Article

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Discovery of tetrahydro-pyrazolo-pyridine as sphingosine 1-phosphate receptor 3 ($S1P_3$)-sparing $S1P_1$ agonists active at low oral doses.

Emmanuel H. Demont,[†,ʃ] James M. Bailey,[†] Rino A. Bit,[†] Jack A. Brown,[†] Colin A. Campbell,[†] Nigel Deeks,[†] Simon J. Dowell,[§] Colin Eldred,[†] Pam Gaskin,[†] James R. J. Gray,[†] Andrea Haynes,[†], David J. Hirst,[†] Duncan S. Holmes,[†] Umesh Kumar,[†] Mary A. Morse,[†] Greg J. Osborne,[§] Jessica F. Renaux,[†] Gail A. L. Seal,[†] Chris A. Smethurst,[†] Simon Taylor,[†,ʃ] Robert Watson,[†] Robert Willis[†] and Jason Witherington[†]*

[†]Immuno Inflammation Therapeutic Area Unit, [§]Platform Technology and Science, GlaxoSmithKline, Gunnels Wood Road, Stevenage, SG1 2NY, UK;

KEYWORDS : FTY720, fingolimod, S1P, agonism, lymphopenia, multiple sclerosis.

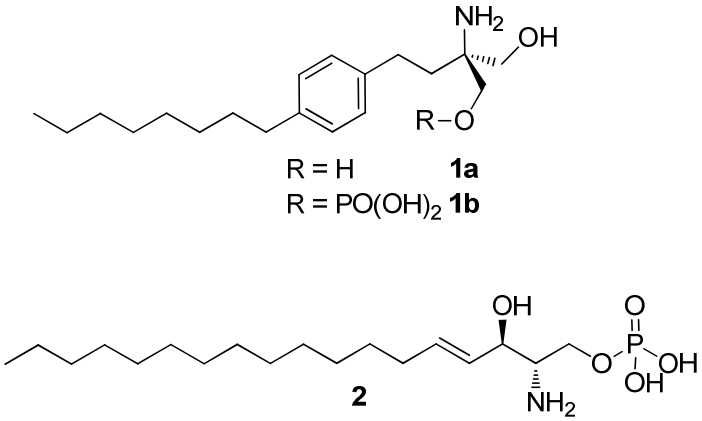
ABSTRACT: FTY720 is the first oral small molecule approved for the treatment of people suffering of relapsing remitting multiple sclerosis. It is a potent agonist of the $S1P_1$ receptor but its lack of selectivity against the $S1P_3$ receptor has been linked to most of the cardiovascular side effects observed in the clinic. These findings have triggered intensive efforts towards the identification of a second generation of $S1P_3$-sparing $S1P_1$ agonists. We have recently disclosed

a series of orally active tetrahydroisoquinoline (THIQ) compounds matching these criteria. In this paper we describe how we defined and implemented a strategy aiming at the discovery of selective structurally distinct follow-up agonists. This effort culminated with the identification of a series of orally active tetrahydro-pyrazolo-pyridines.

INTRODUCTION

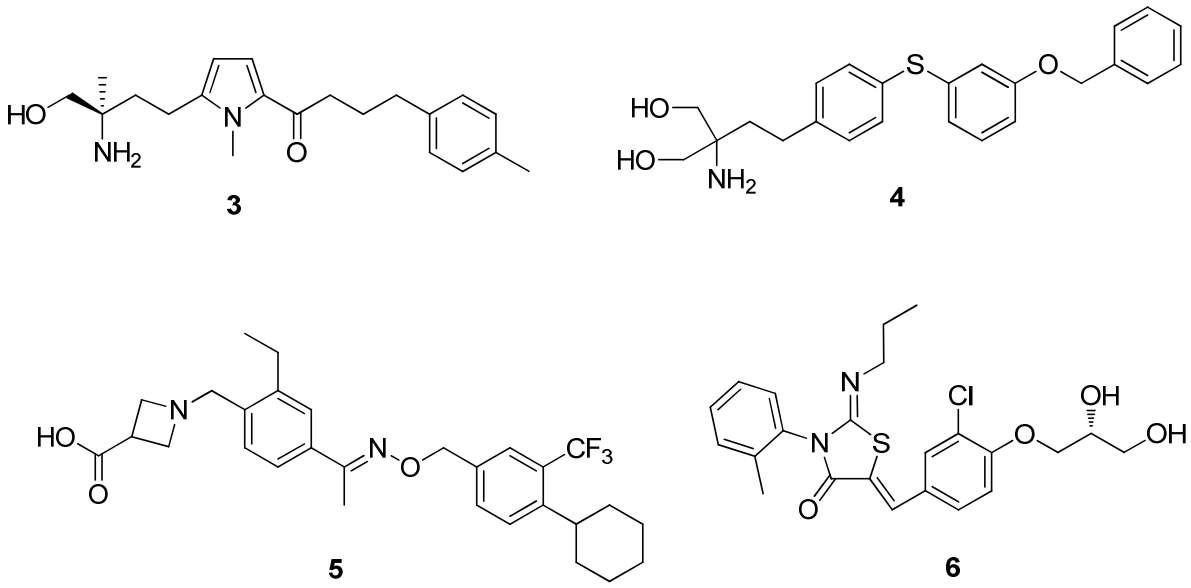
Fingolimod **1a**[1,2] (FTY720, Figure 1) is the first oral disease modifying small molecule to be marketed for the treatment of patients suffering from relapsing-remitting multiple sclerosis. It is phosphorylated in-vivo in an enantioselective manner [3,4] to give **1b** (FTY720P), a potent agonist of four ($S1P_1$, $S1P_{3-5}$) of the five receptors of the sphingolipid S1P **2**.[5,6] Its clinical efficacy is attributed to its ability to block the egress of peripheral lymphocytes from secondary lymphoid organs, a phenomenon purely driven by the $S1P_1$ receptor.[7,8] On the other hand, the side effects observed in the clinic (bradycardia, AV block, macular edema) have been linked mostly,[9-11] but not only,[12] to $S1P_3$ agonism.

Figure 1. Structures of **1a**, its phosphate **1b** and S1P **2**.



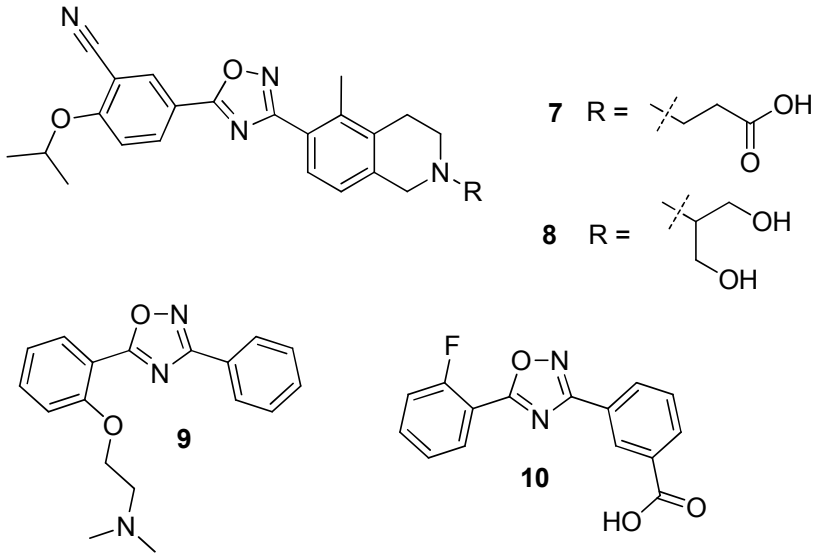
The clinical efficacy of **1a** and the understanding of what is driving its side effects have triggered intensive efforts towards the discovery of $S1P_3$-sparing $S1P_1$ agonists and $S1P_1$ agonists efficacious in pre-clinical models, but with improved selectivity against $S1P_3$, have entered clinical trials, either as pro-drugs (CS-0777 **3**[13,14] and KRP-203 **4**[15] as examples, Figure 2) or as direct agonists such as BAF312[16,17]**5** or ACT-128800[18,19] **6**.

Figure 2. Examples of pro-drugs or direct $S1P_1$ agonists known to have entered clinical trials.



We have recently disclosed [20] the outcome of our own effort, resulting in the identification of $S1P_3$-sparing drug-like $S1P_1$ agonists such as the THIQ **7** [21,22] or its brain-penetrant analogue **8**[23] (Figure 3). These two molecules contain a triaryl motif previously disclosed by Li *et al.*[24] This motif is present in molecules which have entered clinical trials (not as $S1P_1$ agonists) such as Irampanel **9** [25] or PCT124 **10**,[26] suggesting no inherent toxicity associated with this template, even if mutagenicity of 1,2,4 oxadiazole has been reported.[27] This may be part of the reason why it is present on a number of recently disclosed $S1P_1$ agonists.[28-30]

Figure 3: Structures of tri-aryl oxadiazole **7**, **8**, **9** and **10**.

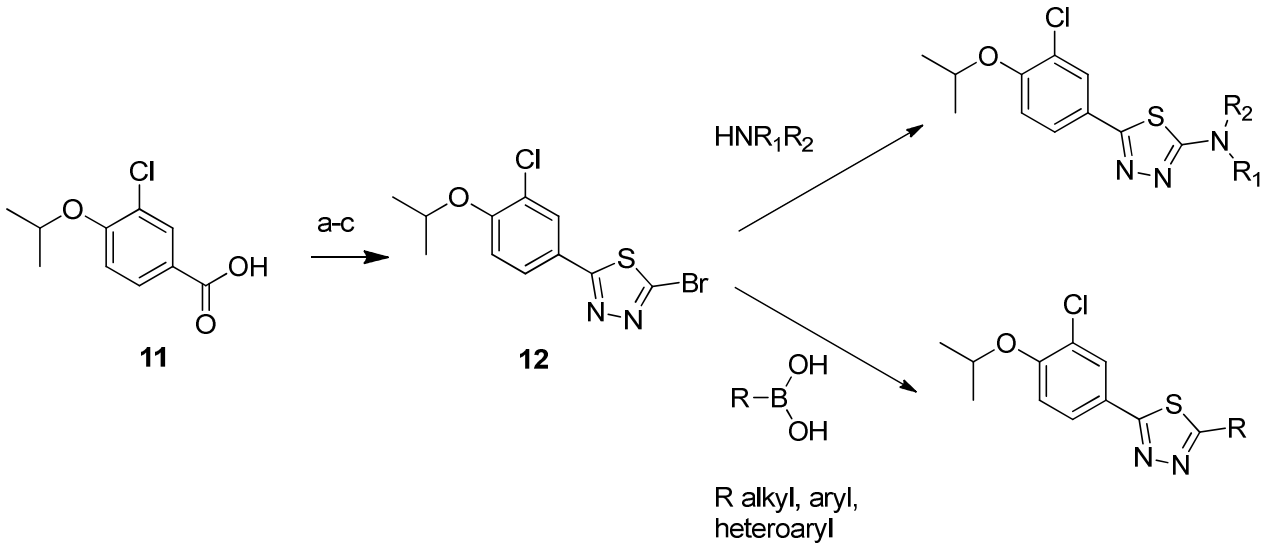


Whilst compounds such as **7** or **8** were progressed towards the clinic, we were interested in identifying a structurally distinct follow-up series. As our internal screening effort did not identify hits of significant interest (lack of potency, drug-likeness and/or tractability), we decided to identify new $S1P_1$ agonists from the triaryl motif shown above. Previous SAR from our group or others[31] had shown that modification of the distal tri-substituted aromatic led to significant drop in potency in most –but not all-[32-34] cases, whilst the oxadiazole central ring could be substituted without significant change in potency or selectivity, with a thiadiazole for example.[35] With these data in hands, we tried identifying a novel motif to replace the bi-cyclic amine incorporated in all our agonists, with the aim of keeping our agonists in what has been recently identified as the chemical space least likely to lead to toxicity (cLogP < 3, PSA > 75 $Å^2$).[36,37] This was most likely to be achieved by introducing subsituents containing heteroatoms, including basic nitrogen and acidic functionality.

The chemistry highlighted in Scheme 1 allowed us to implement this strategy as it was possible to test several hypotheses in a relatively high throughput manner using Buchwald or Suzuki

couplings with aryl bromide **12**, easily obtained from commercially available acid **11**. The nature of the substituent on the distal phenyl were chosen based on previous SAR to contribute to high level of $S1P_1$ agonism[21,31] whilst being robust enough to accommodate a large range of experimental conditions.

Scheme 1. Synthetic route to thiadiazole $S1P_1$ agonists.[(a)]



[(a)] **Reagents and conditions:** (a) $(COCl)_2$, DMF cat., CH_2Cl_2, room temperature, 100%; (b) hydrazinecarbothioamide, $POCl_3$, 90°C, 36%; (c) $CuBr_2$, *t*-BuONO, CH_3CN, room temperature, 89%.

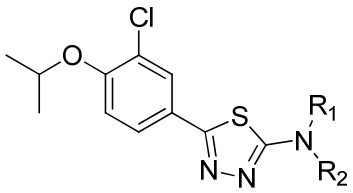
RESULTS AND DISCUSSION

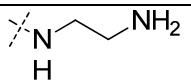
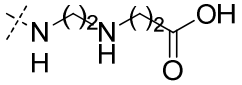
Activities at $S1P_1$ and $S1P_3$ were monitored using either a GTPγS accumulation assay or a β-arrestin recruitment assay. The GTPγS assay is taken as a direct measurement of exchange of guanine nucleotides by the G protein alpha subunit, demonstrating the ability of a given compound to activate the receptor. The β-arrestin assay measures an alternative signalling

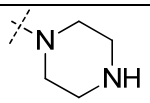
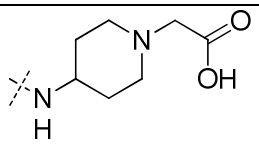
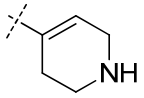
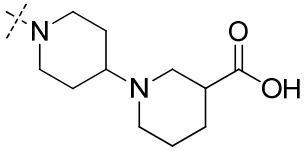
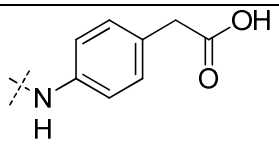
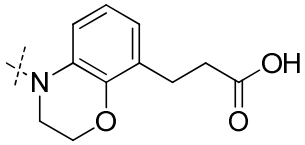
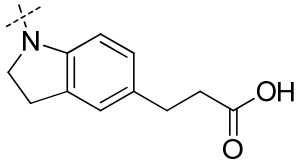
mechanism of the receptor and is taken as a surrogate indicator for the ability of an agonist to internalise the receptor (hence acting as a functional antagonist), a key aspect of the biological mechanism of the $S1P_1$ receptor. As can be seen in Table 1, we did not succeed in obtaining potent ($pEC_{50} > 7$) agonists with alkyl substituent pending from the thiadiazole (compounds **13**-**19**). It was possible to obtain measurable activity with constrained analogues (compare **13**, **14** with **15** and **16**) or with more planar analogues (compare **15** and **17**) but overall, agonists bearing alkyl zwitterionic side-chains were not potent enough to be considered as leads (see agonist **18** as another example).

Only when pi character was introduced or when rigidifying the scaffold was it possible to achieve sub-micromolar activity (compare compound **19** with **16**), rigidifying the template leading in some occasions to significant increased in potency (compounds **20** and **21**). Overall, we felt that this approach was unlikely to deliver leads capable of being optimized to candidate-quality molecules.

Table 1: $S1P_1$ agonists with only two fused aromatics.



Compound	R	$S1P_1$ pEC_{50} (GTPγS) [(a)]
13		< 4.5 (7)
14		< 4.5 (4)

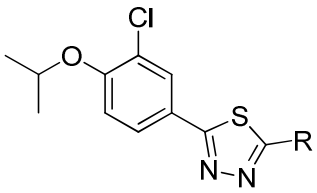
15		5.2 (3)
16		5.6 (2)
17		5.8 (4)
18		5.4 (3)
19		5.9 (2)
20		6.6 (2)
21		7.0 (2)

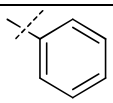
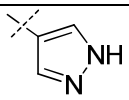
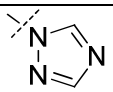
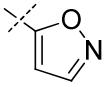
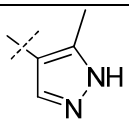
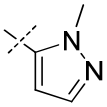
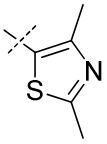
(a) See Experimental Section for details.

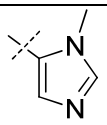
We therefore decided to introduce C or N-linked heteroaryl in this position. If the compounds were to keep a tri-aryl motif, we felt that increased solubility (poor solubility was a general issue with our oxadiazole $S1P_1$ agonists) could be gained by decreasing lipophilicity. Representative examples are shown in Table 2. As can be seen, several heteroaryls were tolerated in this position (compounds **23**-**29**) and no significant activity could be measured against $S1P_3$. They all showed reduced lipophilicity versus their analogous phenyl **22** and ortho-substitution appeared to

be beneficial to activity (compounds **26**-**29**, and **26** versus **23**), a trend already observed in previous series.

Table 2: Profile of $S1P_1$ agonists bearing two heteroaryls.



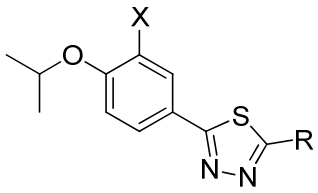
Compound	R	$S1P_1$ pEC_{50} (GTPγS)	$S1P_3$ pEC_{50} (GTPγS)[a]	CHROM LogD @ pH 7.4
22		6.6 (2)	= 4.7[b] (1/3)	8.65
23		6.2 (10)	< 4.5 (10)	5
24		6.3 (3)	< 4.5 (4)	6.18
25		6.0 (2)	< 4.5 (2)	6.93
26		6.9 (8)	< 4.5 (8)	5.45
27		6.6 (2)	< 4.5 (1)	7.04
28		6.6 (3)	< 4.5 (2)	7.76

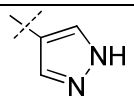
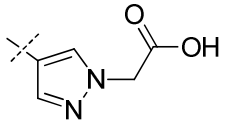
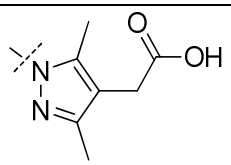
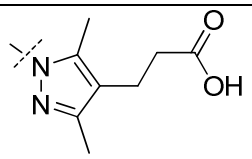
29		7.2 (2)	< 4.5 (2)	5.7

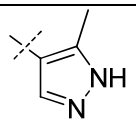
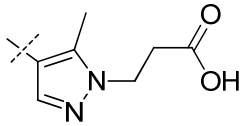
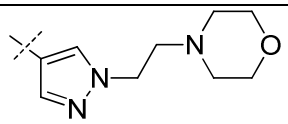
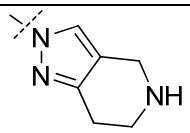
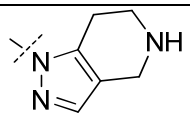
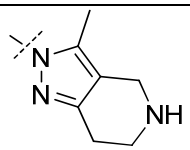
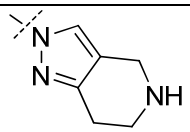
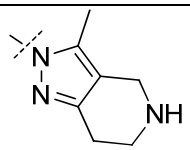
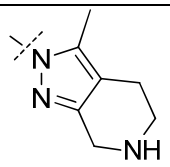
[(a)] See Experimental Section for details; [(b)] One in three occasions.

We then attempted to further increase the potency of our agonists and to further reduce their lipophilicity by introducing amine and/or acidic substituent as this strategy had proven successful in our oxadiazole series with compounds such as **7**. Table 3 highlights key SAR resulting from this effort.

Table 3: tri-aryl thiadiazole amine and acids $S1P_1$ agonists.



Compound	R	X	$S1P_1$ pEC_{50} (GTPγS)	$S1P_1$ pEC_{50} (arrestin) [(a)]	$S1P_3$ pEC_{50} (GTPγS)	CHROM LogD @ pH 7.4
23		Cl	6.2 (10)		< 4.5 (10)	5
30		Cl	4.6 (1)		< 4.5 (4)	2.82
31		Cl	6.0 (7)	5.8 (4)	< 4.5 (7)	3.62
32		Cl	7.4 (7)	7.7 (6)	< 4.5 (9)	4.3 (1)

26		Cl	6.9 (8)		< 4.5 (8)	5.45
33		Cl	5.6 (2)	-	< 4.5 (3)	3.16
34		Cl	5.0 (3)	-	< 4.5 (4)	5.59
35		Cl	6.2 (2)	6.2 (6)	< 4.5 (2)	4.44
36		Cl	6.4 (2)	6.1 (4)	< 4.5 (2)	4.87
37		Cl	6.9 (4)	6.8 (8)	< 4.5 (4)	5.32
38		CN	6.3 (2)	6.9 (6)	< 4.5 (4)	3.37
39		CN	7.0 (2)	7.2 (6)	< 4.5 (4)	3.73
40		CN		6.8 (6)	< 4.5 (4)	4.23

[(a)] See Experimental Section for details.

It was possible to introduce acid side chain substituent to these heteroaryls and significantly increase potency, as long as the chain was at least two carbons long (compare **30** versus **23** and **31** versus **32**), however this strategy was not always sufficient as seen with the loss of activity

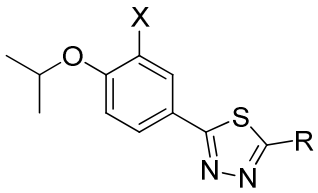
from **26** to the acid **33**. In all cases, the introduction of the acid functionality led to compounds with improvement in lipophilicity.

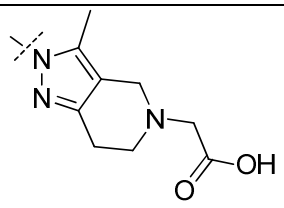
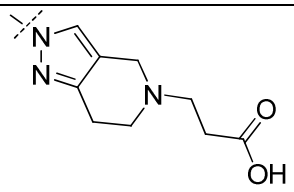
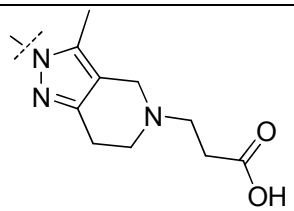
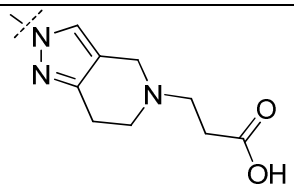
It was also possible to introduce basic side chain; however, the position of the nitrogen was critical for activity (compare **34** with **35** and **36**). The tetrahydro-pyrazolo-pyridine template in agonist **35** was interesting as it allows the introduction of an ortho-substituent on the aromatic ring, leading as previously seen in an increase of potency (compare **37** with **35**). Due to the interesting level of activity seen with these amines, we looked whether SAR observed in the oxadiazole series was transferable. Indeed, replacing the chloro substituent on the distal ring with a nitrile led to similar if not increased potency, and a decreased lipophilicity (compare **38** and **36**, **39** and **37**). It was also possible to shift the basic nitrogen in this bicyclic system without major impact on potency or selectivity (compare **39** and **40**) but this led to increased lipophilicity.

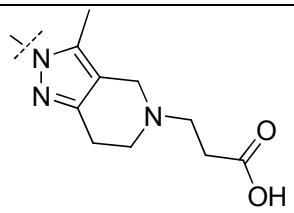
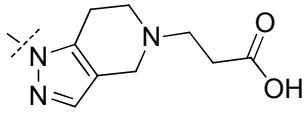
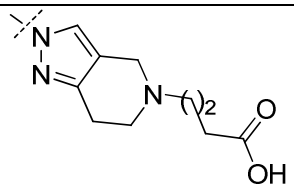
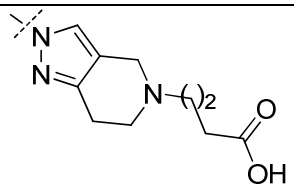
These amines were of particular interest as they allowed the introduction of acid side chain via N-alkylation which had the potential to further increase the potency of our agonists based on previous SAR. Table 4 showed that exquisite potency and selectivity could be reached in doing so, as long as the acid side chain was at least two carbons long (compare **41** with **39**, **42** and **43**). With these zwitterionic $S1P_1$ agonists, the SAR previously observed was confirmed: ortho-substitution of the pyrazole ring led to an increase in potency (compare **43** to **42** and **45** to **44**) and replacing the chlorine on the distal ring by a nitrile led also to an increase in potency and a lowering in lipophilicity (compare **44** to **42** and **45** to **43**). It is worth noting that the position of the constrained amine is important to direct the acid functionality in the right area (**46** is only slightly more potent than **36**) and that increasing the length of the acid chain does not have significant impact on potency and selectivity in the optimal template (compare **47** and **42** as well

as **48** and **44**). Overall, we were able to identify a number of novel selective agonists which activity was similar to **1b** or **7**.

Table 4: Activity of zwitterionic $S1P_1$ agonists.



Compound	R	X	$S1P_1$ pEC_{50} (GTPγS)	$S1P_1$ pEC_{50} (arrestin)	$S1P_3$ pEC_{50} (GTPγS)	CHROM LogD @ pH 7.4
1b	-	-	8.4 (130)	7.7 (44)	8.3 (38)	
7	-	-	7.5 (8)	8.3 (11)	< 4.4 (8)	3.41
41		CN		7.0 (2)	< 4.5 (2)	2.88
42		Cl	7.9 (6)	8.4 (6)	< 4.5 (10)	3.26
43		Cl	8.2 (14)	8.8 (18)	< 4.5 (4)	3.73
44		CN	7.3 (2)	8.3 (6)	< 4.5 (4)	2.58

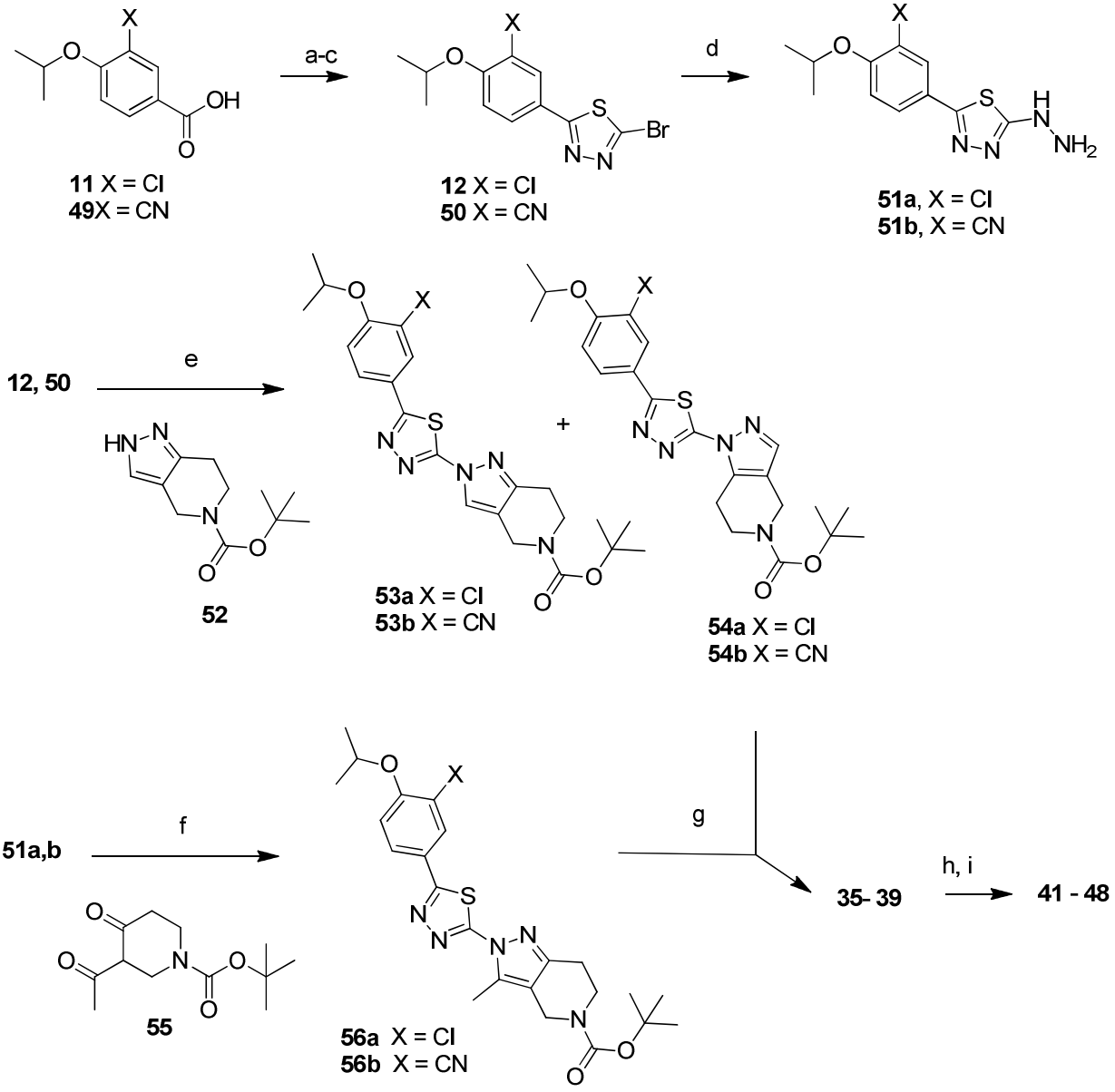
45		CN	7.8 (2)	8.5 (6)	< 4.5 (4)	2.76
46		Cl	6.9 (4)	7.3 (4)	4.7 (2)	3.54
47		Cl	7.9 (2)	8.3 (6)	< 4.5 (2)	3.56
48		CN	-	8.2 (6)	< 4.5 (4)	2.87

CHEMISTRY

The chemistry to access compounds **35**-**48** is highlighted in Scheme 2: 2-Bromo thiadioazole **12** and **50** are obtained from acids **11** and **49** via condensation using hydrazinecarbothioamide followed by diazotation of these amines in the presence of cupric bromide. Displacement of the bromine with hydrazine give compounds **51**. Copper-mediated coupling of aryl bromide **12** and **50** with commercially available pyrazole **52** gives a mixture of regioisomers **53** and **54** which can be separated. Condensation of hydrazine **51** with beta-diketone **55** (easily obtained from 4-keto piperidine) gives compound **56**. Removal of the protecting group of **53**, **54** and **56** gives compounds **35**-**39** which after alkylation and saponification of the intermediate esters give the

zwiterrions **41**-**48**. Compound **40** can be obtained in a similar way using the appropriate regioisomeric beta-diketone analogous to **55**.

Scheme 2: Synthesis of $S1P_1$ agonists **35-39** and **41**-**48**.[(a)]



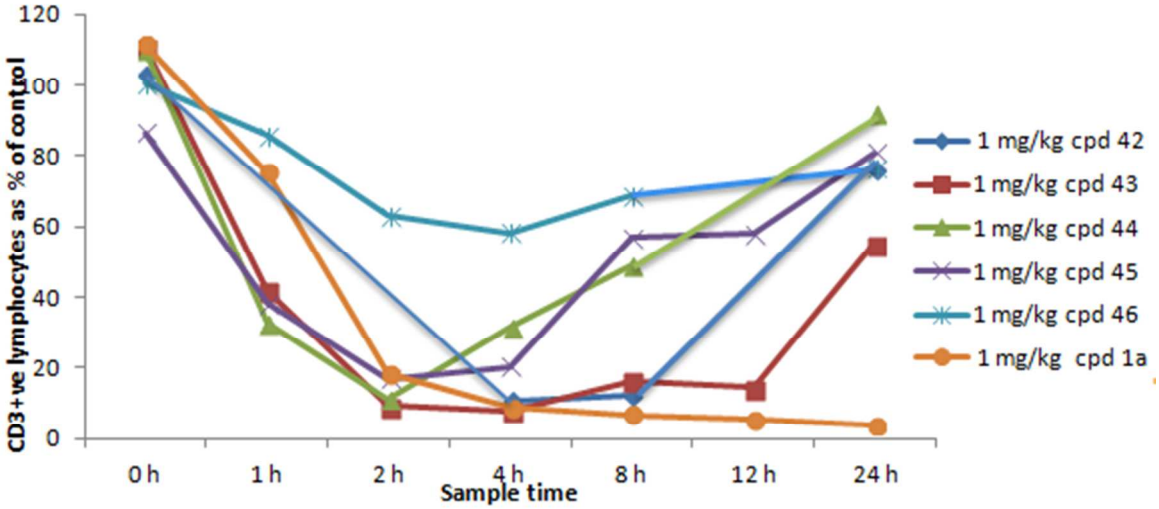
[(a)] **Reagents and conditions**: (a) $(COCl)_2$, DMF cat., CH_2Cl_2, room temperature; (b) hydrazinecarbothioamide, $POCl_3$, 90°C; (c) $CuBr_2$, *t*-BuONO, CH_3CN, room temperature; (d) $NH_2NH_2.H_2O$, CH_3CN, 100°C; (e) **52**, CuI, Cs_2CO_3, DMF, 160°C, μv irradiation; (f) **55**, AcOH,

EtOH, reflux; (g) CF3COOH, CH_2Cl_2, room temperature or HCl, 1,4-dioxan, room temperature; (h) Br(CH_2)nCOOR, K_2CO_3, CH_3CN, 50°C or acrylate, DBU, DMF, room temperature; CF_3COOH, CH_2Cl_2, room temperature or LiOH, THF/water, room temperature.

IN VIVO PHARMACOLOGY

The most potent and selective agonists highlighted in table 4 were screened in our *in-vivo* rat lymphopoenia model at an oral dose of 1 mg/kg, using **1a** as a standard (Figure 4). This model was designed to determine several endpoints; confirming the response predicted from the *in vitro* potency, examining the duration of the effect and providing simultaneous oral pharmacokinetic data[38]. It was therefore effective in differentiating molecules. With the exception of compound **46**, all the agonists showed full effect at 2 or 4 h and were discriminated based on the sustainability of this effect: Compounds **44** and **45** showed only a partial effect at 8 h and compound **43** was still active after 24 h, as opposed to compound **42**.

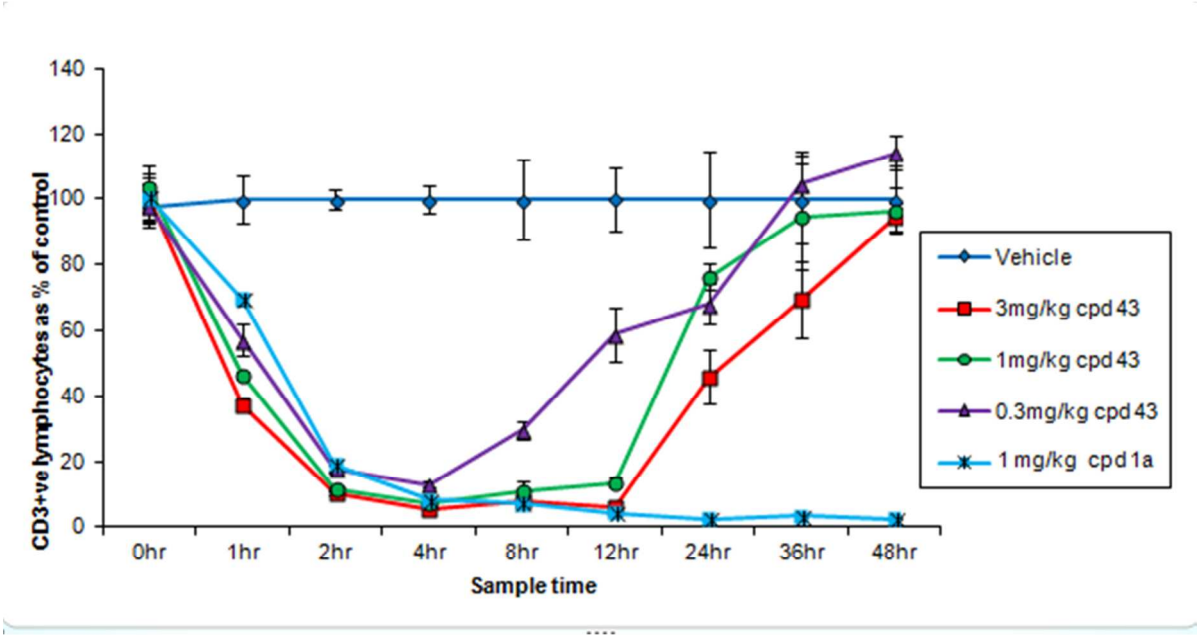
Figure 4: In-vivo effect of selected $S1P_1$ agonists compared to **1a**.[(a)]



[(a)] See supporting information for complete dataset.

Compound **43** was therefore further profiled and its dose response in our rat lymphopoenia assay is shown in Figure 5. This compound proved to be active at an oral dose as low at 0.3 mg/kg with full reversibility after 24 h, in sharp contrast to **1a**; The half-life of **1a** in human is approximately 9 to 10 days[38] and it takes 6 weeks after cessation of dosing for lymphocyte count to return to normal level, which can prove problematic in the case of patients suffering from infection. It is therefore hoped that agonists such as **43**, with an expected much reduced half-life in human will provide a clinical advantage. Also, in the case of **43**, PK sampling taken at all doses demonstrated complete bioavailability, based on i.v. data (*vide infra*). PK-PD analysis established an in-vivo IC_{50} of ca. 6 nM.[39]

Figure 5: Dose response of compound **43** in the lymphopoenia assay.



The full profile of agonist **43** is presented in Table 5. As can be seen, this agonist has good intrinsic properties (cLogP < 3, PSA > 75) and is a partial agonist of the $S1P_5$ receptor with no significant agonism of $S1P_{2\text{-}4}$ receptors or inhibition of the hERG channel. Its poor solubility (<

40 ug/mL in all media) is compensated by good permeability (> 100 nM/s). Compound **43** is highly protein bound in all species, as indicated by the low free fraction in blood. Hepatocyte clearance is highest in dog, lower in cynomolgus monkey and rat and is metabolically stable in mouse. The correlation of in vitro clearance with in vivo blood clearance provides confidence that clearance in humans will be low. Compound **43** did not inhibit the major CYP450 enzymes and no time dependant inhibition was observed in human liver microsomes using CYP3A4 probe substrates midazolam, nifedipine and atorvastatin, suggesting a low risk of drug-drug interactions

Table 5: Properties of agonist **43**.

MW, cLogP, PSA		462, 1.7, 93
CHI LogD @ pH 2.0, 7.4, 10.5		1.03, 2.07, 1.53
$hS1P_1$ pEC_{50} (β-arrestin)		8.8 ± 0.2 (83 ± 9); n = 14
$hS1P_1$ pEC_{50} (receptor internalisation)[(a)]		8.8 ± 0.3 (109 ± 8); n = 5
$hS1P_2$ pEC_{50} (yeast)[(a)]		< 4.5 (0 ± 0.4); n = 4
$hS1P_3$ pEC_{50} (GTPγS)[(a)]		< 4.5 (28 ± 12); n = 9 5.1 (25); n = 1
$hS1P_4$ pEC_{50} (aequorin)[(a)]		< 4.4 (07 ± 6); n = 5
$hS1P_5$ pEC_{50} (aequorin)[(a)]		7.78 ± 0.1 (63 ± 13); n = 5
Solubility @ 24 h (μg/mL) in SGF (pH 1.6), FeSSIF (pH 6.5) and FaSSIF (pH 6.5)	HCl salt	7, 46, 16
	TFA salt	37, 4, 3
Passive Permeability (MDCK, nm/s)		147
hERG pIC_{50} (Dofetalide)		< 4.3 (n = 3)
Hepatocyte CLi (mg/min/g liver; rat, dog, mouse,		3.2, 6. < 0.85, 4.2, 1.8

cyno, human)	
Free fraction (blood, %; rat, dog, mouse, human)	1.4, 1.1, 1.1, 0.8
CYP IC_{50} (μM, 1A2, 2C9, 2C19, 2D6, 3A4VG, 3A4 VR, n =4)	>50, >50, >50, >50, >50, >50,

[(a)] See Experimental Section for details.

Agonist **43** also showed good in-vivo pharmacokinetics (Table 6) and despite a high blood clearance in dog showed good bioavailability in all preclinical species tested. The volume of distribution between 3-5 L/kg in preclinical species indicates distribution into tissues. Using a human PKPD model, the estimated oral dose in human to maintain 60% lymphocyte sequestration is between 6 and 50 mg once daily based on these parameters.[39]

Table 6: In-vivo pharmacokinetics of agonist **43**.

Species	Mouse (Male)	Rat (Male)	Dog (Male)
Strain	CD-1	CD	Beagle
Salt Type	TFA	TFA	HCl
Dose i.v.[(a)], p.o. (mg/kg)	1, 3	1, 3	1, 2
CL_b (i.v., mL/min/kg) % LBF	16 ± 6[(b)] 15%	24 ± 1 25%	33 ± 7 85%
V_{ss} (i.v., L/kg)	3.3 ± 1.2[(b)]	3.7 ± 0.3	5.3 ± 1.5
$T_{1/2}$ (i.v., h)	3.8 ± 1.0[(b)]	4.4 ± 0.5	6.5 ± 3.0
F p.o. (%)	79 ± 8[(c)]	55, 48[(d)]	44 ± 10

Values are mean, n=3 ± SD unless otherwise stated. [(a)] IV dose was 1 h infusion in DMSO (2%, v/v) : Kleptose HPB (10%, w/v) in saline (0.9%, w/v); [(b)] mouse IV PK data is n=5; [(c)] mouse PO PK data is n=4; [(d)] rat PO PK data is

n=2. CL_b: blood clearance; LBF: liver blood flow; Vss: volume of distribution at steady state; $T_{1/2}$: half life; $F_{p.o.}$: oral bioavailability.

Due to its good intrinsic properties, in-vitro and in-vivo pharmacokinetics and efficacy in our lymphopoenia assay, agonist **43** was assessed in a range of toxicity assays: No signals were observed in the Ames test and in the mouse lymphoma assay and agonist **43** was therefore progressed to a 7 days rat oral safety studies at doses of 30, 100 and 300 mg/kg where no findings were observed to prevent further progression. Interestingly, no activation of CYP1A was observed, suggesting that this molecule is not an agonist of the aromatic hydrocarbon receptor, an off-target activity observed with several of our previous oxadiazole agonists.[40]

CONCLUSION

Overall, we have identified a drug-like potent $S1P_3$-sparing $S1P_1$ agonist with good in-vivo efficacy and pharmacokinetics in all pre-clinical species tested. This molecule is structurally significantly distinct from our previous series of candidate quality $S1P_1$ agonists and therefore represents a good follow-up candidate for our program. Further data associated with this molecule will be disclosed in due course.

EXPERIMENTAL SECTION

In vitro pharmacology

Membrane preparation for $S1P_1$ GTPγS assay: All steps were performed at 4°C. Cells were homogenised within a glass Waring blender for 2 bursts of 15 secs in 200 mL of buffer (50 mM

HEPES, 1 mM leupeptin, 25 μg/mL bacitracin, 1 mM EDTA, 1 mM PMSF, 2 μM pepstatin A). The blender was plunged into ice for 5 mins after the first burst and 10-40 mins after the final burst to allow foam to dissipate. The material was then spun at 500 g for 20 mins and the supernatant spun for 36 mins at 48,000 g. The pellet was resuspended in the same buffer as above but without PMSF and pepstatin A. The material was then forced through a 0.6mm needle, made up to the required volume, (usually x4 the volume of the original cell pellet), aliquoted and stored frozen at –80°C.

$S1P_1$ GTPγS assay: $S1P_1$ expressing RH7777 membranes (1.5 μg/well) membranes (1.5 μg/well) were homogenised by passing through a 23G needle. These were then adhered to WGA-coated SPA beads (0.125 mg/well) in assay buffer (HEPES 20 mM, $MgCl_2$ 10 mM, NaCl 100 mM and pH adjusted to 7.4 using KOH 5M). GDP 10 μM FAC and saponin 90 μg/mL FAC were also added.

After 30 minutes precoupling on ice, the bead and membrane suspension was dispensed into white Greiner polypropylene LV 384-well plates (5 μl/well), containing 0.1 μl of compound. 5 μl/well [^{35}S]-GTPγS (0.5 nM for $S1P_1$ or 0.3 nM for $S1P_3$ final radioligand concentration) made in assay buffer was then added to the plates. The final assay cocktail (10.1 μl) was then sealed, spun on a centrifuge, then read immediately on a Viewlux instrument.

Membrane preparation for $S1P_3$ GTPγS assay: All steps were performed at 4°C. Cells were homogenised within a glass Waring blender for 2 bursts of 15 secs in 200 mL of buffer (50 mM HEPES, 1 mM leupeptin, 25 μg/mL bacitracin, 1 mM EDTA, 1 mM PMSF, 2 μM pepstatin A). The blender was plunged into ice for 5 mins after the first burst and 10-40 mins after the final burst to allow foam to dissipate. The material was then spun at 500 g for 20 mins and the

supernatant spun for 3 mins at 48,000 g. The resultant pellet was resuspended in the same buffer without PMSF and pepstatin A but containing 10% w/v sucrose. The membrane suspension was then layered on top of buffer without PMSF and pepstatin A containing 40% w/v sucrose and spun at 100,000 g for 60 mins. The cloudy interface between the 2 sucrose layers was removed and resuspended in buffer without PMSF and pepstatin A. The material was spun at 48,000 g for 45 mins. The resultant cell pellet was resuspended in the required volume in buffer without PMSF and pepstatin A, (usually x4 the volume of the original cell pellet), aliquoted and stored frozen at –80°C.

$S1P_3$ GTPγS assay: $S1P_3$ expressing RBL membranes (0.44 μg/well) purified through a sucrose gradient were homogenised by passing through a 23G needle. These were then adhered to WGA-coated SPA beads (GE Healthcare 0.5 mg/well) in assay buffer (HEPES 20 mM, $MgCl_2$ 10 mM, NaCl 100 mM and pH adjusted to 7.4 using KOH 5M). 2 μg/well of Saponin was added. After 30 minutes precoupling on ice, 5 μM GDP final assay concentration was added to the bead and membrane suspension. The bead, membrane, Saponin and GDP suspension was mixed with [^{35}S]-GTPγS (Perkin Elmer, 0.3 nM final radioligand concentration) made in assay buffer (HEPES 20 mM, $MgCl_2$ 10 mM, NaCl 100 mM and pH adjusted to 7.4 using KOH 5M). The bead, membrane and radioligand suspension was dispensed into white Greiner polypropylene 384-well plates (45 μl/well), containing 0.5 μl of a solution of test compound in 100% DMSO. The final assay cocktail (45.5 μl) was then sealed, spun on a centrifuge, then read on a Viewlux instrument following a 3 hour incubation of plates at room temperature.

$S1P_1$ β-Arrestin recruitment assay: β-Arrestin recruitment assays were carried out using the PathHunter CHO-K1 EDG1 β-Arrestin cell line (DiscoveRx Corporation) in a chemi-

luminescence detection assay. This cell line stably expresses β-Arrestin 2 and $S1P_1$ fused to complementing portions of β-galactosidase ('EA' and 'pro-link', respectively) which associate upon Arrestin recruitment to form functional β-galactosidase enzyme.Cells were grown to 80% confluency in Growth Medium (F12 nutrient HAMS supplemented with 10% heat-inactivated USA FBS, 1% L-glutamax, 800 μg/mL Geneticin and 300 μg/mL Hygromycin). Cells were harvested from the flask using Enzyme Free Cell Dissociation Buffer (Gibco) and washed from flasks with Optimem solution (Gibco). Cells were then centrifuged at 1000 rpm for 2-3 min and resuspended in Assay Buffer (Prepared from Sigma kit H1387 supplemented with 20 mL/L HEPES, 4.7 mL/L $NaHCO_3$, 0.1% pluronic acid F-68 solution, 0.1% BSA and adjusted to pH 7.4 using sodium hydroxide at $1x10^6$ cells/mL. Cells were dispensed into assay plates containing compounds (100nl/well of a solution of test compound in 100% DMSO) at $1x10^4$ cells/well and incubated at 37°C/5% CO_2 for 90 min followed by 15 min at room temperature. 5 μL detection mix (1 part Galacton Star, 5 parts Emerald II, 19 parts Assay Buffer; DiscoveRx) were added per well and the plates incubated at room temperature for 60 min. Luminescence was quantified using a Viewlux plate reader.

$S1P_2$ yeast reporter assay:$S1P_2$ assays were performed using a yeast reporter system as described by Dowell et al [41]. An expression construct containing $S1P_2$ under control of the *GPD* promoter was integrated at the *ura3* locus of *S. cerevisiae* strain as demonstrated by Brown *et al*.[42] Cells were seeded at 0.02 OD_{600} in minimal medium lacking histidine and supplemented with 5mM 3-amino triazole (Sigma, Poole, UK) and 100μM fluorescein-di-(β-D-glucopyranoside) (FDGlu; Invitrogen Molecular Probes). Agonist-mediated growth was measured by conversion of FDGlu to fluorescein as described in Dowell SJ and Brown AJ

(2009) using an Analyst HT plate reader (LJL BioSystems, Sunnyvale, CA; ex. 485nm/em. 535nm) after 24h incubation at 30°C.

$S1P_4$ and $S1P_5$ Aequorin calcium accumulation assays: Aequorin assays were performed using CHO-K1 $S1P_4$ and $S1P_5$ aequorin cell lines purchased from Euroscreen SA (Gosselies, Belgium) and Perkin Elmer (Waltham, MA USA) respectively. In the presence of the cofactor coelenterazine, calcium released on receptor activation results in a conformational change of apo-aequorin and an oxidative decarboxylation reaction producing coelenteramide and a flash luminescence signal at 469 nm.Luminescence was measured in a Lumilux (Perkin Elmer) reader containing internal liquid handling capacity. Frozen cells were revived into DMEM F12 medium containing 10% charcoal-stripped FBS, pre-warmed to 37°C, and all traces of freezing media were removed by centrifugation. Pelleted cells were resuspended in 50 mL of 37°C medium and allowed to recover for 30 min at 37°C. Following recovery, cells were harvested and re-suspended at $2.5x10^6$ cells/mL in loading buffer (prepared from Sigma kit H1387, supplemented with 20 mM HEPES, 4.16 mM $NaHCO_3$, 0.1% pluronic acid F-68 solution and 0.1% BSA) containing 5 μM coelenterazine. Cells were protected from light exposure and incubated at room temperature for 18-24h with gentle rotation. After loading, cells were diluted to $5x10^5$ cells/mL with assay buffer and placed, stirring, in the Lumilux. Instrument automation added 20 μL/well of diluent buffer followed by 20 μL/well of cells ($1x10^5$ cells/well) to compound plates, and agonist luminescent responses were recorded immediately. S1P4 cat # ES-592-A. S1P5 cat # ES-593-A. DMEM F12 cat# either Sigma D6421 or Invitrogen 11320. Charcoal stripped FBS cat # Hyclone SH30068.03. Base buffer: prepared from Sigma kit H1387, supplemented with 20mM HEPES (Sigma H0887) and 4.16mM $NaHCO_3$ (Sigma S8761) and pH'd to 7.4. Loading buffer: Base buffer supplemented with 0.1% pluronic acid F-68 solution (Gibco/Invitrogen 24040-032)

and 0.1% BSA (Calbiochem 126609).Assay buffer/diluent buffer: base buffer supplemented with 0.1% pluronic acid F-68 solution (Gibco/Invitrogen 24040-032).Coelenterazine cat # Invitrogen C6780 or Biotium BT10110-1.

***In vivo* studies.**

Rat Lymphocyte depletion study: 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.

Rat Lymphocyte Reduction studies: Male Lewis rats (300-350g, supplied by Charles River UK Ltd.) had pre-dose blood samples (200µl) removed by direct venepuncture the day prior to oral dosing. On the study day, rats received either vehicle (1% methycellulose 4mLl/kg p.o.) or compound **43** (0.1 – 3 mg/kg p.o.) and had further blood samples taken at 0.25, 0.5, 1, 2, 4, 7, 12, 24, 30, 36, 48 and 54 h post-dose. From each blood sample 50 µL was mixed with 50 µL of water for pharmacokinetic analysis. The remainder of the blood samples were analysed using the Sysmex XT2000iV automatic haematology analyser for lymphocyte counts. At the completion of the study rats were euthanized using a Schedule 1 method.

DMPK Studies: 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. For all *in vivo* studies, the temperature and humidity were nominally maintained at 21°C ± 2°C and 55% ± 10%, respectively. The diet for rodents was 5LF2 Eurodent Diet 14% (PMI Labdiet, Richmond, Indiana) and for dogs was Harlan Teklad 2021C (HarlanTeklad, Madison, WI). There were no known

contaminants in the diet or water at concentrations that could interfere with the outcome of the studies.

Rat PK studies: Male CD rats (supplied by Charles River UK Ltd.) were surgically prepared at GSK with implanted cannulae in the femoral vein (for drug administration) and jugular vein (for blood sampling). Each rat received Duphacillin (100 mg/kg s.c.) and Carprofen (7.5 mg/kg s.c.) as a pre-operative antibiotic and analgesic respectively. Each rat was allowed to recover for at least 2 days prior to dosing. Rats had free access to food and water throughout. Rat PK studies were conducted as a crossover design over 2 dosing occasions, with 4 days between dose administrations. On the first dosing occasion, three rats each received a 1h

intravenous (i.v.) infusion of **43** formulated in DMSO and 10% (w/v) KleptoseTM HPB in saline aq (2%:98% (v/v)) pH 8.0, at a concentration of 0.2 mg/mL to achieve a target dose of 1 mg/kg. On the second dosing occasion, two of the three rats were administered **43** suspended in 1% (w/v) methylcellulose 400 *aq*. at a concentration of 0.6 mg/mL orally, at a target dose of 3 mg/kg. Serial blood samples (*ca.* 90 μL) were collected pre-dose and up to 24 h after the start of the i.v. infusion and post oral dosing. Diluted blood samples were analysed using a specific LC-MS/MS assay (LLQ = 2 ng/mL). At the end of the study the rats were euthanised by administration of sodium pentobarbital (Euthatal^{TM}) through the jugular vein cannula.

Dog PK studies: Three healthy, laboratory-bred, male Beagle dogs (supplied by Harlan Laboratories, UK) were used. The dogs were fasted overnight prior to each dose

administration and fed approximately 4 h after the start of dosing. The dogs had free

access to water throughout the study. This study was conducted as a cross-over design over 2 dosing occasions, with 7 days between dose administrations. On the first dosing occasion, three

dogs each received a 1h intravenous (i.v.) infusion of **43** formulated in DMSO and 10% (w/v) KleptoseTM HPB in saline aq (2%:98% (v/v)), at a concentration of 0.2 mg/mL to achievea target dose of 1 mg/kg. On the second dosing occasion, the same three dogs were

administered **43** suspended in 1% (w/v) methylcellulose 400 *aq*. at a concentration of 1 mg/mL orally, at a target dose of 2 mg/kg. A temporary cannula was inserted into the cephalic vein from which serial blood samples (*ca.* 200 μL) were collected predose and up to 24 hours after the start of dosing intio tubes containing potassium-EDTA anti-coagulant. After collection of the 2 hr timepoint the cannula was removed and later timepoints were taken via direct venepuncture of the jugular vein. Diluted blood samples were analysed for drug concentration using a specific LC-MS/MS assay (LLQ = 1 ng/mL). At the end of each study the dogs were returned to the colony.

Mouse PK Studies: Male CD1 mice (supplied by Charles River UK Ltd.) were used in this study. Mice were surgically prepared with an indewlling cannula in the jugular vein to allow a 1h intravenous (i.v.) infusion administration of **43** at 1 mg/kg. **43** was formulated in DMSO and 10% (w/v) KleptoseTM HPB in saline aq (2%:98% (v/v)) at a concentration of 0.2 mg/mL. A second group of mice (no surgical preparation) received an oral gavage administration of **43** suspended in 1% (w/v) methylcellulose *aq*. at a target dose of 3 mg/kg.. Serial blood samples (*ca.* 25 μL) were collected via the tail vein up to 24 h after the start of the i.v. infusion and post oral dosing. Diluted blood samples were analysed for **43** using a specific LC-MS/MS assay (LLQ = 5 ng/mL).

Blood Sample Analysis

Diluted blood samples (1:1 with water) were extracted using protein precipitation with acetonitrile containing an analytical internal standard. An aliquot of the supernatant was analysed by reverse phase LC-MS/MS using a heat assisted electrospray interface in positive ion mode. Samples were assayed against calibration standards prepared in control blood.

PK Data Analysis from PK studies: PK parameters were obtained from the blood concentration-time profiles using non-compartmental analysis with WinNonlin Professional 4.1a (Pharsight, Mountain View, CA).

Fraction unbound in blood: Control blood from CD rat, CD1 mouse and Beagle dog was obtained on the day of experimentation from in house GSK stock animals. Control human blood was obtained on the day of experimentation from a single non-medicated consenting donor from in house GSK blood donation unit. The human biological samples were sourced ethically and their research use was in accord with the terms of the informed consents. The fraction unbound in blood of each species was determined using rapid equilibrium dialysis technology (RED^{TM} plate (Linden Bioscience, Woburn, MA) at a concentration of 1000 ng/mL. Blood was dialysed against phosphate buffered saline solution by incubating the dialysis units at 37^{o}C for 4 h. Following incubation aliquots of blood and buffer were matrix matched prior to analysis by LC-MS/MS. The unbound fraction was determined using the peak area ratios in buffer and in blood.

In vitro clearance in hepatocytes: The metabolic stability of **43** (0.5 μM) was determined in pooled human , Sprague–Dawley rat, beagle dog, and CD-1 mouse cryopreserved primary hepatocytes (CellzDirect Invitrogen Corporation, Durham, NC, USA). Incubations (final volume 300 μL) were performed at 37°C under an atmosphere of 95%/5% CO_2/O_2 using 0.7 million cells mL^{-1} in Williams Medium E incubation medium, supplemented with maintenance

media (ITS+ (insulin, transferring and selenium complex), antibiotics (penicillin/streptomycin), L-glutamine and HEPES). (Invitrogen, San Diego, CA). 25 µL aliquots of cell suspension were taken at timepoints to 2 h following the start of the incubation and quenched using 100 µL ice-cold acetonitrile containing an analytical internal standard. The samples were centrifuged and the resulting supernatant was analysed by LC-MS/MS. Intrinsic clearance was calculated by non-linear regression analysis of peak area ratio vs time using Grafit v5.0.8 (Erithacus software, UK) to determine the first order elimination rate constant (k). To scale the hepatocyte clearance, a hepatocyte yield of 1.2 x 10^8 cells per gram liver in human, rat and mouse and 2.4 x 10^8 cells per gram liver in the dog were used. [43]

Permeability measurement in MDRI-MDCKII cells: The MDCKII-MDR1 cells (purchased from Netherlands Cancer Institute) were plated from frozen onto Becton Dickinson Falcon™ HTS 24-Multiwell Insert Systems with PET (polyethylene terephthalate) membranes (1 µm pore size and 0.30 cm^2 surface area) at a density of 1.6 x 10^5 cells/cm^2 in cell culture medium (Dulbecco's Modified Eagle Medium (DMEM) with Glutamax, 10% (v/v) Foetal Bovine Serum and 0.5% (v/v) penicillin/streptomycin 10,000 units/mL). The cell monolayers were used for transport studies 3-4 days post seeding. The absorptive membrane permeabilities at pH 7.4 and pH 5.5 were measured in the apical to basolateral (A→B) direction in triplicate sets of wells. Fasted state simulated intestinal fluid (FaSSIF) at pH 5.5 or pH 7.4 was used as the transport medium in the apical compartment, and DMEM (pH 7.4) with 1% HSA in the basolateral compartment. To inhibit P-gp activity both receiver and donor compartments contained 2 µM GF120918 and were preincubated at 37°C for 30 min prior to addition of test compound. For A→B directional transport, 0.45 mL of compound containing solution was added to the A (apical) compartment and 1.3 mL of receiver solution to the B (basolateral) compartment. The

cells were incubated at 37°C with shaking for 90 min. At the end of the incubation, aliquots were taken from donor and receiver compartments and mixed with an aliquot of acetonitrile containing an analytical internal standard. Samples were analysed by LC-MS/MS and permeability was derived from the ratio of analyte peak area/internal standard peak area. The permeability at pH 5.5 or 7.4 (Pexact) was calculated according to Tran *et al*.[44]

Cytochrome P450 inhibition: The effect of test compounds on P450 isoenzymes CYP1A2, CYP2C9, CYP2C19 and CYP2D6 co-expressed in E.Coli with human NADPH reductase (Bactosomes, Cypex UK) was assessed using pre-fluorescent substrates ethoxyresorufin (ER, Sigma), 7-methoxy-4-trifluoromethylcoumarin-3-acetic acid (FCA, GlaxoSmithKline), 3-butyryl-7-methoxycoumarin (BMC, GlaxoSmithKline) and 4-methylaminomethyl-7-methoyxycoumarin (MMMC, GlaxoSmithKline) respectively. The effects on recombinant human CYP3A4 co-expressed in E.Coli with human NADPH reductase (Bactosomes, Cypex UK) was assessed using both 7-benzloxyquinolone (7BQ, Sigma) and diethoxyfluorescein (DEF, GlaxoSmithKline). Compounds (nine concentrations between 0.1 and 100µM) were incubated with recombinant enzyme, the isoform-specific pro-fluorescent substrate and a 0.2mM NADPH regenerating system in a 96-well plate format at 37ºC for ten minutes and the production of each fluorescent metabolite was monitored using a Tecan Spectrafluor plus plate reader (Tecan, UK).

Time-dependent P450 inhibition: The inhibition of recombinant human CYP2D6 and CYP3A4 enzmes in 5 minute increments over 30 min was investigated using fluorescence-based assays and a Cytoflouro plate reader (PerSeptive Biosystems, UK). Test compounds (nine concentrations between 0.1 and 100µM) were incubated with individually expressed recombinant cytochrome P450 enzymes, the isoform-specific pro-fluorescent substrate

(described in the section for Cytochrome P450 inhibition studies above) and a 0.2mM NADPH regenerating system in a 96 well plate format.

Chemistry.

General methods. All solvents were purchased from Sigma Aldrich (Hy-Dry anhydrous solvents) and commercially available reagents were used as received. All reactions were followed by TLC analysis (TLC plates GF254, Merck) or LCMS (liquid chromatography mass spectrometry) using a Waters ZQ instrument. NMR spectra were recorded on a Bruker nanobay 400 MHz or a Bruker AVII+ 600 MHz spectrometers and are referenced as follows: ^{1}H-NMR (400 –or 600- MHZ), internal standard TMS at δ = 0.00; 13C-NMR (100.6 –or 150.9- MHz), internal standard CDCl3 at δ = 77.23 or DMSO-D6 at δ = 39.70. Column chromatography was performed on pre-packed silica gel columns (30-90 mesh, IST) using a biotage SP4. Mass spectra were recorded on Waters ZQ (ESI-MS) and Q-Tof 2 (HRMS) spectrometers. Mass Directed Auto Prep was performed on a Waters 2767 with a MicroMass ZQ Mass Spectrometer using Supelco LCABZ++ column.

Synthetic Methods and Characterization of Compounds. Abbreviations for multiplicities observed in NMR spectra: s; singulet; br s, broad singulet; d, doublet; t, triplet; q, quadruplet; p, pentuplet; spt, septuplet; m, multiplet. The purity of all compounds was determined by LCMS and ^{1}H NMR and was always > 95%.

LCMS. UPLC analysis was conducted on an Acquity UPLC BEH C18 column (50mm x 2.1mm, i.d. 1.7 µm packing diameter) at 40°C. Flow rate was 1mL/min. Formate method: solvents employed were A = 0.1% v/v solution of formic acid in water; B = 0.1% v/v solution of formic acid in acetonitrile. High pH method: the solvents employed were: A = 10 mM

ammonium hydrogen carbonate in water adjusted to pH 10 with ammonia solution; B = acetonitrile. For both methods the gradient employed was:

Time (min)	Flow rate (mL/min)	%A	%B
0	1	99	1
1.5	1	3	97
1.9	1	3	97
2.0	1	0	100

The UV detection was a summed signal from wavelength of 210 nm to 350 nm. Mass spectra were obtained on a Waters ZQ instrument; ionization mode, alternate-scan positive and negative electrospray; scan range 100 to 1000 AMU; scan time: 0.27 sec; inter scan delay 0.10 sec.

MDAP. (Mass-directed automatic preparative purification) Formate method: HPLC analysis was conducted on either a Sunfire C18 column (100 mm x 19 mm, i.d 5µm packing diameter) or a Sunfire C18 column (150 mm x 30 mm, i.d. 5µm packing diameter) at ambient temperature. The solvents employed were: A = 0.1% v/v solution of formic acid in water; B = 0.1% v/v solution of formic acid in acetonitrile. Run as a gradient over either 15 or 25min (extended run) with a flow rate of 20 mL/min (100 mm x 19 mm, i.d 5 µm packing diameter) or 40 mL/min (150 mm x 30 mm, i.d. 5µm packing diameter). High pH method: HPLC analysis was conducted on either an Xbridge C18 column (100 mm x 19 mm, i.d 5µm packing diameter) or a Xbridge C18 column (100 mm x 30 mm, i.d. 5µm packing diameter) at ambient temperature. The solvents employed were: A = 10 mM ammonium bicarbonate in water, adjusted to pH10 with ammonia solution; B = acetonitrile. Run as a gradient over either 15 or 25 min (extended run)

with a flow rate of 20 mL/min (100 mm x 19 mm, i.d 5µm packing diameter) or 40 mL/min (100 mm x 30 mm, i.d 5µm packing diameter).

For both methods the UV detection was a summed signal from wavelength of 210 nm to 350 nm. Mass spectra were obtained on a Waters ZQ instrument; ionization mode, alternate-scan positive and negative electrospray; scan range 100 to 1000 AMU; scan time: 0.50 sec; inter scan delay 0.20 sec.

2-Bromo-5-(3-chloro-4-isopropoxyphenyl)-1,3,4-thiadiazole (12). Step1: A solution of 3-chloro-4-[(1-methylethyl)oxy]benzoic acid (**11**) (60 g, 280 mmol) in CH_2Cl_2 (1 L) at room temperature was treated with DMF (0.5 mL, 6.46 mmol) followed by the portionwise addition of oxalyl chloride (48.9 mL, 559 mmol) over 30 min. A mild effervescence was observed and the resulting mixture was stirred at this temperature for 16 h. The solvent was removed *in vacuo* to give 3-chloro-4-[(1-methylethyl)oxy]benzoyl chloride as yellow oil which solidified as a creamy solid when stored at 4^{o}C (66 g, 100%).LCMS (method formate): Retention time 1.26 min, $[M+H]^+$ = 229, 231 (1 Cl). Mass of methyl ester observed.^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (s, 1H), 8.01 (d, *J* = 8 Hz, 1H), 6.99 (d, *J* = 8 Hz, 1H), 4.71- 4.77 (m, 1H), 1.45 (d, *J* = 8 Hz, 6H). Step 2: A mixture of hydrazinecarbothioamide (29.6 g, 325 mmol) and 3-chloro-4-isopropoxybenzoyl chloride (101 g, 217 mmol) was treated with phosphorus oxychloride (100 g, 650 mmol) and the resulting mixture was heated at 90^{o}C for 3 h then was cooled to room temperature and left still for 16 h before being slowly added to a 1:1 mixture of 10N NaOH aqueous solution (500 mL, 5 mol) and iced water (500 mL) in an ice bath, giving a thick viscous gum. This gum was triturated with hot EtOH (400 mL) in an attempt to crystallise the product, giving a clear supernatant liquid. The supernatant solvent was decanted off into a 500 mL flask and allowed to cool, giving a gummy residue and a clear orange solution, both of which

containing the desired product. The material was recombined and concentrated *in vacuo* to give a yellow solid which was dissolved in EtOAc (800 mL). The organic phase was washed with water (800 mL) then brine (200 mL), dried over $MgSO_4$ and concentrated *in vacuo* to give a beige solid. This residue was dissolved in hot EtOH (200 mL) and the resulting solution was cooled in an ice bath. The beige precipitate formed was collected by filtration and dried under vacuum for 16 h at 40°C to give 5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-amine (21.1 g, 36 %) which was used in the next step without further purification. LCMS (method formate): Retention time 0.94 min, $[M+H]^+$: calculated: 270, 272; found 270, 272 (1 Cl).^{1}H NMR (400 MHz, DMSO-d_6) δ 7.79 (s, 1H), 7.82-7.85 (m, 1H), 7.40 (br s, 2H), 7.25 (d, *J* = 8.8 Hz, 1H), 4.72-4.78 (m, 1H), 1.31 (d, *J* = 8 Hz, 6H).Step 3: Cupric bromide (54.6 g, 245 mmol) and *tert*-butyl nitrite (29.1 mL, 245 mmol) were dissolved in CH_3CN (500 mL) and the resulting mixture was stirred at room temperature for 10 min, then was treated portionwise over 30 min with 5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-amine (30 g, 111 mmol). The resulting dark brown mixture was stirred at room temperature for 1 h, and then was concentrated *in vacuo* to give a black residue. This residue was suspended in EtOAc (500 mL) and the organic phase was washed with a 2N HCl aqueous solution (500 mL). The layers were separated and the organic phase was washed with a 2N HCl aqueous solution (200 mL) then brine (200 mL), dried over $MgSO_4$ and concentrated *in vacuo* to give 2-bromo-5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazole (**12**) (33.2 g, 89%) as a tan solid which was used in the next step without further purification. LCMS (method formate): Retention time 1.33 min; $[M+H]^+$: calculated: 333, 335; found 335 (1 Br). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (d, *J* = 2.0 Hz, 1H), 7.76 (dd, *J* = 8.8, 2.0 Hz, 1H), 7.04 (d, *J* = 8.8 Hz, 1H), 4.66 - 4.72 (m, 1H), 1.44 (d, *J* = 8.0 Hz, 6H).

2-(5-{3-Chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-4,5,6,7-tetrahydro-2*H*-pyrazolo[4,3-*c*]pyridine trifluoroacetate (35). Trifluoroacetic acid (0.142 mL, 1.838 mmol) was added to a solution of 1,1-dimethylethyl 2-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridine-5-carboxylate (**53a**) (35 mg, 0.074 mmol) in CH_2Cl_2 (2 mL) and the resulting mixture was stirred at room temperature for 5 h then was concentrated *in vacuo*. The residue triturated with Et_2O to give 2-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-4,5,6,7-tetrahydro-2H-pyrazolo[4,3-c]pyridine trifluoroacetate (**35**) (35 mg, 97%) as a pale yellow solid. LCMS (formate method): Retention time 1.04 min; $[M+CH_3CN+H]^+$; calculated: 417, 419; found: 417, 419 (1 Cl). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.12 (s, 2H), 8.60 (s, 1H), 8.04 (s, 1H), 7.91 (dd, *J* = 8.0, 2.0 Hz, 1H), 7.36 (d, *J* = 8.0 Hz, 1H), 4.80 - 4.86 (m, 1H), 4.30 (s, 2H), 3.49 (t, *J* = 6.2 Hz, 2H), 3.02 (t, J = 6.2 Hz, 2H), 1.34 (d, *J* = 6.1 Hz, 6H).

1-(5-{3-Chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-4,5,6,7-tetrahydro-1*H*-pyrazolo[4,3-*c*]pyridine trifluoroacetate (36). A solution of 1,1-dimethylethyl 1-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-1,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridine-5-carboxylate (**54a**) (35 mg, 0.074 mmol) in CH_2Cl_2 (2 mL) was treated at room temperature with trifluoroacetic acid (0.14 mL, 1.84 mmol) and the resulting mixture was stirred at this temperature for 5 h. More trifluoroacetic acid (0.14 mL, 1.838 mmol) was added and the resulting mixture was stirred for another 2 h at room temperature then was concentrated *in vacuo* to give, after trituration of the residue with Et_2O, 1-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-4,5,6,7-tetrahydro-1*H*-pyrazolo[4,3-*c*]pyridine trifluoroacetate (**36**) (30 mg, 83%) as a pale yellow solid. LCMS (formate method): Retention time 1.06 min; $[M+H]^+$: calculated: 376, 378; found: 376 (1 Cl).^{1}H NMR (400 MHz, DMSO-d_6)

δ 8.04 (s, 2H), 7.82 - 7.87 (m, 2H), 7.25 (d, *J* = 8.8 Hz, 1H), 4.77 - 4.83 (m, 1H), 4.35 (s, 2H), 3.57 – 3.67 (m, 4H), 1.42 (d, *J* = 8.0 Hz, 6H).

2-(5-{3-Chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-3-methyl-4,5,6,7-tetrahydro-2*H*-pyrazolo[4,3-*c*]pyridine (37). Trifluoroacetic acid (0.5 mL, 6.49 mmol) was added to a solution of 1,1-dimethylethyl 2-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-3-methyl-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridine-5-carboxylate (**56a**) (245 mg, 0.50 mmol) in CH_2Cl_2 (5 mL) at room temperature under nitrogen and the resulting mixture was stirred at this temperature for 1 h. Further trifluoroacetic acid (1 mL, 13.0 mmol) was then added and the resulting mixture was stirred for a further 30 min then was concentrated *in vacuo*. The residue was co-evaporated with CH_2Cl_2 and loaded on a SCX cartridge then eluted with MeOH followed by a 2N NH_3 solution in MeOH. The combined ammonia fractions were concentrated *in vacuo* to give 2-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-3-methyl-4,5,6,7-tetrahydro-2H-pyrazolo[4,3-c]pyridine (**37**) (180 mg, 92%) as a yellow oil.

LCMS (formate method): Retention time 0.93 min; $[M+H]^+$: calculated: 390, 392; found: 390 (1 Cl). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 (d, *J* = 2.3 Hz, 1H), 7.76 (dd, *J* = 8.0, 2.0 Hz, 1H), 7.03 (d, *J* = 8.0 Hz, 1H), 4.66 - 4.69 (m, 1H), 3.89 (s, 2H), 3.16 (t, *J* = 5.8 Hz, 2H), 2.79 (t, J = 6.2 Hz, 2H), 2.57 (s, 3H), 1.44 (d, *J* = 6.0 Hz, 6H).

2-[(1-Methylethyl)oxy]-5-[5-(4,5,6,7-tetrahydro-2*H*-pyrazolo[4,3-*c*]pyridin-2-yl)-1,3,4-thiadiazol-2-yl]benzonitrile trifluoroacetate (38). A solution of 1,1-dimethylethyl 2-(5-{3-cyano-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridine-5-carboxylate (**53b**) (315 mg, 0.675 mmol) in CH_2Cl_2 (7 mL) at room temperature was treated with trifluoroacetic acid (1 mL, 13.0 mmol) and the resulting mixture was stirred at

this temperature for 1.5 h then was concentrated *in vacuo*. The residue was triturated with Et_2O and the precipitate formed was filtered off and dried under vacuum to give 2-[(1-methylethyl)oxy]-5-[5-(4,5,6,7-tetrahydro-2H-pyrazolo[4,3-c]pyridin-2-yl)-1,3,4-thiadiazol-2-yl]benzonitrile trifluoroacetate (**38**) (291 mg, 90%) as a white solid. LCMS (formate method): Retention time 0.85 min; $[M+H]^+$: calculated: 367; found: 367.17. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.20 (br s, 2H), 8.62 (s, 1H), 8.36 (s, 1H), 8.26 - 8.28 (m, 1H), 7.49 (d, *J* = 9.3 Hz, 1H), 4.91 - 4.98 (m, 1H), 4.30 (s, 2H), 3.45 - 3.55 (m, 2H), 3.00 - 3.05 (m, 2H), 1.37 (d, *J* = 6.0 Hz, 6H).

2-[(1-Methylethyl)oxy]-5-[5-(3-methyl-4,5,6,7-tetrahydro-2*H*-pyrazolo[4,3-*c*]pyridin-2-yl)-1,3,4-thiadiazol-2-yl]benzonitrile (39). Trifluoroacetic acid (0.50 mL, 6.52 mmol) was added to a solution of 1,1-dimethylethyl 2-(5-{3-cyano-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-3-methyl-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridine-5-carboxylate (**56b**) (165 mg, 0.343 mmol) in CH_2Cl_2 (2 mL) at room temperature under nitrogen and the resulting mixture was stirred at this temperature for 2 h then was diluted with CH_2Cl_2 (10 mL) and concentrated *in vacuo*. The residue was loaded on a SCX cartridge then eluted with MeOH followed by a 2N NH_3 solution in MeOH. The combined ammonia fractions were concentrated *in vacuo* to give 2-[(1-methylethyl)oxy]-5-[5-(3-methyl-4,5,6,7-tetrahydro-2H-pyrazolo[4,3-c]pyridin-2-yl)-1,3,4-thiadiazol-2-yl]benzonitrile (**39**) (119 mg, 91%) as a yellow solid. LCMS (formate method): Retention time 0.84 min; $[M+H]^+$: calculated: 381; found: 381.18. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.30 (s, 1H), 8.20 - 8.23 (m, 1H), 7.47 (d, *J* = 9.1 Hz, 1H), 4.90-4.96 (m, 1H), 3.69 (s, 2H), 2.93 (t, *J* = 6.2 Hz, 2H), 2.60 (t, J = 6.2 Hz, 2H), 2.57 (s, 3H), 1.37 (d, *J* = 6.0 Hz, 6H).

2-Isopropoxy-5-(5-(3-methyl-4,5,6,7-tetrahydro-2H-pyrazolo[3,4-c]pyridin-2-yl)-1,3,4-thiadiazol-2-yl)benzonitrile hydrochloride (40). Step 1: A solution of *tert*-butyl 3-

oxopiperidine-1-carboxylate (2.0 g, 10 mmol) in THF (40 mL) was cooled to -78ºC under nitrogen and was treated with a 2N lithium diisopropylamide solution in THF (5 mL, 10 mmol) over 2 min. The resulting mixture was stirred at -78ºC under nitrogen for 30 min then acetyl chloride (0.92 mL, 13 mmol) was added dropwise. After complete addition, the reaction mixture was allowed to warm to room temperature over 2 h. Water (25 mL) was added and the mixture extracted with EtOAc (3 x 25 mL). The combined extracts were dried over $MgSO_4$ and concentrated *in vacuo*. Purification of the residue by flash chromatography on silica gel (10-25% EtOAc in *iso*-hexane] gave *tert*-butyl 4-acetyl-3-oxopiperidine-1-carboxylate (426 mg, 18%) as a yellow oil. LCMS (formate method): Retention time 1.07 min; $[M\text{-}tBu+H]^+$: calculated: 186; found: 186.0. Step 2: A mixture of 5-(5-hydrazino-1,3,4-thiadiazol-2-yl)-2-[(1-methylethyl)oxy]benzonitrile(**51b**) (275 mg, 1 mmol) and *tert*-butyl 4-acetyl-3-oxopiperidine-1-carboxylate (400 mg, 1.66 mmol) in EtOH was stirred at room temperature for 24 h then was concentrated *in vacuo*. The residue was dissolved in EtOAc (10 mL). The organic phase was washed twice with water, dried over $MgSO_4$ and concentrated *in vacuo*. Purification of the residue by MDAP gave tert-butyl 2-(5-(3-cyano-4-isopropoxyphenyl)-1,3,4-thiadiazol-2-yl)-3-methyl-4,5-dihydro-2H-pyrazolo[3,4-c]pyridine-6(7H)-carboxylate (10 mg, 2%) as colourless glass. LCMS (formate method): Retention time 1.77 min; $[M+H]^+$: calculated: 481; found: 481.1.^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 - 8.12 (m, 2H), 7.09 (d, J = 8.8 Hz, 1H), 4.73 - 4.80 (m, 1H), 4.63 (br s, 2H), 3.71 (br s, 2H), 2.82 (br s, 2H), 2.70 (s, 3H), 1.47 - 1.51 (m, 15H). Step 3: A solution of tert-butyl 2-(5-(3-cyano-4-isopropoxyphenyl)-1,3,4-thiadiazol-2-yl)-3-methyl-4,5-dihydro-2H-pyrazolo[3,4-c]pyridine-6(7H)-carboxylate (10 mg, 0.02 mmol) in 1,4- dioxan (0.5 mL) was treated with a 4N HCl solution in 1,4-dioxan (0.5 mL) and the resulting mixture was stirred at room temperature for 16 h. Et_2O (5 mL) was added. The precipitate formed was

filtered off, washed with Et_2O and dried under vacuum to give 2-isopropoxy-5-(5-(3-methyl-4,5,6,7-tetrahydro-2H-pyrazolo[3,4-c]pyridin-2-yl)-1,3,4-thiadiazol-2-yl)benzonitrile hydrochloride (**40**) (8 mg, 92%) as an off-white solid. LCMS (formate method): Retention time 0.83 min; $[M+H]^+$: calculated: 381; found: 381.1. ^{1}H NMR (400 MHz, MeOH-d_4) δ 8.21 - 8.26 (m, 2H), 7.39 (d, *J* = 6.6 Hz, 1H), 4.84 - 5.02 (m, 1H), 4.42 (s, 2H), 3.55 - 3.60 (m, 2H), 2.94 - 2.98 (m, 2H), 2.74 (s, 3H), 1.46 (d, *J* = 8.0 Hz, 6H).

2-(2-(5-(3-Cyano-4-isopropoxyphenyl)-1,3,4-thiadiazol-2-yl)-3-methyl-6,7-dihydro-2H-pyrazolo[4,3-c]pyridin-5(4H)-yl)acetic acid trifluoroacetate (41). Step 1: A mixture of 2-[(1-methylethyl)oxy]-5-[5-(3-methyl-4,5,6,7-tetrahydro-2H-pyrazolo[4,3-c]pyridin-2-yl)-1,3,4-thiadiazol-2-yl]benzonitrile hydrochloride (**40**) (500 mg, 1.01 mmol), *tert*-butyl bromoacetate (207 mg, 1.06 mmol) and K_2CO_3 (419 mg, 3.03 mmol) in CH_3CN (15 mL) was stirred at 50ºC for 2 h then was cooled to room temperature and diluted with water (15 mL). The mixture was extracted with EtOAc (2 x 20 mL). The combined organic phases were dried over $MgSO_4$ and concentrated *in vacuo*. . Purification of the residue by flash chromatography on silica gel (0-3% MeOH in CH_2Cl_2) gave *tert*-butyl 2-(2-(5-(3-cyano-4-isopropoxyphenyl)-1,3,4-thiadiazol-2-yl)-3-methyl-6,7-dihydro-2H-pyrazolo[4,3-c]pyridin-5(4H)-yl)acetate (470 mg, 94%) as colourless foam. LCMS (method formate): Retention time 1.02 min, $[M+H]^+$: calculated: 495; found: 495.^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.31 (d, *J* = 2.0 Hz, 1H), 8.23 (dd, *J* = 9.0, 2.3 Hz, 1H), 7.48 (d, *J* = 9.3 Hz, 1H), 4.88 - 4.99 (m, 1H), 3.61 (s, 2H), 3.38 - 3.44 (m, 2H), 2.89 (t, *J* = 5.6 Hz, 2H), 2.68 - 2.75 (m, 2H), 2.58 (s, 3H), 1.46 (s, 9H), 1.38 (d, *J* = 6.0 Hz, 6H).Step 2: Trifluoroacetic acid (3 mL, excess) was slowly added to a stirred solution of *tert*-butyl 2-(2-(5-(3-cyano-4-isopropoxyphenyl)-1,3,4-thiadiazol-2-yl)-3-methyl-6,7-dihydro-2H-pyrazolo[4,3-c]pyridin-5(4H)-yl)acetate (470 mg, 0.95 mmol) in CH_2Cl_2 (10 mL). The resulting mixture was

stirred at room temperature for 4 h then was concentrated *in vacuo*. The residue was co-evaporated twice with toluene then was triturated with Et_2O to give 2-(2-(5-(3-cyano-4-isopropoxyphenyl)-1,3,4-thiadiazol-2-yl)-3-methyl-6,7-dihydro-2H-pyrazolo[4,3-c]pyridin-5(4H)-yl)acetic acid trifluoroacetate (**41**) (467 mg, 89%) as an off-white solid. LCMS (method formate): Retention time 0.86 min, $[M+H]^+$: calculated: 439; found: 439.^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 12.50 (br s, 1H), 8.34 (d, *J* = 2.3 Hz, 1H), 8.25 (dd, *J* = 9.0, 2.1 Hz, 1H), 7.48 (d, *J* = 9.1 Hz, 1H), 4.94 (dt, *J* = 12.1, 6.1 Hz, 1H), 4.30 (br s, 2H), 4.13 (br s, 2H), 3.53 (br s, 2H), 3.03 (t, *J* = 5.7 Hz, 2H), 2.63 (s, 3H), 1.37 (d, *J* = 6.1 Hz, 6H).

3-[2-(5-{3-Chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-2,4,6,7-tetrahydro-5*H*-pyrazolo[4,3-*c*]pyridin-5-yl]propanoic acid (42). A mixture of 2-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-4,5,6,7-tetrahydro-2H-pyrazolo[4,3-c]pyridine trifluoroacetate (**35**) (32 mg, 0.085 mmol), 1,1-dimethylethyl 2-propenoate (0.025 mL, 0.170 mmol) and NEt_3 (0.036 mL, 0.255 mmol) in *n*-BuOH (1.5 mL) and THF (0.5 mL) was stirred at 100°C for 2 h under microwave irradiation then was cooled to room temperature. The mixture was loaded onto a SCX column, then eluted sequentially with MeOH and a 2N NH_3 solution in MeOH. The ammonia fractions were concentrated *in vacuo* and the residue dissolved in DCM (1 mL) and treated with trifluoroacetic acid (9.71 mg, 0.085 mmol). The resulting mixture was stirred at room temperature for 1 h then was concentrated *in vacuo*. Purification of the residue by MDAP gave 3-[2-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridin-5-yl]propanoic acid (**42**) (8 mg, 21%) as a white oily solid. LCMS (method formate): Retention time 0.96 min; $[M+H]^+$: calculated: 448; found: 448.2.^{1}H NMR (600 MHz, DMSO-d_6) δ ppm 12.18 (br s, 1H), 8.37 (s, 1H), 8.03 (d, *J* = 1.8 Hz, 1H), 7.89

(dd, *J* = 8.4, 2.2 Hz, 1H), 7.36 (d, *J* = 9.2 Hz, 1H), 4.79 - 4.87 (m, 1H), 3.57 (s, 2H), 2.74 - 2.83 (m, 6H), 2.46 - 2.50 (m, 2H), 1.35 (d, *J* = 6.2 Hz, 6H).

3-(2-(5-(3-Chloro-4-isopropoxyphenyl)-1,3,4-thiadiazol-2-yl)-3-methyl-6,7-dihydro-2H-pyrazolo[4,3-c]pyridin-5(4H)-yl)propanoic acid hydrochloride (43). Step 1: A solution of 2-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-3-methyl-4,5,6,7-tetrahydro-2H-pyrazolo[4,3-c]pyridine (**37**) (31.4g, 72.5 mmol) in DMF (300 mL) was treated with *tert*-butyl acrylate (13.9 g, 109 mmol) and DBU (33.1 g, 217 mmol) and the resulting mixture was stirred for 16 h at room temperature. The mixture was then diluted with water (700 mL), giving a dense white solid which was collected by filtration and washed with water (100 mL). The product (contaminated with DMF) was dissolved in hot EtOAc (1 L) and the organic phase was washed with water (2 x 300 mL), dried over $MgSO_4$ and concentrated *in vacuo* to give 1,1-dimethylethyl 3-[2-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-3-methyl-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridin-5-yl]propanoate (36.8 g, 98%) as a white solid. LCMS (method formate): Retention time 1.12 min; $[M+H]^+$: calculated: 518; found: 518.0.^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.98 (d, *J* = 2.3 Hz, 1H), 7.76 (dd, *J* = 8.6, 2.3 Hz, 1H), 7.03 (d, *J* = 8.8 Hz, 1H), 4.63 - 4.73 (m, 1H), 3.51 (s, 2H), 2.92 (t, *J* = 7.2 Hz, 2H), 2.82 - 2.85 (m, 4H), 2.66 (s, 3H), 2.53 (t, *J* = 7.3 Hz, 2H), 1.47 (s, 9H), 1.44 (d, *J* = 6.1 Hz, 6H).Step 2: 1,1-Dimethylethyl 3-[2-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-3-methyl-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridin-5-yl]propanoate (36 g, 69.5 mmol) was suspended in a mixture of THF (500 mL) and a 2N HCl aqueous solution (600 mL) and the thick suspension was heated at 50^{o}C for 18 h, giving a white suspension, and then was cooled to room temperature. The solid was filtered off, washed with water (2 x 50 mL) and dried under vacuum at 45^{o}C for 16 h to give 3-[2-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-

3-methyl-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridin-5-yl]propanoic acid hydrochloride (**43**) (30.35 g, 88%) as colourless solid. LCMS (method formate): Retention time 0.96 min; $[M+H]^+$: calculated: 462; found: 462.14.^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 12.75 (br s, 1H), 10.80 (br s, 1H), 8.04 (d, *J* = 2.5 Hz, 1H), 7.90 (dd, *J* = 9.0, 2.5 Hz, 1H), 7.36 (d, *J* = 9.0 Hz, 1H), 4.83 (spt, *J* = 6.0 Hz, 1H), 3.57 - 4.70 (m, 4H), 3.40 - 3.52 (m, 2H), 3.08 (t, *J* = 6.0 Hz, 2H), 2.91 (t, *J* = 7.5 Hz, 2H), 2.65 (s, 3H), 1.34 (d, *J* = 6.0 Hz, 6H).

3-(2-(5-(3-Cyano-4-isopropoxyphenyl)-1,3,4-thiadiazol-2-yl)-6,7-dihydro-2H-pyrazolo[4,3-c]pyridin-5(4H)-yl)propanoic acid (44). Step 1: A solution of 2-[(1-methylethyl)oxy]-5-[5-(4,5,6,7-tetrahydro-2H-pyrazolo[4,3-c]pyridin-2-yl)-1,3,4-thiadiazol-2-yl]benzonitrile (**38**) (80 mg, 0.17 mmol) in DMF (7 mL) at room temperature was treated with DBU (0.075 mL, 0.50 mmol) and ethyl 2-propenoate (0.090 mL, 0.83 mmol) and the resulting mixture was stirred at this temperature for 16 h then was partitioned between EtOAc and water. The layers were separated and the organic phase was washed with water, dried using a phase separator and concentrated *in vacuo*. The residue obtained was triturated with Et_2O to give ethyl 3-(2-(5-(3-cyano-4-isopropoxyphenyl)-1,3,4-thiadiazol-2-yl)-6,7-dihydro-2H-pyrazolo[4,3-c]pyridin-5(4H)-yl)propanoate (19 mg, 24%) as white solid.LCMS (method formate): Retention time 0.96 min; $[M+H]^+$: calculated: 467; found: 467.0.^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.37 (s, 1H), 8.32 (d, *J* = 2.3 Hz, 1H), 8.24 (dd, *J* = 8.8, 2.3 Hz, 1H), 7.47 (d, *J* = 9.1 Hz, 1H), 4.94 (spt, *J* = 6.1 Hz, 1H), 4.07 (q, *J* = 7.1 Hz, 2H), 3.55 (s, 2H), 3.34 (m, 2H), 2.72 - 2.83 (m, 4H), 2.55 (t, *J* = 7.1 Hz, 2H), 1.37 (d, *J* = 6.1 Hz, 6H), 1.18 (t, *J* = 7.1 Hz, 3H). Step 2: A solution of ethyl 3-[2-(5-{3-cyano-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridin-5-yl]propanoate (18 mg, 0.039 mmol) in THF (1 mL) and water (0.5 mL) was treated with LiOH (1.85 mg, 0.08 mmol) and the resulting mixture was stirred at room

temperature. LiOH (0.9 mg, 0.04 mmol) was further added after 2 and 3 h. After 3.5 h, the mixture was diluted with EtOAc and the organic phase was washed with a saturated NH_4Cl aqueous solution. The aqueous layer was extracted twice with CH_2Cl_2 and the combined organic phases were dried using a phase separator and concentrated *in vacuo* to give 3-(2-(5-(3-cyano-4-isopropoxyphenyl)-1,3,4-thiadiazol-2-yl)-6,7-dihydro-2H-pyrazolo[4,3-c]pyridin-5(4H)-yl)propanoic acid (**44**) (17 mg, 100%) as a white solid.LCMS (method formate): Retention time 0.74 min; $[M+H]^+$: calculated: 439; found: 439.0.^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 12.35 (br s, 1H), 8.37 (s, 1H), 8.32 (d, *J* = 2.5 Hz, 1H), 8.24 (dd, *J* = 9.0, 2.5 Hz, 1H), 7.47 (d, *J* = 9.0 Hz, 1H), 4.93 (spt, *J* = 6.0 Hz, 1H), 3.56 (s, 2H), 2.73 - 2.83 (m, 6H), 2.47 (t, *J* = 7.5 Hz, 2H), 1.36 (d, *J* = 6.0 Hz, 6H).

3-[2-(5-{3-Cyano-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-3-methyl-2,4,6,7-tetrahydro-5*H*-pyrazolo[4,3-*c*]pyridin-5-yl]propanoic acid trifluoroacetate (45). Step 1: A solution of 2-[(1-methylethyl)oxy]-5-[5-(3-methyl-4,5,6,7-tetrahydro-2H-pyrazolo[4,3-c]pyridin-2-yl)-1,3,4-thiadiazol-2-yl]benzonitrile (**39**) (45 mg, 0.12 mmol) and 1,1-dimethylethyl 2-propenoate (0.086 mL, 0.59 mmol) in DMF (5 mL) at room temperature under nitrogen was treated with DBU (0.053 mL, 0.355 mmol) and the resulting mixture was stirred at this temperature for 16 h then was concentrated *in vacuo*. The residue was partitioned between CH_2Cl_2 and a saturated $NaHCO_3$ aqueous solution and the layers were separated. The organic phase was dried using a phase separator and concentrated *in vacuo*. Purification of the residue by flash chromatography on silica gel (c-Hexane/EtOAc: 10 to 70% gradient) gave 1,1-dimethylethyl 3-[2-(5-{3-cyano-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-3-methyl-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridin-5-yl]propanoate (46 mg, 76%) as a yellow solid.

LCMS (method formate): Retention time 0.99 min; $[M+H]^+$: calculated: 509; found: 509.0.^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.97 - 8.14 (m, 2H), 7.07 (d, *J* = 8.8 Hz, 1H), 4.70 - 4.79 (m, 1H), 3.50 (s, 2H), 2.90 (t, *J* = 7.3 Hz, 2H), 2.80 - 2.85 (m, 4H), 2.64 (s, 3H), 2.52 (t, *J* = 7.3 Hz, 2H), 1.43 - 1.47 (m, 15H). Step 2: A solution of 1,1-dimethylethyl 3-[2-(5-{3-cyano-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-3-methyl-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridin-5-yl]propanoate (46 mg, 0.090 mmol) in CH_2Cl_2 (2 mL) at room temperature under nitrogen was treated with trifluoroacetic acid (0.348 mL, 4.52 mmol) and the resulting mixture was stirred at this temperature for 7 h, then was diluted with CH_2Cl_2 and concentrated *in vacuo*. Trituration of the residue with c-Hexane/EtOAc 1:1 gave 3-[2-(5-{3-cyano-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-3-methyl-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridin-5-yl]propanoic acid trifluoroacetate (**45**) (25 mg, 48%) as an off white solid. LCMS (method formate): Retention time 0.86 min; $[M+H]^+$: calculated: 453; found: 453.0.^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.32 - 8.35 (m, 1H), 8.23 - 8.28 (m, 1H), 7.45 - 7.51 (m, 1H), 4.88 - 4.98 (m, 1H), 4.21 - 4.36 (m, 2H), 3.40-3.52 (m, 4H), 2.97 - 3.06 (m, 2H), 2.78 - 2.85 (m, 2H), 2.65 (s, 3H), 1.37 (d, *J* = 5.8 Hz, 6H).

3-[1-(5-{3-Chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-1,4,6,7-tetrahydro-5*H*-pyrazolo[4,3-*c*]pyridin-5-yl]propanoic acid (46). A solution of 1-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-4,5,6,7-tetrahydro-1H-pyrazolo[4,3-c]pyridine trifluoroacetate (**36**) (14 mg, 0.037 mmol), *tert*-butyl acrylate (10.8 µL, 0.074 mmol) and NEt_3 (0.02 mL, 0.112 mmol) in *n*-BuOH (1.5 mL) and THF (0.5 mL) was stirred at 100°C for 60 min under microwave irradiation then cooled to room temperature. More *tert*-butyl acrylate (10.8 µL, 0.074 mmol) was added and the mixture was stirred again at 100°C for 60 min under microwave irradiation then cooled to room temperature. The mixture was loaded onto a SCX cartridge then

eluted sequentially with MeOH and a 2N NH_3 solution in MeOH. The ammonia fractions were concentrated *in vacuo* and the residue was dissolved in DCM (1 mL) then treated with trifluoroacetic acid (4.25 mg, 0.037 mmol). The resulting mixture was stirred at room temperature for 16 h then was concentrated *in vacuo*. Purification of the residue with MDAP gave 3-[1-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-1,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridin-5-yl]propanoic acid (**46**) (6 mg, 36%) as a white oily solid.LCMS (method formate): Retention time 0.99 min; $[M+H]^+$: calculated: 448; found: 448.2.^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.39 (s, 1H) 8.03 (d, *J* = 2.3 Hz, 1H), 7.89 (dd, *J* = 8.7, 2.1 Hz, 1H), 7.75 (s, 1H), 7.34 (d, *J* = 8.8 Hz, 1H), 4.82 (spt, *J* = 6.0 Hz, 1H), 3.47 (s, 2H), 3.08 - 3.14 (m, 2H), 2.73 - 2.83 (m, 4H), 2.42 (t, *J* = 7.2 Hz, 2H), 1.34 (d, *J* = 6.1 Hz, 6H).

4-[2-(5-{3-Chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-2,4,6,7-tetrahydro-5*H*-pyrazolo[4,3-*c*]pyridin-5-yl]butanoic acid (47). Step 1: A mixture of 2-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-4,5,6,7-tetrahydro-2H-pyrazolo[4,3-c]pyridine trifluoroacetate (**35**) (100 mg, 0.166 mmol), ethyl 4-bromobutanoate (0.072 mL, 0.497 mmol) and K_2CO_3 (57.2 mg, 0.414 mmol) in DMF (3 mL) was stirred at 80ºC for 1 h then more ethyl 4-bromobutanoate (0.024 mL, 0.166 mmol) was added. The resulting mixture was stirred at 80ºC for another 30 min then cooled to room temperature and partitioned between EtOAc and water. The two layers were separated and the organic phase was washed with water. The layers were separated and the combined organic phases were dried using a phase separator then were concentrated *in vacuo*. The residue was triturated with Et_2O and the solid formed was filtered off and dried under vacuum to give ethyl 4-[2-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridin-5-yl]butanoate (46 mg, 57%) as a cream solid. LCMS (method formate): Retention time 1.04 min; $[M+H]^+$: calculated: 490;

found: 490.18.^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 8.15 (s, 1H) 7.98 (d, *J* = 2.3 Hz, 1H), 7.75 (dd, *J* = 8.6, 2.3 Hz, 1H), 7.03 (d, *J* = 8.6 Hz, 1H), 4.62 - 4.73 (m, 1H), 4.13 (q, *J* = 7.2 Hz, 2H), 3.62 (br s, 2H), 2.80 - 2.94 (m, 4H), 2.62 (br s, 2H), 2.41 (t, *J* = 7.2 Hz, 2H), 1.93 (qt, *J* = 7.2 Hz, 2H), 1.44 (d, *J* = 6.1 Hz, 6H), 1.26 (t, *J* =7.2 Hz, 3H). Step 2: A solution of ethyl 4-[2-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridin-5-yl]butanoate (45 mg, 0.092 mmol) in THF (2 ml) and water (1 mL) was treated with LiOH (4.4 mg, 0.184 mmol) and the resulting mixture was stirred at room temperature for 2 h, then was dissolved with EtOAc. The organic phase was washed sequentially with a saturated NH_4Cl aqueous solution then water. The layers were separated and the organic phase was dried using a phase separator then was concentrated *in vacuo* to give 4-[2-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridin-5-yl]butanoic acid (**47**) (26 mg, 61%) as a white solid.LCMS (method formate): Retention time 0.97 min; $[M+H]^+$: calculated: 462; found: 462.21.^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 12.22 (br s, 1H), 8.36 (s, 1H), 8.02 (d, *J* = 2.3 Hz, 1H), 7.86 - 7.91 (m, 1H), 7.35 (d, *J* = 9.1 Hz, 1H), 4.82 (spt, *J* = 6.1 Hz, 1H), 3.51 (s, 2H), 3.30 - 3.34 (m, 2H), 2.70 - 2.80 (m, 4H), 2.27 (t, *J* = 7 Hz, 2H), 1.74 (qt, *J* = 7 Hz, 2H), 1.34 (d, *J* = 6.1 Hz, 6H).

4-[2-(5-{3-Cyano-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-2,4,6,7-tetrahydro-5*H*-pyrazolo[4,3-*c*]pyridin-5-yl]butanoic acid (48). Step 1: A mixture of 2-[(1-methylethyl)oxy]-5-[5-(4,5,6,7-tetrahydro-2H-pyrazolo[4,3-c]pyridin-2-yl)-1,3,4-thiadiazol-2-yl]benzonitrile trifluoroacetate (**38**) (50 mg, 0.104 mmol), ethyl 4-bromobutanoate (0.045 mL, 0.312 mmol) and K_2CO_3 (36.0 mg, 0.260 mmol) in DMF (3 mL) was stirred at 80ºC for 1 h then cooled to room temperature and partitioned between EtOAc and water. The layers were separated and the organic phase was washed with water then dried using a phase separator and

concentrated *in vacuo* to give ethyl 4-[2-(5-{3-cyano-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridin-5-yl]butanoate (164 mg, 107%) as a yellow oil which was used in the next step without further purification.LCMS (method formate): Retention time 0.93 min; $[M+H]^+$: calculated: 481; found: 481.18. Step 2: A solution of ethyl 4-[2-(5-{3-cyano-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridin-5-yl]butanoate (50 mg, 0.104 mmol) in THF (2 mL) and water (1 mL) was treated with LiOH (5.0 mg, 0.208 mmol) at room temperature and the resulting mixture was stirred at this temperature for 4.5 h. More LiOH (10.0 mg, 0.416 mmol) was added and the resulting mixture was stirred at the same temperature for 16 h then was diluted with EtOAc. The organic phase was sequentially washed with a saturated NH_4Cl aqueous solution and water then dried using a phase separator cartridge and concentrated *in vacuo* to give 4-[2-(5-{3-cyano-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-2,4,6,7-tetrahydro-5*H*-pyrazolo[4,3-*c*]pyridin-5-yl]butanoic acid (**48**) (8 mg, 17%) as a white solid. LCMS (method formate): Retention time 0.79 min; $[M+H]^+$: calculated: 453; found: 453.1. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 12.08 (br s, 1H), 8.37 (s, 1H), 8.32 (d, J = 2.5 Hz, 1H), 8.23 (dd, J = 9.0, 2.5 Hz, 1H), 7.47 (d, J = 9.0 Hz, 1H), 4.93 (spt, J = 6.0 Hz, 1H), 3.54 (s, 2H), 2.77 (s, 4H), 2.53 (t, J = 7.0 Hz, 2H), 2.28 (t, J = 7.0 Hz, 2H), 1.75 (qt, J = 7.0 Hz, 2H), 1.36 (d, J = 6.0 Hz, 6H).

5-(5-Bromo-1,3,4-thiadiazol-2-yl)-2-[(1-methylethyl)oxy]benzonitrile (50). Step 1: A flask was charged with 3-cyano-4-[(1-methylethyl)oxy]benzoic acid (20.9 g, 102 mmol) and hydrazinecarbothioamide 13.9 g, 153 mmol) then phosphorus oxychloride (90 g, 587 mmol) was added. The resulting mixture was stirred at 90°C for 3 h then cooled to room temperature and added very carefully in small portions to a 5M NaOH aqueous solution (500 mL) cooled with an ice bath such that the temperature never rose above 35°C. The resulting mixture was basified to

pH 10 (using a 5M NaOH aqueous solution) then stirred for 30 min. The precipitate formed was collected by filtration and dissolved in DCM (1 L) and MeOH (50 mL). The organic phase was washed with water (500 mL), dried over $MgSO_4$ and concentrated *in vacuo* to give 5-(5-amino-1,3,4-thiadiazol-2-yl)-2-[(1-methylethyl)oxy]benzonitrile (26.3 g, 99%) as pale yellow solid which was used in the next step without further purification. LCMS (method formate): Retention time 0.86 min; $[M+H]^+$ = 261.13. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.01- 8.05 (m, 2H), 7.52 - 7.56 (br s, 2H), 7.37 (d, *J* = 8.0 Hz, 1H), 4.84 - 4.90 (m, 1H), 1.34 (d, *J* = 8.0 Hz, 6H). Step 2: Cupric bromide (19.63 g, 88 mmol) and *tert*-butyl nitrite (10.44 mL, 88 mmol) were dissolved in CH_3CN (400 mL) and the resulting mixture was stirred for 10 min at room temperature. 5-(5-Amino-1,3,4-thiadiazol-2-yl)-2-[(1-methylethyl)oxy]benzonitrile (13 g, 40.0 mmol) was then added in small portions over 30 min. The resulting mixture was stirred for 1 h at room temperature, at 70°C for 2 h then was cooled to room temperature and concentrated *in vacuo*. The residue was dissolved in EtOAc (600 mL) and MeOH (50 mL) and stirred at reflux for 1 h. The insoluble material was filtered through a silica pad and washed with EtOAc (2 x 200 mL). The combined organic phases were washed with a 1M HCl aqueous solution (300 mL), dried over $MgSO_4$ and concentrated *in vacuo*. Purification of the residue by flash chromatography on silica gel (c-Hexane/EtOAc: 0 to 100% gradient) gave 5-(5-bromo-1,3,4-thiadiazol-2-yl)-2-[(1-methylethyl)oxy]benzonitrile (**50**) (8.8 g, 68 %) as a pale yellow solid. LCMS (method formate): Retention time 1.13 min; $[M+H]^+$: calculated: 324, 326; found: 325.7 (1 Br). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07 - 8.12 (m, 2H), 7.10 (d, *J* = 8.8 Hz, 1H), 4.75 - 4.81 (m, 1H), 1.47 (d, *J* = 8.0 Hz, 6H).

2-(3-Chloro-4-isopropoxyphenyl)-5-hydrazinyl-1,3,4-thiadiazole (51a). A suspension of 2-bromo-5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazole (**12**) (33 g, 99 mmol) in *i*-

PrOH (300 mL) was treated under nitrogen with hydrazine hydrate (31.0 mL, 989 mmol) and the resulting mixture was stirred at 105ºC for 16 h then was cooled to room temperature. Water (100 mL) was added and the resulting mixture was concentrated *in vacuo* to approximately half its volume. The solid formed was collected by filtration and dried under vacuum to give 5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2(3H)-one hydrazone (**51a**) (23.6 g, 83 mmol, 84 % yield) as orange solid which was used in the next step without further purification. LCMS (method formate): Retention time 0.97 min; $[M+H]^+$: calculated: 285, 287; found: 285 (1Cl). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.97 (br s, 2H), 7.80 (d, *J* = 2.0 Hz, 1H), 7.64 (dd, *J* = 8.0, 2.0 Hz, 1H), 7.25 (d, *J* = 8.0 Hz, 2H), 4.71- 4.77 (m, 1H), 1.32 (d, *J* = 8 Hz, 6H).

5-(5-Hydrazino-1,3,4-thiadiazol-2-yl)-2-[(1-methylethyl)oxy]benzonitrile (51b). A solution of 5-(5-bromo-1,3,4-thiadiazol-2-yl)-2-[(1-methylethyl)oxy]benzonitrile (**50**) (0.5 g, 1.54 mmoL) and hydrazine hydrate (0.24 mL, 7.7 mmol) in *i*-PrOH (10 mL) was stirred at 100°C under nitrogen. After 2 h, the reaction mixture had solidified and was cooled to room temperature. CH_2Cl_2 (10 mL) was added and the resulting solution was washed with a saturated $NaHCO_3$ aqueous solution. The two layers were separated and the organic phase was dried using a phase separator then was concentrated *in vacuo*. Purification of the residue by flash chromatography on silica gel (CH_2Cl_2/MeOH: 2.5 to 10% gradient) gave 5-(5-hydrazino-1,3,4-thiadiazol-2-yl)-2-[(1-methylethyl)oxy]benzonitrile (**51b**) (272 mg, 64%) as a white solid.LCMS (method formate): Retention time 0.84 min; $[M+H]^+$: calculated: 276; found: 276.13.^{1}H NMR (400 MHz, DMSO-d_6) δ 9.01 (s, 1H), 8.01 - 8.05 (m, 2H), 7.37 (d, *J* = 8.0 Hz, 1H), 5.24 (s, 2H), 4.83 - 4.89 (m, 1H), 1.35 (d, *J* = 8.0 Hz, 6H).

1,1-Dimethylethyl 2-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-2,4,6,7-tetrahydro-5*H*-pyrazolo[4,3-*c*]pyridine-5-carboxylate (53a) and 1,1-dimethylethyl 1-

(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-1,4,6,7-tetrahydro-5*H*-pyrazolo[4,3-*c*]pyridine-5-carboxylate (54a). A mixture of 2-bromo-5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazole (**12**) (2 g, 5.99 mmol), 1,1-dimethylethyl 2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridine-5-carboxylate (CAS#: 230301-11-8, commercially available from Bioblocks, 1.61 g, 7.19 mmol), copper(I) iodide (0.114 g, 0.599 mmol) and Cs_2CO_3 (3.91 g, 11.99 mmol) in DMF (20 mL) was stirred at 160ºC for 1 h under microwave irradiation then was cooled to room temperature and partitioned between water (400 mL) and EtOAc (400 mL). The two layers were separated and the organic phase was washed sequentially with water (200 mL) and brine (200 mL) then dried using a phase separator and concentrated *in vacuo*. Purification of the residue by flash chromatography on silica gel (c-Hexane/EtOAc: 7 to 20% gradient) gave 1,1-dimethylethyl 1-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-1,3a,4,6,7,7a-hexahydro-5H-pyrazolo[4,3-c]pyridine-5-carboxylate (**54a,** 607 mg, 21%) as a cream solid and 1,1-dimethylethyl 2-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridine-5-carboxylate (**53a**, 972 mg, 34%) as a white solid. Compound **54a**: LCMS (method formate): Retention time 1.54 min; $[M+H]^+$: calculated: 476; found 476.^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 7.87 (s, 1H), 7.65 (d, *J* = 8.4 Hz, 1H), 7.49 (s, 1H), 6.94 (d, *J* = 8.6 Hz, 1H), 4.58 (spt, *J* = 6.0 Hz, 1H), 4.40 (br s, 2H), 3.68 (br s, 2H), 3.20 (br s, 2H), 1.41 (s, 9H), 1.34 (d, *J* = 6.1 Hz, 6H).^{13}C NMR (151 MHz, $CDCl_3$) δ ppm 163.5 (s, 1C), 162.7 (s, 1C), 155.7 (s, 1C), 154.5 (s, 1C), 139.9 (s, 1C), 138.9 (s, 1C), 128.9 (s, 1C), 126.8 (s, 1C), 124.4 (s, 1C), 122.6 (s, 1C), 118.0 (s, 1C), 114.8 (s, 1C), 80.0 (s, 1C), 71.9 (s, 1C), 41.0 (s, 1C), 39.9 (s, 1C), 28.2 (s, 3C), 24.3 (s, 1C), 21.7 (s, 2C).Compound **53a**: LCMS (method formate): Retention time 1.51 min; $[M+H]^+$: calculated: 476; found 476.^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 8.19 (s, 1H), 7.95 (d, *J* = 2.0 Hz, 1H), 7.72 (dd, *J* = 8.5, 2.3 Hz,

1H), 7.00 (d, *J* = 8.6 Hz, 1H), 4.65 (spt, *J* = 6.1 Hz, 1H), 4.56 (s, 2H), 3.74 (br s, 2H), 2.84 (br s, 2H), 1.49 (s, 9H), 1.41 (d, *J* = 6.1 Hz, 6H).^{13}C NMR (151 MHz, $CDCl_3$) δ ppm 163.9 (s, 1C), 162.3 (s, 1C), 155.9 (s, 1C), 154.7 (s, 1C), 152.4 (br s, 1C), 129.2 (s, 1C), 126.9 (s, 1C), 124.7 (s, 1C), 123.4 (br s, 1C), 122.9 (s, 1C), 117.7 (br s, 1C), 114.9 (s, 1C), 80.3 (s, 1C), 72.1 (s, 1C), 41.3 (m, 1C), 40.0 (m, 1C), 28.4 (s, 3C), 23.8 (br s, 1C), 21.9 (s, 2C).

1,1-Dimethylethyl 2-(5-{3-cyano-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-2,4,6,7-tetrahydro-5*H*-pyrazolo[4,3-*c*]pyridine-5-carboxylate (53b). A mixture of 5-(5-bromo-1,3,4-thiadiazol-2-yl)-2-[(1-methylethyl)oxy]benzonitrile (**50**) (700 mg, 2.16 mmol), 1,1-dimethylethyl 2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridine-5-carboxylate (CAS#: 230301-11-8, commercially available from Bioblocks, 579 mg, 2.59 mmol), copper(I) iodide (41.1 mg, 0.216 mmol) and Cs_2CO_3 (1407 mg, 4.32 mmol) in DMF (14 mL) was stirred at 160ºC for 90 min under microwave irradiation then was cooled to room temperature and partitioned between water (150 mL) and EtOAc (150 mL). The two layers were separated and the aqueous phase was extracted with EtOAc (150 mL). The combined organic phases were dried using a phase separator and concentrated *in vacuo*. Purification of the residue by flash chromatography on silica gel (c-Hexane/EtOAc: 10 to 35% gradient) gave 1,1-dimethylethyl 2-(5-{3-cyano-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridine-5-carboxylate (**53b**) (315 mg, 31%) as a white solid. LCMS (method formate): Retention time 1.39 min; $[M+H]^+$: calculated: 467; found: 467.2.^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23 (s, 1H), 8.10 - 8.12 (m, 2H), 7.10 (d, *J* = 9.0 Hz, 1H), 4.75 - 4.80 (m, 1H), 4.70 (br s, 2H), 3.77 (br s, 2H), 2.89 (br s, 2H), 1.45 - 1.52 (m, 15H).

1,1-Dimethylethyl 3-acetyl-4-oxo-1-piperidinecarboxylate (55). A mixture of 1,1-dimethylethyl 4-oxo-1-piperidinecarboxylate (23.6 g, 118 mmol) and pyrrolidine (19.6 mL, 237

mmol) in toluene (30 mL) was stirred at 130°C under nitrogen using a Dean Stark trap to remove the water generated. After 5 h the reaction was allowed to cool to room temperature and concentrated *in vacuo* to give a yellow oil. This residue was dissolved in 1,4-dioxan (100 mL), and treated with acetic anhydride (24.6 mL, 261 mmol) and the resulting mixture was allowed to stand at room temperature under nitrogen for 16 h. The orange red solution was then treated with water (30.0 mL, 1.66 mol) and the resulting mixture was stirred at reflux under nitrogen for 3 h then was cooled to room temperature and concentrated *in vacuo* to approximately half of its initial volume. This solution was diluted with EtOAc and washed with water. The layers were separated and the organic phase was washed with a 5% HCl aqueous solution (20 mL), dried over $MgSO_4$ and concentrated *in vacuo* to give 1,1-dimethylethyl 3-acetyl-4-oxo-1-piperidinecarboxylate (**55**) (23.5 g, 82%) as a yellow oil which was used in the next step without further purification. LCMS (method formate): Retention time 0.99 min; $[M\text{-}Boc+H]^+$; calculated 142; found 142.^{1}H NMR (400 MHz, $CDCl_3$) δ 15.70 (s, 1H), 4.20 (br s, 2H), 3.59 (t, J = 5.9 Hz, 2H), 2.45 (t, J = 5.9 Hz, 2H), 2.14 (s, 3H), 1.49 (s, 9H) enol tautomer.

1,1-Dimethylethyl 2-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-3-methyl-2,4,6,7-tetrahydro-5*H*-pyrazolo[4,3-*c*]pyridine-5-carboxylate (56a). A suspension of 1,1-dimethylethyl 3-acetyl-4-oxo-1-piperidinecarboxylate (**55**) (19.6 g, 81 mmol) and 5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2(3H)-one hydrazone (**51a**) (23.1 g, 81 mmol) in EtOH (300 mL) was treated with AcOH (0.5 mL) and the resulting mixture was refluxed for 3 then was allowed to cool to room temperature over 40 min. The solid formed was filtered off and washed with EtOH to give 1,1-dimethylethyl 2-(5-{3-chloro-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-3-methyl-2,4,6,7-tetrahydro-5H-pyrazolo[4,3-c]pyridine-5-carboxylate (**56a**) (19.2 g, 48%) as grey solid. The filtrate was concentrated *in*

vacuo. Purification of the residue by flash chromatography on silica gel (750 g column, 0 to 30% EtOAc in cyclohexane) gave a 1:1 mixture (as assessed buy ^{1}H NMR) of regiosiomers (5.70 g, 14%) as colourless foam. This was dissolved in hot EtOH (100 mL) and cooled overnight, giving a pale pink crystalline solid with the same ratio of regioisomers (as assessed by ^{1}H NMR).LCMS (formate method): Retention time 1.58 min; $[M+H]^+$: calculated: 490, 492; found: 491.9 (1 Cl).^{1}H NMR (400 MHz, $CDCl_3$) δ 7.97 (d, *J* = 2.0 Hz, 1H), 7.76 (dd, *J* = 8.0, 2.0 Hz, 1H), 7.02 (d, *J* = 8.0 Hz, 1H), 4.65 - 4.71 (m, 1H), 4.46 (br s, 2H), 3.71 - 3.76 (m, 2H), 2.81 (br s, 2H), 2.70 (s, 3H), 1.43 - 1.50 (m, 15H).

1,1-Dimethylethyl 2-(5-{3-cyano-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-3-methyl-2,4,6,7-tetrahydro-5*H*-pyrazolo[4,3-*c*]pyridine-5-carboxylate (56b). A mixture of 5-(5-hydrazino-1,3,4-thiadiazol-2-yl)-2-[(1-methylethyl)oxy]benzonitrile (**51b**) (274 mg, 0.995 mmol) and 1,1-dimethylethyl 3-acetyl-4-oxo-1-piperidinecarboxylate (240 mg, 0.995 mmol) in N,N-dimethylacetamide (5 mL) was stirred at 150°C for 1 h under microwave irradiation then cooled to room temperature and concentrated *in vacuo*. Purification of the residue by flash chromatography on silica gel (c-hexane/EtOAc: 10 to 50% gradient) gave 1,1-dimethylethyl 2-(5-{3-cyano-4-[(1-methylethyl)oxy]phenyl}-1,3,4-thiadiazol-2-yl)-3-methyl-2,4,6,7-tetrahydro-5*H*-pyrazolo[4,3-*c*]pyridine-5-carboxylate (**56b**) (165 mg, 34%) as a white solid. LCMS (formate method): Retention time 1.42 min; $[M+H]^+$: calculated: 481; found: 481.9. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.11 - 8.13 (m, 2H), 7.12 (d, *J* = 8.8 Hz, 1H), 4.76 - 4.82 (m, 1H), 4.49 (br s, 2H), 3.73 - 3.80 (m, 2H), 2.83 - 2.85 (m, 2H), 2.73 (s, 3H), 1.54 (s, 9H), 1.49 (d, *J* = 6.0 Hz, 6H).

ASSOCIATED CONTENT:

Supporting Information

The supporting information (lymphocyte reduction for compounds 42-46 is available free of charge on the ACS Publications website at DOI:

AUTHOR INFORMATION

Corresponding Author

* Tel: + 44 1438 764319. Fax: + 44 1438 768302. E-mail: emmanuel.h.demont@gsk.com

Author Contributions

∫ These authors contributed equally to this work

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ABREVIATIONS: S1P, sphingosine 1-phosphate; PK, pharmacokinetics; PD, pharmacodynamic; THIQ, tetrahydroisoquinoline; PSA, polar surface area; CHI, chromatographic hydrophobicity index; CHROM, chromatographic; DMF, N,N-dimethylformamide; DBU, 1,8-Diazabicyclo[5.4.0]undec-7-ene. CL_b: blood clearance; LBF:

liver blood flow; Vss: volume of distribution at steady state; $T_{1/2}$: half life; $F_{p.o.}$: oral bioavailability

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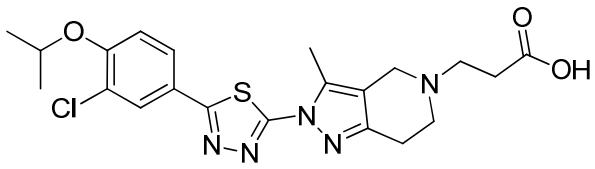
Table of Contents Graphic



43

$S1P_1$ pEC_{50} (β-arrestin) = 8.8
$S1P_3$ pEC_{50} (GTPγS) < 4.5
In vivo IC_{50} = 6 nM
rat Fp.o. = 52%