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Article

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Discovery of A-971432, an Orally Bioavailable Selective Sphingosine-1-Phosphate Receptor 5 ($S1P_5$) Agonists for the Potential Treatment of Neurodegenerative Disorders

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KEYWORDS Selective $S1P_5$ agonist, Neurodegenerative Disorders, A-971432

ABSTRACT $S1P_5$ is one of 5 receptors for sphingosine-1-phosphate and is highly expressed on endothelial cells within the blood-brain barrier, where it maintains barrier integrity in *in vitro* models.[1] Little more is known about the effects of $S1P_5$ modulation due to the absence of tool molecules with suitable selectivity and drug-like properties. We recently reported that molecule A-971432[2] (**29** in this paper) is highly efficacious in reversing lipid accumulation and age-related cognitive decline in rats.[3] Herein we describe the development of a series of selective $S1P_5$ agonists that led to the identification of compound **29**, which is highly selective for $S1P_5$ and has excellent plasma and CNS exposure after oral dosing in pre-clinical species. In order to further

support its suitability for *in vivo* studies of $S1P_5$ biology, we extensively characterized **29**, including confirmation of its selectivity in pharmacodynamic assays of $S1P_1$ and $S1P_3$ function in rats. In addition, we found that **29** improves blood-brain barrier integrity in an *in vitro* model and reverses age-related cognitive decline in mice. These results suggest that $S1P_5$ agonism is an innovative approach with potential benefit in neurodegenerative disorders involving lipid imbalance and/or compromised blood-brain-barrier, such as Alzheimer's disease or Multiple Sclerosis.

INTRODUCTION

The pleiotropic signaling factor sphingosine-1-phosphate is recognized by a family of 5 receptors called $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$.[4] Synthetic agonists of $S1P_1$ drive lymphocyte sequestration and immune suppression.[5] Most notably, the pro-drug FTY720 (Fingolimod) reduces relapse rate and lesion activity in multiple sclerosis, and it also results in cardiovascular events.[6] In addition to agonizing $S1P_1$, the phosphorylated form of FTY720 activates $S1P_3$, $S1P_4$ and $S1P_5$.[5] Many other chemotypes of $S1P_1$ agonists interact with multiple S1P receptors, especially $S1P_5$.[7] $S1P_5$ is most highly expressed in the central nervous system, particularly on oligodendrocytes and brain endothelium.[1,8] Activation of $S1P_5$ on brain endothelial cells enhances barrier integrity and reduces transendothelial migration of monocytes *in vitro*.[1] There is indirect evidence that some of the efficacy of Gilenya in models of multiple sclerosis is from a direct, neuroprotective effect within the CNS.[9] Based on the findings above, we hypothesized that a selective $S1P_5$ agonist may be efficacious for neurodegenerative disorders. If so, the lack of $S1P_1$-driven immune suppression and cardiovascular effects would be advantageous. There are previous reports of selective $S1P_5$ agonists,[10] but our profiling indicates these have insufficient pharmacodynamic properties to establish $S1P_5$ target validation *in vivo* (see Results section). Herein we describe the optimization of the first $S1P_5$ agonists with the selectivity and drug-like properties to interrogate $S1P_5$ biology *in vitro* and *in vivo*.

RESULTS AND DISCUSSION

To test the hypothesis that a selective $S1P_5$ agonist would be efficacious for neurodegenerative disorders it would require a compound with the requisite potency as an $S1P_5$ agonist, selectivity profile, primarily at the other S1P family receptors and PK properties to enable once daily oral dosing. However, at the offset of this project there were no such selective $S1P_5$ agonists reported in the literature and so a medicinal chemistry program was initiated to identify such a compound to enable POC studies. Cognizant of Sir James Black's famous comment that the best way to find a new drug is to start with an existing one, we decided to leverage the experience from our sphingosine-1 phosphate receptor agonist program. This was a very successful collaboration with the University of Virginia based on the $S1P_1$ chemical matter discovered by Macdonald et al at UVA.[11] As part of this project we identified[12] cyclopentyl amino alcohol compound **1**. This compound is phosphorylated on the hydroxyl of the amino alcohol *in vivo* affording a highly potent $S1P_1$ receptor agonist **2**. This meant that to enable *in vitro* SAR in this drug series the amino alcohols had to be phosphorylated in an additional synthetic step prior to screening. To facilitate a more rapid screening cascade a replacement for the amino alcohol "head group" was therefore desired.

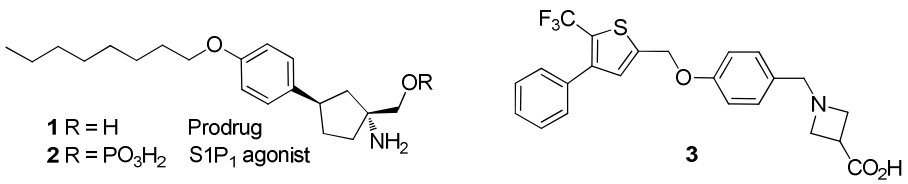
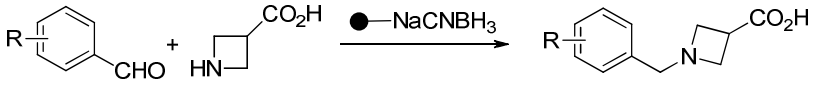


Figure 1. Structures of literature $S1P_1$ agonists

Scientists at Merck[13] had elegantly shown that azetidine-3-carboxylic acid was a suitable direct agonist surrogate for amino alcohol-based prodrugs such as those reported previously. Incorporation of the azetidine onto a high-throughput screening hit afforded selective and orally bioavailable $S1P_1$ r*e*ceptor agonist **3**. Not only would the azetidine carboxylic acid afford compounds that could be screened directly in our *in vitro* assays, it was ideal for exploration via parallel synthesis. It was envisaged that libraries would be constructed from a reductive amination of the azetidine and substituted benzaldehydes in the presence of polymer supported sodium cyanoborohydride in the centralized High Throughput Chemistry group at AbbVie.

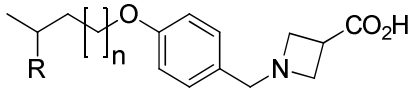
Scheme 1. Reductive amination of an aldehyde with azetidine-3-carboxylic acid using polymer supported $NaCNBH_3$



Prior to library synthesis both a) the robustness of the reductive amination procedure and b) the tolerance for replacement of the cyclopentyl amino alcohol with azetidine carboxylic acid needed to be confirmed. From the ongoing $S1P_1$ program data showed that the alkyloxy tail of compound **2** drove selectivity between $S1P_1$and $S1P_3$. Based on this observation commercially available benzaldehydes with a para alkyl chain of varying length were sourced through Aldrich Market Select[14] to test the reductive amination step. Aldehydes were selected that had both carbon and ether group attachment of the chain to the aryl ring. Reductive amination of the aldehyde with azetidine-3-carboxylic acid was performed using polymer supported $NaCNBH_3$ with the target compounds purified using mass-directed HPLC.[15] To confirm the tolerance for the cyclopentyl amino alcohol replacement with azetidine carboxylic acid, 4-(octyloxy)benzaldehyde was included in this study as this would afford the direct crossover analogue, compound **6**.

This approach generated extensive chemical matter, some of which had failed to progress in the $S1P_1$ project due to a lack of potency on $S1P_1$ and/or selectivity against $S1P_3$ due to its implication in bradycardia.[16] Extensive data mining identified approximately 500 $S1P_1$ project compounds that had failed these potency and/or selectivity criteria. This set was screened in an $S1P_5$ radio-ligand binding assay to identify $S1P_5$-selective agents. This screen, along with existing $S1P_1$ and $S1P_3$ selectivity data, afforded a great launching point for further optimization shown in Table 1.

Table 1. SAR and selectivity of compounds 4 - 10



Compound	n	R	$S1P_1$ RLB IC_{50} (μM)	$S1P_3$ RLB IC_{50} (μM)	$S1P_5$ RLB IC_{50} (μM)	$S1P_5/S1P_1$ selectivity
1	-	-	>30	>10		-
2	-	-	<0.001	0.003	<0.001	1
4	7	H	0.018	0.040	-	-
5	6	H	0.005	0.023	0.001	5
6	5	H	0.002	0.197	0.005	0.4
7	4	H	0.048	5.29	0.009	5
8	3	H	1.18	>10	0.009	>100
9	2	H	3.03	-	0.010	>300
10	1	CH_3	3.46	>10	0.003	>1000

The compounds were screened in assays for $S1P_1$, $S1P_3$ and $S1P_5$ as a representative set of the 5 sphingosine receptors and this identified some striking SAR. Compound **1** clearly demonstrated the requirement for phosphorylation of the amino alcohol for activity with the compound being inactive at both $S1P_1$ and $S1P_3$. In stark contrast, phosphorylated compound **2** was shown to be extremely potent at $S1P_1$, $S1P_3$ and $S1P_5$. Compounds **4** and **5** with decyl and nonyl alkyl chains respectively were equipotent at both $S1P_1$ and $S1P_3$, with compound **5** also being potent at $S1P_5$. Compound **6** is the azetidine analogue of compound **1** with an octyl alkyl chain and maintained a high level of potency at $S1P_1$ and $S1P_5$ but was more than 40 fold selective over $S1P_3$ versus the other 2 S1P receptors tested. This trend continued with compound **7** with a heptyl alkyl chain. This compound maintained its potency at both $S1P_1$ and $S1P_5$ but was now >500-fold selective versus $S1P_3$. Of particular note was compound **8** with a hexyl alkyl chain. This compound maintained a high level of potency against $S1P_5$ but now >1000 fold selective versus $S1P_3$ and had introduced a selectivity window of around 130 versus $S1P_1$. Based on the encouraging data with compound **8**, compound **9** with a pentyl alkyl chain was only screened against $S1P_1$ and $S1P_5$. The compound again maintained a high level of potency against $S1P_5$ but now the selectivity versus $S1P_1$ was > 300 fold. Compound **10** demonstrated that substitution on the alkyl chain was tolerated with the compound maintaining a high potency at $S1P_5$ with an increased selectivity versus $S1P_1$ of >1000. These compounds were determined to be full agonists in a GTPγS activation assay, using $S1P_5$-transfected HEK cell membranes. EC_{50} values were similar between GTPγS and radio-ligand binding formats, and so compound optimization was driven by binding data, with occasional confirmation in the GTPγS assay.

These initial compounds designed around compound **1** had identified a critical structural link to selectivity amongst the S1P family of receptors with an alkyl chain length of 5 or 6 optimal for both $S1P_5$ potency and selectivity. Having identified a selectivity pharmacophore for $S1P_5$, this information was used to design follow up libraries.

As there was no structural information available on the $S1P_5$ receptor to drive compound design it was planned to use parallel synthesis to generate a pharmacophore of the target. To enable this compound **6** was divided into 4 regions shown in Figure 2. Libraries would then be designed to sequentially interrogate these regions using parallel synthesis. Using the SAR from these

libraries compounds combining the best structural motifs would then be designed and synthesized by dedicated synthesis.

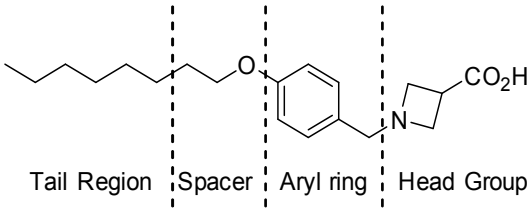
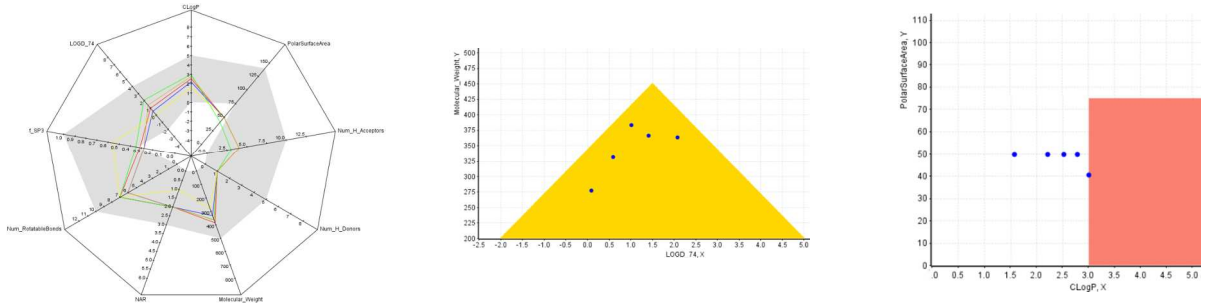


Figure 2. Regions of Compound 6 selected for SAR investigation

Three main criteria were used for selection of the monomers for the libraries. The first was their commercial availability from Aldrich Market Select. Secondly the choice of monomers was based on the Topliss Tree[17]. Monomers were selected to interrogate the effect of both electronics (halogen, methoxy) and steric constraints (methyl, ethyl) on both $S1P_5$ binding and selectivity across the S1P family of receptors. The final criteria was the calculated physicochemical properties of the resulting compounds based on medicinal chemistry guidelines like drug-like properties,[18] Golden Triangle[19] and 3/75.[20]



(a) Drug-like (b) Golden Triangle (c) 3/75 Rule

Figure 4. Representative examples from each library design showing (a) excellent calculated physicochemical properties (b) good projected clearance and oral absorption and (c) predicted reduced risk of toxicity

For clarity only a single representative of each library is shown in Figure 4. From the radar plot visualization it is clearly evident that all the compounds have excellent predicted drug-like properties. The radar plot data is also shown in tabular form below (Table 2). All the compounds were also predicted to have both good clearance and oral absorption using the Golden Triangle. In addition the compounds all have calculated PSA and cLogP that suggest a reduced risk of toxicity based on the 3/75 rule.

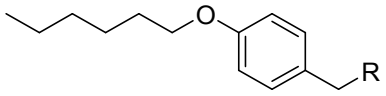
Table 2. Calculated physicochemical properties of compounds 10, 29, 31, 43 and 60

Compound	cLogP	PSA	Mol wt	HBD	HBA	NAR	NRB	FSP_3
10	1.6	50	277.4	1	4	1	7	0.56
29	2.8	50	366.2	1	4	2	6	0.28

31	2.2	50	331.8	1	4	2	6	0.28
43	3.0	41	363.4	1	3	2	7	0.35
60	2.5	50	383.3	1	4	2	7	0.32

The first library was designed to explore the head group region of compound **6**. As lead compound **1** contained the larger cyclopentyl ring, analogues with both pyrrolidine and piperidine replacements of azetidine were considered. It was also recognized that the larger piperidine ring afforded multiple opportunities to vary the carboxylic acid group orientation and analogues exploring this included in the library.

Table 3. SAR and selectivity of compounds 15 - 21



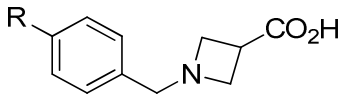
Compound	R	$S1P_5$ RLB IC_{50} (μM)
11		0.075
12		0.026
13		0.125
14		1.210
15		0.016
16		0.028

Racemic pyrrolidine-3-carboxylic acid analogue **11** suggested that ring expansion of the azetidine ring was tolerated while the (R)-isomer **12** revealed important information on the desired stereochemistry of the carboxylic acid group. This was further exemplified with the piperidine-3-carboxylic acid analogues **13** and **14**, the (R)-isomer being more than 10 times more potent than the (S). Compounds incorporating piperidine-4-carboxylic acid afforded the most potent analogues. Compound **15** had an IC_{50} of 16 nM and substituted piperidine **16** only slightly less potent.

Next a library was designed to expand the SAR of the spacer and tail regions of compound **6**. The library interrogated the steric limits by reducing the length of the spacer region between the tail and the aryl ring proximal to the azetidine-3-carboxylic acid. These compounds would afford more conformationally restricted analogues that might have improved oral

bioavailability.[21] A series of phenyl and phenoxy benzaldehydes was sourced again though Aldrich Market Select and reacted with azetidine carboxylic acid.

Table 4. SAR and selectivity of compounds 17 - 22

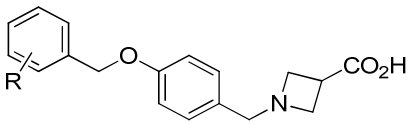


Compound	R	$S1P_1$ RLB IC_{50} (μM)	$S1P_3$ RLB IC_{50} (μM)	$S1P_5$ RLB IC_{50} (μM)	$S1P_5/S1P_1$ selectivity
17	3,4-DiClPh	4.41		0.023	200
18	4-MePh	7.21	>10	0.007	>1000
19	4-EtPh	0.656	>10	0.003	>200
20	3-CF_3Ph	>10	-	0.409	>24
21	3,5-DiClPh	9.81	>10	0.148	66
22	4-ClPhO	>10	-	0.789	>12

Compounds **17**, **18** and **19** with either a 4-substituted or 3,4-disubstituted aryl ring directly attached to the 1-benzylazetidine-3-carboxylic acid were potent at $S1P_5$ with at least a 200-fold window over $S1P_1$. Surprisingly the potency at $S1P_5$ for compound **20** with a trifluoromethyl substituent in the 3-position was greatly reduced. This also resulted in much lower selectivity against $S1P_1$. Compound **21** was included to vary the substitution pattern around the aryl ring and while tolerated resulted in a large reduction in potency at $S1P_5$. Introduction of an oxygen atom linker between the 2 aryl rings was not so promising. Compound **22** based on a biphenyl ether had greatly reduced potency at $S1P_5$.

The previous library established that both straight alkyl chains, like in compound **6**, and more sterically demanding groups, like aryl in compound **18**, were tolerated off the para-position of 1-benzylazetidine-3-carboxylic acid. As a result a follow up library was planned that combined these SAR observations. Incorporating the key observation from the compounds in Table 1 with regard to overall chain length and $S1P_5$ selectivity, it was recognized that the total chain length must be maintained at 6 to 7 atoms. Consequently a library of benzyl ethers was designed and synthesized.

Table 5. SAR and selectivity of compounds 23 - 29



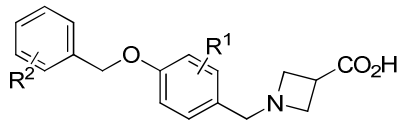
Compound	R	$S1P_1$ RLB IC_{50} (μM)	$S1P_3$ RLB IC_{50} (μM)	$S1P_5$ RLB IC_{50} (μM)	$S1P_5/S1P_1$ selectivity
23	H	>10	>10	0.077	>100
24	2-Me	5.26	-	0.035	151

25	3-Cl	1.78	-	0.010	174
26	3-CF_3	1.06	-	0.004	248
27	4-F	-	-	0.009	-
28	2,4-Cl	0.835	-	0.004	225
29	3,4-Cl	0.362	>10	0.006	60

It was pleasing that compound **23**, the unsubstituted analogue, was as expected potent at $S1P_5$ while being more than a 100-fold selective against both $S1P_1$ and $S1P_3$. Compounds **24**, **25**, **26** and **27** having a mono-substituted aryl ring all had improved potency at $S1P_5$ and increased selectivity versus $S1P_1$ (for **27** $S1P_1$ data was not generated). Similarly the 2,4-dichloro analogue **28** had both impressive $S1P_5$ potency and selectivity versus $S1P_1$. Compound **29** with a 3,4-dicloro substitution motif displayed a very interesting profile. The compound was essentially inactive at $S1P_3$ while having more than a 50 fold window over $S1P_1$.

Suitable benzaldehydes to examine substitution in both the tail and aryl regions simultaneously were then considered. However, the commercial availability of such suitably substituted benzaldehydes was very limited and only a few analogues were secured.

Table 6. SAR and selectivity of compounds 30 - 34



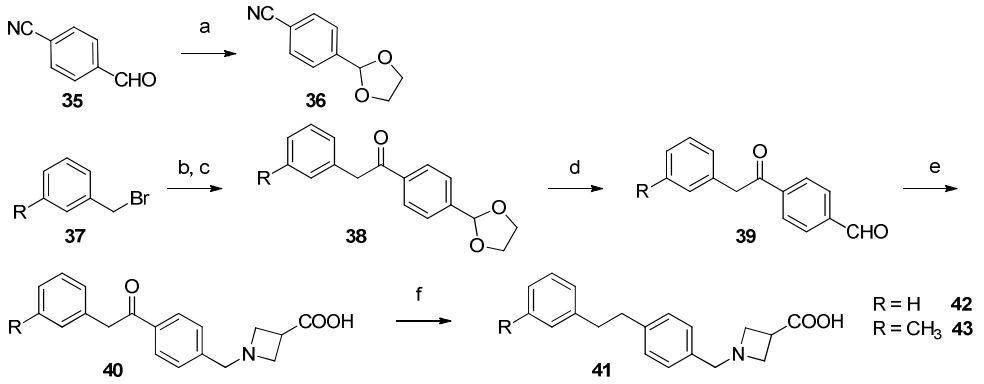
Compound	R^1	R^2	$S1P_1$ RLB IC_{50} (μM)	$S1P_5$ RLB IC_{50} (μM)	$S1P_5/S1P_1$ selectivity
30	2-Me	H	4.17	0.008	>500
31	2-Cl	H	4.23	0.006	>900
32	3-OMe	2,4-Cl	0.12	0.009	13
33	3-NO_2	3,4-Cl	0.22	0.005	44
34	3-OMe	2-Cl	6.23	0.115	54

Compounds **30**, **31**, **32**, **33** and **34** displayed good to excellent potency at $S1P_5$ with a wide selectivity window over $S1P_1$ ranging from 10 to >900 fold. However, with the limited commercial availability of suitable benzaldehydes to enable an extensive parallel synthetic approach it was difficult to draw any meaningful SAR information from these analogues. To afford the SAR from substitution of both aryl rings dedicated synthesis of specific examples was deemed necessary and pursued later in the project (Table 9)

For exploration of the spacer region of compound **6** no suitable benzaldehydes were available from commercial sources and so dedicated synthesis of the key compounds would need to be conducted. The first structural modification to be considered in the spacer region was replacement of the oxygen in the linker between the two aryl rings with carbon. Such a replacement was known to be tolerated for our $S1P_1$ agonists and so it was considered likely it

would also be at least tolerated here. Protection of 4-cyano benzaldehyde **35** with ethylene glycol afforded the dioxolane **36** which underwent a Grignard reaction with commercially available benzyl bromide **37** to yield advanced intermediate **38**. Subsequent deprotection of the dioxolane under acidic conditions afforded the free aldehyde **39** which was reacted with azetidine carboxylic acid under the standard conditions with sodium cyano borohydride. Hydrogenation of ketone **40** in the presence of palladium on carbon gave the desired compounds **41**.

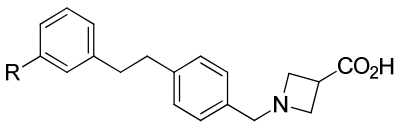
Scheme 2. Synthetic route toward 1,2-diphenylethane analogues 42 and 43



Reagents and conditions: (a) ethane-1,2-diol, PTSA, PhMe; (b) Mg, I_2, Et_2O; (c) **36**, Et_2O; (d) 10% HCl, THF, reflux; (e) azetidine-carboxylic acid, $NaCNBH_3$, MeOH; (f) 1% H_2SO_4/EtOH, Pd/C, H_2.

Surprisingly while compound **42** with the unsubstituted aryl ring to the azetidine head group maintained good potency at $S1P_5$ it was now equipotent at $S1P_1$. This was in contrast to compound **23** which while having a similar potency at $S1P_5$ displayed a selectivity window versus $S1P_1$ of >100-fold. Pleasingly introduction of a meta-electron withdrawing group on the distal aryl ring again established a high selectivity versus $S1P_1$, compound **43** with R = CF_3 being over 250-fold selective.

Table 7. SAR and selectivity of compounds 42 and 43

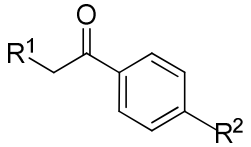


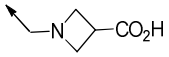
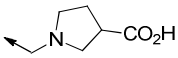
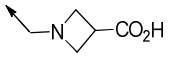
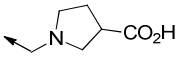
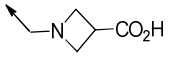
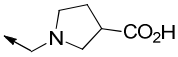
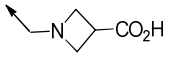
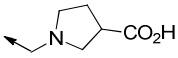
Compound	R	$S1P_1$ RLB IC_{50} (μM)	$S1P_5$ RLB IC_{50} (μM)	$S1P_5/S1P_1$ selectivity
42	H	0.002	0.016	1
43	CF_3	0.221	0.001	260

Having established the synthetic route to compound **40**, which contains a ketone in the linker region, additional ketone containing analogues were pursued. As ketones, particularly aryl ketones, are not considered drug-like molecules this would provide useful internal data around this functional group. The key aldehyde intermediates **39** were synthesized in sufficient quantity

to enable analogues with both azetidine-3-carboxylic acid and pyrrolidine-3-carboxylic acid to be secured.

Table 8. SAR and selectivity of compounds 44 - 51



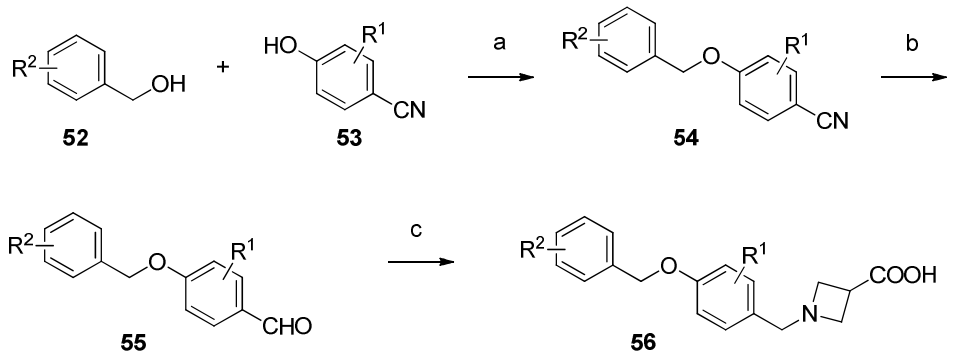
Compound	R^1	R^2	$S1P_5$ MFB IC_{50} (μM)
44	Butyl		0.002
45	Butyl		0.013
46	Pentyl		0.007
47	Pentyl		0.005
48	Ph		0.032
49	Ph		0.277
50	3,4-DiClPh		0.008
51	3,4-DiClPh		0.008

For analogues **44** and **46** with the alkyl chain introduction of the carbonyl moiety had little impact compared to the corresponding analogues with the oxygen in the linker **8** and **7** respectively. Compounds **45** and **47** also demonstrated that increasing the ring size of the amino acid had little effect on potency. The SAR was more striking with the analogues containing an aryl ring off the linker. Compound **48** was slightly less potent than the corresponding des-carbonyl compound **42** whereas compound **49** with the larger pyrrolidine-3-carboxylic acid was >15-fold less potent. Varying the size of the ring of the amino acid head group had little impact on either of the 3,4-dichloro substituted compounds **50** and **51** which both showed similar potency to the corresponding ether linked compound **32**. Such SAR suggests that investigation of the amino acid head group and other structural modifications would need to be done in parallel to identify the optimal design.

The expeditious use of parallel synthesis had furnished a large amount of structural information that drove both $S1P_5$ binding and selectivity across the S1P family of receptors. However, this information was generated in the 4 discreet regions of compound **6** that had been used to enable library design. The final set of compounds was designed by combining this SAR on to the benzyl ether scaffold of compound **23**.

To explore simultaneous substitution on both aryl rings of the benzyl ether scaffold a synthesis was designed starting from the appropriately substituted benzyl alcohols **52** and 4-cyano phenols **53**. These components could be coupled efficiently using a Mitsunobu reaction. The resulting cyano compounds **54** were reduced to the desired aldehydes **55** using DIBAL. Introduction of the azetidine-3-carboxylic acid head group was effected under the standard conditions to afford the desired compounds **56**.

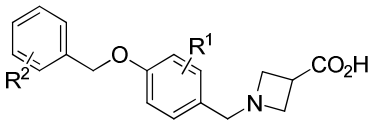
Scheme 3. Synthetic route toward (benzyloxy)benzene analogues 57 - 63



Reagents and conditions: (a) Ph_3P, DIAD, THF; (b) DIBAL, PhMe; (c) azetidine-3-carboxylic acid, $NaCNBH_3$, MeOH.

Compound **57** suggested that substitution on the aryl ring proximal to the azetidine carboxylic acid may drive selectivity over $S1P_1$ while having a slightly negative affect on $S1P_5$ potency. Disappointingly introduction of halogens to both aryl rings furnished flat SAR. 3,4-Dichlorophenyl containing compounds **58** and **59** had demonstrated essentially the same profile as the des-fluoro parent compound **29**. In the 3-trifluoromethylphenyl containing compounds it was a similar story with fluoro-substituted compounds **60** and **61** and chloro-substituted compounds **62** and **63** showing little differentiation from the des-halo parent compound **26**. While introduction of a halogen had not greatly improved potency or selectivity it was pleasing to see that its effect on the cLogP of the compounds was not too detrimental. As a result halogen incorporation was identified as a strategy to block possible sites of metabolism on the proximal to the amino acid head group should this be required later in development.

Table 9. SAR and selectivity of compounds 26, 29, 57 - 63

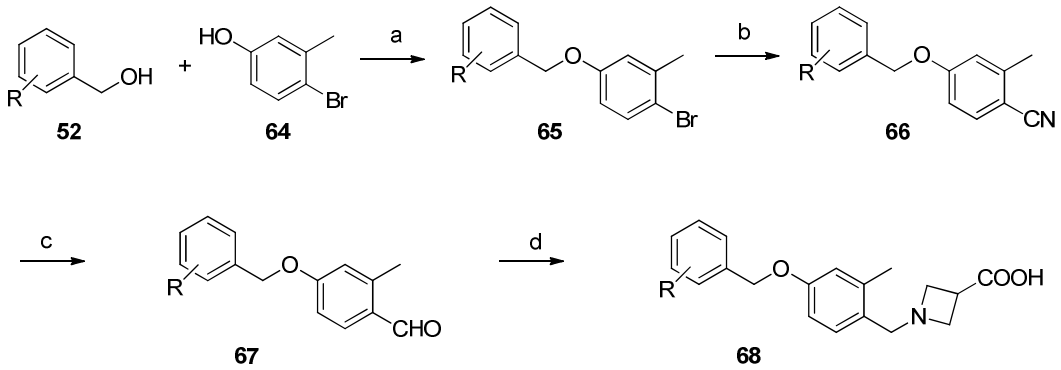


Compound	R^1	R^2	$S1P_1$ MFB IC_{50} (μM)	$S1P_5$ RLB IC_{50} (μM)	$S1P_5/S1P_1$ selectivity	cLogP
29	H	3,4-Cl	0.362	0.006	60	2.8
57	3-F	H	>10	0.041	244	1.6
58	2-F	3,4-Cl	0.472	0.005	90	3.1
59	3-F	3,4-Cl	0.217	0.005	46	3.1

26	H	3-CF_3	1.06	0.004	248	2.2
60	2-F	3-CF_3	0.68	0.002	340	2.5
61	3-F	3-CF_3	0.401	0.002	250	2.5
62	2-Cl	3-CF_3	0.256	0.002	130	3.1
63	3-Cl	3-CF_3	0.109	0.001	160	3.1

Due to the limited commercial availability of alkyl substituted 4-hydroxy phenols an alternative synthetic route was required to investigate methyl analogues of compound **23**. Mitsunobu reaction of appropriately substituted benzyl alcohols **52** with 4-bromo-3-methylphenol **64** gave key bromo intermediates **65** which were subsequently converted to compounds **66** by treatment with copper(I) cyanide. Treatment with DIBAL gave aldehydes **67** which were reacted with azetidine-3-carboxylic acid under the usual conditions to afford target compounds **68**.

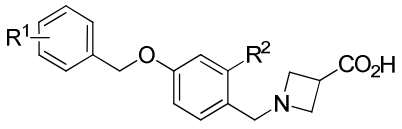
Scheme 4. Synthetic route toward substituted 1-(4-(benzyloxy)-2-methylbenzyl)azetidine-3-carboxylic acids 69 and 70



Reagents and conditions: (a) Ph_3P, DIAD, THF; (b) CuCN, NMP; (c) DIBAL, PhMe; (d) azetidine-3-carboxylic acid, $NaCNBH_3$, MeOH.

Introduction of the methyl substituent resulted in dramatic SAR. While both compounds **69** and **70** were observed to be more potent at $S1P_1$ than their central aryl ring des-methyl analogues **26** and **29** respectively, they were significantly more potent at $S1P_5$. This increased selectivity versus $S1P_1$ is also apparent when considering compound **30**. Compound **30** has an unsubstituted distal aryl ring and the 2-methyl substituent on the central aryl ring as in **69** and **70**. SAR from this set of compounds suggests the importance of substitution on the central aryl ring for driving selectivity while substitution on the distal aryl ring drives potency, at both $S1P_1$ and $S1P_5$.

Table 10. SAR and selectivity of compounds 26, 29, 30, 69 and 70

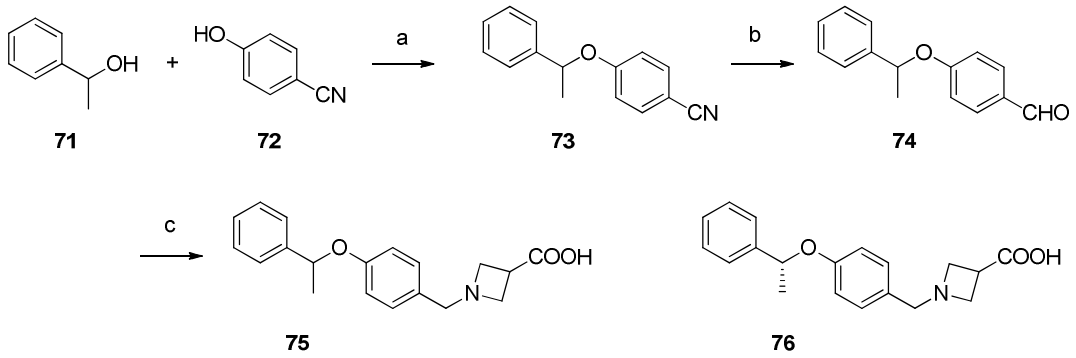


Compound	R^1	R^2	$S1P_1$ MFB IC_{50} (μM)	$S1P_5$ RLB IC_{50} (μM)	$S1P_5/S1P_1$ selectivity

26	3-CF_3	H	1.06	0.004	248
29	3,4-Cl	H	0.362	0.006	60
30	H	Me	4.17	0.008	>500
69	3-CF_3	Me	0.333	0.001	400
70	3,4-Cl	Me	0.166	0.001	150

To complete the SAR around the spacer region of compound **6** substitution from the benzylic carbon was explored. Introduction of a methyl was chosen as this would greatly affect the conformation of the resulting compound and hence alter the trajectory of the tail aryl ring relative to the head group. The synthesis was initially conducted with 1-phenylethanol **71** using the typical 3-step procedure. A Mitsunobu reaction with 4-cyanophenol **72** afforded compound **73** which was then treated with DIBAL to furnish aldehyde **74**. Subsequent reaction with azetidine-3-carboxylic acid gave the desired racemic compound **75**. The enantiomerically pure (R)-isomer **76** was secured using the same synthetic sequence, but using (S)-1-phenylethanol.

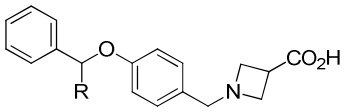
Scheme 5. Synthetic route toward substituted 1-(4-(benzyloxy)-2-methylbenzyl)azetidine-3-carboxylic acids 75 and 76



Reagents and conditions: Ph_3P, DIAD, THF; (b) DIBAL, PhMe; (c) azetidine-3-carboxylic acid, $NaCNBH_3$, MeOH.

Unfortunately substitution off the benzylic carbon was at best tolerated and is likely detrimental. Both the racemate **75** and pure enantiomer **76** had a reduced selectivity window versus $S1P_1$ and neither was more potent than the H analogue **23**.

Table 11. SAR and selectivity of compounds 23, 75 and 76



Compound	R	$S1P_1$ RLB IC_{50} (μM)	$S1P_5$ RLB IC_{50} (μM)	$S1P_5/S1P_1$ selectivity
23	H	>10	0.077	>100
75	Racemic Me	>10	0.255	>39

76	(R)-Me	3.87	0.108	35

The project goal was to identify a compound with the requisite potency as an $S1P_5$ agonist, selectivity profile, primarily at the other S1P family receptors and PK properties to enable once daily oral dosing. The expeditious use of parallel synthesis in combination with the dedicated synthesis of key singletons had facilitated extensive *in vitro* profiling which enabled the construction of a comprehensive pharmacophore around both $S1P_5$ potency and selectivity S1P family of receptors. To permit the additional *in vivo* profiling larger compound inventories would be necessary. Due to project resource constraints it was only possible to scale up a limited number of analogues and so 5 compounds were selected. The compound selection for additional profiling was based on the *in vitro* data using potency at $S1P_5$ and selectivity versus $S1P_1$ with examples selected to ensure the broadest coverage of chemical matter. The compounds selected for scale up are shown in Figure 3: compound **10** with an alkyloxy chain, compound **29** with a substituted distal aryl ring, compound **31** with a substituted central aryl ring, compound **43** with an ethyl linker and compound **60** with substitution on both aryl rings. It should be noted that in addition to this series with the azetidine carboxylic acid head group, three additional compound series of $S1P_5$ selective antagonists were identified and are currently being developed.[22]

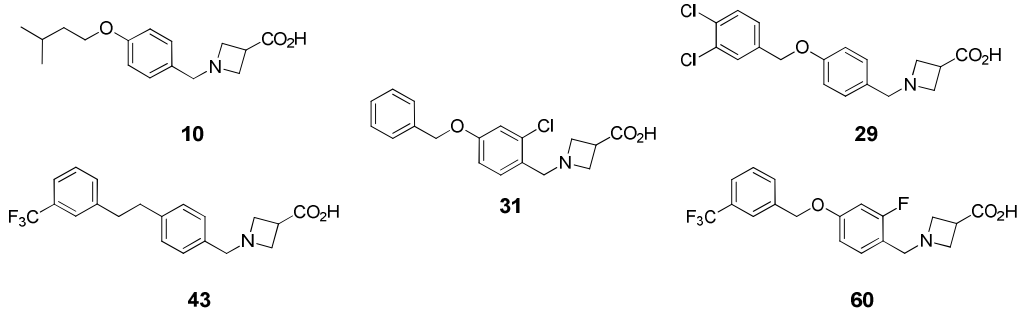


Figure 3. Structures of 5 compounds selected for scale up to enable additional profiling

The 5 compounds were profiled in a high-throughput ADME panel to assess solubility, plasma protein binding, permeability and inhibition of CYP enzymes. All the compounds had good solubility and free fraction. Of note was compound **10** with the branched alkyl chain, that exhibited very low level of plasma protein binding. Despite the incorporation of the azetidine-3-carboxylic acid head group, the compounds displayed reasonable permeability as assessed by the PAMPA assay. Pleasingly none of the compounds were identified as inhibitors of the 4 most common CYP enzymes. All the compounds were then tested in the micronucleus assay and found to be non-clastogenic.

Table 12. High-throughput ADME data for compounds 10, 29, 31, 43 and 60

Compound	Solubility (μM)	PPB (% bound)	PAMPA (cms^{-1})	Inhibition (μM)			
				Cyp1A2	Cyp2C9	Cyp2D6	Cyp3A4
10	46	37	3.2	>20	>20	>20	>20
29	70	95	2.7	>20	>20	>20	>20

31	61	86	4.2	>20	>20	>20	>20
43	73	88	5.3	>20	>20	>20	>20
60	70	87	9.4	16.7	>20	>20	>20

Screening PK was then conducted with the compounds being dosed at 10 mpk po in female Balb/C mice (**29** was tested in CD1 mice). As predicted by the physicochemical properties, all the compounds displayed excellent PK characteristics. The compounds exhibited good $t_{1/2}$ ranging from 3.3 to 5.7 hours, with large maximum concentrations achieved in an hour or less. Most notable was the very large AUC observed with heptanone analogue **43** of 127,000 ng*h/mL.

Table 13. Mouse PK for compounds 10, 29, 31, 43 and 60

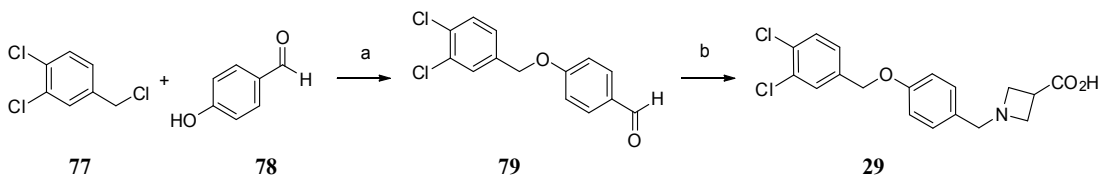
Compound	$t_{1/2}$ (hr)	C_{max} (ng/mL)	T_{max} (ng/mL)	AUC (ng*h/mL)
10	3.3	11,800	0.4	17,300
29*	5.7	2,500	-	35,000
31	4.1	4,230	1.3	16,800
43	4.9	17,900	0.83	127,000
60	4.6	6,480	1.0	37,700

* **29** was tested in CD1 mice

The comprehensive data package generated for compounds **10**, **29**, **31**, **43** and **60** showed them all to be excellent candidates for *in vivo* studies. All the compounds were potent $S1P_5$ agonists and selective across the S1P family of receptors. No issues had been identified from the HT-ADME screen and pleasingly all the compounds had excellent solubility. The PK profiles of the compounds were similarly all suitable for advancing to the planned *in vivo* studies. From these 5 compounds it was decided to advance compound **29** for large-scale synthesis to enable further characterization. The selection of compound **29** was based on it having the longest $t_{1/2}$ which it was thought would be the most appropriate for future studies. It should be noted however that all 5 compounds have profiles suitable for advancing *in vivo*.

While 4-((3,4-dichlorobenzyl)oxy)benzaldehyde **79** was commercially available due to the high cost it was synthesized from more readily available starting materials. A mixture of 3,4-dichlorobenzyl chloride **77**, 4-hydroxybenzaldehyde **78**, potassium carbonate and acetone was refluxed overnight to afford 4-(3, 4-dichlorobenzyloxy)benzaldehyde **79** in 79% yield after filtration and washing. Reaction with azetidine-3-carboxylic acid proceeded smoothly in the presence of sodium cyanoborohydride in methanol at room temperature. The product **29** was again collected by filtration in 60% yield.[2]

Scheme 6. Large Scale Synthetic Route for 1-(4-((3,4-dichlorobenzyl)oxy)benzyl)azetidine-3-carboxylic acid 29



77 78 79 29

Reagents and conditions: (a) K_2CO_3, acetone, reflux; (b) Azetidine-3-carboxylic acid, $NaCNBH_3$, MeOH.

Potency and selectivity SAR was developed with radio-ligand binding assays. To confirm $S1P_5$ agonism, **29** was profiled in two *in vitro* functional assays. FTY720-phosphate was used to define 100% agonism, as it gave more consistent results than the lipid S1P. In a GTPγS assay using membranes from $S1P_5$-transfected HEK cells, **29** induced full agonism and had an EC_{50} = 5.7 nM (Figure 5a). In a forskolin-induced cAMP inhibition assay in $S1P_5$-transfected CHO cells, **29** was also a full agonist, and had an EC_{50} = 4.1 nM (Figure 5b). By comparison, FTY720-phosphate had an EC_{50} of 3.4 and 11 nM in the GTPγS and cAMP assays, respectively.

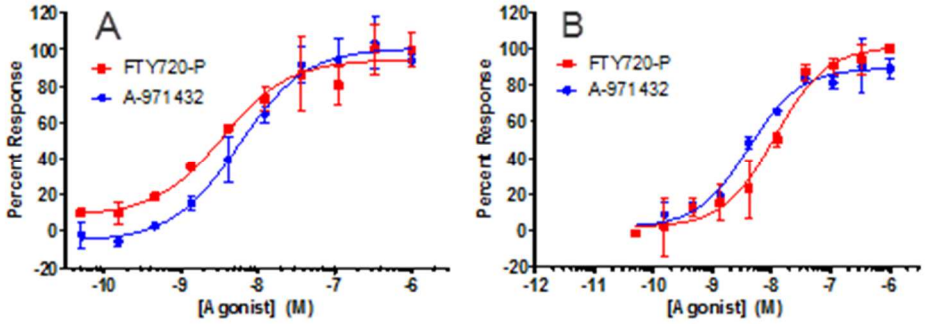


Figure 5. ***In vitro*** **functional $S1P_5$ assays. (a) GTPγS binding in membranes from $S1P_5$-transfected HEK cells. (b) Inhibition of forskolin-induced cAMP in $S1P_5$-transfected CHO cells.**

Compound **29** was profiled in receptor binding assays against the other S1P receptors and found to have no measurable binding up to 10 μM on $S1P_2$, $S1P_3$, and $S1P_4$ (data not shown). At $S1P_1$, **29** has weak potency (0.36 μM). To address agonism versus antagonism at $S1P_1$, we first used an $S1P_1$ GTPγS assay to demonstrate a complete lack of $S1P_1$ activation up to 10 μM **29** (data not shown). We then determined **29** to be a competitive antagonist at $S1P_1$ with a K_B = 0.4 μM in the same system (Supporting information). Lastly, we confirmed a lack of $S1P_1$ activity by dosing **29** in Sprague-Dawley rats and monitoring lymphopenia. We found no change in lymphocyte counts at 5 hours after a single oral dose of 3, 10, 30, or 100 mg/kg (Figure **6**). Plasma exposure was dose-proportional and attained 15,000 ng/ml at the highest dose. These data clearly show **29** is devoid of $S1P_1$ agonism up to the highest doses tested.

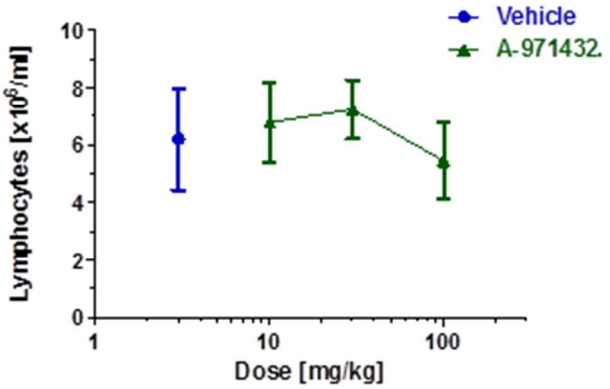


Figure 6. Selectivity of 29 against $S1P_1$ is confirmed by a lack of lymphopenia. Sprague-Dawley rats were administered 10, 30, or 100 mg/kg of 29, or vehicle (0.5% HPMC/0.02% Tween 80) by oral gavage. Five hours later, blood was collected via cardiac puncture under isoflurane anesthesia. Mean number of lymphocytes +/- sem are reported (n = 4). Plasma exposures were determined to be 1,800, 5,000 and 15,000 ng/mL respectively

In rodents, $S1P_3$ agonism results in bradycardia,[16] although in humans these effects are likely mediated by $S1P_1$.[23] To confirm selectivity against $S1P_3$ *in vivo*, we evaluated **29** in cardiovascular safety studies. In anesthetized Sprague-Dawley rats, **29** induced no significant change in heart rate during sequential 30 minute IV infusions at 3, 10, and 30 mg/kg (Figure 7), achieving plasma concentrations of 1,840, 8,660 and 20,300 ng/mL respectively. Overall, the 10 mg/kg dose had minimal hemodynamic effects, and the 30 mg/kg dose produced a 16% increase in mean arterial pressure (not statistically significant) and a 10% decrease in cardiac contractility. These studies confirm *in vivo* that **29** has no activity at $S1P_3$, and when interpreted in the context of efficacy studies to be disclosed separately, also demonstrates a favourable safety margin. While the lack of lymphopenia and bradycardia responses from **29** is implied by its *in vitro* profile, these data demonstrate for the first time a lack of any $S1P_1$–related activity in an $S1P_5$ agonist and support the use of **29** in differentiating $S1P_1$ versus $S1P_5$ biology *in vivo*.

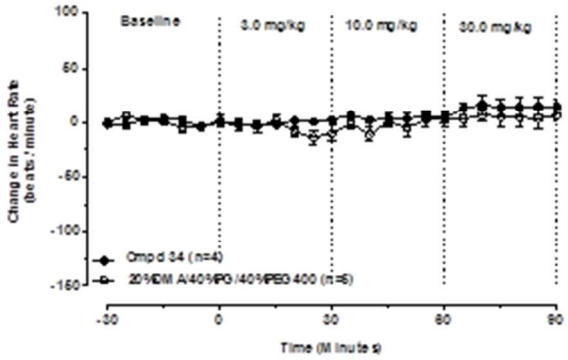


Figure 7. 29 does not cause bradycardia in anesthetized rats. 29 was administered by 3 sequential 30 minute IV infusions at 3, 10 and 30 mg/kg. No effects on heart rate were observed through the highest mean exposure of 20.3 ug/mL. Similarly, no statistically significant effects on mean arterial pressure were observed. Cardiac contractility (dP/dt50)

was decreased 10% (change from baseline) during the end of the third infusion period (P<0.05)

There are two previous reports of $S1P_5$-selective compounds.[10] Our *in vitro* profiling of these molecules showed significantly weaker potency than the agonists we describe. In addition, we were unable to formulate these molecules in a manner resulting in any measurable *in vivo* exposure.

In order to confirm compound **29** was also selective outside the S1P receptor family it was profiled in 2 large screening panels. **29** was found to have an IC_{50} >10 μM at all 129 kinases of an AbbVie internal panel (Supporting information). Of the 79 receptors tested on the CEREP[24] panel, **29** only bound to 2 members at a threshold of >50%, 5-$HT2_a$ (75%) and DA transporter (63%) (Supporting information).

Compound **29** was screened in the Ames test and found to be non-clastogenic. It was negative in the Alarm NMR assay[25] suggesting little likelihood of thiol reactivity, for example with glucuronide. It was also profiled in a dofetilide binding assay and had an IC_{50} of 77 μM.

Pharmacokinetic analyses (Table 14) of **29** in mouse, rat, dog and cynomolgus monkey indicates high oral bio-availability, high exposure, low clearance, a long half-life and at least where tested in rodents, good brain exposure. Despite moderate to high plasma protein binding, total brain exposure of as little as 20 ng/g brain tissue results in full efficacy, in studies to be discussed in subsequent publications.[26] These data indicate **29** can achieve sufficient exposure to saturate $S1P_5$ in peripheral tissues as well as the central nervous system.

Table 14. Pharmacokinetics of 29 in multiple species

Species	Dose (mg/kg)	Sample Analyzed	Protein Binding (%)	IV				PO				
				$t_{1/2}$ (hr)	AUC (ng*h/mL)	CL (L/h/kg)	Vss (L/kg)	$t_{1/2}$ (hr)	Tmax (hr)	Cmax (ng/mL)	AUC (ng*h/mL)	F (%)
BALB/C Mouse	2	Plasma	93	7.6	8,500	0.24	1.9	7.4	2.0	300	4,800	57
BALB/C Mouse	2	Brain	n.d.	9.8	3,200 (Cmax=133 ng/nL	n.d.	n.d.	10	2-24	43	1,600	56
SD Rat	1	Plasma	93	9.0	6,400	0.16	1.3	14	4.3	400	8,700	>100
SD Rat	2	Brain	99.5	n.d.	n.d.	n.d	n.d.	15	8	120	3,100	n.d.
Beagle Dog	1	Plasma	96	9.3	12,000	0.09	1.2	10	1.5	690	11,000	92
Cyno Monkey	1	Plasma	97	3.5	6,400	0.16	0.82	6.7	1.7	650	5,500	86

n.d. = not determined

Compound **29** underwent a 14 day Sprague-Dawley rat tolerability study at 10 and 100 mg/kg, po, qd. One of three females in the 100 mg/kg group was euthanized on Day 8 due to 22%

weight loss, the other females had no weight changes at either dose. All other animals survived to the scheduled necropsy with no adverse clinical signs. Body weights of males on Day 14 were 10% less than vehicle controls at both doses. There were increased liver weights in high dose groups (20% for male, 30% for female) but no corresponding increase in serum ALT. There were no remarkable changes in weights of spleen or thymus, in clinical chemistry, or in hematology, including no changes in lymphocytes. The exposure AUC was 43-54 μg*hr/mL at 10 mg/kg, and 670-820 μg*hr/mL at 100 mg/kg, and was the same at day 2 and at day 14.

Due to the paucity of pharmacology data around $S1P_5$, we profiled **29** in a panel of 41 multiplexed *in vivo* screening assays at Melior Discovery.[27] This panel was designed to efficiently query many biological systems, and should be interpreted as hypothesis-generating, not as 'gold-standard' versions of these assays. **29** was dosed in CD1 mice at 0.3, 3 and 30 mg/kg, qd, po. **29** roughly doubled food intake over a 4 day observation period, but did not result in any change in body weight, compared to vehicle (data not shown). Effects were smallest at the mid-dose. Additional hits that did not achieve significance and/or a dose response include a) anti-convulsant activity in response to pentylenetetrazole challenge, (b) a decrease in MCP-1 following pulmonary antigen challenge, and (c) an increase in urination frequency (Supporting information). No additional hits were found in the other 36 endpoints evaluated. $S1P_5$ agonism does not appear to have pleiotropic effects.

$S1P_5$ is highly expressed in brain endothelium, and its activation has been shown to enhance barrier function in an *in vitro* model of blood-brain barrier integrity. We confirmed this role for $S1P_5$ by showing that **29** increases electrical resistance of hCMEC/D3 cells *in vitro* at 1 μM (Figure 8). This finding confirms previously published data on the impact of $S1P_5$ agonism on endothelial barrier integrity.

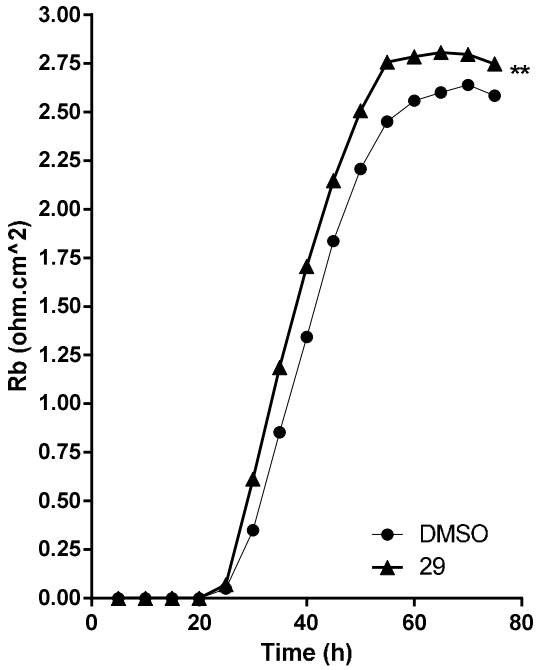


Figure 8. 29 impacts transendothelial electrical resistance as assessed through ECIS in hCMEC/D3 cells; DMSO (n = 4) or 1 μM 29 (n= 2) was added to the cell layer and resistance (ohm.cm^2) was assessed over 75h

As $S1P_5$ is highly expressed in the brain, we explored the impact of $S1P_5$ activation on a functional read-out, namely reversal of age-related cognitive deficits in male C57BL6J mice. After 21 days of treatment with **29**, cognitive function was assessed by spontaneous alternation in the T maze, 225 min after the final dose (estimated Tmax of the compound). Compound **29** reversed the cognitive deficits with an exposure of 41.6 ± 2.5 ng/mL (mean + SEM) and 28.3 ± 1.9 ng/g (mean + SEM)(Figure 9)**.** Further experiments confirmed the pro-cognitive impact of **29** in a dose-dependent manner and that 0.1 mg/kg is the lowest effective dose[3].

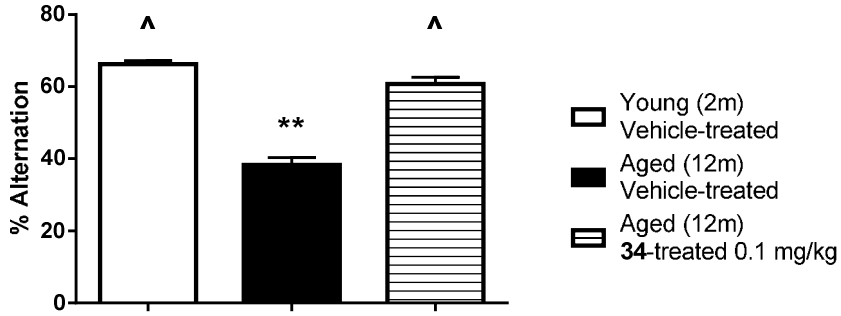


Figure 9. 29 reverses age-related cognitive decline as seen in the spontaneous alternation assay in the T-maze in aged male C57BL6J mice after sub-acute treatment. 29 (0.1 mg/kg) was administered for 21 days, qd. Testing was performed 225 minutes after the final dose. One-way ANOVA: ** $p < 0.05$ in comparison to young vehicle-treated animals, ^ $p < 0.05$ in comparison to aged vehicle-treated animals

CONCLUSIONS

Azetine-3-carboxylic acid was recognized as a bioisostere for the synthetically challenging phosphorylated cyclopentyl amino alcohol. This enabled the application of parallel synthesis at AbbVie's centralized High Throughput Chemistry group to rapidly secure the desired compounds. Diligent data mining of these compounds lead to the discovery of the first $S1P_5$ agonists that are clearly selective within the S1P receptor family. These leads were optimized further using parallel synthesis and dedicated synthesis affording a comprehensive pharmacophore for $S1P_5$ potency and selectivity. Multiple leads with excellent oral exposure were identified with A-971432 (**29**) selected for extensive evaluation. It was confirmed with **29** that the *in vitro* selectivity against $S1P_1$ and $S1P_3$ translated to a lack of lymphopenia and bradycardia, respectively, in rats. **29** was then shown to have highly favorable properties in both kinase and receptor liability panels, Cyp inhibition assays, AMES test, micronucleus test, rat cardiovascular safety, 14-day rat tolerability and the Melior Discovery panel of 41 *in vivo* endpoints. Finally it was demonstrated that **29** enhanced brain endothelial integrity, and previously presented data showed an impact on age-related cognitive decline and lipid accumulation in the central nervous system.[3] We will show in subsequent publications that **29** is highly efficacious in pre-clinical models of neurodegenerative diseases.[26] Compound **29** is available through an MTA for research purposes.

EXPERIMENTAL SECTION

The general synthetic methods used in each General Procedure follow and include an illustration of a compound that was synthesized using the designated General Procedure. Compounds of the present invention were synthesized and their activity assayed as described below. Unless otherwise stated, reagents were purchased from Sigma Aldrich, Acros, Alfa Aesar or the Sigma Aldrich Custom Packaged Reagent service. Reagent/reactant names given are as named on the commercial bottle or as generated by IUPAC conventions or CambridgeSoft® ChemDraw Ultra 9.0.7. Compound names are generated by IUPAC conventions or CambridgeSoft® ChemDraw Ultra 9.0.7.

Analytical data is included within the procedures below, in the illustrations of the general procedures, or in the tables of examples. Unless otherwise stated, all ^{1}H and ^{13}C NMR data were collected on a Varian Mercury Plus 400 MHz or a Bruker AVIII 300 MHz or a Bruker 500 MHz instrument; chemical shifts are quoted in parts per million (ppm). HPLC analytical data are either detailed within the experimental or referenced to the table of LCMS and HPLC conditions, using the lower case letter in Table 16.

Table 15. List of HPLC methods

Method	HPLC Conditions
a	Analytical LCMS was performed on a Waters ZMD mass spectrometer and Alliance HPLC system running MassLynx 3.4 and Openlynx 3.4 software. The ZMD mass spectrometer was operated under positive APCI ionization conditions. The HPLC system comprised a Waters 2795 autosampler sampling from 96-well plates, a Waters 996 diode-array detector and Sedere Sedex-75 evaporative light scattering detector. The column used was a Phenomenex Luna Combi-HTS C8(2) 5µm 100Å (2.1mm × 30mm). A gradient of 10-100% acetonitrile (A) and 0.1% trifluoroacetic acid in water (B) was used, at a flow rate of 2.0mL/min (0-0.1 min 10% A, 0.1-2.6 min 10-100% A, 2.6-2.9 min 100% A, 2.9-3.0 min 100-10% A. 0.5min post-run delay.
b	Analytical LCMS was performed on either a Waters LCT Premier mass spectrometer and 1525 Binary HPLC Pump running MassLynx 4.0 and OpenLynx 4.0 software or a Waters Quattro Ultima mass spectrometer and Agilent 1100 HPLC system controlled by MassLynx 4.0 and OpenLynx 4.0 software. The gradient was 5-60% B in 1.5 min then 60-95% B to 2.5 min with a hold at 95% B for 1.2 min (1.3 mL/min flow rate). Mobile phase A was 10mM ammonium acetate, mobile phase B was HPLC grade acetonitrile. The column used for the chromatography is a 4.6x50 mm MAC-MOD Halo C8 column (2.7 µm particles). Detection methods are DAD and ELSD detection as well as positive/negative electrospray ionization.)
c	Analytical LCMS was performed on Agilent 1200 HPLC/6100 SQ System. Mobile Phase: A: Water (0.05 % TFA) B: Acetonitrile (0.05 % TFA); Gradient Phase: 5 % - 95 % in 1.3 min; Flow rate: 1.6 mL/min; Column: XBridge, 2.5 min; Oven Temp. 50 °C.

d	Analytical LCMS was performed on a Waters ZMD mass spectrometer and Alliance HPLC system running MassLynx 3.4 and Openlynx 3.4 software. The ZMD mass spectrometer was operated under positive APCI ionization conditions. The HPLC system comprised a Waters 2795 autosampler sampling from 96-well plates, a Waters 996 diode-array detector and Sedere Sedex-75 evaporative light scattering detector. The gradient was 5-60% B in 1.5 min then 60-95% B to 2.5 min with a hold at 95% B for 1.2 min (1.3 mL/min flow rate). Mobile phase A was 10mM ammonium acetate, mobile phase B was HPLC grade acetonitrile. The column used for the chromatography is a 4.6x50 mm MAC-MOD Halo C18 column (2.7 µm particles). Detection methods are diode array (DAD) and evaporative light scattering (ELSD) detection as well as positive/negative electrospray ionization.

1-(4-(Hexyloxy)benzyl)azetidine-3-carboxylic acid (8). A mixture of 4-(hexyloxy)benzaldehyde (1950 mg, 9.45 mmol), methanol (20 mL) and AcOH (6 drops) was stirred at ambient temperature for 3 days. The solvent was removed under reduced pressure and the residue dissolved in Methanol (20 mL) and Methanol (20 mL). The resulting solution was divided in to 12 X 2 mL fractions and subjected to purification molecular ion directed prep-HPLC. The combined fractions were evaporated to dryness and dried in vacuo at 60 ℃ for 4 hours. This afforded 1-(4-(hexyloxy)benzyl)azetidine-3-carboxylic acid (683 mg, 2.34 mmol, 24.8 % yield) as an off-white solid. LCMS (Table 16, Method d). Rt = 1.96 min.; MS m/z: 292.16 $(M+H)^+$. Purity 100%. ^{1}H NMR (400 MHz, DMSO) δ ppm 6.85-6.80 (m, 2H), 7.17-7.11 (m, 2H), 3.91 (t, J = 6.52 Hz, 2H), 3.43 (s, 2H), 3.29 (t, J = 7.61 Hz, 2H), 3.10 (t, J = 7.20 Hz, 2H), 2.99 (t, J = 7.34 Hz, 1H), 1.75-1.62 (m, 2H), 1.47-1.23 (m, 6H), 0.87 (ddd, J = 7.14, 3.25, 1.55 Hz, 3H). HRMS: Found 291.1835 $C_{17}H_{25}NO_3$ requires 291.1834.

Compound 4 - 7 and 9 - 35 were synthesized in a similar manner using the appropriate commercially available aldehyde and azetidine-3-carboxylic acid unless otherwise stated following this general procedure. In a 20 mL vial a solution of the aldehyde monomer (1.2eq) dissolved in dichloromethane (1.1 mL) was added, followed by the addition of azetidine-3-carboxylic acid (29mg, 1eq.) dissolved in dichloromethane (0.4 mL), followed by acetic acid (3eq.) dissolved in dichloromethane(0.4mL), followed by MP-Cyanoborohydride resin(3eq.) The mixture was shaken at room temperature for 5 hours.. The reaction was checked by LCMS and concentrated to dryness. The residue was dissolved in 1:1 DMSO:MeOH and purified by preparative HPLC on a Phenomenex Luna C8(2) 5 um 100Å AXIA column (30mm × 75mm). A gradient of acetonitrile (A) and 0.1% trifluoroacetic acid in water (B) was used, at a flow rate of 50mL/min (0-0.5 min 10% A, 0.5-6.0 min linear gradient 10-100% A, 6.0-7.0 min 100% A, 7.0-8.0 min linear gradient 100-10% A). Samples were injected in 1.5mL DMSO:MeOH (1:1). An Agilent 1100 Series Purification system was used, consisting of the following modules: Agilent 1100 Series LCMSD SL mass spectrometer with API-electrospray source; two Agilent 1100 Series preparative pumps; Agilent 1100 Series isocratic pump; Agilent 1100 Series diode array detector with preparative (0.3mm) flow cell; Agilent active-splitter, IFC-PAL fraction collector / autosampler. The make-up pump for the mass spectrometer used 3:1 methanol:water with 0.1% formic acid at a flow rate of 1 mL/min. Fraction collection was automatically triggered when the extracted ion chromatogram for the target mass exceeded the threshold specified in the method. The system was controlled using Agilent Chemstation (Rev B.10.03), Agilent A2Prep, and Leap FractPal software, with custom Chemstation macros for data export. Products were characterized by MS and LCMS (Table 1, Method a).

1-(4-(Decyloxy)benzyl)azetidine-3-carboxylic acid (4). Synthesized from aldehyde 4-(decyloxy)benzaldehyde. LCMS (Table 16, Method d) Rt = 2.36 min.; MS m/z: 348.2. $(M+H)^+$. Purity 100%. ^{1}H NMR (400 MHz, Pyridine) δ ppm 7.35 (d, J = 8.65 Hz, 2H), 7.03-6.98 (m, 2H), 3.89 (t, J = 6.49 Hz, 3H), 3.64-3.42 (m, 7H), 1.69 (dd, J = 8.29, 6.78 Hz, 3H), 1.38 (s, 3H), 1.29-1.11 (m, 14H), 0.82 (dd, J = 7.79, 5.99 Hz, 4H). HRMS: Found 347.2457 $C_{21}H_{33}NO_3$ requires 347.2460.

1-(4-(Nonyloxy)benzyl)azetidine-3-carboxylic acid (5). Synthesized from aldehyde 4-(nonyloxy)benzaldehyde. LCMS (Table 16, Method d). Rt = 2.48 min.; MS m/z: 334.3 $(M+H)^+$. Purity 99%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.30 (m, 2H), 6.91 – 6.84 (m, 2H), 4.16 (t, J = 8.0 Hz, 2H), 4.11 (s, 2H), 3.99 (t, J = 9.6 Hz, 2H), 3.93 (t, J = 6.5 Hz, 2H), 3.44 – 3.31 (m, 1H), 2.05 (s, 3H), 1.81 – 1.69 (m, 2H), 1.41 (d, J = 7.1 Hz, 2H), 1.33 – 1.19 (m, 8H), 0.92 – 0.83 (m, 3H). HRMS: Found 333.2304 $C_{20}H_{31}NO_3$ requires 333.2304.

1-(4-(Octyloxy)benzyl)azetidine-3-carboxylic acid (6). Synthesized from aldehyde 4-(octyloxy)benzaldehyde. LCMS (Table 16, Method d) Rt = 2.00 min.; MS m/z: 320.2 $(M+H)^+$. Purity 96%. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.36 (d, J = 8.65 Hz, 2H), 6.89 (d, J = 8.68 Hz, 2H), 3.46-3.26 (m, 1H), 1.78 (dd, J = 14.69, 6.73 Hz, 2H), 1.55-1.41 (m, 2H), 1.34 (ddd, J = 14.64, 10.47, 2.94 Hz, 8H), 0.91 (t, J = 6.92 Hz, 3H), 4.11 (s, 4H), 4.07-3.87 (m, 4H). HRMS: Found 319.2147 $C_{19}H_{29}NO_3$ requires 319.2147.

1-(4-(Heptyloxy)benzyl)azetidine-3-carboxylic acid (7). Synthesized from aldehyde 4-(heptyloxy)benzaldehyde. LCMS (Table 16, Method d). Rt = 2.07 min.; MS m/z: 306.3 $(M+H)^+$. Purity 100%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 – 7.31 (m, 2H), 6.91 – 6.84 (m, 2H), 4.17 (d, J = 7.7 Hz, 2H), 4.10 (s, 2H), 4.02 – 3.88 (m, 4H), 2.07 (s, 2H), 1.77 (dt, J = 14.5, 6.7 Hz, 2H), 1.43 (td, J = 9.7, 8.6, 4.5 Hz, 2H), 1.38 – 1.23 (m, 6H), 0.95 – 0.84 (m, 3H). HRMS: Found 305.1991 $C_{18}H_{27}NO_3$ requires 305.1991.

1-(4-(Pentyloxy)benzyl)azetidine-3-carboxylic acid (9). Synthesized from aldehyde 4-(pentyloxy)benzaldehyde. LCMS (Table 16, Method a) Rt = 1.36 min.; MS m/z: 278.1 $(M+H)^+$. Purity 100%. HRMS: Found 277.1678 $C_{16}H_{23}NO_3$ requires 277.1678.

1-(4-(Isopentyloxy)benzyl)azetidine-3-carboxylic acid (10). Synthesized from aldehyde 4-(isopentyloxy)benzaldehyde. LCMS (Table 16, Method a) Rt = 1.22 min.; MS m/z: 278 $(M+H)^+$. Purity 100%. HRMS: Found 277.1698 $C_{16}H_{23}NO_3$ requires 277.1678.

1-(4-(Hexyloxy)benzyl)pyrrolidine-3-carboxylic acid (11). Synthesized from aldehyde 4-(hexyloxy)benzaldehyde and pyrrolidine-3-carboxylic acid. LCMS (Table 16, Method a) Rt = 1.35 min.; MS m/z: 306.4 $(M+H)^+$.

(R)-1-(4-(Hexyloxy)benzyl)pyrrolidine-3-carboxylic acid (12). Synthesized from 4-(hexyloxy)benzaldehyde and (R)-pyrrolidine-3-carboxylic acid. LCMS (Table 16, Method a) Rt = 1.35 min.; MS m/z: 306.4 $(M+H)^+$.

(R)-1-(4-(Hexyloxy)benzyl)piperidine-3-carboxylic acid (13). Synthesized from aldehyde 4-(hexyloxy)benzaldehyde and (R)-piperidine-3-carboxylic acid. LCMS (Table 16, Method a) Rt = 1.37 min.; MS m/z: 320.5 $(M+H)^+$.

(S)-1-(4-(Hexyloxy)benzyl)piperidine-3-carboxylic acid (14). Synthesized from aldehyde 4-(hexyloxy)benzaldehyde and (S)-piperidine-3-carboxylic acid. LCMS (Table 16, Method a) Rt = 1.36 min.; MS m/z: 320.4 $(M+H)^+$.

1-(4-(Hexyloxy)benzyl)piperidine-4-carboxylic acid (15). Synthesized from aldehyde 4-(hexyloxy)benzaldehyde and piperidine-4-carboxylic acid. LCMS (Table 16, Method a) Rt = 1.35 min.; MS m/z: 320.4 $(M+H)^+$.

1-(4-(Hexyloxy)benzyl)-3-methylpiperidine-4-carboxylic acid (16). Synthesized from aldehyde 4-(hexyloxy)benzaldehyde 3-methylpiperidine-4-carboxylic acid. LCMS (Table 16, Method a) Rt = 1.40 min.; MS m/z: 334.4 $(M+H)^+$.

1-((3',4'-Dichloro-[1,1'-biphenyl]-4-yl)methyl)azetidine-3-carboxylic acid (17). Synthesized from aldehyde 3',4'-dichloro-[1,1'-biphenyl]-4-carbaldehyde. LCMS (Table 16, Method a) Rt = 1.45 min.; MS m/z: 336 $(M+H)^+$.

1-((4'-Methyl-[1,1'-biphenyl]-4-yl)methyl)azetidine-3-carboxylic acid (18). Synthesized from aldehyde 4'-methyl-[1,1'-biphenyl]-4-carbaldehyde. LCMS (Table 16, Method a) Rt = 1.35 min.; MS m/z: 282 $(M+H)^+$.

1-((4'-Ethyl-[1,1'-biphenyl]-4-yl)methyl)azetidine-3-carboxylic acid (19). Synthesized from aldehyde 4'-methyl-[1,1'-biphenyl]-4-carbaldehyde. LCMS (Table 16, Method a) Rt = 1.34 min.; MS m/z: 296 $(M+H)^+$.

1-((3'-(Trifluoromethyl)-[1,1'-biphenyl]-4-yl)methyl)azetidine-3-carboxylic acid (20). Synthesized from aldehyde 3'-(trifluoromethyl)-[1,1'-biphenyl]-4-carbaldehyde. LCMS (Table 16, Method a) Rt = 1.26 min.; MS m/z: 336 $(M+H)^+$.

1-((3',5'-Dichloro-[1,1'-biphenyl]-4-yl)methyl)azetidine-3-carboxylic acid (21). Synthesized from aldehyde 3',5'-dichloro-[1,1'-biphenyl]-4-carbaldehyde. LCMS (Table 16, Method a) Rt = 1.40 min.; MS m/z: 336 $(M+H)^+$.

1-(4-(4-Chlorophenoxy)benzyl)azetidine-3-carboxylic acid (22). Synthesized from aldehyde 4-(4-chlorophenoxy)benzaldehyde. LCMS (Table 16, Method a) Rt = 1.27 min.; MS m/z: 318 $(M+H)^+$.

1-(4-(Benzyloxy)benzyl)azetidine-3-carboxylic acid (23). Synthesized from aldehyde 4-(benzyloxy)benzaldehyde. LCMS (Table 16, Method a) Rt = 1.16 min.; MS m/z: 298 $(M+H)^+$.

1-(4-((2-Methylbenzyl)oxy)benzyl)azetidine-3-carboxylic acid (24). Synthesized from aldehyde 4-((2-methylbenzyl)oxy)benzaldehyde. LCMS (Table 16, Method a) Rt = 1.30 min.; MS m/z: 310 $(M+H)^+$.

1-(4-((3-Chlorobenzyl)oxy)benzyl)azetidine-3-carboxylic acid (25). Synthesized from aldehyde 4-((3-chlorobenzyl)oxy)benzaldehyde. LCMS (Table 16, Method a) Rt = 1.22 min.; MS m/z: 332 $(M+H)^+$.

1-(4-((3-(Trifluoromethyl)benzyl)oxy)benzyl)azetidine-3-carboxylic acid (26). Synthesized from aldehyde 4-((3-(trifluoromethyl)benzyl)oxy)benzaldehyde. LCMS (Table 16, Method a) Rt = 1.28 min.; MS m/z: 366 $(M+H)^+$.

1-(4-((4-Fluorobenzyl)oxy)benzyl)azetidine-3-carboxylic acid (27). Synthesized from aldehyde 4-((4-fluorobenzyl)oxy)benzaldehyde. LCMS (Table 16, Method a) Rt = 1.25 min.; MS m/z: 316 $(M+H)^+$.

1-(4-((2,4-Dichlorobenzyl)oxy)benzyl)azetidine-3-carboxylic acid (28). Synthesized from aldehyde 4-((2,4-dichlorobenzyl)oxy)benzaldehyde. LCMS (Table 16, Method a) Rt = 1.41 min.; MS m/z: 364 $(M+H)^+$.

1-(4-((3,4-Dichlorobenzyl)oxy)benzyl)azetidine-3-carboxylic acid (29). Synthesized from aldehyde 4-((3,4-dichlorobenzyl)oxy)benzaldehyde. LCMS (Table 16, Method a) Rt = 1.37 min.; MS m/z: 366 $(M+H)^+$.

1-(4-(Benzyloxy)-2-methylbenzyl)azetidine-3-carboxylic acid (30). Synthesized from aldehyde 4-(benzyloxy)-2-methylbenzaldehyde. LCMS (Table 16, Method a) Rt = 1.32 min.; MS m/z: 312 $(M+H)^+$. ^{1}H NMR (300MHz, d4-Methanol) δ 7.45-7.43(2H, d), 7.40-7.37(2H, t), 7.34-7.31(2H, t), 6.98(1H, s), 6.94-6.92(1H, q), 5.12(2H, s), 4.42(2H, s), 4.35-4.29(4H, m), 3.73-3.66(1H, m), 2.42(3H, s)

1-(4-(Benzyloxy)-2-chlorobenzyl)azetidine-3-carboxylic acid (31). Synthesized from aldehyde 4-(benzyloxy)-2-chlorobenzaldehyde. LCMS (Table 16, Method a) Rt = 1.01 min.; MS m/z: 332 $(M+H)^+$. Purity 99%. ^{1}H NMR (300MHz, d4-Methanol) δ 7.51-7.49(1H, d), 7.46-7.44(2H, d), 7.41-7.38(2H, q), 7.35-7.32(1H, q), 7.21-7.20(1H, d), 7.08-7.06(1H, q), 5.15 (2H, s), 4.45(2H, s), 4.22-4.18(4H, m), 3.46-3.41(1H, m). HRMS: Found 331.0974 $C_{18}H_{18}NO_3Cl$ requires 331.0975.

1-(4-((2,4-Dichlorobenzyl)oxy)-3-methoxybenzyl)azetidine-3-carboxylic acid (32). Synthesized from aldehyde 4-((2,4-dichlorobenzyl)oxy)-3-methoxybenzaldehyde. LCMS (Table 16, Method a) Rt = 1.27 min.; MS m/z: 396 $(M+H)^+$.

1-(4-((3,4-Dichlorobenzyl)oxy)-3-nitrobenzyl)azetidine-3-carboxylic acid (33). Synthesized from aldehyde 4-((3,4-dichlorobenzyl)oxy)-3-nitrobenzaldehyde. LCMS (Table 16, Method a) Rt = 1.35 min.; MS m/z: 411 $(M+H)^+$. ^{1}H NMR (400 MHz, CD_3CN) δ 7.77 (d, J = 2.1, 1H), 7.65 (d, J = 1.9, 1H), 7.56 (d, J = 8.3, 1H), 7.51 (dd, J = 2.2, 8.6, 1H), 7.44 - 7.36 (m, 1H), 7.21 (d, J = 8.6, 1H), 5.20 (s, 2H), 3.59 (s, 2H), 3.45 (t, J = 7.6, 2H), 3.30 (t, J = 6.7, 2H), 3.24 (dd, J = 7.2, 13.9, 1H).

1-(4-((2-Chlorobenzyl)oxy)-3-methoxybenzyl)azetidine-3-carboxylic acid (34). Synthesized from aldehyde 4-((2-chlorobenzyl)oxy)-3-methoxybenzaldehyde. LCMS (Table 16, Method a) Rt = 1.27 min.; MS m/z: 360 $(M+H)^+$.

4-(1,3-Dioxolan-2-yl)benzonitrile (36). A mixture of 4-formylbenzonitrile **35** (13.1 g, 0.1 mol), ethylene glycol (62 g, 1 mol), *p*-toluenesulfonic acid monohydrate (1.9 g, 0.01 mol) in 150 mL of toluene were refluxed overnight. After cooling the mixture to room temperature, it was added to 200 mL of ice-cold water and stirred for 15 min. The organic layer was dried over with Na_2SO_4, and the solvent was removed in *vacuo*. Purification on silica-gel column chromatography (PE/EA from 10:1 to 4:1) afforded 4-(1,3-dioxolan-2-yl)benzonitrile **36** as a white solid (14.2 g, yield 81%). LCMS (Table 16, Method c) RT 0.66 min; m/z 176.7 $(M+H)^+$.

4-(2-Phenylacetyl)benzaldehyde. Under N_2, to magnesium turnings (792 mg, 32.98 mmol) and I_2 (7 mg) in Et_2O (5 mL), was added a solution of (bromomethyl) benzene (3.76 g, 21.99 mmol) at room temperature. After stirring for 1 h, the mixture was cooled to 0~15°C. A solution of 4-(1,3-dioxolan-2-yl)benzonitrile **38** (2.89 g, 16.5 mmol) in Et_2O (10 mL) was added dropwise, then refluxed for 1 h. The solution was cooled to room temperature, and treated with ice-water. Subsequently, aqueous 5 M HCl was added, whereby two clear phases were formed. Then the organic phase was separated and the aqueous phase was extracted with EtOAc. The combined organic phase was washed with sat. $NaHSO_3$ and sat. $NaHCO_3$, dried (Na_2SO_4) and concentrated in *vacuo* to get the crude 1-(4-(1,3-dioxolan-2-yl)phenyl)-2-phenylethanone. The crude 1-(4-(1,3-dioxolan-2-yl)phenyl)-2-phenylethanone was dissolved in THF (20 mL) and

10% HCl (30 mL) added to the solution. The reaction mixture was refluxed for 16 h, then cooled to room temperature. EtOAc was added and the organic layer was (Na_2SO_4). After removal of solvent, it was purified by silica-gel column chromatography (PE/EA from 50:1 to 10:1) to afford 4-(2-phenylacetyl)benzaldehyde as a white solid (1.1 g, yield 43%). LCMS (Table 16, Method c) RT 1.57min; m/z 225.1 $(M+H)^+$.

1-(4-(2-Phenylacetyl)benzyl)azetidine-3-carboxylic acid (41). 4-(2-Phenylacetyl)benzaldehyde (133 mg, 0.59 mmol) was added to a stirred solution of azetidine-3-carboxylic acid (60 mg, 0.59 mmol) and HOAc (107 mg, 1.78 mmol) in 10 mL MeOH. The mixture was heated to 40 C. After 15 min, $NaCNBH_3$ (110 mg, 1.78 mmol) was added in a single portion and stirred at 40 °C overnight. After acidifying with 1 M HCl, purification by prep-HPLC afforded 1-(4-(2-phenylacetyl)benzyl)pyrrolidine-3-carboxylic acid (38.7, yield 21%). LCMS (Table 16, Method c) RT 1.23 min; m/z 310.2 $(M+H)^+$. ^{1}H NMR (300MHz, d4-Methanol) δ 8.16-8.14 (2H, d), 7.61-7.59 (2H, d), 7.32-7.24 (5H, m), 4.51-4.50 (2H, s), 4.39-4.36 (4H, m) and 4.35-4.33 (2H, m).

1-(4-Phenethylbenzyl)azetidine-3-carboxylic acid (42). 1-(4-(2-Phenylacetyl)benzyl)azetidine-3-carboxylic acid **41** (200 mg, 0.65 mmol)was dissolved in 1% (V/V) H_2SO_4/EtOH (50mL), to which Pd/C (10 mg) was added and the hydrogenation reaction carried out at room temperature for 32 h. After completion of the reaction, the catalyst was filtered off and ethanol solution neutralized with NaOH, followed by distillation of the solvent. HPLC afforded 1-(4-phenethylbenzyl)azetidine-3-carboxylic acid (100mg , 52%). LCMS (Table 16, Method c) RT 1.35min; m/z 296.2 $(M+H)^+$. ^{1}H NMR (300MHz, d_4-Methanol) δ 7.24-7.23 (2H, d), 7.18-7.16 (2H, d), 7.13-7.10 (2H, t), 7.05-7.03 (2H, t), 7.02 (1H, s), 4.25 (2H, s), 4.19-4.18 (2H, m), 4.17-4.15 (2H, m), 3.22-3.20 (1H, m), 2.87-2.84 (2H, m) and 2.82-2.79 (2H, m).

1-(4-(3-(Trifluoromethyl)phenethyl)benzyl)azetidine-3-carboxylic acid (43). 1-(4-(3-(Trifluoromethyl)phenethyl)benzyl)azetidine-3-carboxylic acid was synthesized using the same procedure as 1-(4-phenethylbenzyl)azetidine-3-carboxylic acid starting from 1-(bromomethyl)-3-(trifluoromethyl)benzene. LCMS (Table 16, Method c) RT 0.63 min; m/z 364.2 $(M+H)^+$. Purity 96%. ^{1}H NMR (300MHz, d_4-Methanol) δ 7.35-7.28 (4H, m), 7.09-7.08 (2H, d), 7.02-7.00 (2H, d), 3.49 (2H, s), 3.43-3.40 (2H, t), 3.25-3.23 (2H, d), 3.12-3.06 (1H, m), 2.90-2.87 (2H, t) and 2.83-2.79 (2H, t). HRMS: Found 363.1444 $C_{20}H_{20}NO_2F_3$ requires 363.1446.

1-(4-Hexanoylbenzyl)azetidine-3-carboxylic acid (44). Prepared using the same procedure as for 1-(4-(2-phenylacetyl)benzyl)azetidine-3-carboxylic acid **51** using 1-bromopentane. LCMS (Table 16, Method c) RT 1.32 min; m/z 290.2 $(M+H)^+$. ^{1}H NMR (300MHz, d4-Methanol) δ 8.07-8.05 (2H, d), 7.59-7.57 (2H, d), 4.37 (2H, s), 4.16-4.14 (4H, m), 3.43-3.40 (1H, m), 3.06-3.03 (2H, t), 1.76-1.70 (2H, m), 1.40-1.35 (4H, m) and 0.96-0.93 (3H, t).

1-(4-Hexanoylbenzyl)pyrrolidine-3-carboxylic acid (45). Prepared using the same procedure as for 1-(4-(2-phenylacetyl)benzyl)azetidine-3-carboxylic acid **51** using 1-bromopentane and pyrrolidine-3-carboxylic acid. LCMS (Table 16, Method c) RT 1.32 min; m/z 304.3 $(M+H)^+$. ^{1}H NMR (300MHz, d4-Methanol) δ 8.06-8.04(2H, d), 7.66-7.64(2H, d), 4.38-4.31 (2H, q), 3.48-3.46 (1H, m), 3.33-3.31 (2H, m), 3.25-3.08 (2H, m), 3.06-3.03 (2H, t), 2.32-2.26 (2H, m), 1.74-1.70 (2H, m), 1.40-1.37 (4H, m) and 0.96-0.93 (3H, t).

1-(4-Heptanoylbenzyl)azetidine-3-carboxylic acid (46). Prepared using the same procedure as for 1-(4-(2-phenylacetyl)benzyl)azetidine-3-carboxylic acid **51** using 1-bromohexane. LCMS (Table 16, Method c) RT 1.39 min; m/z 304.3 $(M+H)^+$. ^{1}H NMR (300MHz, d4-Methanol) δ

8.08-8.06 (2H, d), 7.59-7.58 (2H, d), 4.38 (2H, s), 4.18-4.12 (4H, m), 3.45-3.40 (1H, m), 3.06-3.03 (2H, t), 1.75-1.70 (2H, m), 1.42-1.31 (6H, m) and 0.94-0.92 (3H, t).

1-(4-Heptanoylbenzyl)pyrrolidine-3-carboxylic acid (47). Prepared using the same procedure as for 1-(4-(2-phenylacetyl)benzyl)azetidine-3-carboxylic acid **53** using 1-bromohexane and pyrrolidine-3-carboxylic acid. LCMS (Table 16, Method c) RT 1.40 min; m/z 318.3 $(M+H)^+$. ^{1}H NMR (300MHz, d4-Methanol) δ 8.04-8.02 (2H, d), 7.67-7.65 (2H, d), 4.39-4.33 (2H, q), 3.49 (1H, s), 3.37-3.32 (2H, m), 3.27-3.23 (2H, m), 3.04-3.01 (2H, t), 2.37-2.23 (2H, m), 1.73-1.67 (2H, m), 1.44-1.37 (2H, m), 1.36-1.31 (4H, m) and 0.93-0.90 (3H, t).

1-(4-(2-Phenylacetyl)benzyl)pyrrolidine-3-carboxylic acid (49). Prepared using the same procedure as for 1-(4-(2-phenylacetyl)benzyl)azetidine-3-carboxylic acid **53** using pyrrolidine-3-carboxylic acid. LCMS (Table 16, Method c) RT 1.24 min; m/z 324.2 $(M+H)^+$. ^{1}H NMR (300MHz, d4-Methanol) δ 8.17-8.15(2H, d), 7.68-7.66(2H, d), 7.34-7.30 (2H, t), 7.29-7.27(2H, d), 7.26-7.22(2H, t), 4.53-4.47(2H, t), 4.38(2H, s), 3.77-3.76(6H, m), 2.45-2.34(2H, d).

1-(4-(2-(3,4-Dichlorophenyl)acetyl)benzyl)azetidine-3-carboxylic acid (50). Prepared using the same procedure as for 1-(4-(2-phenylacetyl)benzyl)azetidine-3-carboxylic acid **51** using 4-(bromomethyl)-1,2-dichlorobenzene. LCMS (Table 16, Method c) RT 1.39 min; m/z 378.1 $(M+H)^+$. ^{1}H NMR (300MHz, d4-Methanol) δ 8.05-8.03(2H, d), 7.53-7.51(2H, d), 7.37-7.36 (2H, d), 7.10-7.08(1H, m), 4.40(2H, s), 4.31(2H, s), 4.27-4.22(4H, m), 3.62-3.59(1H, m).

1-(4-(2-(3,4-Dichlorophenyl)acetyl)benzyl)pyrrolidine-3-carboxylic acid (51). Prepared using the same procedure as for 1-(4-(2-phenylacetyl)benzyl)azetidine-3-carboxylic acid **51** using 4-(bromomethyl)-1,2-dichlorobenzeneand pyrrolidine-3-carboxylic acid. LCMS (Table 16, Method c) RT 1.40 min; m/z 392.1 $(M+H)^+$. ^{1}H NMR (300MHz, d4-Methanol) δ 8.14-8.13(2H, d), 7.66-7.65(2H, d), 7.48-7.47(2H, m), 7.23-7.21(1H, q), 4.36-4.28(2H, q), 3.42-3.41(1H, m), 3.30-3.11(4H, m), 2.32-2.24(2H, m).

4-(Benzyloxy)-3-fluorobenzonitrile. Under N_2, DIAD (0.32 g, 1.54 mmol) was treated with Ph_3P (0.41 g, 4.54 mmol) at 0 °C in dry THF(10 mL). The mixture was stirred until there was a precipitate. Then 3-fluoro-4-hydroxybenzonitrile (0.2 g, 1.46 mmol) and benzyl alcohol (0.17 g, 1.54 mmol) were added at the same time. The mixture was warmed to room temperature, and stirred overnight. The reaction mixture was concentrated and purified by silica-gel column chromatography (PE/EA = 4:1) to afford 4-(benzyloxy)-3-fluorobenzonitrile as a white solid (0.28 g, yield 84%). GC-MS RT 10.83 min; m/z 227.1 (M).

4-(Benzyloxy)-3-fluorobenzaldehyde. 4-(Benzyloxy)-3-fluorobenzonitrile (645 mg,2.84 mmol) was dissolved in toluene (10 mL) and cooled to 0 °C. A portion of 1M DIBAL (4.55 mmol, 4.55 mL) in hexane was added dropwise under N_2. The solution was further stirred for 1 hour at 0 °C. Chloroform (12 mL) was then added followed by 10% HCl (30 mL), and the resulting solution stirred at room temperature for 1 h. The organic layer was separated, washed with distilled water, dried with Na_2SO_4 and filtered. After removal of the solvent, the residue was purified by silica-gel column chromatography (PE/EA = 4:1) to afford 4-(benzyloxy)-3-fluorobenzaldehyde as a white solid (0.62 g, yield 95%). LCMS (Table 16, Method c) RT 1.26 min; m/z 231.1 $(M+H)^+$.

1-(4-(Benzyloxy)-3-fluorobenzyl)azetidine-3-carboxylic acid (57). 4-(Benzyloxy)-3-fluorobenzaldehyde (653 mg, 2.84 mmol) was added to a stirred solution of azetidine-3-carboxylic acid (287 mg, 2.84 mmol) and HOAc (536 mg, 8.52 mmol) in 5 mL MeOH. The

mixture was heated to 40 ℃. After 15 min, $NaCNBH_3$ (512 mg, 8.52 mmol) was added in a single portion and stirred at 40 ℃ overnight. After acidifying with 1M HCl, the residue was purified by Prep-HPLC affording 1-(4-(benzyloxy)-3-fluorobenzyl)azetidine-3-carboxylic acid (571 mg, yield 63%). LCMS (Table 16, Method c) RT 0.95min; m/z 316.2 $(M+H)^+$. ^1H NMR (300MHz, d4-Methanol): δ 7.44-7.43 (2H, d), 7.39-7.36 (2H, m), 7.33-7.30 (1H, m), 7.28-7.25 (1H, m), 7.23-7.22 (1H, d), 7.20-7.18 (1H, m), 5.19 (2H, s), 4.32 (2H, s), 4.29-4.25(4H, m) and 3.68-3.63 (1H, m).

1-(4-((3,4-Dichlorobenzyl)oxy)-2-fluorobenzyl)azetidine-3-carboxylic acid (58). Prepared using the same procedure as for 1-(4-(benzyloxy)-3-fluorobenzyl)azetidine-3-carboxylic acid **60** using 4-(bromomethyl)-1,2-dichlorobenzene and 2-fluoro-4-hydroxybenzonitrile. LCMS (Table 16, Method c) RT 1.10min; m/z 384.1 $(M+H)^+$. ^1H NMR (300MHz, d4-Methanol) δ 7.52 (1H, s), 7.45-7.44 (1H, d), 7.36-7.32 (1H, t), 7.29-7.27 (1H, d), 6.87-6.84 (2H, q), 5.04 (2H, s), 4.33 (2H, s), 4.27-4.20 (4H, m) and 3.59-3.56 (1H, m).

1-(4-((3,4-Dichlorobenzyl)oxy)-3-fluorobenzyl)azetidine-3-carboxylic acid (59). Prepared using the same procedure as for 1-(4-(benzyloxy)-3-fluorobenzyl)azetidine-3-carboxylic acid **60** using 4-(bromomethyl)-1,2-dichlorobenzene. LCMS (Table 16, Method c) RT 1.11min; m/z 384.1 $(M+H)^+$. ^1H NMR (300MHz, d4-Methanol) δ 7.64 (1H, s), 7.57-7.55 (1H, d), 7.41-7.40 (1H, d), 7.31-7.29 (1H, d), 7.24-7.23 (2H, q), 5.21 (2H, s), 4.31 (2H, s), 4.22-4.21 (4H, d) and 3.50-3.33 (1H, m).

1-(2-Fluoro-4-((3-(trifluoromethyl)benzyl)oxy)benzyl)azetidine-3-carboxylic acid (60). Prepared using the same procedure as for 1-(4-(benzyloxy)-3-fluorobenzyl)azetidine-3-carboxylic acid **59** using 1-(bromomethyl)-3-(trifluoromethyl)benzene and 2-fluoro-4-hydroxybenzonitrile. LCMS (Table 16, Method c) RT 1.08min; m/z 384.2 $(M+H)^+$. Purity 97%. ^1H NMR (300MHz, d4-Methanol) δ 7.75 (1H, s), 7.72-7.70 (1H, d), 7.65-7.63 (1H, d), 7.60-7.57 (1H, t), 7.45-7.42 (1H, t), 6.98-6.96 (2H, q), 5.22 (2H, s), 4.42 (2H, s), 4.36-4.29 (4H, m) and 3.70-3.63 (1H, m). HRMS: Found 383.1145 $C_{19}H_{17}NO_3F_4$ requires 383.1145.

1-(3-Fluoro-4-((3-(trifluoromethyl)benzyl)oxy)benzyl)azetidine-3-carboxylic acid (61). Prepared using the same procedure as for 1-(4-(benzyloxy)-3-fluorobenzyl)azetidine-3-carboxylic acid **60** using 1-(bromomethyl)-3-(trifluoromethyl)benzene. LCMS (Table 16, Method c) RT 1.08min; m/z 384.2 (M+H)+. 1H NMR(300MHz, d4-Methanol): δ 7.76 (1H, s), 7.73-7.71 (1H, d), 7.65-7.64 (1H, d), 7.61-7.58 (1H, t), 7.31-7.28 (1H, m), 7.27-7.25 (1H, d), 7.23-7.21 (1H, m), 5.23 (2H, s), 4.33 (2H, s), 4.30-4.26 (4H, m) and 3.69-3.63 (1H, m).

1-(2-Chloro-4-((3-(trifluoromethyl)benzyl)oxy)benzyl)azetidine-3-carboxylic acid (62). Prepared using the same procedure as for 1-(4-(benzyloxy)-3-fluorobenzyl)azetidine-3-carboxylic acid **57** using 1-(bromomethyl)-3-(trifluoromethyl)benzene and 2-chloro-4-hydroxybenzonitrile. LCMS (Table 16, Method c) RT 1.12min; m/z 400.1 $(M+H)^+$. ^1H NMR (300MHz, d4-Methanol) δ 7.66 (1H, s), 7.62-7.61 (1H, d), 7.55-7.53 (1H, d), 7.50-7.47 (1H, t), 7.43-7.42 (1H, d), 7.15-7.14 (1H, d), 7.01-6.98 (1H, q), 5.12 (2H, s), 4.44 (2H, s), 4.30-4.23 (4H, m) and 3.62-3.58 (1H, m).

1-(3-Chloro-4-((3-(trifluoromethyl)benzyl)oxy)benzyl)azetidine-3-carboxylic acid (63). Prepared using the same procedure as for 1-(4-(benzyloxy)-3-fluorobenzyl)azetidine-3-carboxylic acid **57** using 1-(bromomethyl)-3-(trifluoromethyl)benzene and 3-chloro-4-hydroxybenzonitrile. LCMS (Table 16, Method c) RT 1.12min; m/z 400.1 $(M+H)^+$. ^1H NMR (300MHz, d4-Methanol) δ 7.82 (1H, s), 7.77-7.76 (1H, d), 7.67-7.65 (1H, d), 7.63-7.60 (2H, q),

7.42-7.40 (1H, q), 7.27-7.26 (1H, d), 5.33 (2H, s), 4.36 (2H, s), 4.33-4.28 (4H, m) and 3.72-3.66 (1H, m).

1-Bromo-4-(3,4-dichlorobenzyloxy)-2-methylbenzene. Under N_2, DIAD (908 mg, 4.49 mmol) was treated with Ph_3P (1.18 g, 4.49 mmol) at 0 °C in dry THF(20 mL). The mixture was stirred until there was a precipitate. Then 4-bromo-3-methylphenol (0.8 g g, 4.23 mmol) and 3,4-dicholorobenzyl alcohol (795 mg, 4.49 mmol) were added at the same time. The mixture was warmed to room temperature, and stirred overnight. After concentrating, purification by silica-gel column chromatography (PE/EA = 4:1) afforded 1-bromo-4-(3,4-dichlorobenzyloxy)-2-methylbenzene as a white solid (0.94 g, yield 64%). GC-MS: m/z 345.9 (M), RT: 13.96 min. ^{1}H NMR (300MHz, $CDCl_3$): δ 7.52 (1H, s), 7.46-7.44 (1H, d), 7.42-7.40 (1H, d), 7.25-7.23 (1H, q), 6.85-6.84 (1H, d), 6.66-6.64 (1H, q), 4.97 (2H, s) and 2.36 (3H, s).

4-(3,4-Dichlorobenzyloxy)-2-methylbenzonitrile. A mixture of 1-bromo-4-(3,4-dichlorobenzyloxy)-2-methylbenzene (0.94 g, 207 mmol) and CuCN (0.56 g, 6.2 mmol) in N-methyl-2-pyrrolidone (10 mL) was heated to 160 °C for 32 h. After cooling slightly, the warm mixture was washed with 5% NaCN and extracted with ether. The combined extract was washed with 5% NaCN, water, brine and dried (Na_2SO_4). After removal of solvent in *vacuo*, the residue was purified by silica-gel column chromatography (PE/EA = 10:1) to afford 4-(3,4-dichlorobenzyloxy)-2-methylbenzonitrile as a white solid (496 mg, yield 63%). LCMS (Table 16, Method c) RT 1.46 min; m/z 292.0 $(M+H)^+$.

4-(4,5-Dichloro-2-methylbenzyloxy)benzaldehyde. 4-(3,4-dichlorobenzyloxy)-2-methylbenzonitrile (644 mg, 2.2 mmol) was dissolved in toluene (35 mL) and cooled to 0 ° C. A portion of 1M DIBAL (3.5 mmol, 3.5 mL) in hexane was added dropwise under N_2. The solution was stirred for an hour at 0 °C. $CHCl_3$ (40 mL) was then added followed by 10% HCl (30 mL), and the solution was stirred at room temperature for 1 h. The organic layer was separated, washed with distilled water, dried (Na_2SO_4) and filtered. After removal of the solvent, the residue was purified by silica-gel column chromatography (PE/EA = 4:1) to afford 4-(4,5-dichloro-2-methylbenzyloxy)benzaldehyde as a white solid (345 mg, yield 53%). LCMS Method c) RT 1.85 min; m/z 295.0 $(M+H)^+$.

1-(2-Methyl-4-((3-(trifluoromethyl)benzyl)oxy)benzyl)azetidine-3-carboxylic acid (69). Prepared using the same procedure as for 1-(4-(3,4-dichlorobenzyloxy)-2-methylbenzyl)azetidine-3-carboxylic acid **75** using 1-(bromomethyl)-3-(trifluoromethyl)benzene. LCMS (Table 16, Method c) RT 1.43min; m/z 380.2 $(M+H)^+$.^{1}H NMR (300MHz, d4-Methanol) δ 7.59(1H, s), 7.56-7.54 (1H, d), 7.48-7.46 (1H, d), 7.44-7.41 (1H, t), 7.19-7.17 (1H, d), 6.82 (1H, d), 6.82-6.76 (1H, q), 5.03 (2H, s), 4.22 (2H, s), 4.06-4.05 (4H, d), 3.30-3.29 (1H, m) and 2.25(3H, s).

1-(4-(3,4-Dichlorobenzyloxy)-2-methylbenzyl)azetidine-3-carboxylic acid (70). 4-(4,5-dichloro-2-methylbenzyloxy)benzaldehyde (345 mg, 1.17 mmol) was added to a stirred solution of azetidine-3-carboxylic acid (118 mg, 1.17 mmol) and HOAc (211 mg, 3.51 mmol) in 5 mL CH_3OH. The mixture was heated to 40 °C. After 15 min, $NaCNBH_3$ (218 mg, 3.51 mmol) was added in a single portion and stirred at 40 °C overnight. After being acidified with 1M HCl prep HPLC afforded 1-(4-(3,4-dichlorobenzyloxy)-2-methylbenzyl)azetidine-3-carboxylic acid **75** (201 mg, yield 45%). LCMS (Table 16, Method c) RT 1.47 min; m/z 380.1 $(M+H)^+$. ^{1}H NMR (300MHz, d_4-Methanol) δ 7.49-7.48 (1H, d), 7.42-7.40 (1H, d), 7.26-7.24 (1H, q), 7.22-7.20 (2H,

d), 6.84-6.83 (1H, d), 6.80-6.78 (1H, q), 4.97 (2H, s), 4.25 (2H, s), 4.09-4.07 (4H, d), 3.35-3.28 (1H, m) and 2.29 (3H, s).

4-(1-Phenylethoxy)benzonitrile (73). Under N_2, DIAD (1.06 g, 5.25 mmol) was treated with Ph_3P (1.38 g, 5.25 mmol) at 0 ℃ in dry THF(15 mL). The mixture was stirred until there was a precipitate. Then 4-hydroxybenzonitrile **77** (630 mg, 5.25 mmol) and DL-1-phenylethanol **76** (611 mg, 5 mmol) were added at the same time. The mixture was warmed to room temperature, and stirred overnight. Concentrated and purified by silica-gel column chromatography (PE/EA from 15:1 to 10:1) to afford 4-(1-phenylethoxy)benzonitrile **78** as a white solid (0.62 g, yield 55%). GC-MS: m/z 223.1 (M), RT: 11.05 min. ^{1}H NMR (300MHz, $CDCl_3$): δ 7.49-7.47 (2H, d), 7.36-7.32 (4H, q), 7.29-7.27 (2H, d), 5.37-5.33 (1H, q) and 1.67-1.66 (3H, d).

4-(1-Phenylethoxy)benzaldehyde (74). 4-(1-Phenylethoxy)benzonitrile **76** (200 mg, 0.86 mmol) was dissolved in toluene (10 mL) and cooled to 0 °C. A portion of 1M DIBAL (1.44 mmol, 1.44 mL) in hexane was added dropwise under N_2. The solution was stirred for another hour at 0 °C. $CHCl_3$ (12mL) was then added followed by 10% HCl (30 mL), and the solution was stirred at room temperature for 1 h. The organic layer was separated, washed with distilled water, dried with Na_2SO_4 and filtered. The solvent was almost completely removed. Purified by silica-gel column chromatography (PE/EA = 4:1) to afford 4-(1-phenylethoxy)benzaldehyde **77** as a white solid (85 mg, yield 42%).

1-(4-(1-Phenylethoxy)benzyl)azetidine-3-carboxylic acid (75). 4-(1-phenylethoxy)benzaldehyde **74** (1.06 g, 4.7 mmol) was added to a stirred solution of azetidine-3-carboxylic acid (476 mg, 4.7 mmol) and HOAc (850 mg, 14.1 mmol) in 5 mL CH_3OH. The mixture was heated to 40 ℃. After 15 min, $NaCNBH_3$ (889 mg, 14.1 mmol) was added in a single portion and stirred at 40 ℃ overnight to get compound 1-(4-(1-phenylethoxy)benzyl)azetidine-3-carboxylic acid (400 mg, yield 27%). LCMS (Table 16, Method c) RT 1.00 min; m/z 312.1 $(M+H)^+$. ^{1}H NMR (300MHz, d_4-Methanol): δ 7.27-7.26 (2H, d), 7.22-7.16 (4H, m), 7.17-7.11 (1H, t), 6.85-6.83 (2H, d), 5.34-5.31 (1H, q), 4.11(2H, s), 4.03-4.02 (4H, d), 4.32-3.25 (1H, m) and 1.51-1.49 (3H, d).

(R)-1-(4-(1-Phenylethoxy)benzyl)azetidine-3-carboxylic acid (76). (R)-1-(4-(1-phenylethoxy)benzyl)azetidine-3-carboxylic acid was synthesized using the same procedure as 1-(4-(1-phenylethoxy)benzyl)azetidine-3-carboxylic acid **75** starting from the chiral material (S)-1-phenylethanol. The configuration conversion ratio for the Mitsunobu step is 86:14(*R*:*S*). Purification by prep-chiral-HPLC afforded (S)-1-(4-(1-phenylethoxy)benzyl)azetidine-3-carboxylic acid **76**. LCMS (Table 16, Method c) RT 0.97min; m/z 312.2 $(M+H)^+$. ^{1}H NMR (300MHz, d_4-Methanol) δ 7.39-7.38 (2H, d), 7.34-7.29 (4H, m), 7.26-7.23 (1H, t), 6.98-6.96 (2H, d), 5.47-5.43 (1H, q), 4.26-4.22 (6H, m), 3.66-3.61 (1H, m) and 1.63-1.61 (3H, d). e.e. value: 95.4.

4-(3,4-Dichlorobenzyloxy)benzaldehyde (79). A 2 L round bottomed flask was charged with 4-hydroxybenzaldehyde **78** (150 g, 1.22 mol) and potassium carbonate (254.64 g, 1.84 mol) in acetone (1 L) and was added 3,4-dichlorobenzyl chloride **77** (240 g, 1.22 mol) portion wise. The reaction mixture was then heated to reflux for overnight. The reaction completion was monitored by TLC and then cooled to room temperature. The cooled reaction mixture was then poured into a beaker containing cold water to get precipitate. The solid obtained was filtered, washed with water (2 X 250 ml) and dried. The solid product was then suspended in methanol (1 L) and stirred for 15 min at room temperature. The methanol was filtered off and the solid

product dried to yield 272 g (79%) of 4-(3,4-dichlorobenzyloxy)benzaldehyde **79**. ^{1}H NMR (500 MHz, d6-DMSO) δ 9.85 (s, 1H), 7.90 (d, 2H), 7.78 (s, 1H), 7.69 (d, 1H), 7.43 (m, 1H), 7.2 (d, 2H) and 5.22 (s, 2H).

1-(4-(3,4-Dichlorobenzyloxy)benzyl)azetidine-3-carboxylic acid (29) - Large Scale. A 3 L round bottomed flask was charged with 4-(3, 4-dichlorobenzyloxy)benzaldehyde **79** (80 g, 0.28 mol) and azetidine-3-carboxylic acid (30.4 gm, 0.28 mol) was suspended in methanol (2 L), then acetic acid (8 mL) was added to the reaction mixture and stirred for 1 h. Sodium cyanoborohydride (9.6 g, 0.15 mol) was added portion wise and stirred over night at room temperature. The completion of the reaction was monitored by TLC. Solid was filtered, washed with methanol (2 x 250 mL) and dried to yield 62 g (60%) of 1-(4-(3,4-dichlorobenzyloxy)benzyl)azetidine-3-carboxylic acid as white solid. LCMS (Table 16, Method b) RT 1.81min; MS m/z: 366.1 $(M+H)^{+}$. Purity 100%. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.15 (m, 1H), 3.45 (m, 2H), 3.6 (m, 2H), 3.75 (s, 2H), 5.08 (s, 2H), 6.94 (d, 2H), 7.25 (d, 2H), 7.40 (d, 1H), 7.60 (d, 1H), 7.68 (s, 1H). ^{13}C NMR (125 MHz, d6-DMSO) δ 33.7, 56.0, 60.8, 67.6, 114.6, 127.7, 129.3, 129.8, 130.3, 130.6, 131.1, 138.3, 157.2, 174.3. IR (KBr pellet) 3421.1, 2923.6, 1612.2, 1512.8, 1248.7 cm^{-1}. MP: 165.6 - 166.1 °C. HRMS: Found 365.0583 $C_{18}H_{17}NO_2Cl_2$ requires 365.0585.

Inhibition of [^{33}P]S1P Binding to S1P Receptors

Radio-ligand binding was carried out using membranes from transiently transfected HEK cells overexpressing $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ or $S1P_5$. All compounds were dissolved in DMSO and serial dilutions were carried out in DMSO prior to addition to assay buffer. Final assay DMSO concentrations were 1 or 0.5 % (v/v). [^{33}P]S1P was purchased from Perkin Elmer and used at 50 pM in all assays. Frozen membranes were thawed and resuspended in assay buffer containing 50 mM HEPES pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$ and 0.1% fatty acid free BSA. Membrane was added to give 5-10 μg of membrane per well. Non-specific binding was determined in the presence of cold 1 μM S1P. Incubations were carried out at room temperature for 45-60 min; except that S1P2 binding was incubated 90 min at 4°C. After incubation, samples were filtered onto GF/B or GF/C filtration plates using a Packard 96 well harvester. Plates were dried before adding Microscint to each well, sealed and counted on a Topcount (Perkin-Elmer). Radioactivity counts are converted to percent activity, with samples containing [^{33}P]S1P as zero inhibition and samples containing [^{33}P]S1P plus 1 μM S1P as 100% inhibition. IC_{50}'s were determined by fitting the percent activity data to the equation percent activity = 100%-(100%/(1+([inhibitor]/IC_{50}))) using non-linear least-means-squares curve fitting.

S1P Receptor GTPγS Assays

The [^{35}S]GTPγS binding assay was performed using both scintillation proximity assay and filtration methods. Both formats are advantageously run in 96 well plates and utilize membranes from stable CHO human cell lines overexpressing $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ or $S1P_5$. Compound stocks were made up to 10 mM using DMSO and serial dilutions were carried out using 100% DMSO. Compounds were transferred to 96 well plates to yield a final DMSO concentration of 1 or 0.5 % (v/v) for all assays. Frozen membranes were thawed and diluted in assay buffer containing of 20 mM HEPES pH 7.4, 0.1% fatty acid-free BSA, 100 mM NaCl, 5 mM $MgCl_2$ and 10 μM GDP. For the SPA assay, membranes are premixed with WGA-SPA beads to yield a final concentration per well of 5 μg membrane and 500 μg of bead. For the filtration assay, membranes are added directly to the incubation plate at 5 μg per well. The assay begins with the

addition of 50 µl of the membrane or membrane/bead mixture to each well of the assay plate. Next, 50 µl of 0.4 nM [^{35}S]GTPγS is added to each well and incubated for 30 min. For the SPA assay the plates are spun and then read on the Topcount. For the filtration assay the plate is harvested onto GF-C filtration plates using a Packard 96 well harvester.

Cardiovascular Safety Study Methods

Male Sprague-Dawley rats (327-400 g) were anesthetized with the long acting barbiturate Inactin (100 mg/kg i.p.), and instrumented with vascular catheters located in femoral arteries (to measure mean arterial pressure and heart rate and to collect blood samples). An additional transducer-tip catheter was located in the left ventricle for measurement of left ventricular pressure (dP/dt at 50 mmHg), a marker of cardiac contractility. Two femoral vein catheters were inserted to deliver drug and saline (to maintain hydration). Following stabilization for at least 1 hour, a 30 minute baseline period was recorded followed by infusion of , **29** at 3, 10 and 30 mg/kg/30 minutes in 20% DMA/40% PG/40% PEG-400 vehicle (1 mL/kg); results with drug-treated animals (n=4) were compared to those treated with vehicle (n=5). Statistical analysis was by unpaired t tests. Plasma samples were taken at the end of each infusion period for exposure determinations.

PK methods lymphopenia

Female Sprague Dawley rats (Charles River Labs) were administered various doses of compound suspended in 0.5% HPMC/0.02% Tween 80 or vehicle alone by oral gavage. Five hours later, blood was collected via cardiac puncture under isoflurane anesthesia into tubes containing heparin. Whole blood was analyzed for lymphocyte count using a Cell-Dyn Hematology Analyzer (Abbott Diagnostics). Lymphocyte count is reported as mean +/- SEM (n = 4 or 6 per group). An aliquot of blood was centrifuged and plasma was collected and stored at -80°C until analyzed by mass spectrometry for drug concentration. Animals were housed in an AAALAC accredited SPF facility on a 12/12 hour light cycle and provided food and water ad libitum. All procedures involving animals were reviewed and approved by the internal IACUC.

Blood Brain Barrier Model

The assay was performed as previously described[1]. In short, transendothelial electrical resistance in confluent monolayers of hCMEC/D3 cells was measured using electrical cell-substrate impedance sensing model ZTheta (Applied BioPhysics, NY, USA) and 8W10E+ arrays. 300 µL Cell suspension (1.0 × 105 cells) was added to each well in Endothelial Cell Basal Medium-2 supplemented with 2.5% human serum and 5 ηg/ml bFGF. Cells were seeded in medium containing dimethyl sulfoxide (DMSO, n =4) or **29** (10-6M, n = 2). Prior to seeding, the collagen-coated ECIS arrays were equilibrated with growth medium and Rb values were calculated using ECIS software version 1.2.55.0 PC.

Spontaneous Alternation T-maze assay

Male C57BL/6J mice of 2 months (n = 11) and 12 months old (n = 20) were used for the study. They were purchased from (Janvier; Le Genest St Isle – France). Body weight was 27.4 ± 0.4 (mean ± SEM) and 33.0 ± 0.5 grams at the start of the experiment for 2 and 12 months old mice, respectively. Animals were group-housed with 5 to7 animals per cage, containing bedding material (Mixal, Safe, Epinay-sur-Orge, France) and maintained in a room with controlled temperature (21-22°C) with relative humidity range of 43 - 70 % and a reversed light-dark cycle (12h/12h; lights on: 17:30 - 05:30; lights off: 05:30 – 17:30). Food (A04; Safe, Epinay-sur-Orge, France) and water were available ad libitum.

Animals were randomly assigned to a group, and experiments were conducted blinded to treatment. Sub-acute treatment consisted of single daily po administrations (10 mL/kg) for 21 days before the T-maze. On the day of the T-maze (day 21), the treatment was conducted 225 min before the trial (Tmax). The T-maze apparatus was made of gray Plexiglas with a main stem (55 cm long x 10 cm wide x 20 cm high) and two arms (30 cm long x 10 cm wide x 20 cm high) positioned at 90 degree angle relative to the main stem. A start box (15 cm long x 10 cm wide) was separated from the main stem by a sliding door. The experimental protocol consists of one single session, which starts with 1 "forced-choice" trial, followed by 14 "free-choice" trials. A session was terminated as soon as 14 free-choice trials have been performed or 15 min have elapsed, whatever event occurred first. The percentage of alternation over the 14 free-choice trials was determined for each mouse and was used as an index of working memory performance. This percentage was defined as entry in a different arm of the T-maze over successive trials (i.e., left–right–left–right, etc). Analysis of variance (ANOVA) was performed on data with a post-hoc Tukey where appropriate. A p-value < 0.05 was considered significant.

SUPPORTING INFORMATION

Supporting information is available at http://pubs.acs.org

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ABBREVIATIONS

AAALAC: Association for Assessment and Accreditation of Laboratory Animal Care, ALT: Alanine Aminotransferase, DIAD: Diisopropyl Azodicarboxylate, ECIS: Electrical Cell-Substrate Impedence Sensing, Et_2O: Diethyl Ether, EtOH: Ethanol, FSP_3: Fraction SP_3 Carbon, HEPES: 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid, HPMC: Hypromellose, IACUC: Institutional Animal Care and Use Committees, IUPAC: International Union of Pure and Applied Chemists, MeOH: Methanol, MFB: Membrane Filtration Binding, NAR: Number of Aromatic Rings, NRB: Number of Rotatable Bonds, PE/EA: Petroleum Ether/Ethyl Acetate, PhMe: Toluene, PTSA: Para-Toluene Sulphonic Acid, PTZ: Pentylenetetrazole, RLB: Radio-Ligand Binding, S1P: Sphingosine-1-Phosphate, $S1P_1$: Sphingosine-1-Phosphate Receptor 1, $S1P_2$: Sphingosine-1-Phosphate Receptor 2, $S1P_3$: Sphingosine-1-Phosphate Receptor 3, $S1P_4$: Sphingosine-1-Phosphate Receptor 4, $S1P_5$: Sphingosine-1-Phosphate Receptor 5, SEM:

Standard Error of the Mean, SPA: Scintillation Proximity Assay, TEER: Transendothelial Electrical Resistance.

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