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Discovery, synthesis and SAR analysis of novel selective small molecule $S1P_4$-R agonists based on a (2*Z*,5*Z*)-5-((pyrrol-3-yl)methylene)-3-alkyl-2-(alkylimino)thiazolidin-4-one chemotype

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ABSTRACT

High affinity and selective $S1P_4$ receptor ($S1P_4$-R) small molecule agonists may be important proof-of-principle tools used to clarify the receptor biological function and effects to assess the therapeutic potential of the $S1P_4$-R in diverse disease areas including treatment of viral infections and thrombocytopenia. A high-throughput screening campaign of the Molecular Libraries-Small Molecule Repository was carried out by our laboratories and identified (2*Z*,5*Z*)-5-((1-(2-fluorophenyl)-2,5-dimethyl-1*H*-pyrrol-3-yl)methylene)-3-methyl-2-(methylimino) thiazolidin-4-one as a promising $S1P_4$-R agonist hit distinct from literature $S1P_4$-R modulators. Rational chemical modifications of the hit allowed the identification of a promising lead molecule with low nanomolar $S1P_4$-R agonist activity and exquisite selectivity over the other $S1P_{1-3,5}$-Rs family members. The lead molecule herein disclosed constitutes a valuable pharmacological tool to explore the effects of the $S1P_4$-R signaling cascade and elucidate the molecular basis of the receptor function.

Sphingosine-1-phosphate (S1P) is a bioactive lysophospholipid metabolite formed from ceramide and sphingosine in various cells including mast cells, platelets, and macrophages in response to diverse stimuli such as growth factors, cytokines, G-protein-coupled receptors (GPCRs) agonists and antigens. S1P regulates a broad variety of cellular signaling pathways resulting in calcium homeostasis, actin polymerization, cell proliferation, motility and survival. The biological responses of S1P are mediated by the activation of cell membrane GPCRs as well as by the modulation of incompletely characterized intracellular targets. The GPCRs responsive to S1P include $GPR_{3,6,12}$ receptor family, and five receptor subtypes of the endothelial differentiation genes (Edg) family termed $S1P_{1-5}$ (formally Edg-1, -5, -3, -6, and -8).[1–5]

Recently, $S1P_{1-5}$ receptor ($S1P_{1-5}$-Rs) family has gained growing attention due to its physiological and pathophysiological role in immune function, cardiovascular system and cancer invasion.[2] S1P receptor agonists have been demonstrated to alter immunological responses and inhibit lymphocyte recirculation.[3–8] Noteworthy, numerous high affinity $S1P_1$-R molecule agonists are currently in preclinical and clinical trials as immunosuppressive drug candidates for the treatment of autoimmune diseases. Amongst them, $S1P_{1,3-5}$-Rs pan-agonist Fingolimod (FTY720) has been recently approved by the FDA as orally active prodrugfor the treatment of multiple sclerosis.[9,10]

Understanding S1P-modulated pathways in the endothelial and vascular smooth muscle cells is a critical ongoing area of investigation. Importantly, S1P-dependent activation of vascular endothelial $S1P_{1,3}$-Rs promotes vasorelaxation responses and antagonizes vasoconstriction by activation of the endothelial isoform of nitric oxide synthase and subsequent production of nitric oxide via the small GTP-binding cytoskeleton signaling protein Rac 1. In contrast, $S1P_{2,3}$-Rs in smooth muscle cells can elicit vasoconstriction responses through the activation of the small G-protein RhoA signaling cascade, particularly at high concentrations of S1P.[4,11]

Interestingly, molecules targeting S1P-metabolizing enzymes have been recently proposed as innovative potential therapeutics for viral infections.[12]

Additionally, sphingosine kinase 1 (SphK1)/S1P signaling cascade has been recently linked to the transcription factor hypoxia-inducible factor 1α (H1F-1α) in distinct tumor cell lines. Given the key role of H1F-1α in the adaptive changes to hypoxia

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including angiogenesis and metastasis, SphK1/S1P pathway has been proposed as an innovative target for therapeutic intervention in oncology, thus demanding further studies to fully elucidate the function of S1P and its target receptors upon Sphk1 activation.[13]

Amongst $S1P_{1-5}$-Rs, the $S1P_4$-R couples to $G\alpha_i$, $G\alpha_o$ and $G_{\alpha 12/13}$ proteins leading to the stimulation of MAPK/ERK signaling pathways, as well as PLC and Rho-Cdc42 activation.[14,15]

$S1P_4$-R is predominantly expressed in lymphocyte-containing tissues including the thymus, spleen, bone marrow, appendix, peripheral leukocytes, lung, and shows approximately 100–600-fold reduced binding affinity for S1P compared to the other S1P-R family members.[16]

Although poorly characterized, the contribution of $S1P_4$-R to the immune response is becoming increasingly evident. It has been demonstrated that S1P induces migratory response of murine T-cell lines expressing both $S1P_1$-R and $S1P_4$-R mRNA; in D10.G4.1 and EL-4.IL-2 murine cells, S1P-induced migration was significantly inhibited by treatment with (*S*)-FTY720-phosphate, a potent agonist at $S1P_1$-R and $S1P_4$-R. Additionally, S1P-induced T-cell migration involved the activation of Rho family small GTPase, Cdc 42 and Rac, in murine CHO cells co-expressing $S1P_4$-R and $S1P_1$-R on the cell surface. These results have suggested that the association of $S1P_4$-R and $S1P_1$-R may play an important role in the migratory and recirculation response of T-cells toward S1P.[1]Additional studies demonstrated that the intratracheal delivery of the synthetic sphingosine analogs with mixed activity over $S1P_{1,3-5}$-Rs efficiently inhibited the T-cell response to influenza virus infection by impeding the accumulation of dendritic cells (DCs) in draining lymph nodes. The inhibitory effects were not observed upon specific chemical activation of $S1P_1$-R, and persisted in $S1P_3$-R null mice. Based on these findings and on the evidence that $S1P_4$-R is highly expressed in DCs in contrast to $S1P_5$-R expression, it has been hypothesized that the $S1P_4$-R modulation in the lung may be effective at controlling the immunopathological response to viral infections.[17,18]

Moreover, while the S1P effects on the systemic vasoregulation are predominantly mediated by $S1P_{1,2,3}$-Rs subtypes, the $S1P_4$-R has been demonstrated to have a key role in S1P-induced vasoconstriction in the rat pulmonary circulation by activating Rho kinase.[19]

Both in vitro and in vivo experiments in animal models have recently indicated an additional potential therapeutic application of $S1P_4$-R molecule modulators in the terminal differentiation of megakaryocytes. Notably, the application of $S1P_4$-R antagonist might be exploited for inhibiting potentially detrimental reactive thrombocytosis, whereas $S1P_4$-R agonists represent a potential therapeutic approach for stimulating platelet repopulation after thrombocytopenia.[20]

To date, despite the $S1P_4$-R therapeutic potential, the in vivo function of the target receptor remains largely unknown. Indeed, the limited number of known selective $S1P_4$-R small molecule antagonists is restricted to the molecules recently disclosed by our research group,[21,22] whereas promiscuous selectivity profile is found for the $S1P_4$-R agonists reported in the literature. Amongst them, benzymidazole derivative **1** was reported as a potent $S1P_4$-R agonist with low nanomolar partial agonist (pa) activity on $S1P_{1,5}$-Rs subtypes (Fig. 1).[23] The constrained azacyclic analog of FTY720 **2** was described as a potent non selective $S1P_{4,5}$-Rs agonist (Fig. 1).[24] Remarkably, the indole-alanine derivative **3** has been reported as apotent $S1P_4$-R agonist with good selectivity against the other S1P family receptors (Fig. 1).[25] However, **3** and its structurally related compounds have been also studied as high affinity modulators of glycine recognition site on the *N*-methyl-D-aspartate receptor complex.[26]

In an effort to discover novel and selective $S1P_4$-R agonists, a high-throughput screening (HTS) campaign of the Molecular Libraries-Small Molecule Repository (MLSMR) was carried out by our laboratories and identified the hit (2*Z*,5*Z*)-5-((1-(2-fluorophenyl)-2,5-dimethyl-1*H*-pyrrol-3-yl)methylene)-3-methyl-2-(methylimino)thiazolidin-4-one **4** endowed with moderate $S1P_4$-R agonist activity, modest selectivity against $S1P_{1,5}$-Rs and no activity over $S1P_{2,3}$-Rs at concentrations up to 25 μM (Fig. 1).

The structural integrity and biological activity of the original hit **4** were confirmed by re-synthesizing the title compound (Scheme 1). Reaction of commercially available dimethylthiourea **7I** with ethylchloroacetate **8** provided 3-methyl-2-(methylimino)-thiazolidin-4-one **9I** which was then reacted with pyrrole-3-carbaldehyde **14**. The (2*Z*,5*Z*)-configuration of 2-methylimino and the olefinic bond of **4** was verified by $^1H,^1H$-NOESY experiment.[27]

To start our SAR studies on **4**, we prepared a series of derivatives varying the polar thiazolidin-4-one head while maintaining the 2-fluorophenyl coil and the 2,5-dimethylpyrrol-3-yl spacer as constant moieties. Representative examples of the explored modifications are represented by compounds **15a**–**15n** (Scheme 1, Table 1). **15a–15e**, **15g**, **15j–15n** were synthesized using the appropriate commercially available thioureas (**7II**–**7VII**, **7IX**–7**XIII**) and **8** to give the corresponding thizolidin-4-ones (**9II–9VII**, **9IX–9XIII**). The synthesis of compound **15h** involved the preparation of the thiourea precursor (**7VIII**) from methyl isothiocyanate (**5**) and 2-ethanolamine (**6**), followed by the synthesis of the corresponding thiazolidin-4-one(**9VIII**). **15i** was synthesized from oxazolidin-4-one (**12**) obtained by reaction of dimethyl urea (**10**) with chloro acetylchloride (**11**), while compound **15f** was easily obtained from commercially available 3-methylthiazolidine-2,4-dione (**13**). Final condensation of the aforementioned five-membered ring intermediates (**9II–9XIII**, **12**, **13**) with **14** furnished the desired products **15a**–**15n**.

The biological results of the obtained molecules are listed in Table 1.[28]

When the methyl group was removed from either position 3 (**15a**) or both positions 2 and 3 (**15b**), the potency decreased substantially. Moreover, the 3-unsubstituted 2-phenylimino analog (**15d**) was devoid of activity, and significantly reduced potency was found for the corresponding 2-(pyridin-2-yl) derivative (**15e**) as well as for the 3-methyl-2-phenylimino analog (**15c**). Compounds containing bulky alkylic groups at both positions 2 and 3 were devoid of potency (**15l**–**15n**). By contrast, similar activity to the hit was found in the presence of ethyl groups at the same positions (**15g**). Noteworthy, the analog containing the polar 2-hydroxyethyl substituent at position 2 (**15h**) was only 1.5-less potent than the hit. Taken all together, these data suggest that bulky alkylic groups with different polarity are tolerated at position 2, while position 3 may be involved in a lipophilic interaction within a smaller binding pocket. Replacement of the 2-(methylimino)thiazolidin-4-one head with thiazolidine-2,4-dione (**15f**) or oxazolidin-4-one (**15i**) led to complete loss of activity. Interestingly, conformationally restricted analogs (**15j**, **15k**) were also inactive. To note, the 2-alkylimino functionality of **15j** and**15k** is locked into the *E*-geometry suggesting that the *Z*-configuration at position 2 may be a binding requirement.

Successively, we synthesized compounds **19a–19x** (Scheme 2, Table 2)in order to optimize the lipophilic aryl coil while keeping the thiazolidin-4-one polar head and the 2,5-dimethylpyrrol-3-yl spacer as in the hit and **15g**. The synthesis of these derivatives is depicted in Scheme 2. Condensation of hexadione **16** with the appropriate amine **17** furnished the corresponding N-substituted-2,5-dimethylpyrrole, which yielded the pyrrol-3-carbaldehyde derivative **18** via Vilsmeier–Haack reaction. Condensation of **18** with the opportune thiazolidin-4-one (**9I** or **9VII**) afforded the desired products **19a–19x**. The biological responses of the obtained compounds are listed in Table 2.[28]

When the *ortho*-fluorine of the hit was replaced with either chlorine, bromine or methyl group (**19a**, **19b**, **19c**), no activity

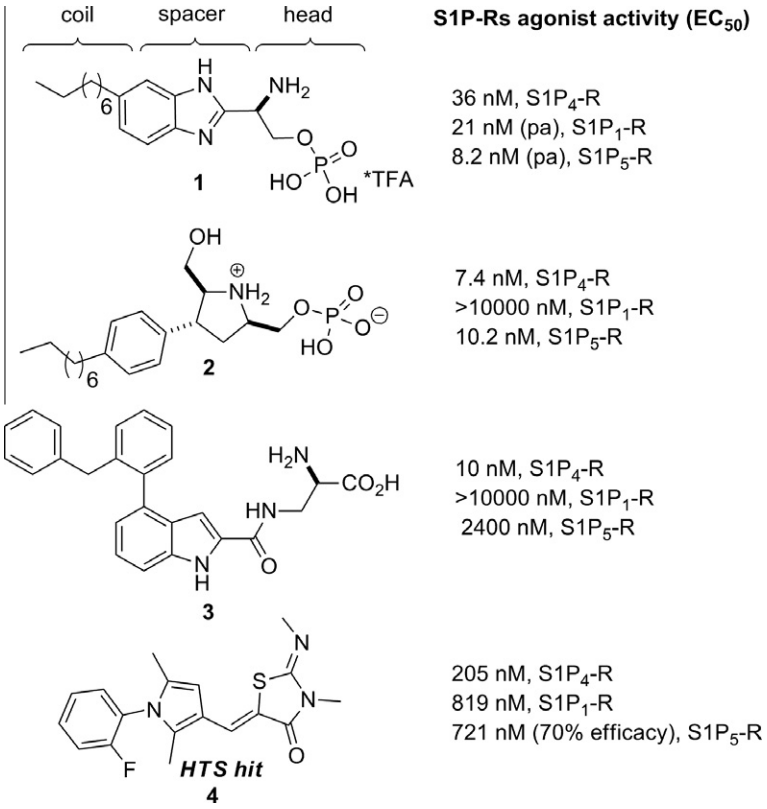
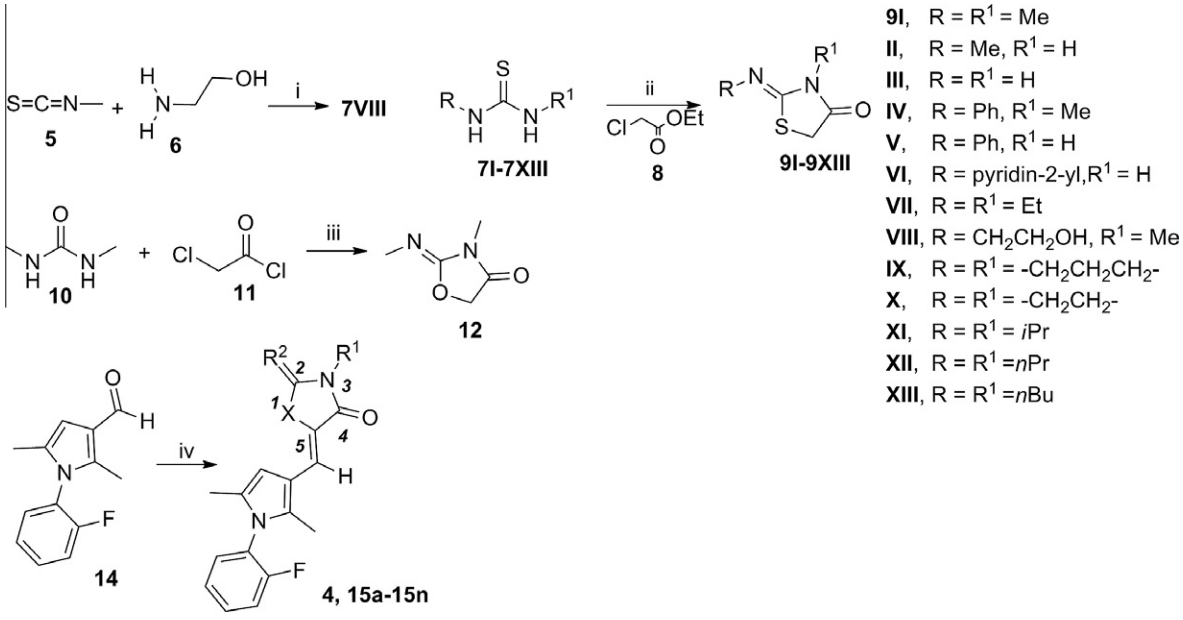


Figure 1. $S1P_4$-R agonist modulators.



Scheme 1. Synthesis of analogs **15a**–**15n**. Reagents and conditions: (i) **5** (1 equiv), **6** (1.05 equiv), CH_2Cl_2, 0 °C to rt, (ii) **7I**–**7XIII** (1 equiv), **8** (2 equiv), NaOAc (5 equiv), EtOH, 60 °C, overnight; (iii) **10** (1 equiv), **11** (1.1 equiv), tolune, 100 °C, 5 h; (iv) **9I**–**9XIII** or **12** or **13** (1 equiv), **14** (1 equiv), piperidine (1.5 equiv), EtOH, 60 °C, 4 h.

was observed. Interestingly, the 3-fluorophenyl isomer (**19d**) was eight-fold less potent, while the 4-fluorophenyl derivative (**19e**) showed only two-fold lower potency than the hit. Compounds containing variously disubstituted phenyl rings including but not limited to the examples herein reported (**19f–h, 19j**) were found to be inactive or have significantly reduced activity, thus suggesting that polar and bulky groups in this region are detrimental for the activity. Consistent with this hypothesis, naphthalenyl derivatives (**19n, 19o**) were inactive. Interestingly, amongst the disubstituted phenyl rings, the 4-chloro-2-fluorophenyl analog (**19k**) was only two-fold less potent and the 2,4-difluorophenyl analog (**19i**) was equipotent to the hit, while the unsubstituted phenyl derivative (**19l**) was only 2–3-fold less potent than **15g**. The fluorine was therefore identified as suitable bioisoster of the hydrogen

Table 1
S1P$_4$-R agonist activity of compounds **15a–15n** (EC_{50} nM)

Compd	R^2	R^1	X	EC_{50}[a] (nm)
15a	NMe	H	S	3600
15b	NH	H	S	NA
15c	NPh	Me	S	2800
15d	NPh	H	S	NA
15e	N(pyridin-2-yl)	H	S	2100 (70%)[b]
15f	O	Me	S	NA
15g	NEt	Et	S	250
15h	NCH_2CH_2OH	Me	S	306
15i	NMe	Me	O	NA
15j	$NCH_2CH_2CH_2$		S	NA
15k	NCH_2CH_2		S	NA
15l	N*i*Pr	*i*Pr	S	NA
15m	N*n*Pr	*n*Pr	S	NA
15n	N*n*Bu	*n*Bu	S	NA

[a] Data are reported as mean of $n = 3$ determinations.
[b] Percentage of response. NA = not active at concentrations up to 25 μM.

Table 2
S1P$_4$-R agonist activity of compounds **19a–19x** (EC_{50} nM)

Compd	Ar	R	R^1	EC_{50}[a] (nm)
19a	2-Chlorophenyl	Me	Me	NA
19b	2-Bromophenyl	Me	Me	NA
19c	2-Methylphenyl	Me	Me	NA
19d	3-Fluorophenyl	Me	Me	1600 (60%)[b]
19e	4-Fluorophenyl	Me	Me	433
19f	3-Cyano-4-fluoro-phenyl	Me	Me	3200 (50%)[b]
19g	4-Methoxy-3-(trifluoromethyl) phenyl	Me	Me	NA
19h	2-Methoxy-5-methyl phenyl	Me	Me	NA
19i	2,4-Difluorophenyl	Me	Me	210
19j	2,4-Dimethylphenyl	Me	Me	NA
19k	4-Chloro-2-fluorophenyl	Me	Me	440
19l	Phenyl	Et	Et	602
19m	Pyridin-2-yl	Me	Me	1800
19n	Naphthalen-1-yl	Me	Me	NA
19o	Naphthalen-1-yl	Et	Et	NA
19p	3-Fluoro-pyridin-2-yl	Me	Me	3100 (70%)[b]
19q	3-Fluoro-pyridin-2-yl	Et	Et	5400 (70%)[b]
19r	Benzyl	Me	Me	262
19s	2-Flurobenzyl	Me	Me	72
19t	2,6-Difluorobenzyl	Me	Me	92
19u	2,6-Difluorobenzyl	Et	Et	314
19v	2,4-Diflurobenzyl	Me	Me	147
19w	1-Phenyleth-1-yl	Me	Me	660 (20%)[b]
19x	1-Phenyleth-1-yl	Et	Et	NA

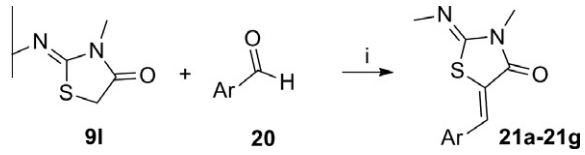
[a] Data are reported as mean of $n = 3$ determinations.
[b] Percentage of response. NA = not active at concentrations up to 25 μM.

atom at positions 2,4 of the phenyl ring. By contrast, replacement of 2-fluophenyl with the basic pyridinyl ring (**19m**) resulted in nine-fold decreased potency than the hit; reduced activity compared to the hit and **15g**, was also found for 3-fluoropyridinyl derivatives (**19p–19q**). Elongation of the hydrophobic coil by insertion of a methylene at the pyrrole nitrogen was tolerated as observed for the benzyl (**19r**), 2-flurobenzyl (**19s**), 2,6-difluorobenzyl (**19t–19u**) and 2,4-difluorobenzyl (**19v**) analogs. However, the introduction of a methyl group at the benzylic carbon negatively affected the potency (**19w**, **19x**) probably as a result of a steric clash within the binding site or due to conformational changes in the molecule.

The study of the 2,5-dimethylpyrrol-3-yl spacer was carried out conserving the head 3-methyl-2-(methylimino)thiazolidin-4-one as in the hit, and selecting the easily accessible coil fragments from the active molecules. The synthesis of these derivatives is outlined in Scheme 3. The condensation of thiazolidin-4-one **9l** with commercially available carbaldehydes **20** under the conditions previously described furnished the desired products **21a–21g** that were submitted for biological activity (Table 3).[28]

The 5-methylpyrazol-4-yl derivative (**21c**) was 18-fold less potent than the hit. Complete loss of activity was observed for the 3,5-dimethyl and the unsubstituted pyrazol-4-yl analogs(**21a–b**) as well as for the 2,5-unsubstituted pyrrol-3-yl (**21d–21f**) and the 2-methyl indol-3-yl (**21g**) analogs, thus indicating that the 2,5-dimethylpyrrol-3-yl moiety is an essential molecular feature for the receptor binding.

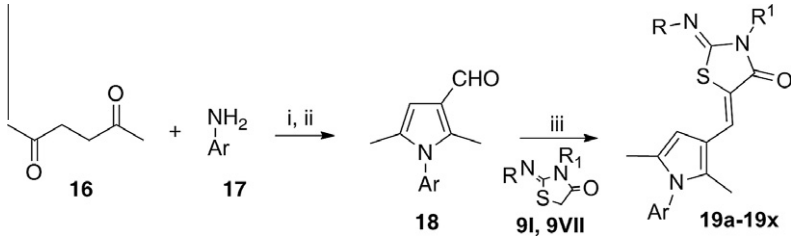
The functional activity of the most active compounds was tested against S1P$_{1\text{-}3,5}$-Rs subtypes (Table 4).The phenyl 3-ethyl-2-(ethylimino)thiazolidin-4-one derivative **19l** showed increased S1P$_{1,5}$-Rs activity, while retaining selectivity towards S1P$_{2\text{-}3}$-Rs compared to the hit. Interestingly, the benzyl derivatives **19r–19v** showed high to low nanomolar activity for S1P$_{1,5}$-Rs; some activity versus S1P$_2$-R and S1P$_3$-R was also observed for compounds **19r–19t**. The



Scheme 3. Synthesis of analogs **21a–21g**. Reagents and conditions: (i) **9l** (1 equiv), **20** (1 equiv), piperidine (1.5 equiv), EtOH, 60 °C, 4 h.

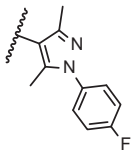
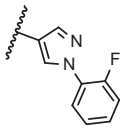
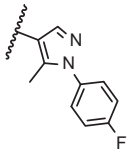
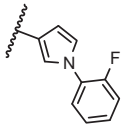
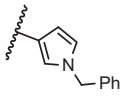
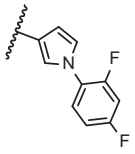
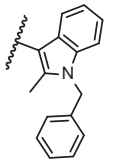
4-fluorophenyl derivative **19e** displayed two-fold decreased potency against S1P$_1$-R, while keeping similar activity pattern for S1P$_{2,3,5}$-Rs compared to the hit. Significantly improved selectivity profile was found for the 2-fluorophenyl 3-methyl-2-((2-hydroxyethyl)imino) derivative **15h** which showed only a weak activity for S1P$_1$-R and S1P$_5$-R subtypes. Similarly, the 4-chloro-2-fluorophenyl derivative **19k** showed only modest activity against S1P$_{1,5}$-Rs. Interestingly, the introduction of ethyl groups at the thiazolidin-4-one head of the hit conferred to **15g** exquisite selectivity against all S1P$_{1\text{-}3,5}$-Rs subtypes. Additionally, a good potency/selectivity profile was observed in the presence of 2,4-difluorophenyl as lipophilic coil with **19i** displaying high selectivity against S1P$_1$-R (21-fold), and no activity against S1P$_{2\text{-}3,5}$-Rs up to 25 μM.

The SAR at the polar head were further investigated by the synthesis of derivatives **24a–24g** (Scheme 4, Table 5) containing



Scheme 2. Synthesis of analogs **19a–19x**. Reagents and conditions: (i) **16** (1 equiv) **17** (1 equiv), sulfamic acid (0.05 equiv), rt, 3 h; (ii) $POCl_3$ (2 equiv), DMF, 0–60 °C, 3 h; (iii) **18** (1 equiv), **9l** or **9VII** (1 equiv) piperidine (1.5 equiv), EtOH, 60 °C, 4 h.

Table 3
$S1P_4$-R agonist activity of compounds **21a–21g** (EC_{50} nM)

Compd	Ar	EC_{50}[a] (nm)
21a		NA
21b		NA
21c		3800
21d		NA
21e		NA
21f		NA
21g		NA

[a] Data are reported as mean of $n = 3$ determinations. NA = not active at concentrations up to 25 μM.

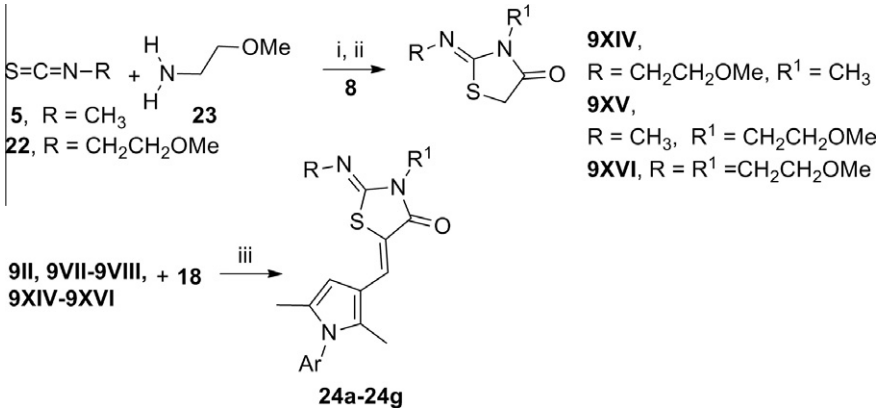
the 2,5-dimethylpyrrol-3-yl moiety and either the benzyl or 2,4-difluorophenyl nucleus, the latter selected as the most suitable lipophilic coil in terms of receptor biological profile. Compounds **24a–24c** were synthesized as previously described starting from the opportune thiazolidin-4-ones (**9II**, **9VII–9VIII**, Scheme 1) and the pyrrol-3-carbaldehydes (**18**, Scheme 2). Analogously, the synthesis of **24d–24g** was accomplished starting from the appropriate isothiocyanate (**5**, **22**) and 2-methoxyethanamine (**23**) to provide the corresponding thiazolidin-4-ones **9XIV–9XVI** which were successively reacted with appropriate pyrrol-3-carbaldehyde (**18**) to give the final products (Scheme 4). The $S1P_4$-R functional activity of the monomethyl **24a** and the diethyl **24b** 2,4-difluorophenyl derivatives paralleled those of the 2-fluorophenyl series **15a** and **15g**. The benzyl 2-(2-hydroxyethyl)iminoderivative **24c** had similar potency compared to the corresponding 2-fluorophenyl analog **15h**. Interestingly, the 2-(2-methoxyethyl)imino analog **24d** showed six-fold increased activity compared to **24c**, thus prompting us to synthesize the 2,4-difluorophenyl analog **24f** (**CYM50308**).[29] Notably, **24f** was respectively 4- and 30-fold more potent than **19i** and the regioisomer **24g**.[30] These findings support our working hypothesis that larger polar groups are better tolerated at position 2 of the thizolidin-4-one head, as further corroborated by the lack of activity shown by the di-substituted 3-(2-methoxyethyl)-2-(2-methoxyethyl)imino derivative **24e**. To test for selectivity, **24b, 24d** and **24f** were assayed on $S1P_{1\text{-}3,\ 5}$-Rs subtypes (Table 6). The 2,4-difluorophenyl 3-ethyl-2-(ethylimino) derivative **24b** showed decreased selectivity against $S1P_{1,5}$-Rs compared to the dimethyl (**19i**) and the 2-fluorophenyl (**15g**) counter parts. The benzyl 3-methyl-2-(2-methoxyethyl)imino derivative **24d** was selective against $S1P_{1\text{-}3}$-Rs but displayed only three-fold selectivity versus $S1P_5$-R. Remarkably, the 2,4-difluorophenyl 2-(2-methoxyethyl)imino derivative **24f** elicited exquisite selectivity profile displaying 37-fold selectivity against $S1P_5$-R and no appreciable activity over $S1P_{1\text{-}3}$-Rs subtypes at concentrations up to 25 μM.

In summary, we have reported the discovery, synthesis and SAR analysis of novel selective small moleculeS$1P_4$-R functional agonists based on a (2*Z*,5*Z*)-5-((pyrrol-3-yl)methylene)-3-alkyl-2-(alkylimino)thiazolidin-4-one chemotype structurally unrelated to the known $S1P_4$-R modulators. Systematic SAR studies of the MLSMR hit **4**, endowed with moderate $S1P_4$-R potency, high selectivity over $S1P_{2,3}$-Rs but submicromolar activity towards both $S1P_{1,5}$-Rs, led to the identification of a full spectrum selective $S1P_4$-R agonist compound **24f** (**CYM50308**). Indeed, the disclosed lead molecule **24f** displayed low nanomolar $S1P_4$-R agonist activity and exquisite selectivity over the other S1P-Rs subtypes. Noteworthy, **24f** provides a novel valuable pharmacological tool to explore the effects of the $S1P_4$-R signaling cascade and elucidate the molecular basis of the in vivo receptor function. Details of further research efforts will be communicated in due course.

Table 4
$S1P_{1\text{-}5}$-Rs selectivity counter screen of selected compounds

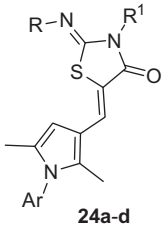
Compd	EC_{50}[a] (nM)				
	$S1P_4$	$S1P_1$	$S1P_2$	$S1P_3$	$S1P_5$
4	205	819	NA	NA	721 (70%)[b]
15g	250	NA	NA	NA	NA
15h	306	20% (8.3 μM)[c]	NA	NA	60% (8.3 μM)[c]
19e	433	1800 (70%)[b]	NA	NA	652 (50%)[b]
19i	210	4470	NA	NA	NA
19k	440	3010	NA	NA	3400
19l	602	68	NA	NA	237
19r	262	720	3650	NA	144
19s	72	250	60% (25 μM)[c]	25% (25 μM)[c]	51
19t	92	34	120% (25 μM)[c]	70%(25 μM)[c]	34
19u	314	18	NA	NA	22
19v	147	287	NA	NA	37

[a] Data are reported as mean of $n = 3$ determinations.
[b] Percentage of response.
[c] Concentration producing the reported percentage of response. NA = not active at concentrations up to 25 μM.



Scheme 4. Synthesis of analogs **24a**–**24g**. Reagents and conditions: (i) **5** or **22** (1 equiv), **23** (1.05 equiv), CH_2Cl_2, 0 °C to rt, 2 h; (ii) **8** (2 equiv), EtOH, 60 °C, overnight; (iii) **9II**, **9VII**–**9VIII**, **9XIV**–**9XVI** (1 equiv), **18** (1 equiv), piperidine (1.5 equiv), EtOH, 60 °C, 4 h.

Table 5
$S1P_4$-R agonist activity of compounds **24a**–**24g** (EC_{50} nM)



24a-d

Compd	R	R^1	Ar	EC_{50}[a] (nm)
24a	Me	H	2,4-Difluorophenyl	1000
24b	Et	Et	2,4-Difluorophenyl	104
24c	CH_2CH_2OH	Me	Benzyl	396
24d	CH_2CH_2OMe	Me	Benzyl	68
24e	CH_2CH_2OMe	CH_2CH_2OMe	Benzyl	NA
24f	CH_2CH_2OMe	Me	2,4-Difluorophenyl	56
24g	Me	CH_2CH_2OMe	2,4-Difluorophenyl	1700

[a] Data are reported as mean of $n = 3$ determinations. NA = not active at concentrations up to 25 μM.

Table 6
$S1P_{1-5}$-Rs selectivity counter screen of compounds **24b**, **24d**, **24f** (EC_{50} nM).

Compd	EC_{50}[a] (nm)				
	$S1P_4$	$S1P_1$	$S1P_2$	$S1P_3$	$S1P_5$
24b	104	816	NA	NA	6180
24d	68	85%[c]	NA	NA	196 (80%)[b]
24f	56	NA	NA	NA	2100

[a] Data are reported as mean for $n = 3$ determinations.
[b] Percentage of response.
[c] Percentage of inhibition at 25 μM. NA = not active at concentrations up to 25 μM.

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27. $^1H,^1H$-NOESY spectrum was acquired using spectrometer Bruker DRX-600 equipped with a 5 mm DCH cryoprobe. NOE interaction was not observed between the methyl groups located at position 2 and 3 of the thiazolidin-4-one nucleus, thus indicating *Z* configuration of the 2-methylimino bond (numeration as in Scheme 1). The H atom of the olefinic bond at position 5 (numeration as in Scheme 1) gave rise to a cross-peak with the methyl group at position 2 but not with the H atom at position 4 of the 2,5-dimethylpyrrol-3-yl nucleus; *Z* configuration was therefore assigned to the aforementioned bond.
28. The biological assays were performed using Tango $S1P_4$-BLA U2OS cells containing the human Endothelial Differentiation Gene 6 (EDG6; $S1P_4$-R) linked to a GAL4-VP16 transcription factor via a TEV protease site. The cells also express a beta-arrestin/TEV protease fusion protein and a beta-lactamase (BLA) reporter gene under the control of a UAS response element. Stimulation of the $S1P_4$-R by agonist causes migration of the fusion protein to the GPCR, and through proteolysis liberates GAL4-VP16 from the receptor. The liberated

VP16-GAL4 migrates to the nucleus, where it induces transcription of the BLA gene. BLA expression is monitored by measuring fluorescence resonance energy transfer (FRET) of a cleavable, fluorogenic, cell-permeable BLA substrate. As designed, test compounds that act as $S1P_4$-R agonists will activate $S1P_4$-R and increase well FRET. Compounds were tested in triplicate at a final nominal concentration of 25 μM.

29. The regiochemistry and (2*Z*,5*Z*)-configuration of **24f** were verified respectively by Heteronuclear Multiple Bond Coherence (HMBC) and $^{1}H,^{1}H$-NOESY experiments performed on spectrometer Bruker DRX-600 equipped with a 5 mm DCH cryoprobe. In $^{1}H,^{13}C$-HMBC spectrum the α-protons of the 2-(2-methoxyethyl)imino substituent only coupled to carbon C2 of the 3-methyl-2-((2-methoxyethyl)imino)thiazolidin-4-one scaffold. No NOE effects were observed between the α-protons of the 2-(2-methoxyethyl)imino and the 3-methyl substituent of the 3-methyl-2-(2-methoxyethyl)imino)thiazolidin-4-one core; the proton of the exocyclic olefinic bond at position 5 gave rise to a cross-peak only with the 2-methyl group of the pyrrol-3-yl ring (numeration as in Scheme 1).
30. The regiochemistry of **24g** was verified by $^{1}H,^{13}C$-HMBC experiment performed using spectrometer Bruker DRX-600 equipped with a 5 mm DCH cryoprobe: the α-protons of the 3-(2-methoxyethyl) substituent coupled to both carbons C2 and C4 of the 3-(2-methoxyethyl)-2-(methylimino)thiazolidin-4-one nucleus (numeration as in Scheme 1).