC-MS t_R: 3.16 min; $[\alpha]_D^{27}$ = −4.86° (0.20, DMSO).

*6-([1,1′-Biphenyl]-4-ylmethyl)-3-((1S,2S)-2-hydroxycyclohexyl)-thieno[2,3-d]pyrimidin-4(3H)-one (**9**).* 5-([1,1′-Biphenyl]-4-ylmethyl)-2-amino-*N*-((1*S*,2*S*)-2-hydroxycyclohexyl)thiophene-3-carboxamide (**49**) (91 mg, 0.22 mmol) was dispersed in formamide (20 mL), before heating at 180 °C for 4 h. LC-MS analysis indicated conversion was complete. TLC analysis (EtOAc/PE 1:1, plate run twice) indicated the starting material and product both had an R_f ∼ 0.4, with only the starting material staining positive with ninhydrin. The mixture was cooled, then poured onto ice/water, and the resulting precipitate collected by filtration (vacuum), and further washed with water. The crude product was purified by FCC (eluent EtOAc/PE 0:100 to 60:40, wet load in DCM), to give 52 mg (56%) of an off-white solid. ^{1}H NMR (DMSO-d_6) δ 8.39 (s, 1H), 7.75−7.56 (m, 4H), 7.50−7.42 (m, 2H), 7.42−7.31 (m, 3H), 7.21 (s, 1H), 4.92 (s, 1H), 4.41 (s, 1H), 4.25 (s, 2H), 3.91 (s, 1H), 2.15−1.50 (m, 4H), 1.48−1.11 (m, 4H); ^{13}C NMR (DMSO-d_6) δ 162.0, 156.8, 145.9, 142.1, 139.8, 138.9, 138.6, 129.2, 128.9, 127.4, 127.0, 126.6, 123.7, 119.6, 69.1, 35.3, 35.2, 30.6, 25.1, 23.9; m/z MS (TOF ES$^+$) 417.3 [MH]$^+$; HRMS - $C_{25}H_{25}N_2O_2S$ [MH]$^+$ calcd 417.1637; found 417.1613; LC-MS t_R: 3.98 min; $[\alpha]_D^{27}$ = +17.77° (0.35, DMSO).

*Methyl 2-Amino-3,4-dimethylbenzoate (**15**).*[12] 2-Amino-3,4-dimethylbenzoic acid (**14**) (4.92 g, 29.8 mmol) and conc. H_2SO_4 (3 mL) were dissolved in MeOH (50 mL) and boiled under reflux for 48 h. LC-MS analysis indicated approximately half of the starting material had been converted. Additional H_2SO_4 (1 mL) was added, and heating continued. After a total of 6 days, the mixture was cooled to RT, and concentrated under reduced pressure. The residue was neutralized with sat. $NaHCO_{3(aq)}$, and the resulting precipitate collected by filtration (vacuum), and washed with water. After drying, 4.452 g (83%) of an off-white solid was obtained. ^{1}H NMR ($CDCl_3$) δ 7.69 (d, J = 8.2 Hz, 1H), 6.58 (d, J = 8.2 Hz, 1H), 6.18 (s, 2H), 3.86 (s, 3H), 2.30 (s, 3H), 2.13 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 169.2, 147.8, 142.8, 128.4, 122.0, 119.4, 109.5, 51.7, 21.3, 13.0; m/z MS (TOF ES$^+$) 180.2 [MH]$^+$; LC-MS t_R: 3.48 min.

*Methyl 2-Amino-5-bromo-3,4-dimethylbenzoate hydrobromide (**16**).* Methyl 2-amino-3,4-dimethylbenzoate (**15**) (3.40 g, 19.0 mmol) was dissolved in 1,4-dioxane/CCl_4 (1:1, 100 mL), and cooled to 0 °C

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over an ice bath, after wrapping the flask in aluminum foil to exclude light. To the cooled mixture, was added a solution of bromine (3.03 g, 18.97 mmol, 1.0 equiv) in 1,4-dioxane/CCl_4 (1:1, 20 mL) in a dropwise fashion. The mixture was stirred for 2 h at 0 °C, before addition of Et_2O, and collection of the resulting precipitate by filtration (vacuum). After washing with further Et_2O and drying, 5.96 g (93%) of an off-white solid was obtained. ^{1}H NMR (DMSO-d_6) δ 7.79 (s, 1H), 4.40 (s, 3H), 3.79 (s, 3H), 2.33 (s, 3H), 2.10 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 167.2, 148.7, 140.7, 130.7, 123.8, 109.7, 108.3, 51.7, 20.5, 14.4; m/z MS (TOF ES$^+$) 258.0 $[MH]^+$; LC-MS t_R: 3.71 min.

*Ethyl 2-Amino-4-methylbenzoate (**18**).*[10] 2-Amino-4-methylbenzoic acid (**17**) (2.10 g, 13.9 mmol) was dissolved in EtOH (50 mL) with conc. H_2SO_4 (1 mL), before boiling under reflux for 2 h. LC-MS analysis indicated little progression, so further conc. H_2SO_4 (1 mL) was added, and heating continued for 65 h. The mixture was cooled, then concentrated under reduced pressure. The resulting residue was neutralized with sat. $NaHCO_{3(aq)}$, then extracted with DCM (3 × 30 mL). The combined organic layers were concentrated to give a brown oil, which was further purified by FCC (eluent EtOA/PE 0:100 to 10:90), to give 1.87 g (75%) of pale yellow oil. ^{1}H NMR ($CDCl_3$) δ 7.85 (d, J = 8.1 Hz, 1H), 6.96 (s, 1H), 6.77 (d, J = 8.1 Hz, 1H), 4.36 (q, J = 7.2 Hz, 2H), 2.34 (s, 3H), 1.39 (t, J = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.2, 147.7, 145.2, 131.3, 119.8, 118.5, 110.5, 60.6, 21.8, 14.5; ^{1}H NMR; m/z MS (TOF ES$^+$) $C_{10}H_{14}NO_2$ 180.1 $[MH]^+$; LC-MS t_R: 3.82 min.

*Ethyl 2-Acetamido-4-methybenzoate (**19**)..*[10,11] Ethyl 2-amino-4-methylbenzoate (**18**) (1.87 g, 10.4 mmol) and TEA (1.74 mL, 12.5 mmol, 1.2 equiv) were dissolved in DCM (50 mL) and cooled to 0 °C under an atmosphere of nitrogen. Acetyl chloride (0.81 mL, 11.5 mmol, 1.1 equiv) was added and then the mixture was allowed to warm to RT, before stirring overnight. LC-MS analysis indicated incomplete conversion after this time, so further TEA (1.74 mL, 12.5 mmol, 1.2 equiv) and acetyl chloride (0.81 mL, 11.5 mmol, 1.1 equiv) were added and stirring continued for a further 4 h. The mixture was washed with water (50 mL), then sat. $NaHCO_{3(aq)}$ (50 mL), then concentrated under reduced pressure, to give 2.56 g of yellow solid (quantitative). ^{1}H NMR ($CDCl_3$) δ 11.10 (s, 1H), 8.53 (s, 1H), 7.92 (d, J = 8.2 Hz, 1H), 6.88 (ddd, J = 8.2/1.7/0.6 Hz, 1H), 4.35 (q, J = 7.1 Hz, 2H), 2.39 (s, 3H), 2.22 (s, 3H), 1.40 (t, J = 7.1 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 169.2, 168.5, 145.9, 141.7, 130.8, 123.5, 120.7, 112.6, 61.3, 25.7, 22.3, 14.4; m/z MS (TOF ES$^+$) 176.2 $[M\text{-}OEt]^+$; LC-MS t_R: 3.79 min.

*Ethyl 2-Acetamido-5-bromo-4-methylbenzoate (**20**).* Ethyl 2-acetamido-4-methybenzoate (**19**) (2.55 g, 11.5 mmol) was dissolved in acetic acid (8 mL) at RT, with stirring. A solution of bromine (1.84 g, 11.5 mmol, 1.0 equiv) in acetic acid (2 mL) was added in a dropwise fashion. After 48 h of stirring, LC-MS indicated partial progression, therefore another portion of bromine (0.30 mL, 921 mg, 5.76 mmol, 0.5 equiv) was added. After a further 72 h of stirring, further progression was evident, with the appearance of a minor peak indicating formation of the dibromo product. The mixture was stirred with 5% $Na_2S_2O_{3(aq)}$ (20 mL) for 30 min, then sat. $NaHCO_3$ (150 mL) added. This was extracted with DCM (3 × 50 mL), and the combined organic extracts washed further with sat. $NaHCO_{3(aq)}$ (50 mL), before concentration under reduced pressure. Purification of the crude material was attempted by FCC (eluent DCM), however, both starting material and product were found to coelute. FCC was reattempted (eluent DCM/PE 50:50 to 70:30, then 100:0) to give 687 mg of yellow solid (23%, brsm) and a further 995 mg of mixture containing starting material and desired product. ^{1}H NMR ($CDCl_3$) δ 10.99 (s, 1H), 8.65 (s, 1H), 8.16 (s, 1H), 4.37 (q, J = 7.1 Hz, 2H), 2.43 (s, 3H), 2.22 (s, 3H), 1.42 (t, J = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.2, 167.4, 145.4, 140.7, 134.1, 122.3, 117.6, 114.5, 61.8, 25.7, 23.8, 14.3; m/z MS (TOF ES$^+$) 300.2 $[MH]^+$; LC-MS t_R: 4.11 min.

*2-Amino-5-bromo-3-methylbenzoic Acid Hydrobromide (**22**).*[29] 2-Amino-3-methylbenzoic acid (**13**) (2.08 g, 13.8 mmol) was dissolved in acetic acid (8 mL) and stirred at RT. A solution of bromine (2.20 g, 13.8 mmol, 1.0 equiv) in acetic acid (2 mL) was added in a dropwise fashion, followed by washings of DCM (5 mL). The mixture was stirred at RT for 1 h, then diluted with Et_2O. The resulting precipitate was collected by filtration (vacuum) and washed with Et_2O. After drying, 4.07 g (95%) of a pale beige solid was obtained as the hydrobromide salt. ^{1}H NMR (DMSO-d_6) δ 9.19 (s, 3H), 7.68 (dd, J = 2.5/0.4 Hz, 1H), 7.33 (dd, J = 2.5/0.8 Hz, 1H), 2.10 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 168.9, 148.8, 136.4, 130.6, 126.4, 111.0, 104.8, 17.3; m/z MS (TOF ES$^+$) 230.0 $[MH]^+$; LC-MS t_R: 3.62 min.

*2-Amino-5-bromo-4-methylbenzoic Acid (**23**).*[30] Ethyl 2-acetamido-5-bromo-4-methylbenzoate (**20**) (672 mg, 2.24 mmol) was dissolved in 5 M $NaOH_{(aq)}$ (20 mL) and EtOH (30 mL). The resulting mixture was heated at 90 °C under a reflux condenser for 20 h. LC-MS analysis at this indicated hydrolysis was complete. The mixture was cooled to RT, then concentrated under reduced pressure to remove EtOH. The aqueous slurry was acidified with 2 M $HCl_{(aq)}$, to pH 2 resulting in formation of a precipitate. This was collected by filtration (vacuum), then washed with water and dried, to give 415 mg (81%) of a beige solid. ^{1}H NMR (DMSO-d_6) δ 7.77 (s, 1H), 11.41−6.62 (m, 2H), 6.72 (d, J = 0.6 Hz, 1H), 2.22 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 168.3, 150.7, 142.8, 133.7, 118.3, 109.4, 107.8, 22.7; m/z MS (TOF ES$^+$) 230.1 $[MH]^+$; LC-MS t_R: 3.56 min.

*2-Amino-5-bromo-3,4-dimethylbenzoic Acid (**24**).*[13] Methyl 2-amino-5-bromo-3,4-dimethylbenzoate hydrobromide (**16**) (5.93 g, 17.5 mmol) was dispersed in THF/water (1:1, 100 mL), and the flask atmosphere purged with nitrogen. To this was added LiOH·H_2O (3.67 g, 87.4 mmol, 5.0 equiv), and the mixture stirred at RT for 24 h. LC-MS analysis after this time indicated complete hydrolysis had occurred. The mixture was concentrated under reduced pressure to remove THF and MeOH, and then acidified with excess 2 M $HCl_{(aq)}$. The resultant precipitate was collected by filtration (vacuum), and washed with water, before allowing to dry on the filter bed overnight. This was then taken up in EtOAc (300 mL) and washed with brine (80 mL), before drying over $MgSO_4$. Concentration of dried organic layer under reduced pressure gave 4.00 g (94%) of a pale brown solid. ^{1}H NMR (DMSO-d_6) δ 7.78 (br s, 2H), 7.78 (s, 1H), 2.32 (s, 3H), 2.09 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 168.9, 149.0, 140.3, 131.2, 123.5, 109.3, 109.1, 20.4, 14.3; m/z MS (TOF ES$^+$) 244.1 $[MH]^+$; LC-MS t_R: 3.33 min.

*2-Amino-5-bromo-N-((1S,2S)-2-hydroxycyclohexyl)benzamide (**25**).* 2-Amino-5-bromobenzoic acid (**21**) (1.08 g, 5.00 mmol) and (1*S*,2*S*)-2-aminocyclohexanol hydrochloride (834 mg, 1.1 equiv) were coupled according to General Procedure A, to give 1.21 g (77%) of precipitate as a yellow solid, requiring no further purification. ^{1}H NMR (DMSO-d_6) δ 8.02 (d, J = 8.1 Hz, 1H), 7.70 (d, J = 2.4 Hz, 1H), 7.24 (dd, J = 8.8/2.4 Hz, 1H), 6.64 (d, J = 8.8 Hz, 1H), 6.47 (s, 2H), 4.65 (d, J = 5.2 Hz, 1H), 3.63−3.49 (m, 1H), 3.46−3.36 (m, 1H), 2.02−1.72 (m, 2H), 1.71−1.43 (m, 2H), 1.19 (d, J = 6.6 Hz, 4H); ^{13}C NMR (DMSO-d_6) δ 167.3, 148.6, 133.8, 130.5, 118.2, 117.2, 105.0, 71.0, 54.9, 34.6, 31.3, 24.6, 24.2; m/z MS (TOF ES$^+$) 313.1 $[MH]^+$; LC-MS t_R: 3.17 min.

*2-Amino-5-bromo-N-((1S,2S)-2-hydroxycyclohexyl)-3-methylbenzamide (**26**).* 2-Amino-5-bromo-3-methylbenzoic acid hydrobromide (**22**) (2.00 g, 6.43 mmol) and (1*S*,2*S*)-2-aminocyclohexanol hydrochloride (1.07 g, 1.1 equiv) were coupled according to General Procedure A, to give 2.00 g (95%) of precipitate as a pale yellow solid, requiring no further purification. ^{1}H NMR (DMSO-d_6) δ 8.03 (d, J = 8.1 Hz, 1H), 7.58 (d, J = 2.3 Hz, 1H), 7.22 (d, J = 1.7 Hz, 1H), 6.24 (s, 2H), 4.64 (d, J = 5.2 Hz, 1H), 3.68−3.47 (m, 1H), 3.46−3.34 (m, 1H), 2.07 (s, 3H), 1.98−1.71 (m, 2H), 1.72−1.51 (m, 2H), 1.34−1.06 (m, 4H); ^{13}C NMR (DMSO-d_6) δ 167.7, 146.6, 134.1, 128.2, 125.7, 117.2, 105.1, 70.9, 54.9, 34.6, 31.3, 24.5, 24.2, 17.3; m/z MS (TOF ES$^+$) 327.1 $[MH]^+$; LC-MS t_R: 3.62 min.

*2-Amino-5-bromo-N-((1S,2S)-2-hydroxycyclohexyl)-4-methylbenzamide (**27**).* 2-Amino-5-bromo-4-methylbenzoic acid (**23**) (386 mg, 1.68 mmol) and (1*S*,2*S*)-2-aminocyclohexanol hydrochloride (281 mg, 1.1 equiv) were coupled according to General Procedure A, to give 508 mg (92%) of precipitate as a beige solid, requiring no further purification. ^{1}H NMR (DMSO-d_6) δ 7.95 (d, J = 8.0 Hz, 1H), 7.74 (s, 1H), 6.64 (d, J = 0.5 Hz, 1H), 6.43 (s, 2H), 4.62 (d, J = 5.2 Hz, 1H), 3.68−3.45 (m, 1H), 3.45−3.34 (m, 1H), 2.20 (s, 3H), 1.97−1.71 (m,

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2H), 1.70−1.49 (m, 2H), 1.36−1.06 (m, 4H); ^{13}C NMR (DMSO-d_6) δ 167.2, 148.9, 140.0, 131.2, 118.1, 115.0, 108.0, 70.9, 54.83, 34.6, 31.3, 24.5, 24.2, 22.4; m/z MS (TOF ES$^+$) 327.1 [MH]$^+$; LC-MS t_R: 3.57 min.

*2-Amino-5-bromo-N-((1S,2S)-2-hydroxycyclohexyl)-3,4-dimethylbenzamide (**28**).* 2-Amino-5-bromo-3,4-dimethylbenzoic acid (**24**) (1.00 g, 4.10 mmol) and (1*S*,2*S*)-2-aminocyclohexanol hydrochloride (684 mg, 1.1 equiv) were coupled according to General Procedure A, to give 1.32 g (94%) of precipitate as an off-white solid, requiring no further purification. ^{1}H NMR (DMSO-d_6) δ 7.99 (d, J = 8.0 Hz, 1H), 7.66 (s, 1H), 6.27 (s, 2H), 4.62 (d, J = 5.2 Hz, 1H), 3.66−3.49 (m, 1H), 3.46−3.34 (m, 1H), 2.31 (s, 3H), 2.06 (s, 3H), 1.97−1.73 (m, 2H), 1.73−1.50 (m, 2H), 1.34−1.08 (m, 4H); ^{13}C NMR (DMSO-d_6) δ 167.8, 146.7, 137.5, 128.7, 123.1, 115.4, 109.7, 70.9, 54.9, 34.6, 31.3, 24.5, 24.2, 20.1, 14.3; m/z MS (TOF ES$^+$) 341.1 [MH]$^+$; LC-MS t_R: 3.31 min.

*6-Bromo-3-((1S,2S)-2-hydroxycyclohexyl)quinazolin-4(3H)-one (**29**).* 2-Amino-5-bromo-*N*-((1*S*,2*S*)-2-hydroxycyclohexyl)benzamide (**25**) (1.18 g, 3.8 mmol) was dispersed in DMF-DMA (12 mL) and heated, with stirring at 85 °C for 3 h. LC-MS analysis indicated that the dimethylamine addition product was the major component of the reaction mixture, with elimination to the desired product progressing slowly. Further DMF-DMA (5 mL) was added, and the reaction temperature was increased to 115 °C, with heating continued for 72 h (progress monitored by LC-MS). The mixture was cooled to RT and carefully quenched with water (very exothermic), and the resulting precipitate collected by filtration (vacuum) before washing with water. After drying under air, the crude precipitate was recrystallized from EtOH to 767 mg (63%) of a yellow solid. ^{1}H NMR ($CDCl_3$) δ 8.69 (s, 1H), 8.38 (d, J = 2.2 Hz, 1H), 7.83 (dd, J = 8.7/2.3 Hz, 1H), 7.70 (d, J = 8.7 Hz, 1H), 4.81−4.43 (m, 1H), 4.14−3.91 (m, 1H), 2.30−2.16 (m, 1H), 2.11−1.97 (m, 1H), 1.96−1.65 (m, 3H), 1.63−1.32 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 160.6, 148.9, 144.7, 137.8, 129.7, 128.7, 123.4, 121.3, 61.5, 35.7, 31.1, 25.4, 24.5; m/z MS (TOF ES$^+$) 323.1 [MH]$^+$; LC-MS t_R: 3.21 min.

*6-Bromo-3-((1S,2S)-2-hydroxycyclohexyl)-8-methylquinazolin-4(3H)-one (**30**).* 2-Amino-5-bromo-*N*-((1*S*,2*S*)-2-hydroxycyclohexyl)-3-methylbenzamide (**26**) (1.50 g, 4.58 mmol) was dispersed in triethylorthoformate (30 mL) under an atmosphere of nitrogen. The mixture was heated under a reflux condenser, under nitrogen at 100 °C for 19 h. LC-MS analysis indicated partial conversion had occurred, so the temperature was increased to 150 °C for 96 h and monitored by LC-MS. The mixture was cooled to RT before quenching with a small amount of water (with care). On addition of water, a biphasic mixture was formed, so EtOAc was added, and the water layer decanted. The organic layer was then dried over $MgSO_4$, before concentration under reduced pressure to dryness. The crude residue was purified by FCC (eluent EtOAc/PE 0:100 to 100:0), to give 996 mg (65%) of a pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 8.48 (s, 1H), 8.06 (dd, J = 2.4/0.5 Hz, 1H), 7.87 (dd, J = 2.3/0.9 Hz, 1H), 4.95 (d, J = 5.3 Hz, 1H), 4.38 (s, 1H), 3.94 (s, 1H), 2.53 (s, 3H), 2.14−1.56 (m, 5H), 1.47−1.20 (m, 3H); ^{13}C NMR (DMSO-d_6) δ 159.6, 148.3, 145.0, 138.4, 136.8, 125.9, 121.8, 118.8, 67.9, 35.1, 29.7, 25.0, 23.9, 16.7; m/z MS (TOF ES$^+$) 337.1 [MH]$^+$; LC-MS t_R: 3.77 min.

*6-Bromo-3-((1S,2S)-2-hydroxycyclohexyl)-7-methylquinazolin-4(3H)-one (**31**).* 2-Amino-5-bromo-*N*-((1*S*,2*S*)-2-hydroxycyclohexyl)-4-methylbenzamide (**27**) (481 mg, 1.46 mmol) was dispersed in formamide (4 mL) in a 10 mL microwave vial, before sealing and heating at 120 °C with stirring. LC-MS analysis after this time indicated no reaction progression, so the temperature was increased to 150 °C, and stirring continued for 4 h. LC-MS analysis indicated the reaction was complete, so the mixture was cooled to RT overnight, before diluting with water (30 mL), then extracting with EtOAc (3 × 30 mL). The combined organic layers were washed with water (30 mL) and brine (30 mL), before concentration under reduced pressure. The crude residue was recrystallized from EtOH, to give 339 mg (69%) of pale brown solid. Concentration of the mother liquor gave 141 mg of impure product. ^{1}H NMR ($CDCl_3$) δ 8.50 (s, 1H), 8.32 (s, 1H), 7.60−7.51 (m, 1H), 4.61 (s, 1H), 4.04 (s, 1H), 2.50 (s, 3H), 2.33−2.17 (m, 1H), 2.10−1.68 (m, 4H), 1.67−1.30 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 160.4, 145.6, 145.5, 145.1, 130.3, 128.3, 123.8, 121.1, 72.1, 35.7, 31.0, 25.4, 24.5, 23.8; m/z MS (TOF ES$^+$) 337.1 [MH]$^+$; LC-MS t_R: 3.64 min.

*6-Bromo-3-((1S,2S)-2-hydroxycyclohexyl)-7,8-dimethylquinazolin-4(3H)-one (**32**).* 2-Amino-5-bromo-*N*-((1*S*,2*S*)-2-hydroxycyclohexyl)-3,4-dimethylbenzamide (**28**) (1.28 g, 3.75 mmol) was dispersed in DMF-DMA (12 mL) under an atmosphere of nitrogen, and heated at 85 °C for 6 h, before LC-MS analysis indicated conversion was complete. The mixture was cooled to RT, and carefully quenched with water. The resulting precipitate was collected by filtration (vacuum), washed further with water, and dried to give 1.25 g (95%) of an off-white solid. ^{1}H NMR (DMSO-d_6) δ 8.46 (s, 1H), 8.14 (s, 1H), 4.93 (d, J = 5.4 Hz, 1H), 4.36 (s, 1H), 3.94 (s, 1H), 2.59 (s, 3H), 2.49 (s, 3H), 2.17−1.54 (m, 5H), 1.52−1.06 (m, 3H); ^{13}C NMR (DMSO-d_6) δ 159.6, 147.9, 144.7, 141.5, 136.0, 126.3, 122.9, 120.8, 68.7, 35.1, 26.8, 25.0, 23.9, 20.4, 14.1; m/z MS (TOF ES$^+$) 351.1 [MH]$^+$; LC-MS t_R: 3.51 min.

*6-((6-Chloropyridin-3-yl)methyl)-3-((1S,2S)-2-hydroxycyclohexyl)-quinazolin-4(3H)-one (**33**).* 6-Bromo-3-((1*S*,2*S*)-2-hydroxycyclohexyl)quinazolin-4(3*H*)-one (**29**) (170 mg, 0.53 mmol) underwent Negishi coupling according to General Procedure B, to give 129 mg (66%) of a pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 8.40 (d, J = 2.1 Hz, 1H), 8.37 (s, 1H), 8.01 (d, J = 1.8 Hz, 1H), 7.74 (dd, J = 8.2/2.5 Hz, 1H), 7.70 (dd, J = 8.4/2.1 Hz, 1H), 7.61 (d, J = 8.3 Hz, 1H), 7.44 (dd, J = 8.2/0.5 Hz, 1H), 4.91 (d, J = 5.3 Hz, 1H), 4.39 (s, 1H), 4.15 (s, 2H), 3.94 (s, 1H), 2.12−1.57 (m, 5H), 1.48−1.20 (m, 3H); ^{13}C NMR (DMSO-d_6) δ 160.3, 149.9, 148.8, 148.3, 146.0, 140.1, 139.0, 136.1, 134.9, 127.4, 125.7, 124.2, 121.7, 68.9, 36.6, 35.2, 27.6, 25.0, 23.9; m/z MS (TOF ES$^+$) 370.2 [MH]$^+$; LC-MS t_R: 3.18 min; $[\alpha]_D^{27}$ = +7.78° (0.43, DMSO).

*6-((6-Chloropyridin-3-yl)methyl)-3-((1S,2S)-2-hydroxycyclohexyl)-8-methylquinazolin-4(3H)-one (**34**).* 6-Bromo-3-((1*S*,2*S*)-2-hydroxycyclohexyl)-8-methylquinazolin-4(3*H*)-one (**30**) (462 mg, 1.37 mmol) underwent Negishi coupling according to General Procedure B, to give 390 mg (74%) of pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 8.40 (d, J = 2.2 Hz, 1H), 8.39 (s, 1H), 7.85 (d, J = 1.4 Hz, 1H), 7.73 (dd, J = 8.2/2.5 Hz, 1H), 7.58 (s, 1H), 7.44 (d, J = 8.2 Hz, 1H), 4.91 (d, J = 5.4 Hz, 1H), 4.38 (s, 1H), 4.10 (s, 2H), 3.93 (s, 1H), 2.50 (s, 3H), 2.21−1.53 (m, 5H), 1.51−1.12 (m, 3H); ^{13}C NMR (DMSO-d_6) δ 160.5, 149.9, 148.2, 144.9, 144.5, 140.1, 138.4, 136.2, 135.6, 135.2, 124.2, 123.4, 121.6, 68.6, 36.6, 35.2, 30.5, 25.0, 24.0, 17.0; m/z MS (TOF ES$^+$) 384.2 [MH]$^+$; LC-MS t_R: 3.63 min.

*6-((6-Chloropyridin-3-yl)methyl)-3-((1S,2S)-2-hydroxycyclohexyl)-7-methylquinazolin-4(3H)-one (**35**).* 6-Bromo-3-((1*S*,2*S*)-2-hydroxycyclohexyl)-7-methylquinazolin-4(3*H*)-one (**31**) (328 mg, 0.97 mmol) underwent Negishi coupling according to General Procedure B, to give 334 mg (90%) of an off-white solid. ^{1}H NMR (DMSO-d_6) δ 8.35 (s, 1H), 8.31 (d, J = 2.1 Hz, 1H), 7.84 (s, 1H), 7.59 (dd, J = 8.2/2.5 Hz, 1H), 7.50 (s, 1H), 7.48−7.38 (m, 1H), 4.90 (d, J = 5.4 Hz, 1H), 4.37 (s, 1H), 4.15 (s, 2H), 3.93 (s, 1H), 2.35 (s, 3H), 2.07−1.48 (m, 5H), 1.47−1.14 (m, 3H); ^{13}C NMR (DMSO-d_6) δ 160.2, 150.0, 148.2, 146.4, 146.3, 143.6, 140.0, 137.6, 135.1, 128.0, 126.4, 124.2, 119.7, 68.9, 35.2, 34.4, 30.4, 25.1, 24.0, 19.7; m/z MS (TOF ES$^+$) 384.2 [MH]$^+$; LC-MS t_R: 3.55 min.

*6-((6-Chloropyridin-3-yl)methyl)-3-((1S,2S)-2-hydroxycyclohexyl)-7,8-dimethylquinazolin-4(3H)-one (**36**).* 6-Bromo-3-((1*S*,2*S*)-2-hydroxycyclohexyl)-7,8-dimethylquinazolin-4(3*H*)-one (**32**) (250 mg, 0.71 mmol) underwent Negishi coupling according to General Procedure B. After stirring for 24 h, LC-MS analysis indicated only partial conversion had occurred. Further $Pd(P(^tBu)_3)_2$ (11 mg, 0.02 mmol, 0.03 equiv) were added and stirring continued for a further 24 h. After this time the mixture was heated to 55 °C for an additional 24 h. After this time, the reaction had not progressed any further, so the mixture was cooled over an ice bath and quenched with a small amount of water, before dilution with water (20 mL). The aqueous slurry was extracted with EtOAc (3 × 20 mL), and the combined organic layers washed with brine (20 mL) before concentration under reduced pressure. The crude product was purified by FCC (eluent EtOAc/PE 60:40 to 100:0), to give 164 mg of the starting material (**36**) and 73 mg (75% brsm) of the desired product as an off-white

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glassy solid. ^{1}H NMR ($CDCl_3$) δ 8.19 (d, J = 2.1 Hz, 1H), 8.11 (s, 1H), 7.81 (s, 1H), 7.27 (dd, J = 8.6/2.8 Hz, 1H), 7.17 (d, J = 8.2 Hz, 1H), 4.49 (s, 1H), 4.16−3.85 (m, 3H), 3.10 (s, 1H), 2.47 (s, 3H), 2.21 (d, J = 15.6 Hz, 4H), 2.00−1.70 (m, 4H), 1.61−1.29 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 162.1, 149.7, 149.5, 144.5, 143.6, 142.1, 139.0, 136.8, 134.6, 134.3, 125.1, 124.2, 119.5, 71.6, 37.0, 35.5, 31.0, 25.4, 24.5, 16.9, 13.7; m/z MS (TOF ES$^+$) 398.2 [MH]$^+$; LC-MS t_R: 3.41 min.

*2-Amino-5-bromo-N-((1S,2S)-2-hydroxycyclohexyl)nicotinamide (**38**).* 2-Amino-5-bromopyridine-3-carboxylic acid (**37**) (1.09 g, 5.00 mmol) and (1*S*,2*S*)-2-aminocyclohexanol hydrochloride (834 mg, 1.1 equiv) were coupled according to General Procedure A, to give 1.47 g (94%) of precipitate as an off-white solid, requiring no further purification. ^{1}H NMR (DMSO-d_6) δ 8.22 (d, J = 8.2 Hz, 1H), 8.14 (d, J = 2.4 Hz, 1H), 8.12 (d, J = 2.4 Hz, 1H), 7.17 (s, 2H), 4.70 (d, J = 5.0 Hz, 1H), 3.71−3.46 (m, 1H), 3.48−3.21 (m, 1H), 2.01−1.72 (m, 2H), 1.72−1.46 (m, 2H), 1.41−0.99 (m, 4H); ^{13}C NMR (DMSO-d_6) δ 166.0, 157.5, 150.9, 138.4, 111.8, 104.0, 71.0, 55.1, 34.4, 31.2, 24.5, 24.2; m/z MS (TOF ES$^+$) 314.1 [MH]$^+$; LC-MS t_R: 3.32 min.

*6-Bromo-3-((1S,2S)-2-hydroxycyclohexyl)pyrido[2,3-d]pyrimidin-4(3H)-one (**39**).* 2-Amino-5-bromo-*N*-((1*S*,2*S*)-2-hydroxycyclohexyl)-nicotinamide (**38**) (515 mg, 1.64 mmol) was suspended in formamide (4.5 mL) in a 10 mL microwave vial, before sealing the tube. The mixture was heated to 180 °C for 1.5 h, then allowed to stir at RT overnight, before a further period of heating at 180 °C for 2 h. The mixture was cooled to RT, then quenched with water. The resulting precipitate was collected by filtration (vacuum) to give 168 mg of brown solid. The aqueous filtrate was extracted with EtOAc (3 × 30 mL), and the combined organic extracts washed with brine (30 mL). TLC analysis (EtOAc) indicated that product was still trapped in the aqueous layer. The aqueous layer was saturated with NaCl, before re-extraction with EtOAc (2 × 30 mL) then MeOH/EtOAc (1:9, 30 mL). The combined organic layers were washed with brine (30 mL), then dried over Na_2SO_4 before concentrating under reduced pressure, to give an additional 366 mg of yellow solid. The crude solids were combined, and purified by FCC (eluent MeOH/DCM 0:100 to 6:94), to give 233 mg (44%) of pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 9.05 (d, J = 2.6 Hz, 1H), 8.68 (s, 1H), 8.65 (d, J = 2.6 Hz, 1H), 5.01 (d, J = 5.0 Hz, 1H), 4.35 (s, 1H), 3.96 (s, 1H), 2.12−1.50 (m, 5H), 1.49−1.10 (m, 3H); ^{13}C NMR (DMSO-d_6) δ 160.2, 156.4, 156.0, 149.3, 137.5, 118.1, 117.3, 68.3, 34.9, 30.1, 25.0, 23.9; m/z MS (TOF ES$^+$) 324.1 [MH]$^+$; LC-MS t_R: 3.35 min.

*6-((6-Chloropyridin-3-yl)methyl)-3-((1S,2S)-2-hydroxycyclohexyl)-pyrido[2,3-d]pyrimidin-4(3H)-one (**40**).* 6-Bromo-3-((1*S*,2*S*)-2-hydroxycyclohexyl)pyrido[2,3-*d*]pyrimidin-4(3*H*)-one (**39**) underwent Negishi coupling according to General Procedure B. After quenching, the reaction mixture was concentrated under reduced pressure (prior LC-MS analysis indicated extractive workup was not suitable, due to solubility of the product in the aqueous layer). The crude product was purified by FCC (eluent MeOH/DCM 0:100 to 6:94, slow gradient over 20 column volumes) to give 33 mg (14%) of an off-white glassy solid. An additional 107 mg (37%) of yellow glassy solid was also isolated form the column, eluting before the desired product, and found to be 6-bromo-7-((6-chloropyridin-3-yl)methyl)-3-((1*S*,2*S*)-2-hydroxycyclohexyl)-7,8-dihydropyrido[2,3-*d*]pyrimidin-4(3*H*)-one. This was the major product of the reaction, resulting from nucleophilic attack by the zincate 7-position of the pyrido[2,3-*d*]pyrimidin-4(3*H*)-one ring system. ^{1}H NMR (CD_3OD) δ 8.90 (s, 1H), 8.58 (s, 1H), 8.49 (d, J = 2.4 Hz, 1H), 8.36 (d, J = 2.2 Hz, 1H), 7.74 (dd, J = 8.3/2.5 Hz, 1H), 7.42 (d, J = 8.2 Hz, 1H), 4.46 (s, 1H), 4.25 (s, 2H), 4.08 (s, 1H), 3.35 (s, 3H), 2.27−1.66 (m, 5H), 1.63−1.31 (m, 3H); ^{13}C NMR (CD_3OD) δ 162.7, 157.2, 156.7, 151.0, 150.8, 150.8, 141.5, 137.9, 137.0, 136.3, 125.8, 118.5, 70.6, 36.2, 35.4, 30.9, 26.4, 25.3; m/z MS (TOF ES$^+$) 371.2 [MH]$^+$; LC-MS t_R: 3.36 min.

*Ethyl 3-([1,1′-Biphenyl]-4-yl)acrylate (**44**).*[6] Phenylboronic acid (**41**) (2.47 g, 20.3 mmol, 1.25 equiv), 4-bromobenzaldehyde (**42**) (3.00 g, 16.2 mmol), (ethoxycarbonylmethylene)-triphenylphosphorane (**43**) (8.47 g, 24.3 mmol, 1.5 equiv), $PdCl_2(PPh_3)_2$ (398 mg 0.57 mmol, 0.035 equiv), and PPh_3 (298 mg, 1.13 mmol, 0.07 equiv) were dispersed in degassed DME (64 mL) and degassed 2 M $Na_2CO_{3(aq)}$ (32 mL). The mixture was heated at 70 °C for 22.5 h, then at 100 °C for 3 h, followed by 85 °C overnight. LC-MS analysis over this time indicated the Wittig reaction progressed at a faster rate than the Suzuki coupling. The mixture was cooled, then dilute with water (200 mL), before extracting with Et_2O (3 × 100 mL). The combined organic layers were washed with brine (100 mL), before concentration under reduced pressure. The residue was diluted with Et_2O/PE to effect precipitation of triphenylphosphine oxide, the majority of which was removed by filtration (vacuum). The resulting filtrated was reconcentrated under reduced pressure, and purified by FCC (eluent Et_2O/PE 0:100, followed by 8:92, then 10:90) to give ethyl (*Z*)-3-([1,1′-biphenyl]-4-yl)acrylate as 677 mg (17%) of a clear colorless oil and ethyl (*E*)-3-([1,1′-biphenyl]-4-yl)acrylate as 2.78 g (68%) of a white solid. Total yield 3.46 g (84%, *E*/*Z* 4:1).

E-isomer.[14] ^{1}H NMR ($CDCl_3$) δ 7.73 (d, J = 16.0 Hz, 1H), 7.67−7.56 (m, 6H), 7.52−7.42 (m, 2H), 7.42−7.34 (m, 1H), 6.48 (d, J = 16.0 Hz, 1H), 4.28 (q, J = 7.1 Hz, 2H), 1.36 (t, J = 7.1 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 167.2, 144.3, 143.1, 140.3, 133.6, 129.0, 128.7, 128.0, 127.7, 127.2, 118.3, 60.7, 14.5; m/z MS (TOF ES$^+$) 253.2 [MH]$^+$; LC-MS t_R: 3.83 min.

Z-isomer.[15] ^{1}H NMR ($CDCl_3$) δ 7.71 (d, J = 8.3 Hz, 2H), 7.65−7.55 (m, 4H), 7.49−7.40 (m, 2H), 7.40−7.31 (m, 1H), 6.97 (d, J = 12.7 Hz, 1H), 5.97 (d, J = 12.7 Hz, 1H), 4.21 (q, J = 7.1 Hz, 2H), 1.28 (t, J = 7.1 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 166.4, 142.9, 141.9, 140.7, 133.9, 130.6, 129.0, 127.7, 127.2, 126.8, 119.8, 60.5, 14.3; m/z MS (TOF ES$^+$) 253.1 [MH]$^+$; LC-MS t_R: 3.83 min.

*Ethyl 3-([1,1′-Biphenyl]-4-yl)propanoate (**45**).*[14,16] Ethyl 3-([1,1′-biphenyl]-4-yl)acrylate (**44**) (3.39 g, 13.4 mmol, *E*/*Z* isomers recombined) was dissolved in EtOAC (150 mL). Pd/C (10%, 300 mg, 0.1 wt eq) as a slurry in water (0.5 mL) was added and the mixture degassed by sonication. The vessel was evacuated and filled with hydrogen three times, then stirred under an atmosphere of hydrogen (balloon) for 4 h at RT. LC-MS analysis indicated complete consumption of starting material. The reaction mixture was filtered through a bed of Celite, with washings of EtOAc, before concentrating the filtrate under reduced pressure, to give 3.40 g (99%) of clear colorless oil. ^{1}H NMR ($CDCl_3$) δ 7.60−7.55 (m, 2H), 7.52 (d, J = 8.1 Hz, 2H), 7.43 (dd, J = 7.5 Hz, 2H), 7.37−7.30 (m, 1H), 7.28 (d, J = 8.0 Hz, 2H), 4.15 (q, J = 7.1 Hz, 2H), 3.00 (t, J = 7.8 Hz, 2H), 2.75−2.54 (m, 2H), 1.25 (t, J = 7.1 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 173.1, 141.1, 139.8, 139.4, 128.9, 127.4, 127.3, 127.2, 60.6, 36.0, 30.8, 14.4; m/z MS (TOF ES$^+$) 255.2 [MH]$^+$; LC-MS t_R: 3.78 min.

*3-([1,1′-Biphenyl]-4-yl)propanal (**46**).*[16] Ethyl 3-([1,1′-biphenyl]-4-yl)propanoate (**45**) (2.18 g, 8.57 mmol) was dissolved in dry toluene (35 mL) under an atmosphere of nitrogen. The solution was degassed under a stream of nitrogen, before cooling to −78 °C in a dry ice/acetone bath. A solution of 1 M DIBALH in toluene (17 mL, 17 mmol, 2.0 equiv) was added in dropwise, and the mixture stirred at −78 °C for 1.25 h. TLC analysis (DCM) indicated disappearance of the starting material, so the mixture was quenched with care, with dropwise addition of MeOH, while maintaining the temperature at −78 °C. Once quenched, the mixture was allowed to warm to RT, and stirred for 15 min, before addition of sat. Rochelle's solution (50 mL) and stirring for 30 min. The resulting mixture was then extracted with Et_2O (3 × 50 mL), and the combined organic layers washed with brine (50 mL). On concentration under reduced pressure, 2.01 g of milky white oil was obtained with an odor reminiscent of cinnamaldehyde. The crude product was purified by FCC (eluent EtOAc/PE 0:100 to 30:70), to give 1.58 g (88%) of a white solid. ^{1}H NMR ($CDCl_3$) δ 9.86 (t, J = 1.3 Hz, 1H), 7.61−7.55 (m, 2H), 7.55−7.50 (m, 2H), 7.47−7.40 (m, 2H), 7.38−7.31 (m, 1H), 7.28 (d, J = 8.4 Hz, 2H), 3.01 (t, J = 7.5 Hz, 2H), 2.89−2.78 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 201.7, 141.0, 139.6, 139.5, 128.9, 128.9, 127.5, 127.3, 127.2, 45.4, 27.9; m/z MS (TOF ES$^+$) no mass peaks observed; LC-MS t_R: 3.95 min.

*2-Cyano-N-((1S,2S)-2-hydroxycyclohexyl)acetamide (**48**).* Cyanoacetic acid (**47**) (419 mg, 4.92 mmol) and (1*S*,2*S*)-2-aminocyclohexanol hydrochloride (821 mg, 5.41 mmol) were coupled according to General Procedure A. After stirring in water/sat. $NaHCO_{3(aq)}$, no precipitate was evident, and the organic extracts of this aqueous slurry contained mainly 1,1,3,3-tetramethylurea by-product. The aqueous layer was concentrated to dryness under

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reduced pressure, and the resulting residue taken up in MeCN, and stirred at RT for 30 min. The inorganic solid mass was removed by filtration (vacuum) and the resulting filtrate concentrated under reduced pressure to give 1.60 g of yellow solid. This was recrystallized from EtOAc to give 400 mg of a yellow crystalline solid. The mother liquor was reconcentrated and purified by FCC (eluent EtOAc/PE 50:50 to 100:0) to give a further 237 mg of an off-white solid. Total yield 637 mg (71%). ^{1}H NMR (DMSO-d_6) δ 8.07 (d, J = 7.9 Hz, 1H), 3.57 (d, J = 0.7 Hz, 2H), 3.45−3.28 (m, 1H), 3.26−3.13 (m, 1H), 1.93−1.70 (m, 2H), 1.69−1.46 (m, 2H), 1.35−0.94 (m, 4H); ^{13}C NMR (DMSO-d_6) δ 161.6, 116.4, 71.0, 54.9, 33.9, 30.8, 25.5, 24.1, 23.7; m/z MS (TOF ES$^-$) 181.1 [M − H]$^-$; LC-MS t_R: 2.40 min.

*5-([1,1′-Biphenyl]-4-ylmethyl)-2-amino-N-((1S,2S)-2-hydroxycyclohexyl)thiophene-3-carboxamide (**49**).* 3-([1,1′-Biphenyl]-4-yl)propanal (**46**) (334 mg, 1.59 mmol), 2-cyano-*N*-((*1S,2S*)-2-hydroxycyclohexyl)acetamide (**48**) (289 mg, 1.59 mmol, 1.0 equiv), sulfur (51 mg, 1.59 mmol, 1.0 equiv) and TEA (0.22 mL, 1.59 mmol, 1.0 equiv) were dispersed in EtOH (1.6 mL) in a 10 mL microwave vial. The mixture was sonicated at RT for 5 min, before flushing the atmosphere with nitrogen and sealing the vial. The mixture was heated at 60 °C for 6 h, at which time LC-MS analysis indicated the reaction was complete. The mixture was cooled to RT, before pouring onto ice/water. The resulting brown solid was collected by filtration (vacuum) and washed with water, then air-dried. The crude product was purified by FCC (eluent EtOAc/PE 10:90 to 100:0) to give 367 mg (57%) of red solid. ^{1}H NMR (DMSO-d_6) δ 7.80−7.56 (m, 4H), 7.53−7.40 (m, 2H), 7.40−7.28 (m, 3H), 7.24 (d, J = 7.9 Hz, 1H), 7.05 (s, 2H), 6.97 (s, 1H), 4.54 (s, 1H), 3.92 (s, 2H), 3.67−3.43 (m, 1H), 3.33 (s, 1H), 1.96−1.71 (m, 2H), 1.70−1.48 (m, 2H), 1.45−0.97 (m, 4H); ^{13}C NMR (DMSO-d_6) δ 165.4, 160.2, 134.0, 139.9, 138.2, 128.9, 128.9, 127.3, 126.8, 126.6, 122.4, 122.0, 106.9, 71.4, 54.3, 34.9, 34.6, 31.6, 24.6, 24.2; m/z MS (TOF ES$^+$) 407.2 [MH]$^+$; HRMS $C_{24}H_{27}N_2O_2S$ [MH]$^+$ calcd 407.1793; found 407.1797; LC-MS t_R: 3.92 min; $[\alpha]_D^{27}$ = +20.58° (0.14, DMSO).

Intact Cell Radioligand Binding Assays. Flp-In Chinese hamster ovary (CHO) cells expressing the human muscarinic acetylcholine M_1 (hM_1 mAChR) were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with fetal bovine serum (FBS) (ThermoTrace (Melbourne, Australia) and 0.2 mg/mL hygromycin-B (Roche, Mannheim, Germany). The cells were plated at 10^4 cells per well in 96-well Isoplates (PerkinElmer). Prior to assay the growth medium was removed and the attached cells were used to perform radioligand binding studies in the presence of 0.2 nM [^{3}H]NMS and varying concentrations of acetylcholine (Sigma, St. Louis, MO) and PAMs in a total volume of 200 μL of binding buffer (10 mM HEPES, 145 mM NaCl, 1 mM $MgSO_4{\cdot}7H_2O$, 10 mM glucose, 5 mM KCl, 2 mM $CaCl_2$, 1.5 mM $NaHCO_3$, pH 7.4). The binding reaction mixtures were incubated for 1 h at 37 °C, in a humidified incubator and terminated by rapid removal of radioligand followed by two 100 μL washes with ice-cold 0.9% NaCl buffer. Radioactivity was determined by addition of 100 μL Microscint scintillation liquid (PerkinElmer Life Sciences) to each well and counting in a MicroBeta plate reader (PerkinElmer Life Sciences).

IP-One Accumulation Assays. The IP-One assay kit (Cisbio, France) was used for the direct quantitative measurement of myoinositol 1 phosphate (IP_1) in FlpIn CHO cells stably expressing the hM_1 mAChR. The cells were detached and resuspended in IP_1 stimulation buffer (10 mM Hepes, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose, 50 mM LiCl, pH 7.4). The stimulations were performed in 384-well Proxy-plates (PerkinElmer) in a total volume of 14 μL, in the absence or presence of increasing concentrations of ACh and the PAMs, at cell density of 10^6 million cells/ml for 1 h at 37 °C, 5% CO_2. The reactions were terminated by addition of 6 μL of lysis buffer containing HTRF reagents (the anti-IP1 Tb cryptate conjugate and the IP1-D2 conjugate), followed by incubation for 1 h at RT. The emission signals were measured at 590 and 665 nm after excitation at 340 nm using an Envision multilabel plate reader (PerkinElmer), and the signal was expressed as the HTRF ratio: F = ((fluorescence$_{665nm}$/fluorescence$_{590nm}$) × 10^4).

Data Analysis. All data were analyzed using Prism 6.01 (GraphPad Software, San Diego, CA). Binding-interaction studies with allosteric ligands were fitted to the following allosteric ternary complex model (eq 1):[31]

$$Y = \frac{B_{max}[A]}{[A] + \left(\frac{K_A K_B}{\alpha'[B] + K_B}\right)\left(1 + \frac{[I]}{K_I} + \frac{[B]}{K_B} + \frac{\alpha[I][B]}{K_I K_B}\right)} \quad (1)$$

where Y is percentage (vehicle control) binding, B_{max} is the total number of receptors, [A], [B], and [I] are the concentrations of radioligand, allosteric modulator, and the orthosteric ligand, respectively, K_A, K_B, and K_I are the equilibrium dissociation constants of the radioligand, allosteric modulator, and orthosteric ligand, respectively. α' and α are the binding cooperativities between the allosteric ligand and [^{3}H]NMS and the allosteric modulator and the agonist acetylcholine, respectively. Saturation binding experiments were used to determine the value of pK_A for [^{3}H]NMS (pK_A = 9.70 ± 0.01, K_A = 0.2 nM). Values of α (or α') > 1 denote positive cooperativity; values < 1 (but > 0) denote negative cooperativity, and value = 1 denotes neutral cooperativity. For the majority of compounds, a complete inhibition of [^{3}H]NMS binding by the allosteric modulator was observed, consistent with a very high level of negative cooperativity. In these cases to allow fitting of the data, logα' was fixed to −3 to reflect this high negative cooperativity. The dissociation constant of ACh (K_I) was not fixed in these analyses but rather determined for each separate experiment. No difference was observed in the value of K_I between experiments.

Concentration−response curves for the interaction between the allosteric ligand and the orthosteric ligand in the IP-One accumulation assays were globally fitted to the following operational model of allosterism and agonism (eq 2):[32]

$$E = \{E_m(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n\} / \{([A]K_B + K_A K_B + [B]K_A + \alpha[A][B])^n + (\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n\} \quad (2)$$

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 are operational measures of orthosteric and allosteric ligand efficacy, respectively, α is the binding cooperativity parameter between the orthosteric and allosteric ligand, β denotes the magnitude of the allosteric effect of the modulator on the efficacy of the orthosteric agonist, and n denotes the transducer slope that describes the underlying stimulus-response coupling of the ligand-occupied receptor to the signal pathway. This parameter was constrained to be shared between all curves within a fitted data set for each interaction study, and in all instances was not significantly different from unity. In many instances, the individual model parameters of eq 2 could not be directly estimated via the nonlinear regression algorithm by analysis of the functional data alone due to parameter redundancy. To facilitate model convergence, therefore, we fixed the equilibrium dissociation constant of each ligand to that determined from the whole cell binding assays. For compounds where no agonism was observed, logτ_B was fixed to −3.

All affinity, potency, and cooperativity values were estimated as logarithms, and statistical comparisons between values were by one-way analysis of variance using a Tukey's multiple comparison post test to determine significant differences between mutant receptors and the WT M_1 mAChR. A value of $p < 0.05$ was considered statistically significant.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

brsm, based on recovery of starting material; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMF-DMA, *N*,*N*-dimethylformamide, dimethylacetal; FCC, flash column chromatography; HCTU, *O*-(1*H*-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; min, minutes; PE, petroleum spirits 40−60; TEA, triethylamine

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