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**2,8-Diazaspiro[4.5]decan-8-yl)pyrimidin-4-amine Potent CCR4 Antagonists Capable of
Inducing Receptor Endocytosis.**

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Abstract

A number of potent 2,8-diazaspiro[4.5]decan-8-yl)pyrimidin-4-amine CCR4 antagonists binding to the extracellular allosteric site were synthesised. (*R*)-*N*-(2,4-Dichlorobenzyl)-2-(2-(pyrrolidin-2-ylmethyl)-2,8-diazaspiro[4.5]decan-8-yl)pyrimidin-4-amine (***R***)-(18a) has high affinity in both the [¹²⁵I]-TARC binding assay with a p*K*_i of 8.8, and the [³⁵S]-GTPγS functional assay with a pIC₅₀ of 8.1, and high activity in the human whole blood actin polymerisation assay (pA₂ = 6.7). The most potent antagonists were also investigated for their ability to induce endocytosis of CCR4 and were found to internalise about 60% of the cell surface receptors, a property which is not commonly shared by small molecule antagonists of chemokine receptors.

1. Introduction

Chemokines are a group of about 50 small, basic proteins of 8 – 10 kDa, which together with their receptors mainly regulate the recruitment of leukocytes into inflammatory sites.

Chemokines exert their effects through the activation of G protein-coupled receptors situated on the cell surface. Ten CC chemokine receptors have been identified so far named as CC-chemokine receptor 1, 2, 3 etc. Most chemokine receptors recognise more than one chemokine and several chemokines bind to more than one receptor.¹ CC chemokine receptor 4 (CCR4) is the only receptor identified so far for the macrophage-derived chemokine (MDC, CCL22) and thymus and activation-regulated chemokine (TARC, CCL17), and shown to be highly expressed in the thymus. T helper 2 (Th2) cytokines in inflamed tissues lead to eosinophilia, high levels of serum IgE and mast cell activation, all of which contribute to the pathogenesis of allergic diseases.² Upon exposure to allergen, dendritic cells within tissue secrete CCL22 and CCL17, which can recruit Th2 cells from the circulation. The T cells can then migrate along this chemokine gradient to the dendritic cells. The latter migrate from the inflamed tissue to local lymph nodes where CCL22 and CCL17 may recruit further T cells. Elevated levels of CCL17 and CCL22 as well as accumulation of CCR4-positive cells were observed in lung biopsy samples from patients with atopic asthma following allergen challenge.³ CCR4 is also expressed by immune suppressive regulatory T cells,^{4,5} and a minor subset of Th17 cells.^{6,7} Hence CCR4 antagonists represent a novel therapeutic intervention in diseases where CCR4 is involved, such as asthma,⁸ lung disease,⁴ atopic dermatitis,⁹ allergic bronchopulmonary aspergillosis,¹⁰ leukemia,¹¹ colon cancer,¹² inflammatory bowel disease,⁵ the mosquito-borne tropical diseases, such as Dengue fever,¹³ and allergic rhinitis.¹⁴ In addition, CCR4 antagonists were used as molecular adjuvants in vaccines.¹⁵⁻¹⁸ Finally, CCR4 monoclonal antibodies were recently explored for CCR4⁺ T cell leukemia and one such antibody, Mogamulizumab (20 mg injection), was launched in 2012 in Japan for the

treatment of relapsed or refractory adult T cell leukemia/lymphoma.^{19,20} In December 2014 approval for additional indication for chemotherapy-native CCR4-positive adult T-cell leukemia-lymphoma (ATL) of Mogamulizumab was granted in Japan. The launch of the humanised monoclonal antibody Mogamulizumab underlines the value of generating cheaper small molecule CCR4 antagonists in this area. Progress in the discovery of small-molecule CCR4 antagonists was reviewed by Purandare and Somerville in 2006.²¹ A number of other CCR4 antagonists have appeared in the literature since the publication of this review.²²⁻³⁸ These antagonists appear to belong to two chemotypes. The first chemotype, exemplified by compounds **1** – **7**, includes lipophilic amines, such as Bristol Myers Squibb (BMS) compounds **1**²² and **2**,²⁴ Astellas compounds **3**²⁷ and **4**,²⁹ the Daiichi Sankyo compound **5**,³¹ **6**³³ and **7**.³⁴ The second chemotype, exemplified by sulfonamides **8** – **10**, includes AstraZeneca pyrazine **8**,³⁵ the Ono pyrazine **9**,³⁶ and the GlaxoSmithKline indazole **10**.^{37,38} (Fig. 1). Indazole **10** was the first small molecule candidate to be progressed to human studies; however, the compound suffered from low solubility and weak potency.³⁹ Our group has recently published our efforts to identify novel sulfonamide templates for lead optimisation studies.^{40,41}

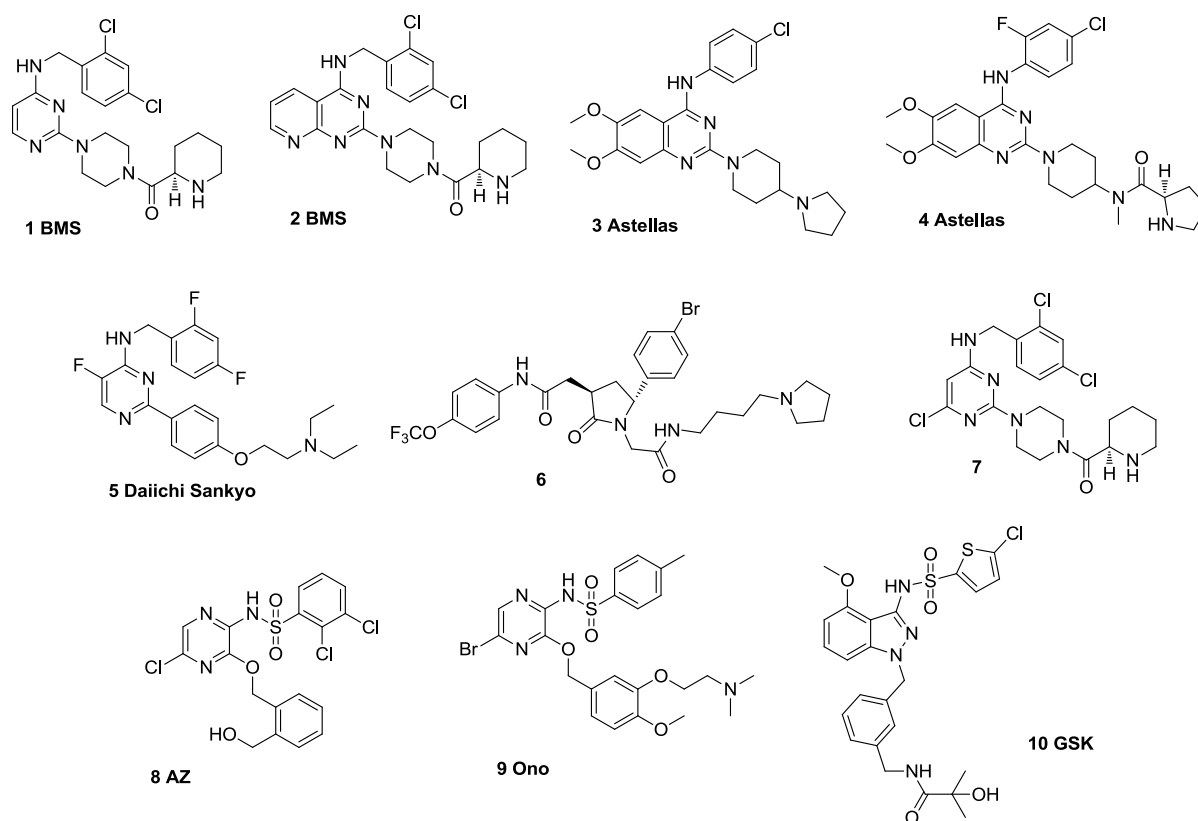


Figure 1. Structures for some recently published CCR4 antagonists. Numbers refer to compounds described in text.

Moreover, our group has recently reported that antagonism of human CCR4 can be achieved through three distinct binding sites on the receptor.⁴² Sulfonamides **8** – **10** were shown to bind at an intracellular allosteric binding site, arbitrarily named at GSK as site II, which is different from the binding site (site I), where lipophilic amine antagonists, such as **1**, **2** and **4** bind. Glu 290, which is found in Helix VII, is the anchor point for site I – perhaps the only residue strongly believed to interact with the chemokine, and these basic site I antagonists. The two allosteric binding sites I and II are distinct from each other and from the orthosteric binding site where CCL17 and CCL22 bind. Similar findings for a CCR4 and CCR5 intracellular binding site were reported by the AstraZeneca group.⁴³ Our group has so far reported only on sulfonamide (site II) intracellular CCR4 antagonists,^{37,38,40,41} herein we report our efforts on identifying basic site I allosteric antagonists.

2. Chemistry

At the time that this work was initiated X-ray crystal structures of G Protein Coupled Receptors were not reported, however, the structures of antagonists **1** – **3** were published, together with a CCR4 homology model based on bovine rhodopsin.²⁷ Our initial lead, the spirocyclic pyrimidine proline amide (\pm)-**11a**, was overlaid on BMS pyrimidine homoproline amide **1** docked in the homology model, and showed that the basic nitrogen atoms of the proline and homoproline moieties of (*R*)-**1** and (*R*)-**11a** respectively adopted a similar position in the receptor, although the proline nitrogen was further away from the core (Fig. 2).

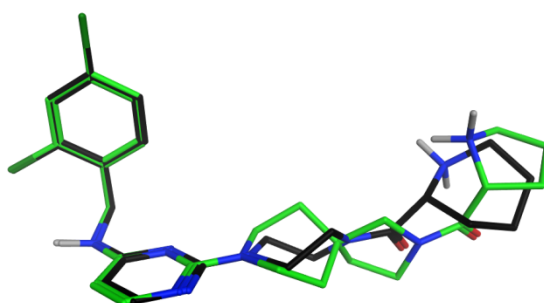
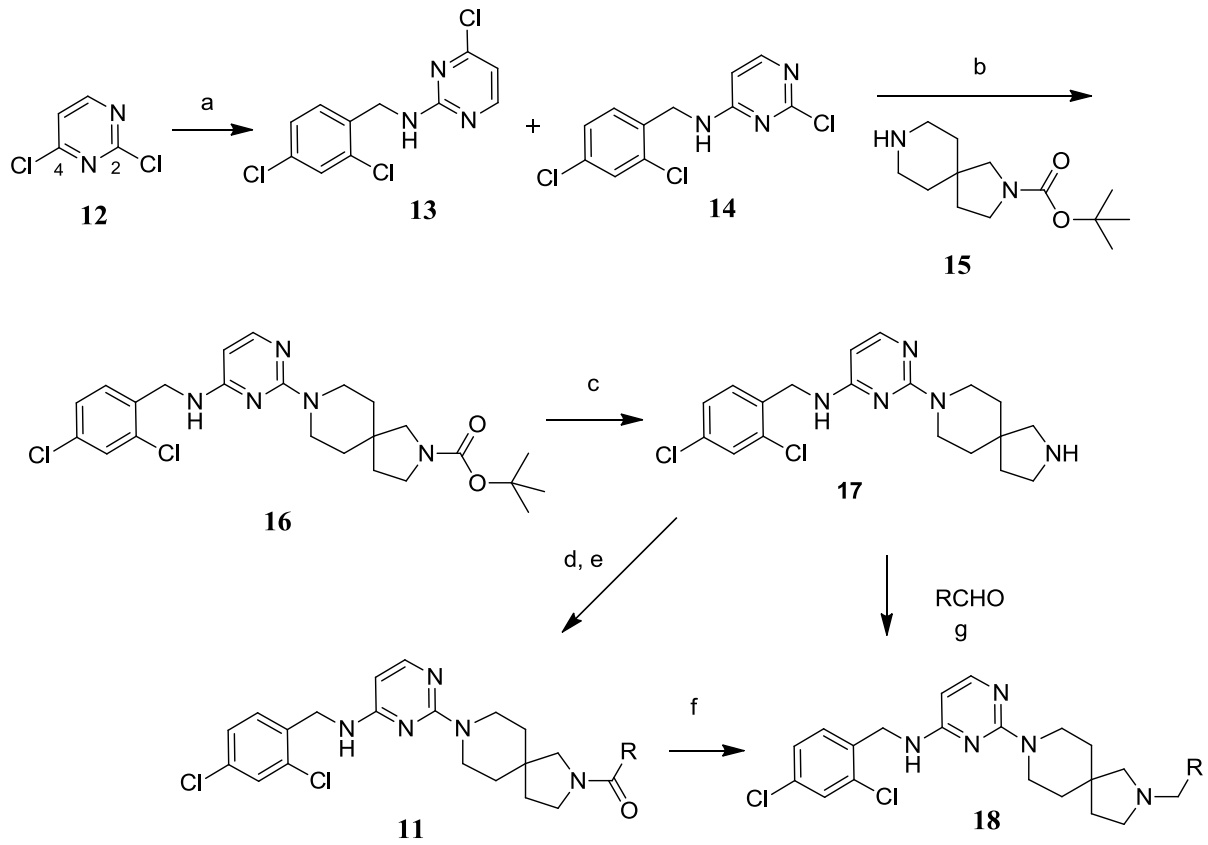


Figure 2. Overlay of compound (*R*)-**1** (black) with compound (*R*)-**11a** (green).

Compounds **11a-h** were prepared according to the route outlined in Scheme 1 starting from 2,4-dichloropyrimidine **12** and 2,4-dichlorobenzylamine to give after chromatography the regioisomeric chloropyrimidines **13** and **14** in 21 and 49% yield respectively. The amine was expected to react predominantly at the more electrophilic C4-position to give **14** as the major regioisomer. The structure of **14** was confirmed by irradiation of the benzylic NH proton (8.42 ppm), which produced an NOE enhancement of the methylene protons (4.54 ppm) and

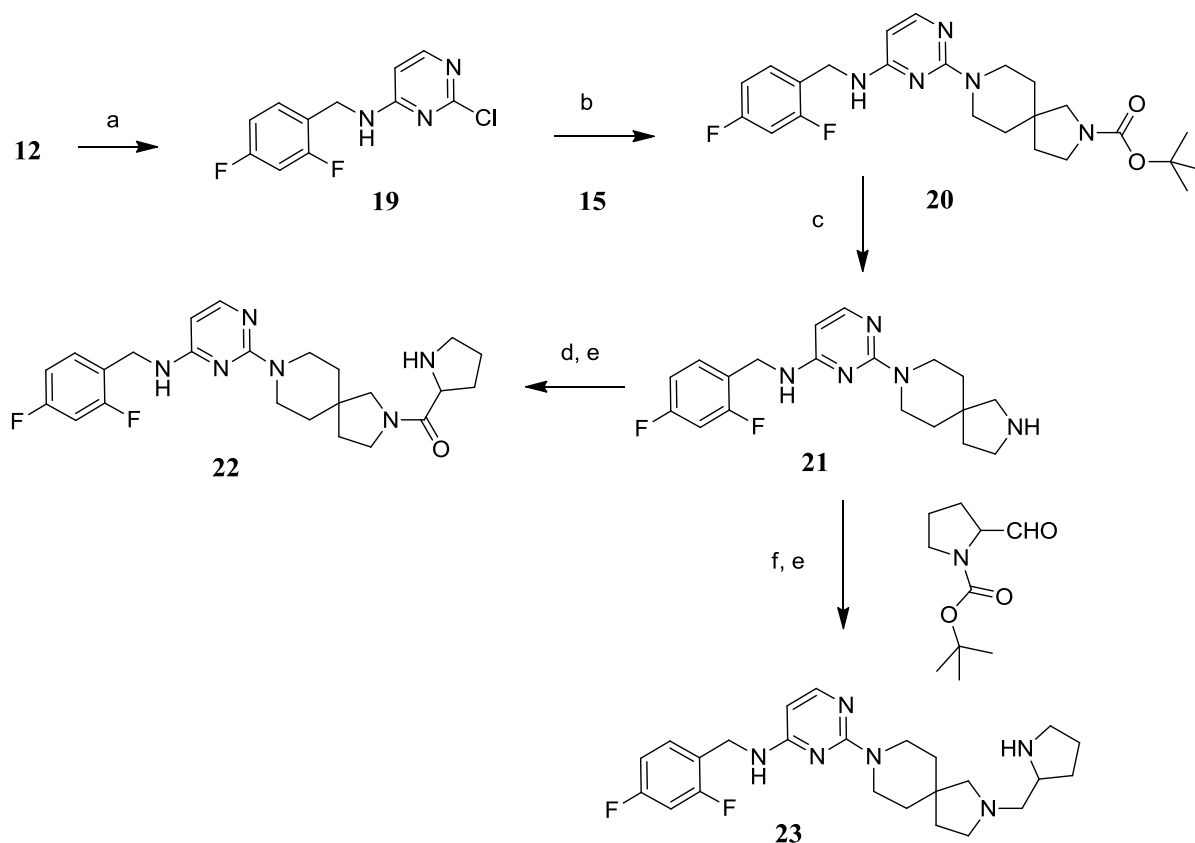
the pyrimidyl proton at the 5-position (6.58 ppm). The latter compound was reacted with commercially available BOC-2,8-diazaspiro[4.5]decane **15** in refluxing isopropyl alcohol to give spirocyclic pyrimidine **16** in 90% yield. The BOC protecting group was removed following treatment with hydrogen chloride in dioxane (99%), and the resulting amine **17** was acylated with a variety of acids in the presence of *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylethaniminium hexafluorophosphate *N*-oxide (HATU) in DMF. In the cases where the acid was a BOC-protected amino acid the resulting acylation product was deprotected with TFA to give the target compounds **11a-h**. The amides **11a,b** were reduced with either lithium aluminum hydride in THF or with borane in THF to give the diamines **18a,b**. Alternatively, racemic **18a** was obtained from **17** and *N*-BOC-2-formylpyrrolidine by reductive amination in the presence of sodium triacetoxyborohydride. The two enantiomers of **18a** were resolved by preparative chiral HPLC, and characterised by comparison with material obtained from the reduction of the enantiomerically pure amides **11a**.



	R		R
a		e	
b		f	
c		g	
d		h	

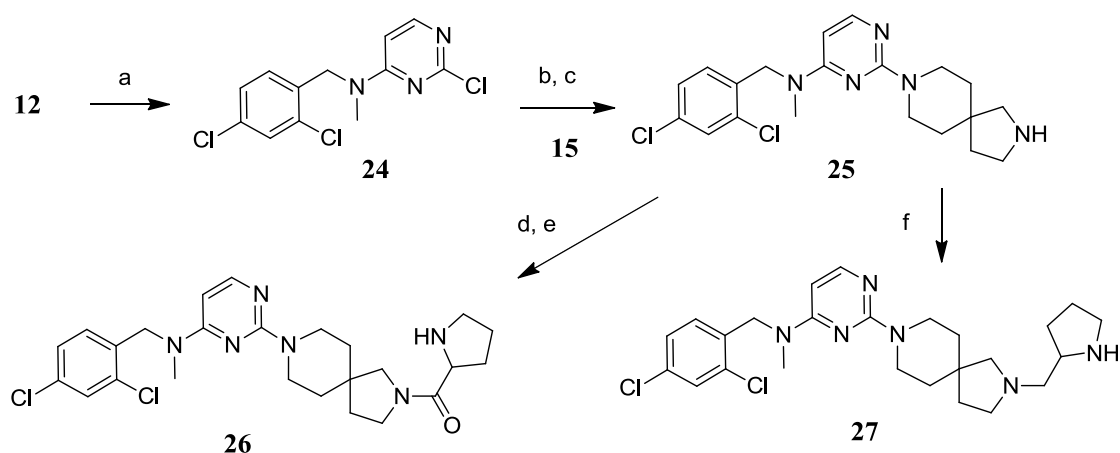
Scheme 1. *Reagents and Conditions:* a) 2,4-dichlorobenzylamine (1.1 equiv.), DIPEA, 1,2-dichloroethane, 20 °C, 24 h; b) **15**, isopropyl alcohol, 90 °C, 48 h; c) 4M HCl in dioxane, 0.5 h; d) RCO₂H (or BOC-protected amino acid), HATU, DIPEA, DMF, 1 h; e) TFA, dichloromethane (in the case of BOC protected amino acids); f) LiAlH₄, THF, 0 °C, 18 h; g) NaBH(OAc)₃, THF.

The racemic 2,4-difluoro analogues of **11a** and **18a** (**22** and **23** respectively) were prepared from **12** by analogous synthesis as shown in scheme 2.



Scheme 2. *Reagents and Conditions:* a) 2,4-difluorobenzylamine (1.1 equiv.), DIPEA, 1,2-dichloroethane, 20 °C, 24 h; b) **15**, isopropyl alcohol, 90 °C, 48 h; c) 4M HCl in dioxane, 0.5 h; d) (±)-N-BOC-proline, HATU, DIPEA, DMF, 1 h; e) TFA/dichloromethane or neat TFA; f) N-BOC-2-formylpyrrolidine, NaBH(OAc)₃, THF, 4Å molecular sieves.

Finally, the *N*-methyl analogues of **11a** and **18a** (**26** and **27** respectively) were prepared from **12** by a similar route as shown in Scheme 3.



Scheme 3. Reagents and Conditions: a) *N*-methyl-(2,4-dichlorobenzyl)amine (1.1 equiv.), DIPEA, 1,2-dichloroethane, 20 °C, 24 h; b) **15**, isopropyl alcohol, 90 °C, 48 h; c) 4M HCl in dioxane, 0.5 h; d) (±)-*N*-BOC-proline, HATU, DIPEA, DMF, 1 h; e) TFA, dichloromethane; f) *N*-BOC-2-formylpyrrolidine, NaBH(OAc)₃.

3. Results and Discussion

The antagonist activity at human CCR4 of the compounds shown in Table 1 was determined by [¹²⁵I]-TARC radioligand binding assay.⁴⁴ This assay used recombinant CCR4 expressing CHO cell membranes adhered to WGA-coated Leadseeker scintillation proximity assay (SPA) beads. The SPA beads contained scintillants that emit light when stimulated by emitted radiation, for example, during binding of the radiolabelled ligand to CCR4 bringing it in close proximity to the bead, and the output was measured on a scintillation counter. The [¹²⁵I]-TARC binding assay measured the inhibition of radiolabelled CCL17 binding directly

to CHO-CCR4 membranes in the presence of an antagonist, and the affinity was expressed as the pK_i . Another assay using isolated human peripheral blood mononuclear cells (PBMCs) was used as a secondary screen to determine potency against the native receptor for the more potent compounds in the primary assay.⁴² The assay quantified cytoskeletal reorganisation (formation of filamentous (F-) actin) which occurs in a variety of cells in response to chemoattractants and is a prelude to chemotaxis. This was achieved by staining the F-actin with a fluorescent derivative of phalloidin, which binds with high affinity and specificity to the interface between actin monomers in F-actin. The response was measured as an increase in the fluorescence intensity of the target cell population in a flow cytometer and was expressed as a pA_2 . Calculated partition coefficient (clogP), chromatographic $\log D_{7.4}$ (chrom $\log D$ at pH 7.4) and ChemiLuminescent Nitrogen Detection (CLND) kinetic solubility are included for all test compounds in this study. The high throughput CLND solubility assay involved addition of aqueous buffer to a test compound DMSO solution over a period of time until the compound precipitated. Compounds **1** and **9** were used as standards and are included in Table 1.

The uncapped spirocyclic amine **17** had weak affinity (pK_i 6.5) compared to the standards. The racemic proline amide **11a** was ten-fold more potent than **17**, but was still weaker than the reference compound **1**. Both enantiomers of the homoproline analogue **11b** had similar affinity to (\pm)-**11a**, but were more lipophilic (clogP 4.3, chrom $\log D_{7.4}$ 3.7) and had similar solubility. The β -amino acid amides **11c** and **11d** had slightly reduced affinity compared to (\pm)-**11a**. These findings paralleled the SAR reported for the BMS series.²² The sarcosine amide **11e**, although less lipophilic than the analogues investigated so far (chrom $\log D_{7.4}$ 3.2), offered no additional advantage (affinity or solubility). The cyclohexyl amide **11f** had the lowest affinity of all compounds in Table 1, lowest solubility and highest lipophilicity (clog P 5.0 and chrom $\log D_{7.4}$ 7.2). The 2-pyridyl amide **11g** was similarly disappointing as it

offered no advantage for either affinity or solubility. The morpholine amide **11h** was made as a less basic analogue to (\pm)-**11a**, however, it was still lipophilic ($\log D_{7.4}$ 3.8) and had only slightly increased solubility. Unfortunately this compound was not tested in the binding assay. The difluoro analogue **22**, although less lipophilic than **11a**, was like the *N*-methyl analogue **26**, equipotent to **11a**. All of the amides discussed so far had at least ten-fold lower affinity than **1** and were therefore rejected. These compounds (**11a-h**, **22** and **26**) were quite rigid because of the inflexibility of the spirocyclic scaffold and the amide linker group, leaving only one rotatable bond, between the heterocyclic ring and the acyl group, restricting thus the movement of the basic nitrogen of the pyrrolidine/piperidine ring. It was envisaged that reduction of the amide group to produce the diamines **18**, **23** and **27** would produce a more flexible arrangement, allowing for a better interaction with the conserved glutamic acid 290 residue located in the site I allosteric binding site.²⁷ Diamines **18a**, **18b**, **23** and **27** were synthesised as described earlier and assayed in the [¹²⁵I]-TARC SPA binding assay. They were found to be between 0.9 and 1.4 log units more active than the respective amides making these analogues at least as active as **1**. The two enantiomers of **18a** had identical affinity as the racemate, but had reduced solubility. It was not possible to measure the pK_a of the 1,2-diamino group spectrophotometrically, however, they were expected to be more basic than their α -amino amide analogues. The Chemaxon calculated pK_a for the proline nitrogen of **11a** was 9.8, whereas it was 10.9 for **18a**. For comparison the calculated pK_a for the homoproline nitrogen of **1** was 8.9, making these analogues very unlikely to have any permeability or oral absorption. Compounds (\pm)-**18a**, **23** and **27** were examined in the [³⁵S]-GTP γ S functional assay, described in our earlier publications, and compared with **1**, and the two site II antagonists **9** and **10**. In this assay all four site I antagonists were performing at the upper limit of the functional assay, and were found to be equipotent with a pIC₅₀ of 8.1. Analogues (\pm)-**18a** and **27** were progressed to the actin polymerisation in human PBMCs

assay, where (\pm)-**18a** was found to be as active as **1**, whereas **27** was less potent ($pA_2 = 6.0$). By comparison the site II antagonists **9** and **10** had $pA_2 = 6.6$ and 6.2 respectively, indicating that they were capable of crossing the cell lipid bilayer in this assay to reach their binding site.³⁸ Following the very encouraging potency of (\pm)-**18a** in the human blood assay, it was progressed to a pharmacokinetic study *in vivo* in the rat. Compound (\pm)-**18a** (dosed at 1 mg/kg) was found to have negligible oral bioavailability, low clearance of 15 mL/min/kg (18% liver blood flow), moderate to high volume of distribution (5 L/kg) and a long half-life of 4 h. In addition the human plasma protein binding was 93.2%.

4. Receptor endocytosis studies

CCL22 and CCL17 were shown to induce CCR4 receptor endocytosis (internalisation from the cell surface of human T_H2 cells), resulting in a loss of functional responsiveness.⁴⁵ Internalisation of the receptor following agonist exposure is thought to be a means of receptor desensitisation in GPCRs.⁴⁶ Endocytosis is an ATP-dependent process, which is largely inhibited at 4°C. Hence the antibody staining for the receptor endocytosis assays was performed at 4°C in all cases in order to confirm that there was receptor endocytosis and not simply inhibition of antibody binding by the test compounds.⁴⁷ Our group has confirmed that binding to the orthosteric site with CCL22 or CCL17 induced concentration dependent reduction in cell surface expression of CCR4 receptors ($pEC_{50} = 8.77 \pm 0.08$ and 7.98 ± 0.23 respectively) on HUT78 cells.⁴⁷ CCL22 induced almost complete receptor internalisation, whereas CCL17 caused only 50%. The ability to induce endocytosis suggests that these CCR4 antagonists are in fact biased ligands with antagonist activity in the primary functional assays, but agonist activity in the receptor internalisation assay.⁴⁷ However as receptor internalisation will prevent activation by chemokines this is really an antagonistic effect. So we will continue to refer to these compounds as CCR4 antagonists. Furthermore, four potent CCR4 antagonists (**2**, **4**, **8** and **9**) which were shown to bind to two distinct allosteric sites on

the CCR4 receptor (compounds **2** and **4** bind to site I; compounds **8** and **9** to site II) were investigated for induction of receptor endocytosis. The study showed that only the site I antagonists were capable of invoking internalisation (**2**: $pEC_{50} = 8.0 \pm 0.2$ and **4**: $pEC_{50} = 6.4 \pm 0.2$). The most potent antagonists from the spiro-pyrimidine series, compounds (*R*)-**18a**, (*S*)-**18a**, (\pm)-**23** and (\pm)-**27** were tested to assess their ability to invoke endocytosis of the CCR4 receptors on HUT78 cells (Table 2). The antagonists were incubated with HUT78 cells for 30 min at 37 °C and the CCR4 expression levels were evaluated by flow cytometry. The pEC_{50} is presented in Table 2 together with the degree of inhibition of receptor expression shown as a percentage. As observed previously, the natural agonist CCL22 inhibited cell surface expression almost completely. The two enantiomers of **18a** were found to be almost equipotent, with the *R* enantiomer showing a marginal advantage (*R*)-**18a** $pEC_{50} = 8.2$ and (*S*)-**18a** $pEC_{50} = 7.9$. The respective reduction in the number of cell surface CCR4 was 64% and 56%. The racemic difluoro analogue **23** also triggered internalisation of the receptor with a similar pEC_{50} and similar reduction in the number of receptors to that of the two enantiomers of **18a**, however, the NMe analogue **27**, which was the most potent spiro-pyrimidine inhibitor in the SPA assay, had a lower pEC_{50} value in the HUT78 cells with over 70% of cell surface CCR4 internalised. We have shown previously that site II antagonists do possess activity in the F- actin polymerisation assay in isolated human PBMCs, and hence cross the cell membrane to reach their binding site.⁴² Therefore, the finding that site I compounds are capable of causing endocytosis, whereas site II are not, highlights an important aspect of CCR4 biology and not simply a difference in crossing the lipid bilayer of cell membranes. Furthermore, it is important to note that this extremely interesting property of endocytosis is not a property commonly associated with small molecule antagonists. Two reports of small molecule CCR3 and CXCR3 agonists emerged out of antagonists screens before publication of our findings with CCR4.^{48, 49}

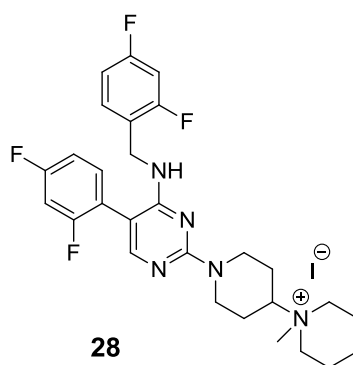


Figure 3. Structure of Kirin CCR4 antagonist K777

Recently the Kirin group reported that their CCR4 antagonist K777, the quaternary ammonium iodide **28** (Fig. 3) was capable of inducing CCR4 endocytosis at a concentration of 10 nM with a 50% reduction of cell surface CCR4.⁵⁰ Furthermore, these authors suggested that **28** binds to one of the allosteric sites, but they were not able to define the exact binding site. Considering the structure of **28** together with our earlier report that **2** and **4** bind to site I and cause CCR4 endocytosis,⁴⁷ we speculate that **28** also binds to site I. We have also considered CCR4 antagonists that were reported to possess *in vivo* activity, such as **5** inhibiting ovalbumin-induced airway inflammation in guinea pigs,³¹ compounds **1**²² and **2**²⁴ in murine allergic inflammation models, and we note that all these compounds are site I CCR4 antagonists and that **2** and **18a** cause receptor internalisation. In contrast site II antagonists, such as **8** and **9** do not cause receptor internalisation, and none has been reported to possess any *in vivo* activity. We therefore speculate whether the observed *in vivo* activity of site I CCR4 antagonists might be a consequence of their ability to induce endocytosis of the receptor.

Having shown that site I CCR4 antagonists have activity in endocytosis assays the question arises whether these compounds should still be called antagonists? We prefer to call them as biased signalling ligands as they have two properties, antagonism of orthosteric agonist drive

in F-actin, GTP γ S and chemotaxis assays, and agonism of receptor internalisation. It is still correct to call them antagonists with respect to the functional effects.

All of the site I antagonists are basic compounds with low permeabilities and unfavourable pharmacokinetic profiles. Therefore, further investigations are required to identify CCR4 site I antagonists with improved pharmacokinetic properties. The future directions and strategies for drug discovery in the field of small molecule antagonists and monoclonal antibodies targeting CCR4 have recently been reviewed by Pease.⁵¹

5. Conclusion

A number of 2,8-diazaspiro[4.5]decan-8-yl)pyrimidin-4-amine derivatives were synthesized and screened as CCR4 antagonists. Compound (\pm)-**18a** has high affinity in the [¹²⁵I]-TARC binding assay with a p*K*_i of 8.8, and the [³⁵S]-GTP γ S functional assay with a p*C*₅₀ of 8.1. Furthermore, it has high activity in the human whole blood actin polymerisation assay (p*A*₂ = 6.7). The most potent antagonists were investigated for their ability to induce endocytosis of CCR4 and were found to partially internalise the receptor with p*E*₅₀ of about 8. The two enantiomers of **18a** were found to have identical binding affinities, and these were paralleled in the internalisation assay. It is suggested that the *in vivo* activity of those allosteric CCR4 antagonists binding to site I might be due to their ability to induce internalisation of the receptor.

6. Experimental Section

Organic solutions were dried over anhydrous Na₂SO₄ or MgSO₄. TLC was performed on Merck 0.25 mm Kieselgel 60 F₂₅₄ plates. Products were visualised under UV light and/or by staining with aqueous KMnO₄ solution. LCMS analysis was conducted on either System A

an Acquity UPLC BEH C18 column (2.1 mm × 50 mm i.d. 1.7 μm packing diameter) eluting with 0.1% formic acid in water (solvent A), and 0.1 % formic acid in acetonitrile (solvent B), using the following elution gradient 0.0 – 1.5 min 3 – 100 % B, 1.5 – 1.9 min 100% B, 1.9 – 2.0 min 3% B, at a flow rate of 1 mLmin⁻¹ at 40 °C. The UV detection was an averaged signal from wavelength of 210 nm to 350 nm, and mass spectra were recorded on a mass spectrometer using alternate-scan electrospray positive and negative mode ionization (ES+ve and ES-ve); or System B an Agilent Sunfire C₁₈ column (30 mm × 4.6 mm ID, 3.5 μm packing diameter) eluting with 0.1% trifluoroacetic acid in water (solvent A), and 0.1% trifluoroacetic acid in MeCN (solvent B) using the following elution gradient 0.0 – 4.2 min 3 – 100% B, 4.2 – 4.8 min 100% B, 4.8 – 5.0 min 100 – 3 % B at a flow rate of 3 mL min⁻¹ at 30 °C. Column chromatography was performed on Flashmaster II, an automated multi-user flash chromatography system, utilizing disposable, normal phase, SPE cartridges (2 g to 100 g). Mass-directed auto-preparative HPLC (MDAP) for Method A was conducted on a Sunfire C18 column (150 mm × 30 mm i.d. 5 μm packing diameter) at ambient temperature eluting with 0.1 % TFA in water (solvent A) and 0.1 % TFA in acetonitrile (solvent B), using an appropriate elution gradient over 15 min at a flow rate of 40 mL min⁻¹ and detecting at 210 – 350 nm at room temperature; for Method B was conducted on an Xbridge C18 column (150 mm × 30 mm i.d. 5 μm packing diameter) at ambient temperature eluting with 10 mM ammonium bicarbonate in water adjusted to pH 10 with ammonia (solvent A) and acetonitrile (solvent B), using an appropriate elution gradient over 15 min at a flow rate of 40 mL min⁻¹ and detecting at 210 – 350 nm at room temperature. Mass spectra were recorded on Micromass ZMD mass spectrometer using electro spray positive and negative mode, alternate scans. The software used was *MassLynx* 3.5 with *OpenLynx* and *FractionLynx* options. ¹H NMR spectra were recorded at 400 MHz, unless otherwise stated. The chemical shifts are expressed in ppm relative to tetramethylsilane. High resolution positive ion mass spectra

were acquired on a Micromass Q-ToF 2 hybrid quadrupole time-of-flight mass spectrometer.

Optical rotations were measured with an Optical Activity AA100 digital polarimeter.

Analytical chiral HPLC was conducted on Chiralpak column (250 mm × 4.6 mm) eluting with an appropriate ratio of EtOH-heptane for 30 min at room temperature, flow rate 1 mL min⁻¹ injection volume 15 μL detecting at 215 nm. The purity of all compounds screened in the biological assays was examined by LCMS analysis and was found to be ≥ 95%, unless otherwise specified. All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals. The human biological samples were sourced ethically and their research use was in accord with the terms of the informed consents.

6.1 (R)-(8-(4-((2,4-Dichlorobenzyl)amino)pyrimidin-2-yl)-2,8-diazaspiro[4.5]decan-2-yl)(pyrrolidin-2-yl)methanone (R)-(11a)

A mixture of **17** (100 mg, 0.22 mmol), (*R*)-*N*-BOC-proline (52 mg, 0.24 mmol), HATU (54.3 mg, 0.14 mmol) and DIPEA (0.122 mL, 0.7 mmol) in DMF (1 mL) was stirred at ambient temperature for 1 h. The reaction mixture was purified by MDAP (Method A) the appropriate fractions were evaporated under reduced pressure. The residue was dissolved in DCM (0.5 mL) and TFA (0.5 mL) and stirred for 0.5 h. The solvent was evaporated under reduced pressure and the residue was dissolved in MeOH (0.5 mL) and passed down an aminopropyl cartridge (0.5 g). The cartridge was eluted with MeOH and the filtrate was evaporated under reduced pressure to give (*R*)-**11a** (85 mg, 79%) as a colourless gum: $[\alpha]_{\text{D}}^{20} = +36$ ($c = 0.93$ in MeOH); ¹H NMR δ (400 MHz, CD₃OD) 7.70 (d, $J = 6.0$ Hz, 1H), 7.43 (d, $J = 2.0$ Hz, 1H), 7.33 (d, $J = 8.0$ Hz, 1H), 7.26 (dd, $J = 8.0, 2.0$ Hz, 1H), 5.85 (d, $J = 6.0$ Hz, 1H), 4.58 (s, 2H), 3.85 – 3.41 (m, 9H), 3.20 – 3.05 (m, 1H), 2.85 – 2.69 (m, 1H), 2.28 – 2.04 (m, 1H), 1.90 (t, $J = 7.0$ Hz, 1H), 1.86 – 1.70 (m, 3H), 1.72 – 1.58 (m, 1H), 1.51 – 1.41 (m, 4H); the two

exchangeable protons were not observed; ^{13}C NMR δ (126 MHz, CD_3OD) 174.4, 174.3, 164.3, 162.7, 155.7, 137.7, 135.0, 134.9, 134.2, 131.2, 130.1, 128.3, 96.8, 60.3, 60.0, 57.2, 56.9, 48.3, 45.9, 45.8, 43.0, 42.8, 42.7, 42.6, 40.8, 37.1, 35.3, 35.2, 35.1, 35.0, 31.4, 31.3, 27.4 (the additional peaks were observed due to rotamers); ^{13}C NMR δ (101 MHz, $\text{DMSO-}d_6$, 393.2 K) 171.7, 161.9, 160.8, 154.7, 135.9, 132.5, 131.4, 129.8, 127.8, 126.4, 94.3, 58.4, 54.9, 47.9, 46.4, 43.2 (2C), 33.3 (2C), 28.9, 25.4; IR ν_{max} (neat) 3302, 2925, 2868, 1634, 1588, 1492, 1450 cm^{-1} ; Anal. Calcd. for $\text{C}_{24}\text{H}_{31}\text{Cl}_2\text{N}_6\text{O}$ = 489.1931. Found = 489.1925 $[\text{M}+\text{H}]^+$; LCMS (System A, UV, ES) RT = 0.67 min, $[\text{M}+\text{H}]^+$ = 489, 491, 493 100% purity.

6.2 (S)-(8-(4-((2,4-Dichlorobenzyl)amino)pyrimidin-2-yl)-2,8-diazaspiro[4.5]decan-2-yl)(pyrrolidin-2-yl)methanone (S)-(11a)

Was prepared by a similar method to its enantiomer to give (21 mg, 56%) as a colourless gum: $[\alpha]_{\text{D}}^{20} = -37$ ($c = 1.08$ in MeOH); LCMS (System A, UV, ES) RT = 0.67 min, $[\text{M}+\text{H}]^+$ = 489, 491, 493 100% purity. Other spectroscopic data were identical to its enantiomer.

6.3 (R)-(8-(4-((2,4-Dichlorobenzyl)amino)pyrimidin-2-yl)-2,8-diazaspiro[4.5]decan-2-yl)(piperidin-2-yl)methanone (R)-(11b)

Was prepared from **17** (30 mg, 0.076 mmol) and (*R*)-1-(*tert*-butoxycarbonyl)piperidine-2-carboxylic acid (19 mg, 0.084 mmol) according to the procedure described for the preparation of (*R*)-**11a** to give (*R*)-**11b** (15 mg, 39%) as a colourless gum: $[\alpha]_{\text{D}}^{20} = +12$ ($c = 1.03$ in MeOH); ^1H NMR δ (400 MHz, CD_3OD) 7.71 (d, $J = 6.0$ Hz, 1H), 7.43 (t, $J = 2.0$ Hz, 1H), 7.34 (d, $J = 8.0$ Hz, 1H), 7.26 (dd, $J = 8.0, 2.0$ Hz, 1H), 5.86 (d, $J = 6.0$ Hz, 1H), 4.58 (s, 2H), 3.87 – 3.62 (m, 4H), 3.61 – 3.42 (m, 4H), 3.29 – 3.19 (m, 1H), 3.14 – 3.02 (m, 1H), 2.72 – 2.55 (m, 1H), 1.89 (t, $J = 7.0$ Hz, 2H), 1.80 (t, $J = 7.0$ Hz, 2H), 1.68 – 1.54 (m, 2H), 1.52 – 1.31 (m, 6H); the two exchangeable protons were not observed; ^{13}C NMR δ (126 MHz, CD_3OD) 174.0, 173.9, 164.2, 162.7, 162.6, 155.7, 137.7, 134.9, 134.8, 134.2, 131.2, 130.1,

128.3, 96.8, 58.9, 58.7, 57.3, 56.5, 46.3, 46.2, 45.9, 45.5, 42.9, 42.8, 42.7, 42.6, 40.8, 37.1, 35.3, 35.2, 35.1, 35.0, 30.4, 30.3, 27.2, 25.2, 25.1 (the additional peaks were observed due to rotamers); Anal. Calcd. for $C_{25}H_{33}Cl_2N_6O$ = 503.2087. Found = 503.2075 $[M+H]^+$; LCMS (System A, UV, ES) RT = 0.68 min, $[M+H]^+$ = 503, 505, 507, 100% purity.

6.4 (S)-(8-(4-((2,4-Dichlorobenzyl)amino)pyrimidin-2-yl)-2,8-diazaspiro[4.5]decan-2-yl)(piperidin-2-yl)methanone (S)-(11b)

Was prepared from **17** (30 mg, 0.076 mmol) and (*S*)-1-(*tert*-butoxycarbonyl)piperidine-2-carboxylic acid (19 mg, 0.084 mmol) according to the procedure described for the preparation of (*R*)-**11a** to give (*S*)-**11b** (14 mg, 36%) as a colourless gum: $[\alpha]_D^{20} = -11$ ($c = 1.04$ in MeOH); LCMS (System A, UV, ES) RT = 0.68 min, $[M+H]^+$ = 503, 505, 507, 100% purity. Other spectroscopic data identical to its enantiomer.

6.5 (±)-(8-(4-((2,4-Dichlorobenzyl)amino)pyrimidin-2-yl)-2,8-diazaspiro[4.5]decan-2-yl)(pyrrolidin-3-yl)methanone (11c)

Was prepared from **17** (30 mg, 0.076 mmol) and (±)-*N*-BOC-pyrrolidine-3-carboxylic acid (18 mg, 0.084 mmol) according to the same procedure described for the preparation of (*R*)-**11a** to give (±)-**11c** (12 mg, 32%) as a colourless gum: 1H NMR δ (400 MHz, CD_3OD) 7.70 (d, $J = 6.0$ Hz, 1H), 7.43 (br.s, 1H), 7.33 (d, $J = 8.0$ Hz, 1H), 7.25 (dd, $J = 8.0, 2.0$ Hz, 1H), 5.85 (d, $J = 6.0$ Hz, 1H), 4.58 (s, 2H), 3.84 – 3.61 (m, 4H), 3.60 – 3.38 (m, 4H), 3.18 – 3.08 (m, 1H), 3.08 – 2.95 (m, 3H), 2.90 – 2.79 (m, 1H), 2.14 – 2.00 (m, 1H), 1.95 – 1.85 (m, 2H), 1.84 – 1.77 (m, 1H), 1.54 – 1.41 (m, 4H); the two exchangeable protons were not observed; ^{13}C NMR δ (126 MHz, CD_3OD) 176.2, 176.0, 164.3, 162.7, 155.7, 137.7, 134.9, 134.2, 131.2, 130.1, 128.3, 96.8, 57.9, 56.7, 51.5, 51.4, 48.3, 48.2, 46.4, 45.6, 44.1, 43.8, 42.9, 42.8, 42.7, 42.6, 40.9, 37.1, 35.3, 35.3, 35.1, 35.0, 31.6 (the additional peaks were observed due to

rotamers); Anal. Calcd. for $C_{24}H_{31}Cl_2N_6O$ = 489.1931. Found = 489.1917 $[M+H]^+$; LCMS (System A, UV, ES) RT = 0.67 min, $[M+H]^+$ = 489, 491, 493, 100% purity.

6.6 (±)-(8-(4-((2,4-Dichlorobenzyl)amino)pyrimidin-2-yl)-2,8-diazaspiro[4.5]decan-2-yl)(piperidin-3-yl)methanone (11d)

Was prepared from **17** (30 mg, 0.076 mmol) and (±)-1-(*tert*-butoxycarbonyl)piperidine-3-carboxylic acid (19 mg, 0.084 mmol) according to the procedure described for the preparation of (**R**)-**17a** to give (±)-**11d** (23 mg, 60%) as a colourless gum: 1H NMR δ (500 MHz, CD_3OD) 7.70 (dd, J = 6.0, 2.5 Hz, 1H), 7.43 (s, 1H), 7.37 – 7.31 (m, 1H), 7.26 (d, J = 8.0 Hz, 1H), 5.85 (br. s, 1H), 4.58 (s, 2H), 3.82 – 3.61 (m, 4H), 3.59 – 3.50 (m, 1H), 3.50 – 3.38 (m, 2H), 3.06 – 2.87 (m, 2H), 2.80 – 2.50 (m, 3H), 1.94 – 1.84 (m, 2H), 1.80 (t, J = 7.0 Hz, 1H), 1.75 – 1.59 (m, 2H), 1.58 – 1.24 (m, 5H), 1.01 – 0.81 (m, 1H); the two exchangeable protons were not observed; ^{13}C NMR δ (126 MHz, CD_3OD) 175.5, 175.4, 162.7, 134.9, 134.8, 134.2, 132.6, 131.2, 130.1, 130.0, 128.3, 69.3, 57.7, 56.6, 46.9, 46.1, 45.4, 42.9, 42.8, 42.7, 42.6, 40.9, 40.3, 37.1, 35.3, 35.2, 35.1, 31.8, 30.3, 28.3, 26.2, 25.1, 24.2, 14.6, 11.6 (the additional peaks were observed due to rotamers); Anal. Calcd. for $C_{25}H_{33}[^{35}Cl]_2N_6O$ = 503.2087. Found = 503.2078 $[M+H]^+$; LCMS (System A, UV, ES) RT = 0.65 min, $[M+H]^+$ = 503, 505, 507, 98% purity.

6.7 1-(8-(4-((2,4-Dichlorobenzyl)amino)pyrimidin-2-yl)-2,8-diazaspiro[4.5]decan-2-yl)-2-(methylamino)ethanone (11e)

Was prepared from **17** (30 mg, 0.076 mmol) and *N*-BOC-sarcosine (15 mg, 0.084 mmol) according to the procedure described for the preparation of (**R**)-**11a** to give **11e** (23 mg, 44%) as the bis-TFA salt; 1H NMR δ (400 MHz, CD_3OD) 7.62 (dd, J = 7.0, 2.0 Hz, 1H), 7.50 (d, J = 2.0 Hz, 1H), 7.41 – 7.36 (m, 1H), 7.35 – 7.29 (m, 1H), 6.20 (dd, J = 7.0, 2.0 Hz, 1H), 4.72 (s, 2H), 3.98 (s, 2H), 3.87 – 3.63 (m, 5H), 3.63 – 3.50 (m, 2H), 3.46 – 3.35 (m, 1H), 2.75 (s,

3H), 1.99 (t, $J = 7.0$ Hz, 1H), 1.90 (t, $J = 7.0$ Hz, 1H), 1.66 (br. s, 4H); the four exchangeable protons were not observed; Anal. Calcd. for $C_{22}H_{29}[^{35}Cl]_2N_6O = 463.1774$. Found = 463.1776 $[M+H]^+$; LCMS (System A, UV, ES) RT = 0.60 min, $[M+H]^+ = 463, 465, 99\%$ purity. The free base was formed by dissolving the salt in methanol (0.5 mL) and by passing through an aminopropyl cartridge (0.5 g). The cartridge was washed with additional methanol, and the filtrate was concentrated to afford **11e** (15 mg, 42%) as a white solid: 1H NMR δ (400 MHz, DMSO- d_6) 7.74 (d, $J = 4.5$ Hz, 1H), 7.59 (d, $J = 2.0$ Hz, 2H), 7.40 (dd, $J = 8.0, 2.0$ Hz, 1H), 7.35 (d, $J = 8.0$ Hz, 1H), 5.82 (br. s, 1H), 4.50 (d, $J = 5.0$ Hz, 2H), 3.78 – 3.63 (m, 2H), 3.59 – 3.47 (m, 4H), 3.45 (t, $J = 7.5$ Hz, 2H), 3.20 (br. s, 2H), 2.27 (d, $J = 2.5$ Hz, 3H), 1.77 (t, $J = 7.0$ Hz, 1H), 1.67 (t, $J = 7.0$ Hz, 1H), 1.44 – 1.23 (m, 4H); Anal. Calcd. for $C_{22}H_{29}[^{35}Cl]_2N_6O = 463.1774$. Found = 463.1776 $[M+H]^+$; LCMS (System A, UV, ES) RT = 0.61 min, $[M+H]^+ = 463, 465, 467, 100\%$ purity.

6.8 Cyclohexyl(8-(4-((2,4-dichlorobenzyl)amino)pyrimidin-2-yl)-2,8-diazaspiro[4.5]decan-2-yl)methanone (11f)

Was prepared from **17** (30 mg, 0.076 mmol) and cyclohexanecarboxylic acid (12 mg, 0.084 mmol) according to the procedure described for the preparation of **11a** to give **11f** (20 mg, 52%) as a colourless gum: 1H NMR δ (400 MHz, CD_3OD) 7.73 (dd, $J = 6.0, 2.0$ Hz, 1H), 7.46 (t, $J = 2.0$ Hz, 1H), 7.37 (d, $J = 8.0$ Hz, 1H), 7.29 (dd, $J = 8.0, 2.0$ Hz, 1H), 5.88 (d, $J = 6.0$ Hz, 1H), 4.61 (s, 2H), 3.84 – 3.63 (m, 4H), 3.62 – 3.53 (m, 1H), 3.50 (t, $J = 7.0$ Hz, 1H), 3.42 (s, 1H), 2.62 – 2.43 (m, 1H), 1.91 (t, $J = 7.0$ Hz, 1H), 1.87 – 1.67 (m, 6H), 1.59 – 1.12 (m, 10H); the exchangeable proton was not observed; ^{13}C NMR δ (126 MHz, CD_3OD) 177.6, 177.5, 164.2, 162.5, 162.48 155.5, 137.6, 134.8, 134.78, 134.1, 131.1, 130.0, 128.2, 96.8, 57.7, 56.5, 45.9, 45.3, 43.8, 43.7, 42.7, 42.7, 42.6, 42.5, 40.7, 37.0, 35.2, 35.0, 34.9, 30.1, 30.0, 27.0, 26.8, 26.7 (the additional peaks were observed due to rotamers); Anal. Calcd. for

$C_{26}H_{34}[^{35}Cl]_2N_5O = 502.2135$. Found = 502.2118 $[M+H]^+$; LCMS (System A, UV, ES) RT = 1.01 min, $[M+H]^+ = 502, 504, 506$, 100% purity.

6.9 (8-(4-((2,4-Dichlorobenzyl)amino)pyrimidin-2-yl)-2,8-diazaspiro[4.5]decan-2-yl)(pyridin-2-yl)methanone (11g)

Was prepared from **17** (30 mg, 0.076 mmol) and pyridine-2-carboxylic acid (10 mg, 0.084 mmol) according to the procedure described for the preparation of **11a** to give **11g** (18 mg, 47%) as a colourless gum: 1H NMR (500 MHz, CD_3OD) 8.62 (d, $J = 4.5$ Hz, 1H), 8.04 – 7.92 (m, 1H), 7.81 – 7.65 (m, 2H), 7.55 – 7.49 (m, 1H), 7.46 (dd, $J = 8.5, 2.0$ Hz, 1H), 7.39 – 7.32 (m, 1H), 7.31 – 7.23 (m, 1H), 5.91 – 5.80 (m, 1H), 4.60 (d, $J = 15.0$ Hz, 2H), 3.84 – 3.77 (m, 1H), 3.74 (t, $J = 7.0$ Hz, 2H), 3.70 – 3.50 (m, 5H), 1.95 – 1.83 (m, 2H), 1.64 – 1.39 (m, 4H), the exchangeable NH proton was not observed; 1H NMR δ (400 MHz, $DMSO-d_6$, 393 K) 8.59 (d, $J = 5.0$ Hz, 1H), 7.89 (dt, $J = 8.0, 1.5$ Hz, 1H), 7.77 (d, $J = 6.0$ Hz, 1H), 7.70 (d, $J = 8.0$ Hz, 1H), 7.48 (d, $J = 2.0$ Hz, 1H), 7.46 – 7.39 (m, 2H), 7.37 – 7.28 (m, 1H), 7.01 (br. s, 1H), 5.84 (d, $J = 6.0$ Hz, 1H), 4.55 (br. s, 2H), 3.75 – 3.57 (m, 6H), 3.50 (s, 2H), 1.82 (t, $J = 7.0$ Hz, 2H), 1.49 (br. s, 4H); ^{13}C NMR δ (126 MHz, CD_3OD) 169.0, 168.9, 162.7, 162.6, 155.3, 149.7, 149.6, 139.0, 139.0, 134.9, 134.2, 131.2, 130.1, 130.1, 128.3, 128.3, 126.7, 126.6, 124.7, 124.7, 60.0, 57.5, 46.3, 43.2, 42.8, 42.6, 40.9, 37.6, 35.4, 34.9 (the additional peaks were observed due to rotamers); Anal. Calcd. for $C_{25}H_{27}[^{35}Cl]_2N_6O = 497.1618$. Found = 497.1610 $[M+H]^+$; LCMS (System A, UV, ES) RT = 0.86 min, $[M+H]^+ = 497, 499, 501$, 98% purity.

6.10 (\pm)-(8-(4-((2,4-Dichlorobenzyl)amino)pyrimidin-2-yl)-2,8-diazaspiro[4.5]decan-2-yl)(morpholin-3-yl)methanone (11h)

Was prepared from **17** (30 mg, 0.076 mmol) and (\pm)-4-(*tert*-butoxycarbonyl)morpholine-3-carboxylic acid (19 mg, 0.084 mmol) according to the procedure described for the

preparation of **11a** to give **11h** (20 mg, 52%) as a colourless oil: ^1H NMR δ (400 MHz, CD_3OD) 7.70 (d, $J = 6.0$ Hz, 1H), 7.44 (br. s, 1H), 7.33 (d, $J = 8.0$ Hz, 1H), 7.26 (dd, $J = 8.0, 1.5$ Hz, 1H), 5.86 (d, $J = 6.0$ Hz, 1H), 4.58 (br. s, 2H), 4.02 – 3.88 (m, 1H), 3.84 – 3.42 (m, 9H), 3.40 – 3.24 (m, 3H, (obscured by CD_3OD), 2.96 – 2.88 (m, 2H), 1.89 (t, $J = 7.0$ Hz, 1H), 1.80 (t, $J = 7.0$ Hz, 1H), 1.56 – 1.29 (m, 4H); the two exchangeable protons were not observed; ^{13}C NMR δ (126 MHz, CD_3OD) 170.8, 170.7, 164.2, 162.7, 155.7, 137.7, 134.9, 134.9, 134.2, 131.2, 130.1, 128.3, 96.8, 69.8, 68.5, 57.9, 57.8, 57.4, 56.6, 46.0, 45.6, 45.4, 45.3, 43.0, 42.8, 42.7, 42.6, 40.8, 37.1, 35.3, 35.2, 35.1, 34.9 (the additional peaks were observed due to rotamers); Anal. Calcd. for $\text{C}_{24}\text{H}_{31}[^{35}\text{Cl}]_2\text{N}_6\text{O}_2 = 505.1880$. Found = 505.1878 $[\text{M}+\text{H}]^+$; LCMS (System A, UV, ES) RT = 0.62 min, $[\text{M}+\text{H}]^+ = 505, 507, 509$, 100% purity.

6.11 4-Chloro-*N*-(2,4-dichlorobenzyl)pyrimidin-2-amine (13) and 2-chloro-*N*-(2,4-dichlorobenzyl)pyrimidin-4-amine (14)

2,4-Dichlorobenzylamine (1.0 mL, 7.4 mmol) was added to a stirring solution of 2,4-dichloropyrimidine (1.0 g, 6.7 mmol) and DIPEA (1.8 mL, 10.1 mmol) in anhydrous DCE (10 mL) and the reaction mixture was stirred at 20°C under a nitrogen atmosphere for 24 h. LCMS showed the presence of a mixture of regioisomers (System A, RT = 1.10 min, 63% and 1.25 min, 27%). The reaction mixture was diluted with water (50 mL) and extracted with DCM (3 × 50 mL). The combined organic extracts were washed with brine (50 mL), dried using a hydrophobic frit and evaporated under reduced pressure. The residue was purified by column chromatography on a silica column, using a gradient of 0-100% ethyl acetate-cyclohexane over 40 min. The appropriate fractions were combined and evaporated under reduced pressure to afford the two regioisomers as white solids: less-polar isomer **13** (400 mg, 21%): ^1H NMR δ (400 MHz, $\text{DMSO}-d_6$) 8.33 – 8.11 (m, 2H), 7.60 (d, $J = 2.0$ Hz, 1H),

7.43 – 7.28 (m, 2H), 6.74 (d, $J = 5.0$ Hz, 1H), 4.51 (d, $J = 5.0$ Hz, 2H); ^{13}C NMR δ (126 MHz, DMSO- d_6) 162.1, 160.1, 159.9, 135.6, 132.9, 132.1, 129.6, 128.6, 127.3, 109.8, 41.7; IR ν_{max} (neat) 3269, 1597, 1561, 1531, 1449, 1412 cm^{-1} ; Anal. Calcd. for $\text{C}_{11}\text{H}_9[^{35}\text{Cl}]_3\text{N}_3 = 287.9857$. Found = 287.9860, $[\text{M}+\text{H}]^+$; LCMS (System A, UV, ES) RT = 1.25 min, $[\text{M}+\text{H}]^+ = 288, 290, 292, 294$ 100% purity; and the more-polar isomer **14** (950 mg, 49%): ^1H NMR δ (400 MHz, DMSO- d_6) 8.42 (t, $J = 4.5$ Hz, 1H), 7.97 (d, $J = 5.0$ Hz, 1H), 7.63 (d, $J = 2.0$ Hz, 1H), 7.45 – 7.29 (m, 2H), 6.58 (d, $J = 5.0$ Hz, 1H), 4.54 (d, $J = 4.5$ Hz, 2H); ^{13}C NMR δ (101 MHz, DMSO- d_6) 163.4, 159.7, 155.8, 134.8, 133.3, 132.5, 130.6, 128.7, 127.4, 105.2, 41.0; IR ν_{max} (neat) 3263, 1591, 1563, 1467, 1337 cm^{-1} ; Anal. Calcd. for $\text{C}_{11}\text{H}_9[^{35}\text{Cl}]_3\text{N}_3 = 287.9857$. Found = 287.9860, $[\text{M}+\text{H}]^+$; LCMS (System A, UV, ES) RT = 1.10 min, $[\text{M}+\text{H}]^+ = 288, 290, 292$, 100% purity.

6.12 *tert*-Butyl 8-((2,4-dichlorobenzyl)amino)pyrimidin-2-yl)-2,8-diazaspiro[4.5]decane-2-carboxylate, hydrochloride salt (16)

tert-Butyl 2,8-diazaspiro[4.5]decane-2-carboxylate (**15**) (1.67 g, 6.93 mmol) was added to a stirring solution of **14** (2.00 g, 6.93 mmol) in *i*-PrOH (9 mL) and the mixture was heated to 90 °C for 48 h. The reaction mixture was allowed to cool to 20 °C and diethyl ether (10 mL) was added. The resulting solid was collected by filtration, washed with more diethyl ether and dried overnight to give **16** (3.3 g, 90%) as a white solid: ^1H NMR δ (400 MHz, CD_3OD) 7.61 (d, $J = 7.0$ Hz, 1H), 7.51 (d, $J = 2.0$ Hz, 1H), 7.41 (d, $J = 8.0$ Hz, 1H), 7.33 (dd, $J = 8.0, 2.0$ Hz, 1H), 6.20 (d, $J = 7.0$ Hz, 1H), 4.72 (s, 2H), 3.87 – 3.61 (m, 4H), 3.47 – 3.37 (m, 2H), 3.24 (s, 2H), 1.85 (t, $J = 7.0$ Hz, 2H), 1.66 – 1.58 (m, 4H), 1.47 (s, 9H); the two exchangeable protons were not observed; ^{13}C NMR δ (126 MHz, MeOD- d_4) 164.0, 156.7, 153.8, 142.1, 135.5, 135.4, 135.3, 132.1, 130.5, 128.7, 99.2, 81.2, 81.1, 57.2, 56.3, 45.6, 45.3, 43.8, 43.7, 43.4, 42.0, 41.2, 36.7, 35.6, 34.9, 28.9 (the additional peaks were observed due to rotamers);

IR ν_{\max} (neat) 3230, 2922, 2962, 1687, 1655, 1619, 1581, 1406 cm^{-1} ; Anal. Calcd. for $\text{C}_{24}\text{H}_{32}[^{35}\text{Cl}]_2\text{N}_5\text{O}_2 = 492.1928$. Found = 492.1918, $[\text{M}+\text{H}]^+$; LCMS (System A, UV, ES) RT = 1.04 min, $[\text{M}+\text{H}]^+ = 492, 494, 496$, 100% purity.

6.13 *N*-(2,4-Dichlorobenzyl)-2-(2,8-diazaspiro[4.5]decan-8-yl)pyrimidin-4-amine (17)

Compound **16** (455 mg, 0.860 mmol) was treated with 4M HCl in dioxane (3.0 mL) and the mixture was stirred at ambient temperature for 0.5 h. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in DCM (20 mL), re-evaporated under reduced pressure, and dried in vacuo to give **17**·2HCl as a white solid (400 mg, 99%): ^1H NMR δ (400 MHz, CD_3OD) 7.67 (d, $J = 7.0$ Hz, 1H), 7.50 (d, $J = 2.0$ Hz, 1H), 7.44 (d, $J = 8.0$ Hz, 1H), 7.35 (dd, $J = 8.0, 2.0$ Hz, 1H), 6.28 (d, $J = 7.0$ Hz, 1H), 4.74 (s, 2H), 3.92 – 3.81 (m, 2H), 3.80 – 3.71 (m, 2H), 3.47 (t, $J = 7.5$ Hz, 2H), 3.24 (s, 2H), 2.06 (t, $J = 7.5$ Hz, 2H), 1.86 – 1.66 (m, 4H); the four exchangeable protons were not observed; ^{13}C NMR δ (126 MHz, CD_3OD) δ 164.0, 153.7, 142.1, 135.3, 135.2, 132.1, 130.4, 128.7, 99.3, 55.3, 45.6, 43.7, 43.3, 42.6, 35.6, 34.9; IR ν_{\max} (neat) 3431, 3355, 2869, 2827, 1650, 1617, 1574, 1467, 1410 cm^{-1} ; Anal. Calcd. for $\text{C}_{19}\text{H}_{24}[^{35}\text{Cl}]_2\text{N}_5 = 392.1403$. Found = 392.1408, $[\text{M}+\text{H}]^+$; LCMS (System A, UV, ES) RT = 0.56 min, $[\text{M}+\text{H}]^+ = 392, 394, 396$, 100% purity. The free base was obtained by passing down an aminopropyl cartridge (40 g) eluting with MeOH and concentrating under reduced pressure: ^1H NMR δ (400 MHz, CD_3OD) 7.71 (d, $J = 6.0$ Hz, 1H), 7.44 (d, $J = 2.0$ Hz, 1H), 7.33 (d, $J = 8.0$ Hz, 1H), 7.26 (dd, $J = 8.0, 2.0$ Hz, 1H), 5.87 (d, $J = 6.0$ Hz, 1H), 4.59 (s, 2H), 3.78 – 3.65 (m, 2H), 3.64 – 3.54 (m, 2H), 3.35 (s, 2H), 3.19 (t, $J = 7.5$ Hz, 2H), 2.93 (s, 2H), 1.82 (t, $J = 7.5$ Hz, 2H), 1.49 (t, $J = 5.5$ Hz, 4H); the two exchangeable protons were not observed; Anal. Calcd. for $\text{C}_{19}\text{H}_{24}[^{35}\text{Cl}]_2\text{N}_5 = 392.1403$. Found = 392.1408, $[\text{M}+\text{H}]^+$; LCMS (System A, UV, ES) RT = 0.55 min, $[\text{M}+\text{H}]^+ = 392, 394, 396$, 95% purity.

6.14 (R)-N-(2,4-Dichlorobenzyl)-2-(2-(pyrrolidin-2-ylmethyl)-2,8-diazaspiro[4.5]decan-8-yl)pyrimidin-4-amine (R)-(18a)

LiAlH₄ (0.20 mL of a 1 M solution in diethyl ether, 0.20 mmol) was added to a solution of (R)-**11a** (75 mg, 0.14 mmol) in THF (1 mL) at 0 °C, and the reaction mixture was stirred at room temperature for 18 h. Water (8 μL) was added, followed by aqueous sodium hydroxide (8 μL of 2 M solution) and more water (24 μL). The reaction was stirred at room temperature for 0.5 h, anhydrous MgSO₄ was then added and the mixture was stirred for an additional 15 min, before being filtered under vacuum. The filtrate was then partitioned between EtOAc (10 mL) and water (10 mL). The organic layer was separated, and the aqueous layer was extracted with more EtOAc (3 × 10 mL). The combined organic layers were dried using a hydrophobic frit and concentrated under reduced pressure. The crude product was taken up in DMSO (1 mL) and purified by MDAP (Method A). The solvent was evaporated and the solid was taken up in MeOH (1 mL) and passed through an aminopropyl cartridge (1 g). The cartridge was washed with additional methanol and the eluant was evaporated under reduced pressure to give (R)-**18a** (34 mg, 50%) as a colourless gum: $[\alpha]_D^{20} = -5.1$ (c = 0.47 in MeOH); Chiral HPLC RT = 36.5 min, 99.4% chiral purity (Chiralpak AD, 250 × 4.6 mm, 5% EtOH-heptane (containing 0.1% isopropylamine), flow rate of 1 mL/min); ¹H NMR (500 MHz, CD₃OD) 7.68 (d, *J* = 6.0 Hz, 1H), 7.42 (d, *J* = 1.5 Hz, 1H), 7.33 (d, *J* = 8.0 Hz, 1H), 7.24 (dd, *J* = 8.0, 1.5 Hz, 1H), 5.83 (d, *J* = 6.0 Hz, 1H), 4.57 (s, 2H), 3.79 – 3.43 (m, 4H), 3.19 – 3.07 (m, 1H), 3.00 – 2.89 (m, 1H), 2.83 – 2.75 (m, 1H), 2.71 – 2.64 (m, 1H), 2.63 – 2.56 (m, 1H), 2.56 – 2.33 (m, 4H), 1.97 – 1.87 (m, 1H), 1.81 – 1.71 (m, 2H), 1.66 (t, *J* = 7.0 Hz, 2H), 1.56 – 1.21 (m, 5H); the two exchangeable NH protons were not observed; ¹³C NMR δ (126 MHz, CD₃OD) 164.2, 162.7, 155.7, 137.7, 134.9, 134.2, 131.2, 130.1, 128.3, 96.5, 67.5, 62.8, 58.4, 55.2, 46.8, 43.1, 43.0, 41.8, 38.8, 37.8, 31.4, 25.9; IR ν_{max} (neat) 3300, 2922, 1587, 1491, 1448, 1338 cm⁻¹; Anal. Calcd. for C₂₄H₃₃[³⁵Cl]₂N₆ = 475.2138. Found =

457.2134; $[M+H]^+$; LCMS (System A, UV, ES) RT = 0.57 min, $[M+H]^+$ = 475, 477, 479, 97% purity.

6.15 (S)-N-(2,4-Dichlorobenzyl)-2-(2-(pyrrolidin-2-ylmethyl)-2,8-diazaspiro[4.5]decan-8-yl)pyrimidin-4-amine (S)-(18a)

$[\alpha]_D^{20} = +8.5$ ($c = 0.50$ in MeOH); Chiral HPLC RT = 41.7 min, 97% chiral purity (Chiralpak AD, 250 × 4.6 mm, 5% ethanol-heptane [0.1% isopropylamine], flow rate of 1 mL/min); other spectroscopic data were identical to its enantiomer (**R**)-**18a**.

6.16 N-(2,4-Dichlorobenzyl)-2-(2-(pyrrolidin-2-ylmethyl)-2,8-diazaspiro[4.5]decan-8-yl)pyrimidin-4-amine (±)-18a

(*R*)-*tert*-Butyl 2-formylpyrrolidine-1-carboxylate (0.08 mL, 0.4 mmol) was added to a mixture of **17** (165 mg, 0.42 mmol) and 4A molecular sieves (100 mg) in DCM (3 mL) and the reaction was stirred at ambient temperature, under a nitrogen atmosphere for 30 min. Acetic acid (3 μ L, 0.042 mmol) and sodium triacetoxyborohydride (107 mg, 0.51 mmol) were added and the reaction mixture was stirred for an additional 3 h. The mixture was partitioned between DCM (50 mL) and water (50 mL), the organic layer was separated, washed with water (3 × 50 mL), dried using a hydrophobic frit and concentrated under reduced pressure. TFA (1.5 mL) was then added to the residue and the mixture was stirred at ambient temperature for 1 h. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in DMSO (2 mL) and purified by MDAP (Method A). The solvent was evaporated and the solid was taken up in MeOH (1 mL), passed through an aminopropyl cartridge (1 g), washed with MeOH and the filtrate was evaporated under reduced pressure to afford (±)-**18a** (84 mg, 42%) as a white solid: LCMS (Method A), UV, ES) RT = 0.61 min, $[M+H]^+$ = 475, 477, 479, 96% purity. Analytical chiral HPLC RT=35.4 min and 40.6 min (1:1) on a Chiralpak AD column (250 × 4.6 mm), eluting with 5% EtOH–

heptane (containing 0.2% isopropylamine), flow-rate 1 mL/min), indicating racemisation occurred during the reductive amination. A portion of (\pm)-**18a** (28 mg) was resolved by preparative chiral HPLC on a Chiralpak AD-H (250 mm \times 30 mm) column eluting isocratically with 10% EtOH – hexane (containing 0.2% isopropylamine) over 40 min, flow-rate 45 mL/min collecting fractions with RT=14-16 min and 17-19 min respectively. The appropriate fractions were concentrated to give the two enantiomers (5 mg, 18%) for each enantiomer.

6.17 (\pm)-*N*-(2,4-Dichlorobenzyl)-2-(2-(piperidin-2-ylmethyl)-2,8-diazaspiro[4.5]decan-8-yl)pyrimidin-4-amine (18b)

Borane.THF complex (0.08 mL of a 1 M solution in THF, 0.08 mmol) was added to a stirring solution of **11b** (16 mg, 0.016 mmol) in THF (0.2 mL) and the reaction mixture was stirred at ambient temperature for 18 h. MeOH (1 mL) and HCl (0.5 mL of a 6 M aqueous solution) were slowly added and the reaction was heated to reflux for 30 min. The reaction mixture was concentrated, taken up in DMSO (1 mL) and purified by MDAP (Method B). The appropriate fractions were combined and the solvent was evaporated under reduced pressure to afford **18b** (4 mg, 51%) as a white solid: ^1H NMR δ (400 MHz, CD_3OD) 7.70 (d, $J = 6.0$ Hz, 1H), 7.45 (d, $J = 2.0$ Hz, 1H), 7.35 (d, $J = 8.0$ Hz, 1H), 7.27 (dd, $J = 8.0, 2.0$ Hz, 1H), 5.85 (d, $J = 6.0$ Hz, 1H), 4.59 (s, 2H), 3.78 – 3.63 (m, 2H), 3.60 – 3.48 (m, 2H), 3.05 (d, $J = 12.0$ Hz, 1H), 2.77 – 2.59 (m, 3H), 2.58 – 2.35 (m, 4H), 2.24 (dd, $J = 12.0, 4.0$ Hz, 1H), 1.81 (d, $J = 12.0$ Hz, 1H), 1.73 – 1.60 (m, 4H), 1.55 – 1.37 (m, 6H), 1.18 – 1.03 (m, 1H); the two exchangeable NH protons were not observed; LCMS (System B, UV, ES) RT = 1.69 min, $[\text{M}+\text{H}]^+ = 489, 491, 493, 100\%$ purity.

6.18 2-Chloro-*N*-(2,4-difluorobenzyl)pyrimidin-4-amine (19)

Was prepared from **12** (1.0 g, 6.7 mmol), 2,4-difluorobenzylamine (0.79 mL, 6.7 mmol) and DIPEA (1.8 mL, 10 mmol) according to the procedure described for the preparation of **14** to give **18** (1.26 g, 73%) as a white solid: ^1H NMR δ (400 MHz, CD_3OD) 7.88 (br. s, 1H), 7.52 – 7.34 (m, 1H), 7.03 – 6.82 (m, 2H), 6.45 (d, $J = 6.0$ Hz, 1H), 4.58 (br. s, 2H); the exchangeable NH proton was not observed; ^{19}F NMR δ (376 MHz, CD_3OD) (-112.8)-(-113.5) (m, 1F), (-115.9)-(-116.4) (m, 1F); LCMS (System A, UV, ES) RT = 0.94 min, $[\text{M}+\text{H}]^+ = 256, 258, 100\%$ purity. LCMS (System A, UV, ES) RT = 0.92 min, $[\text{M}+\text{H}]^+ = 256, 258, 100\%$ purity.

6.19 *tert*-Butyl 8-(4-((2,4-difluorobenzyl)amino)pyrimidin-2-yl)-2,8-diazaspiro[4.5]decane-2-carboxylate, hydrochloride salt (20)

Was prepared from **15** (188 mg, 0.782 mmol) and **19** (200 mg, 0.782 mmol) according to the procedure described for the preparation of **16** to give **20** (245 mg, 63%) as a white solid: ^1H NMR δ (400 MHz, CD_3OD) 7.57 (d, $J = 7.0$ Hz, 1H), 7.49 – 7.33 (m, 1H), 7.05 – 6.86 (m, 2H), 6.12 (d, $J = 7.0$ Hz, 1H), 4.66 (s, 2H), 3.88 – 3.60 (m, 4H), 3.48 – 3.39 (m, 2H), 3.26 (s, 2H), 1.87 (t, $J = 7.0$ Hz, 2H), 1.70 – 1.59 (m, 4H), 1.47 (s, 9H); the two exchangeable protons were not observed; ^{19}F NMR (376 MHz, CD_3OD) (-112.5) – (-112.8) (m, 1F), (-115.7) – (-116.0) (m, 1F); LCMS (System A, UV, ES) RT = 0.97 min, $[\text{M}+\text{H}]^+ = 460, 100\%$ purity.

6.20 *N*-(2,4-Difluorobenzyl)-2-(2,8-diazaspiro[4.5]decan-8-yl)pyrimidin-4-amine, dihydrochloride salt (21)

Was prepared from **20** (245 mg, 0.494 mmol) and HCl (3.0 mL of a 4 M solution in dioxane, 12.0 mmol) according to the procedure described for the preparation of **17** to give **21** (213 mg, 100%) as a colorless gum: ^1H NMR δ (400 MHz, CD_3OD) 7.60 (d, $J = 7.0$ Hz, 1H), 7.51 – 7.35 (m, 1H), 7.06 – 6.85 (m, 2H), 6.16 (d, $J = 7.0$ Hz, 1H), 4.67 (s, 2H), 3.93 – 3.82 (m, 2H), 3.80 – 3.70 (m, 2H), 3.45 (t, $J = 7.5$ Hz, 2H), 3.21 (s, 2H), 2.05 (t, $J = 7.5$ Hz, 2H), 1.77

(t, $J = 5.6$ Hz, 4H); the four exchangeable protons were not observed; ^{19}F NMR δ (376 MHz, MeOD- d_4) (-112.5) – (-112.7) (m, 1F), (-115.8) – (-116.0) (m, 1F); LCMS (System A, UV, ES) RT = 0.50 min, $[\text{M}+\text{H}]^+ = 360$, 100% purity.

6.21 (±)-(8-(4-((2,4-Difluorobenzyl)amino)pyrimidin-2-yl)-2,8-diazaspiro[4.5]decan-2-yl)(pyrrolidin-2-yl)methanone (22)

Was prepared from **21** (30 mg, 0.069 mmol) and (±)-*N*-BOC-proline (15 mg, 0.069 mmol) according to the procedure described for the preparation of **11a** to give **22** (19 mg, 60%) as a colourless gum: ^1H NMR (500 MHz, CD_3OD) 7.68 (d, $J = 6.0$ Hz, 1H), 7.44 – 7.32 (m, 1H), 6.97 – 6.79 (m, 2H), 5.81 (d, $J = 6.0$ Hz, 1H), 4.53 (s, 2H), 3.84 – 3.75 (m, 2H), 3.75 – 3.64 (m, 2H), 3.63 – 3.43 (m, 3H), 3.41 – 3.32 (m, 2H), 3.19 – 3.09 (m, 1H), 2.81 – 2.71 (m, 1H), 2.26 – 2.13 (m, 1H), 1.92 (t, $J = 7.0$ Hz, 1H), 1.87 – 1.71 (m, 3H), 1.70 – 1.59 (m, 1H), 1.57 – 1.46 (m, 4H); the two exchangeable NH protons were not observed; ^{13}C NMR δ (126 MHz, DMSO- d_6 , 393.4 K) 171.8, 161.9, 160.8, 160.0 (2C), 154.2, 129.9, 122.5, 109.9, 102.4, 93.9, 58.2, 54.6, 46.0, 42.8, 39.9 (2C), 36.2, 33.1 (2C), 28.6, 25.1 (2 aromatic carbons missing due to weak signal); ^{19}F NMR δ (376 MHz, CD_3OD) (-113.5) – (-115.3) (m, 1F), (-116.0) – (-117.9) (m, 1F); Anal. Calcd. for $\text{C}_{24}\text{H}_{31}\text{F}_2\text{N}_6\text{O} = 457.2522$. Found = 457.2515; $[\text{M}+\text{H}]^+$; LCMS (System A, UV, ES) RT = 0.54 min, $[\text{M}+\text{H}]^+ = 457$, 100% purity.

6.22 (±)-*N*-(2,4-Difluorobenzyl)-2-(2-(pyrrolidin-2-ylmethyl)-2,8-diazaspiro[4.5]decan-8-yl)pyrimidin-4-amine (23)

A mixture of **21** (free base) (36 mg, 0.10 mmol) and 1-BOC-2-formylpyrrolidine (20 mg, 0.10 mmol) in THF (1 mL) was treated with 4A molecular sieves (20 mg) and sodium triacetoxyborohydride (25.5 mg, 0.12 mmol) and the mixture was stirred at 25 °C overnight under nitrogen. The reaction mixture was filtered through celite and the filtrate was concentrated under reduced pressure. TFA (0.5 mL) was added to the residue and the

reaction stirred at room temperature for 30min, and then concentrated under reduced pressure. The residue was purified by MDAP (Method A) appropriate fractions were evaporated under reduced pressure and the residue was dissolved in MeOH and applied to an aminopropyl cartridge (1g). The cartridge was washed with MeOH and the solution was concentrated under nitrogen in a blow-down unit. The concentrate was freeze-dried to give **23** (20 mg, 45%) as a beige coloured solid: ^1H NMR (400 MHz, CD_3OD) 7.68 (d, $J = 6.0$ Hz, 1H), 7.45 – 7.32 (m, 1H), 7.00 – 6.80 (m, 2H), 5.81 (d, $J = 6.0$ Hz, 1H), 4.55 (s, 2H), 3.80 – 3.67 (m, 2H), 3.66 – 3.55 (m, 2H), 3.28 – 3.14 (m, 1H), 3.05 – 2.94 (m, 1H), 2.85 (td, $J = 10.0, 7.0$ Hz, 1H), 2.77 – 2.37 (m, 6H), 2.06 – 1.90 (m, 1H), 1.86 – 1.75 (m, 2H), 1.71 (t, $J = 7.0$ Hz, 2H), 1.58 – 1.46 (m, 4H), 1.46 – 1.37 (m, 1H); the two exchangeable NH protons were not observed; LCMS (System B, UV, ES) RT = 1.45 min, $[\text{M}+\text{H}]^+ = 443$, 99% purity.

6.23 2-Chloro-*N*-(2,4-dichlorobenzyl)-*N*-methylpyrimidin-4-amine (**24**)

Prepared from **12** (0.50 g, 3.4 mmol), *N*-methyl-(2,4-dichlorobenzyl)amine (0.64 g, 3.4 mmol) and DIPEA (1.3 mL, 7.4 mmol) according to the procedure described for the preparation of **14** to give **24** (0.39 g, 38%) as a white solid: ^1H NMR δ (400 MHz, CD_3OD) 8.04 (br. s, 1H), 7.52 (d, $J = 2.0$ Hz, 1H), 7.31 (dd, $J = 8.0, 2.0$ Hz, 1H), 7.12 (d, $J = 8.0$ Hz, 1H), 6.66 (br. s, 1H), 4.89 (s, 2H (obscured by HOCD_3)), 3.13 (br. s, 3H); LCMS (System A, UV, ES) RT = 1.28 min, $[\text{M}+\text{H}]^+ = 302, 304, 306$, 100% purity.

6.24 *N*-(2,4-Dichlorobenzyl)-*N*-methyl-2-(2,8-diazaspiro[4.5]decan-8-yl)pyrimidin-4-amine (**25**)

A mixture of **24** (200 mg, 0.661 mmol), **15** (159 mg, 0.661 mmol) and isopropyl alcohol (1 mL) were stirred at 90 °C for 24 h. The reaction was cooled to ambient temperature, concentrated under reduced pressure and treated with HCl (1.0 mL of a 4 M solution in dioxane, 4.0 mmol). The reaction was stirred at ambient temperature for 30 min and was then

partitioned between EtOAc (30 mL) and saturated aqueous sodium bicarbonate solution (30 mL). The organic layer was separated, dried using a hydrophobic frit and concentrated under reduced pressure to give **25** (180 mg, 67%) as a pale brown oil: $^1\text{H NMR } \delta$ (400 MHz, CD_3OD) 7.86 (d, $J = 6.0$ Hz, 1H), 7.49 (d, $J = 2.0$ Hz, 1H), 7.28 (dd, $J = 8.0, 2.0$ Hz, 1H), 7.07 (d, $J = 8.0$ Hz, 1H), 6.00 (d, $J = 6.0$ Hz, 1H), 4.84 (br. s, 2H), 3.81 – 3.66 (m, 2H), 3.65 – 3.52 (m, 2H), 3.14 (s, 3H), 2.98 (t, $J = 7.0$ Hz, 2H), 2.72 (s, 2H), 1.76 – 1.59 (m, 2H), 1.53 – 1.39 (m, 4H); the exchangeable NH proton was not observed; LCMS (System B, UV, ES) RT = 1.82 min, $[\text{M}+\text{H}]^+ = 407, 409, 411$, 87% purity.

6.25 (\pm) -**(8-(4-((2,4-Dichlorobenzyl)(methyl)amino)pyrimidin-2-yl)-2,8-diazaspiro[4.5]decan-2-yl)(pyrrolidin-2-yl)methanone (26)**

Was prepared from **25** (94 mg, 0.23 mmol) and (\pm) -*N*-BOC-proline (50 mg, 0.23 mmol) according to the procedure described for the preparation of **11a** to give **26** (31 mg, 26%) as a colourless gum: $^1\text{H NMR } \delta$ (400 MHz, CD_3OD) 7.87 (d, $J = 6.0$ Hz, 1H), 7.49 (t, $J = 2.0$ Hz, 1H), 7.27 (dd, $J = 8.0, 2.0$ Hz, 1H), 7.06 (d, $J = 8.0$ Hz, 1H), 6.01 (d, $J = 6.0$ Hz, 1H), 4.84 (br. s, 2H), 3.89 – 3.43 (m, 8H), 3.23 – 3.08 (m, 4H), 2.88 – 2.72 (m, 1H), 2.28 – 2.11 (m, 1H), 1.91 (t, $J = 7.0$ Hz, 1H), 1.88 – 1.73 (m, 3H), 1.73 – 1.59 (m, 2H), 1.49 (br. s, 4H); the exchangeable NH proton was not observed; LCMS (System B, UV, ES) RT = 1.86 min, $[\text{M}+\text{H}]^+ = 503, 505, 507$, 100% purity.

6.26 (\pm) -***N*-(2,4-Dichlorobenzyl)-*N*-methyl-2-(2-(pyrrolidin-2-ylmethyl)-2,8-diazaspiro[4.5]decan-8-yl)pyrimidin-4-amine (27)**

Was prepared from **25** (90 mg, 0.22 mmol) and (\pm) -*N*-BOC-2-formylpyrrolidine (44 mg, 0.22 mmol) according to the procedure described for the preparation of **23** to give **27** (35 mg,

32%) as a white solid: $^1\text{H NMR } \delta$ (400 MHz, CD_3OD) 7.85 (d, $J = 6.0$ Hz, 1H), 7.49 (d, $J = 2.0$ Hz, 1H), 7.27 (dd, $J = 8.0, 2.0$ Hz, 1H), 7.06 (d, $J = 8.0$ Hz, 1H), 5.99 (d, $J = 6.0$ Hz, 1H), 4.83 (br. s, 2H), 3.74 – 3.49 (m, 4H), 3.30 – 3.19 (m, 1H), 3.13 (s, 3H), 3.01 (td, $J = 10.0, 7.0$ Hz, 1H), 2.87 (td, $J = 10.0, 7.0$ Hz, 1H), 2.77 – 2.67 (m, 1H), 2.67 – 2.35 (m, 5H), 2.03 – 1.92 (m, 1H), 1.88 – 1.76 (m, 2H), 1.69 (t, $J = 7.0$ Hz, 2H), 1.55 – 1.35 (m, 5H); the exchangeable NH proton was not observed; LCMS (System B, UV, ES) RT = 1.72 min, $[\text{M}+\text{H}]^+ = 489, 491, 493$, 100% purity.

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Table 1. In vitro data pK_i for [125 I]-TARC binding assay, calculated logP, measured chrom logD at pH 7.4, CLND solubility and human whole blood actin polymerisation assay pA_2 .

Cmpd	[125I]-TARC	clogP	Chrom	CLND^a	hWB
	pK_i		logD	Solub.	pA_2
	\pm SEM			(μg/mL)	
	(n)				
1	8.6 \pm 0.1	4.5	3.5	140	6.76 \pm 0.03
	(3)				(80)
9	8.5 \pm 0.1	4.4	2.9	193	6.6 \pm 0.1
	(8)				(24)
17. 2HCl	6.5 \pm 0.1	4.0	3.5	144	ND
	(2)				
(\pm)-11a	7.6 \pm 0.1	3.8	3.5	79	ND
	(4)				
(R)-11a	7.8 \pm 0.3	3.8	3.4	180	ND
	(8)				
(S)-11a	7.0 \pm 0.4	3.8	3.3	165	ND
	(4)				
(R)-11b	7.6 \pm 0.3	4.3	3.7	172	ND
	(4)				
(S)-11b	7.7 \pm 0.2	4.3	3.7	170	ND
	(4)				
(\pm)-11c.TFA	7.3 \pm 0.2	3.3	3.3	339	ND
	(2)				
(\pm)-11d	7.3 \pm 0.2	3.3	3.5	186	ND

	(2)				
11e.2TFA	6.9±0.2	3.1	3.2	138	ND
	(2)				
11f	6.2±0.1	5.0	7.2	3	ND
	(2)				
11g	7.0±0.1	3.7	5.3	17	ND
	(2)				
(±)-11h	ND	3.4	3.8	233	ND
(±)-18a	8.8±0.5	4.3	4.4	270	6.7±0.3
	(4)				(2)
(S)-18a	8.4±0.2	4.3	4.3	56	ND
	(4)				
(R)-18a	8.3±0.1	4.3	4.2	16	ND
	(4)				
(±)-18b	8.6±0.1	4.9	4.6	182	ND
	(2)				
(±)-22	7.6±0.1	2.6	2.9	175	ND
	(2)				
(±)-23	8.5±0.3	3.2	3.2	160	ND
	(5)				
(±)-26	7.6±0.2	4.2	4.5	190	ND
	(2)				
(±)-27	9.0±0.2	4.7	5.3	141	6.0±0.2
	(5)				(2)

Table 2. The effectiveness of (*R*)-**18a**, (*S*)-**18a**, (\pm)-**23** and (\pm)-**27** to internalise the CCR4 receptor, and the percentage reduction in expression of cell-surface receptors.

Cmpd	pEC₅₀	% of CCR4 receptors internalised
CCL22	8.8 \pm 0.1 (n=5)	98 \pm 7
(<i>S</i>)-18a	7.9 \pm 0.2 (n=5)	56 \pm 10
(<i>R</i>)-18a	8.2 \pm 0.2 (n=5)	64 \pm 10
(\pm)-23	7.8 \pm 0.2 (n=5)	62 \pm 4
(\pm)-27	6.8 \pm 0.2 (n=5)	74 \pm 6

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