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Identification and synthesis of potent and selective pyridyl-isoxazole based agonists of sphingosine-1-phosphate 1 ($S1P_1$)

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ABSTRACT

The synthesis and structure–activity relationship (SAR) of a series of pyridyl-isoxazole based agonists of $S1P_1$ are discussed. Compound **5b** provided potent in vitro activity with selectivity, had an acceptable pharmacokinetic profile, and demonstrated efficacy in a dose dependent manner when administered orally in a rodent model of arthritis.

Sphingosine-1-phosphate (S1P) is the endogenous ligand for the sphingosine-1-phophate receptors ($S1P_{1-5}$).[1] The interaction between S1P and the S1P receptors plays a fundamental physiological role in a number of processes including vascular stabilization, heart development, lymphocyte homing, and tumor related angiogenesis.[2] Agonism of $S1P_1$, in particular, has been shown to play a significant role in lymphocyte trafficking from the thymus and secondary lymphoid organs, resulting in immunosuppression.[3] In support of this, S1P Kinase knock-out (KO) mice have been shown to significantly reduce lymphocyte egress from the thymus and the lymph nodes.[4] Additionally, lymphocyte egress was significantly impaired in $S1P_1$ conditional KO mice,[3,5] as well as with the treatment of a selective $S1P_1$ agonist.[6] Ultimately, entrapped lymphocytes are unable to reach targeted tissues (Fig. 1).

Fingolimod (**1**, FTY720) (Fig. 2) has been validated as an immunological therapeutic agent in a variety of preclinical models of autoimmune disease, demonstrating immunosuppression with a reduction in circulating lymphocytes.[2b] Additionally, fingolimod (**1**) was shown to be rapidly phosphorylated by sphingosine-1 phosphate kinase 2 (S1PK2) to form the phosphorylated species (**2**, FTY720-P) which acts as a potent agonist on the $S1P_{1,3,4,5}$ subtypes (Fig. 2).[7] In 2010, **1** was approved as a first line therapy for the treatment of multiple sclerosis. In spite of its clinical success, several concerns emerged during clinical trials including cardiovascular effects (bradycardia and blood pressure elevation), macular edema, and a prolonged half-life in human.[8]

Consistent with other research groups,[9,10] our efforts have been directed toward identifying an orally active, direct-acting small molecule $S1P_1$ full agonists for use in autoimmune diseases with selectivity for $S1P_1$ over $S1P_3$ and improved PK properties relative to fingolimod (**1**) to minimize potential host defense issues. Agonism of $S1P_3$ was originally thought to be the source of the cardiovascular effects observed with **1**, based on rodent studies,[11] but more recent clinical studies with $S1P_3$ sparing agonists have shown that the effects seen in humans are dependent, at least in part, on $S1P_1$ activation.[12] Since $S1P_3$ agonism does not appear to contribute to the efficacy, selectivity remains a desirable attribute.

The key structural features of fingolimod (**1**) include an aminodiol polar head group which is phosphorylated by S1PK2, a 1,4-disubstituted phenyl ring which acts as a rigid linker, and a lipophilic tail which is important for interacting with the hydrophobic binding pocket of the S1P receptors.[13,14] We recently disclosed the identification of BMS-520 (**3**) as a potent and selective $S1P_1$ agonist

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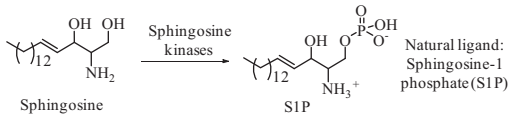


Figure 1. Structures of sphingosine and sphingosine 1-phosphate (S1P).

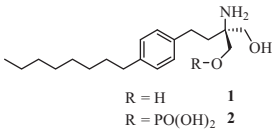


Figure 2. Structures of fingolimod (**1**) and fingolimod-P (**2**).

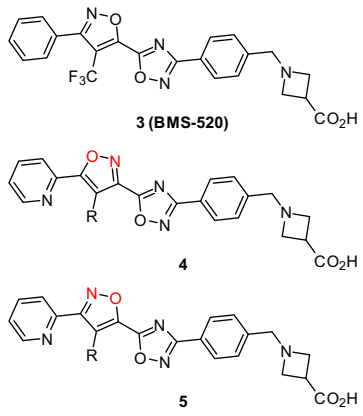


Figure 3. Discovery of pyridyl-isoxazole based agonists of $S1P_1$.

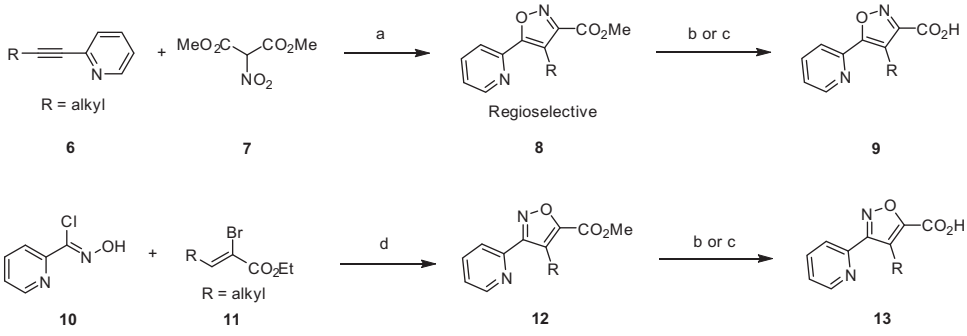
with a phenyl-isoxazole as lipophilic tail fragment.[15] In this Letter, we describe our efforts toward exploring pyridyl-isoxazoles as novel lipophilic tail fragments. This effort resulted in highly potent and selective $S1P_1$ full agonists derived from both C3-linked isomers (**4**) and C5-linked isomers (**5**) (Fig. 3).

The general synthetic approaches for C-4 alkyl pyridyl-isoxazoles carboxylic acids **9** and **13**, used in the preparation of compounds **4** and **5**, are outlined in Scheme 1. Pyridyl-isoxazole carboxylates **8**, used in the preparation of isoxazoles agonists **4**, were accessed via a regioselective [3+2] dipolar cycloaddition reaction between acetylene pyridines **6** and the dipole generated in situ from dimethyl 2-nitromalonate **7** under microwave conditions at 170 °C in the presence of an ionic liquid, 1-butyl-3-methylimidazoliumhexafluorophosphate. Subsequent hydrolysis of the *t*-butyl ester afforded carboxylic acids **9**. Pyridyl-isoxazole carboxylates **12**, used in the preparation of isoxazoles agonist **5**, were prepared by coupling bromoenotes **11** with the dipole generated in situ from *N*-hydroxypicolinimidoyl chloride **10** in the presence of triethylamine to form pyridyl-isoxazole carboxylates as a mixture of two regioisomers. Chromatographic separation provided the desired regioisomers **12** in low yield (~10%) which were then hydrolyzed to afford pyridyl-isoxazole carboxylic acids **13**. Pyridyl-isoxazoles **4** and **5** were prepared by coupling **17** with isoxazoles **9** or **13**, followed by cyclodehydration and deprotection of the azetidine carboxylic acid, as depicted in Scheme 2.

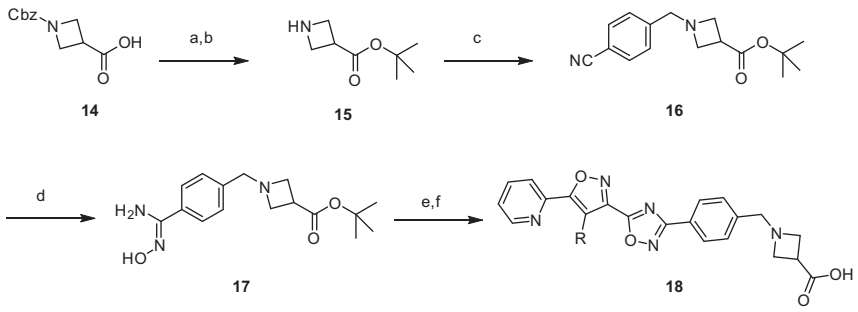
As access to intermediate **12** (R = CF_3) was limited due to poor yields, an improved synthetic route was developed to prepare compound **5b**, as described in Scheme 3. This approach involved a [3+2]-dipolar cycloaddition reaction between the dipole generated from *N*-hydroxypicolinimidoyl chloride **19**, in the presence of trimethylamine, and ethyl propiolate to afford pyridyl isoxazole carboxylate **20**. Subsequent condensation with *N′*-hydroxy-4-methylbenzimidamide under basic conditions provided intermediate **21**. A selective palladium catalyzed bromination of **21** provided **22** in good yield, which was then treated with methyl 2,2-difluoro-2-(fluorosulfonyl)acetate to afford trifluoromethyl pyridyl-isoxazole **23** in 45% yield. Compound **23** was brominated with NBS to give **24**, and the resulting bromide was displaced with **15** followed by the deprotection of the azetidine carboxylic acid to provide **5b**.

Pyridyl-isoxazoles **4** and **5** were evaluated in a functional GTPγS assay[16] looking at both human $S1P_1$ and $S1P_3$ receptor agonism, as summarized in Table 1. In general, all compounds were potent $S1P_1$ full agonists and highly selective over $S1P_3$. With desirable intrinsic potency, each compound was evaluated in vivo in a rat blood lymphocyte reduction (BLR) pharmacodynamic/pharmacokinetic (PD/PK) assay[17] in which healthy rats were dosed orally with a 1.0 mg/kg dose. Blood samples were drawn at 4 h and 24 h time points to assess the reduction in circulating lymphocytes relative to control and to evaluate plasma concentration for each compound. In this assay, all pyridyl-isoxazoles (**4a–c** and **5a–b**) demonstrated >50% lymphocyte reduction in plasma at 24 h with C5-linked pyridyl-isoxazoles **5a** and **5b** providing a near maximal response relative to the C3-linked analogs **4a–c** (e.g. **4b** vs **5a**). Additionally, when **5b** was dosed at a 0.3 mg/kg, ~50% lymphopenia was still maintained at 24 h. When compared to isoxazole **3**, **5b** was equipotent and selective, provided a similar plasma concentration at 4 h, but had reduced exposure at 24 h (54 nM vs 703 nM).[15] This attenuated exposure at the later time point could potentially be advantageous since a potent compound with a shorter half-life compared with **1** could mitigate potential host–defense issues upon drug discontinuation.

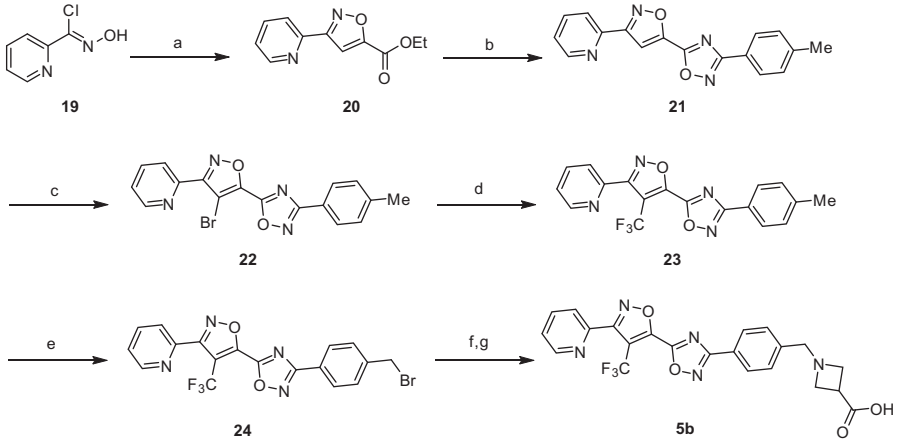
With a desirable potency and selectivity profile and a clean liability profile (Table 2), **5b** was further evaluated in vivo. In



Scheme 1. Reagents and conditions: (a) 1-butyl-3-methyl-1*H*-imidazol-3-ium hexafluorophosphate, toluene, Microwave, 170 °C, 10%; (b) LiOH–H_2O, MeOH/H_2O; (c) 1 N aqueous NaOH, MeOH, microwave, 100 °C; (d) Et_3N, CH_2Cl_2, ~10%.

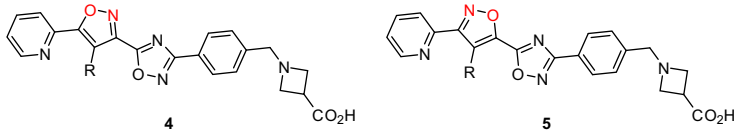


Scheme 2. Reagents and conditions: (a) *t*-BuOH, DMAP, EDCI, CH_2Cl_2, 81%; (b) 10% Pd/C, EtOAc, 92%; (c) 4-cyanobenzaldehyde, $Na(CN)BH_3$; MeOH, 89%; (d) $HONH_2$–HCl; $NaHCO_3$, *t*-BuOH, 55%; (e) **9** or **13**, EDCI, HOBt, DMF, 53–86%; (f) TFA.



Scheme 3. Reagents and conditions: (a) ethyl propiolate, Et_3N, CH_2Cl_2, 90%; (b) *N*′-hydroxy-4-methyl benzenecarboximidamide, NaH or Cs_2CO_3, DMF, 86%; (c) NBS, $Pd(OAc)_2$, MeCN, 87%; (d) methyl 2,2-difluoro-2-(fluorosulfonyl)acetate, CuI, HMPA, DMF, 45%; (e) NBS, AIBN, 90%; (f) **15**, Et_3N, DMF, 75%; (g) TFA.

Table 1
SAR of the C3-linked pyridyl-isoxazoles (**4**) and C5-linked pyridyl-isoxazoles (**5**)



						Rat Blood Lymphocyte Reduction Assay – 1.0 mg/kg			
#	R	*h*S1P$_1$ GTPgS EC$_{50}$ nM	*h*S1P$_3$ GTPgS EC$_{50}$ nM	S1P$_3$/S1P$_1$ Sel.	Lymphocyte reduction 4 h (%)	Concn (nM)	Lymphocyte reduction 24 h (%)	Concn (nM)	4 h/24 h concn ratio
4a	Et	3.4	1900	550×	84	972	58	64	15
4b	Pr	0.82	2100	2500×	81	292	53	9.9	30
4c	^{i}Pr	1.00	8200	8200×	84	686	64	31	22
5a	Pr	0.21	630	3000×	82	318	85	26	12
5b	CF_3	0.71	2900	4100×	85	2510	80	54	47
3	CF_3	0.47	1600	3400×	78	2134	91	703	3.0

Concn: plasma concentrations at 4 and 24 h timepoints.

multi-species pharmacokinetic (PK) studies (Table 3), **5b** exhibited good oral absorption and low clearance in mouse, rat, and dog with low volume of distribution and acceptable half-lives. Cyno, on the other hand, was a clear outlier relative to the other species displaying low bioavailability, high clearance, and a short half-life. With a reasonable PK profile, **5b** was evaluated in a rat adjuvant induced arthritis model of human rheumatoid arthritis.[18] In this model, arthritis was induced by the subcutaneous injection of Complete Freund's Adjuvant containing heat-killed *Mycobacterium butyricum* into the base of the tail of male Lewis rats. Pyridyl-isoxazole **5b** was administered at doses of 0.03. 0.1, 0.5, and 3 mg/kg po/QD, along with fingolimod (**1**) at 1 mg/kg po/QD, for 21 days starting at day 0 (Fig. 4). Baseline measurements of hind paw volume were obtained by water displacement plethysmometry. Additional paw volume measurements were performed over the next three weeks, and the increases in paw volume above baseline were calculated.

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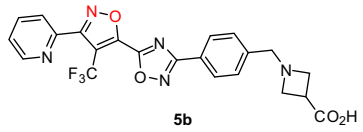
Table 2
Partial in vitro profiling data for isoxazole **5b**



5b

Parameter	Result
Human $S1P_4/S1P_1$ selectivity	3.2×
Human $S1P_5/S1P_1$ selectivity	3.2×
Protein binding (bound)	99.5% human 99.4% mouse 97.6% rat 72.6% dog 93.5% monkey
Mutagenicity	Ames negative
hERG (patch clamp)	13% @ 10 μM 34% @ 30 μM
Na^+ (patch clamp)	<5% @ 10 μM (1 and 4 Hz)
Ca^+ (patch clamp)	12% @ 10 μM
CYP[a] inhibition (IC_{50})	>40 μM 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4
Liver microsomal stability (% remaining)	*h* 88, *r* 100, *m* 100

[a] CYP = cytochrome P450.

Table 3
Multi-species PK parameters of compound **5b**

Parameter	Mouse	Rat	Cyno[a]	Dog
po dose (mg/kg)	5	5	1	1
iv dose (mg/kg)	2	2	1	1
$T_{1/2}$ (h), iv	4.5	4.8	1.1	6.1
Cl (mL/min/kg), iv	0.3	1.0	14	2.6
% hepatic flow	<1	1.4	32	8.4
V_{ss} (L/kg), iv	0.1	0.4	0.5	0.7
F_{po} (%)	70	100	12	87
Peak/trough ratio	2.8	26	>400	7.4

[a] Cynomolgus monkey.

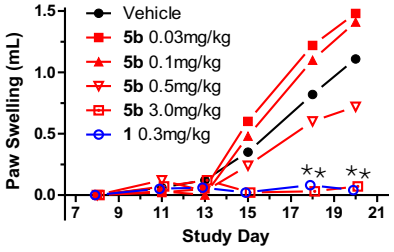


Figure 4. Effect of once daily dosing of **5b** vs **1** (FTY720) on paw volume in rat AA. $^*p$ <0.05 vs vehicle.

As seen in Figure 4, doses of 0.5 and 3.0 mg/kg provided dose-dependent reduction of paw swelling relative to the vehicle treated rats, with the 3.0 mg/kg dose providing complete suppression of disease development.

In conclusion, a potent and selective series of pyridyl-isoxazole based $S1P_1$ agonists has been identified. Compound **5b** was identified as a highly potent drug-like agonist of $S1P_1$ with significant selectivity over $S1P_3$. With a desirable potency and selectivity profile and a clean liability profile, **5b** was advanced into in vivo studies, providing a differentiated and potentially more desirable PK profile compared with fingolimod **1**. Additionally, **5b** demonstrated robust efficacy in a pre-clinical, chronic model of rheumatoid arthritis at a dose of 3.0 mg/kg QD. Overall, isoxazoles **5b** and **3** have demonstrated that an isoxazole based lipophilic fragment can provide highly desirable agonists of $S1P_1$.

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16. *hS1P-1 GTPg35S binding assay:* Compounds were loaded in a 384 Falcon v-bottom plate (0.5 ml/well in a 3-fold dilution). Membranes prepared from S1P1/CHO cells or EDG3-Ga15-bla HEK293T cells were added to the compound plate (40 ml/well, final protein 3 mg/well) with multidrop. [^{35}S]GTP (1250 Ci/mmol, Perkin Elmer) was diluted in assay buffer: 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 10 mM GDP, 0.1% fatty acid free BSA, and 10 mg/ml Saponin to 0.4 nM. 40 ml of the [^{35}S] GTP solution was added to the compound plate with a final concentration of 0.2 nM. The reaction was kept at room temperature for 45 min. At the end of incubation, all the mixtures in the compound plate were transferred to a 384 well FB filter plates via GPCR robot system. The filter plate was washed with water 4 times by using the modified manifold Embla plate washer and dried at 60 °C for 45 min.

30 ml of MicroScint 20 scintillation fluid was added to each well for counting at Packard TopCount. EC_{50} is defined as the agonist concentration that corresponds to 50% of the Y_{max} (maximal response) obtained for each individual compound tested.

17. *Blood lymphocyte reduction assay (BLR) in rat:* Lewis rats were dosed orally with test article (as a solution or suspension in the vehicle) or vehicle alone (polyethylene glycol 300, 'PEG300'). Blood was drawn at 4 h and 24 h by retro-orbital bleeding. Blood lymphocyte counts were determined on an ADVIA 120 Hematology Analyzer (Siemens Healthcare Diagnostics). The results were measured as a reduction in the percentage of circulating lymphocytes as compared to the vehicle treated group at the 4 h and 24 h measurement. The results represent the average results of all animals within each treatment group (n = 3–4).
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