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# Synthesis and evaluation of alkoxy-phenylamides and alkoxy-phenylimidazoles as potent sphingosine-1-phosphate receptor subtype-1 agonists

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ABSTRACT

In the design of potent and selective sphingosine-1-phosphate receptor agonists, we were able to identify two series of molecules based on phenylamide and phenylimidazole analogs of FTY-720. Several designed molecules in these scaffolds have demonstrated selectivity for S1P receptor subtype 1 versus 3 and excellent in vivo activity in mouse. Two molecules PPI-4621 (**4b**) and PPI-4691 (**10a**), demonstrated dose responsive lymphopenia, when administered orally.



The discovery of the sphingosine-1-phosphate (S1P) receptor class of G-protein coupled receptors (GPCRs) has opened up exciting and highly competitive areas of research and development. Signaling through members of this cluster of five receptors ($S1P_{1-5}$) with the endogenous natural ligand S1P can induce multiple effects on cardiovascular and immune system function and other yet poorly defined effects on additional physiological systems.[1]

FTY-720 is a new molecular entity undergoing clinical evaluation. This prodrug is currently in phase III clinical studies for multiple sclerosis and has completed clinical trials in solid organ transplant rejection prevention.[2] The prodrug FTY-720, upon in vivo phosphorylation, converts to a potent non-selective S1P receptor agonist ($S1P_{1,3-5}$) that has profound immunomodulatory activity through direct modulation of in lymphocyte trafficking.[3] This immunomodulatory activity is due to triggering of S1P receptor subtype 1 ($S1P_1$) signaling cascades.[4] In contrast, it is postulated that agonists of S1P receptor subtype 3 ($S1P_3$) have been associated with deleterious side effects including bradycardia.[5] We therefore sought to discover potent $S1P_1$ receptor agonists with low potency on $S1P_3$ ($S1P_3$-sparing) in order to retain the positive therapeutic properties and reduce the side effect profile of non-selective S1P receptor agonists like FTY-720.

Our effort to explore new chemical entities in the S1P agonist area started with investigations of S1P and FTY-720 based analogs. A preliminary exploration of S1P related structures has demonstrated that an amide insertion in the S1P structure is well tolerated (Fig. 1a).[6] Most recently, Clemens and co-workers have demonstrated this by replacing the ethylene component of FTY-720 with an amide bond to allow for active molecules across S1P receptor subtypes 1, 3, 4, and 5 (Fig. 1b).[7] We chose the amide inserted analog of FTY-720 as an entry point for medicinal chemistry and determination of a structure–activity relationship (SAR). With a simple synthetic strategy in hand (Scheme 1), one could envision the use of an aniline moiety to take advantage of the amino acid chiral pool as a potential agonist head-piece (Fig. 2) to establish a rapid SAR of the region. This allowed for utility of a mono alcohol species to avoid issues with mono phosphate synthesis from prochiral diols. We envisioned introduction of a phenolic oxygen at the lipophilic tail section to facilitate convergent synthesis and expedited tail modifications and thus it was important to gain a thorough understanding of the effects this modification would exert on the in vivo biological activity of these analogs. The ether insertion would allow for the utility of a commercial library of

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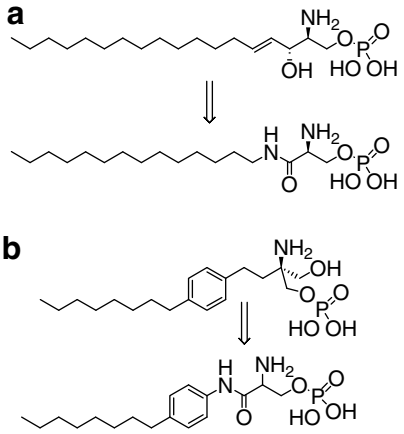
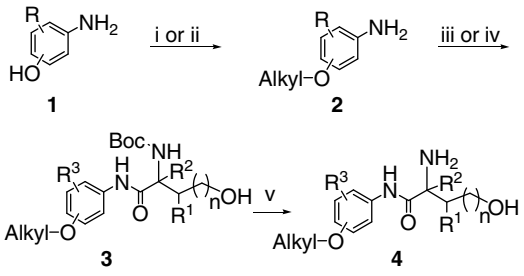


**Figure 1.** (a) S1P and its corresponding amide analog; (b) phospho-FTY-720 and its corresponding amino acid based analog.



**Scheme 1.** Reagents: (i) alkyl bromide, $Cs_2CO_3$, NaI, DMF; (ii) alkyl bromide, KO$^t$Bu, NaI, acetone; (iii) *N*-Boc-amino acid, EDC, HOBt, DIPEA, $CH_2Cl_2$; (iv) *N*-Boc-amino acid, HATU, DIPEA, DMF; (v) TFA, $CH_2Cl_2$.

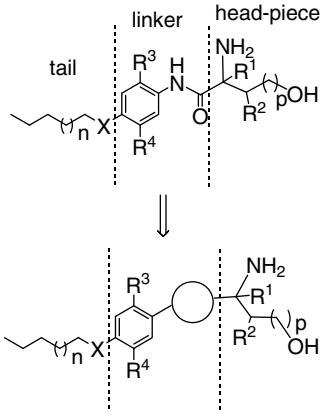
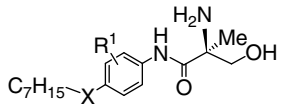


**Figure 2.** Structure–activity relationship study based on in vivo efficacy: $p = 0$ or 1; $R^1$ and $R^2$ were selected from H or Me (chiral center *S* or *R*); $R^3$ or $R^4$ are selected from H, F, Cl, Me, OMe, or $CO_2Me$, X is selected from $CH_2$ or O; and $n = 1$–6.

substituted 4-aminophenols or precursors to develop an extensive SAR around both the linker and lipophilic tail sections. Furthermore, we envisioned replacement of the amide bond with a more rigid imidazole ring (Fig. 2) as a potential alternative scaffold with the goals of establishing SAR around the linker section and ultimately achieving an active $S1P_1$ agonist with in vivo potency and a moderate degree of selectivity for S1P receptor subtype 1 over 3.

In pursuit of obtaining a preliminary SAR in phenylamide derivatives of FTY-720 we have generated a number of compounds

**Table 1**
Percent lymphopenia obtained upon 10 mg/kg oral (PO) administration of the alcohol at 6 h post-dosing in mice[11]

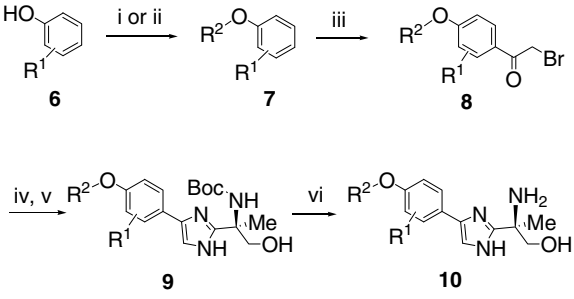


| Alcohol | X | $R^1$ | Percent lymphopenia |
|---|---|---|---|
| **5a** | $-CH_2-$ | H | 36 |
| **4a** | –O– | H | 72 |
| **4b** | $-CH_2O-$ | H | 78 |
| **4c** | $-CH_2O-$ | 2-F | 65 |
| **4d** | $-CH_2O-$ | 3-F | 66 |

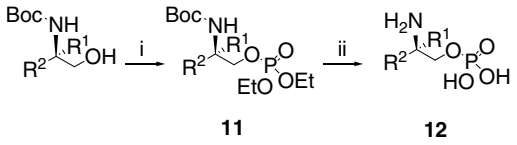
based on synthetic (Scheme 1). Alkylation of the hydroxyl group of a substituted aminophenol **1** was achieved using alkyl bromide and a catalytic amount of NaI in the presence of either $Cs_2CO_3$ in DMF (60 °C) or KO$^t$Bu in acetone (50 °C). The amino group of the desired intermediate was then acylated with the desired Boc-protected amino acid using either *N*-ethylcarbodiimide (EDC), 1-hydroxybenzotriazole (HOBt), and *N*,*N*-diisopropylethylamine (DIPEA) or O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and DIPEA. The final compound was obtained in good yields from Boc deprotection of the intermediate with 30% trifluoroacetic acid (TFA).

Based on the short synthetic strategy (Scheme 1), commercial availability of the starting material, and in vivo lymphopenia, a preliminary SAR was obtained as illustrated in Figure 1. This SAR demonstrated that the *S*-chirality of the amine was essential for compound activity with α-methyl-serine providing better in vivo activity than serine, *homo*-serine or threonine.[8] Incorporation of an oxygen in the alkyl tail section of the molecule provided better in vivo activity in comparison to the des-oxy analog (Table 1). Further investigation of alkyl ether tail modifications (C5 through C10) provided moderate to good in vivo activity with C8 (**4b**, PPI-4621) demonstrating optimal in vivo activity. Fluorine substitution on the aromatic linker was the only tolerated substitution.

Our next approach was modification of the amide linker region to apply a rigid imidazole ring to explore the SAR relative to the azole series of molecules. The designed compounds were synthesized as described in Scheme 2. Substituted phenols were alkylated with the appropriate alkyl bromide using KO$^t$Bu in acetone and a catalytic amount of NaI at 50 °C, or in a microwave at 80 °C using KO$^t$Bu in THF. Friedel-Crafts acylation of the corresponding phenyl ether provided the bromoacetophenone precursor. Reaction of the bromoacetophenone with *N*-protected-amino acid provided the



**Scheme 2.** Reagents and conditions: (i) alkylbromide, KO$^t$Bu, NaI, acetone; (ii) alkylbromide, KO$^t$Bu, THF, microwaved, 45 min, 80 °C; (iii) 1—bromoacetyl bromide, $AlCl_3$, $CH_2Cl_2$; (iv) *N*-Boc-amino acid, $Cs_2CO_3$, DMF; (v) $AcONH_4$, toluene or xylenes, reflux; (vi) TFA, $CH_2Cl_2$.




**Scheme 3.** Reagents: (i) diethyl phosphorochloridate, $Et_3N$, $CH_2Cl_2$; (ii) TMSBr, $CH_2Cl_2$.

amino acid ester intermediate which, upon intramolecular cyclization in the presence of excess ammonium acetate, produced the desired phenylimidazole. The phenylimidazole was either deprotected to remove the Boc group using 30% TFA in $CH_2Cl_2$, or was phosphorylated as illustrated in Scheme 3.

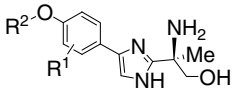
The imidazole analogs of FTY-720 showed excellent in vivo activity analogous to the corresponding amide analogs (Table 2). The C8 analog (**10a**, PPI-4691) showed slightly better in vivo activity than the C7 analogs. Similar to the amide counter-part, only fluoro substitution was tolerated on the phenyl group.

In order to determine the binding activity and compound selectivity against human $S1P_1$ and $S1P_3$ receptors, the desired phosphates were synthesized based on the synthetic strategy illustrated in Scheme 3. Reaction of Boc-protected amino-alcohol with excess diethyl chlorophosphate in the presence of triethylamine gave phospho-triester **11** which upon treatment with excess bromotrimethylsilane afforded the desired final phosphate **12**.

Binding activity of S1P and synthesized phosphate agonists were measured using a modified version of the [$^{33}$P]S1P binding assay described by Davis and co-workers.[9] S1P and compound **12a** showed similar binding activity at $S1P_1$ as reported in Table 3. Compounds **12b**–**12e** showed similar binding activity at $S1P_1$ with two- to threefold improvement over **12a**. This demonstrated the change from amide to imidazole allowed for SAR information transfer to obtain potent S1P agonists. Compounds **12a**–**12e** demonstrated weaker but similar binding activity at $S1P_3$, allowing for moderate to good selectivity of eight- to 42-fold. Overall, relatively weaker binding activity was observed for the amide series at receptor subtypes 4 and 5. The [$^{35}$S]GTPγS functional assay was used to further investigate the receptor subtype selectivity of both series of molecules. The functional assay binding activity was measured as described by Davis and co-workers.[10] Agonist **12e** was observed to be three times more potent than S1P itself with a relatively moderate selectivity of eightfold for $S1P_1$ over $S1P_3$ (Table 4). Fluoro-substitution on the phenyl ring appeared to have little or no effect on the agonist activity or selectivity. A further three- to fivefold improvement in $S1P_1$ receptor activity was observed when the amide group was replaced with an imidazole moiety (compounds **12d** and **12e**). Even though the $S1P_3$ activity for the amide and imidazole analogs remained the same, the improved activity at $S1P_1$ for the corresponding imidazole analogs led to three- to fivefold improvement in agonist selectivity for $S1P_1$ over $S1P_3$. Therefore, based on the functional assay against human $S1P_1$ and $S1P_3$ receptors, both amide and imidazole series of the S1P agonist demonstrated excellent $S1P_1$ receptor agonist activity with moderate to good overall selectivity for S1P receptor subtypes 1 over 3.

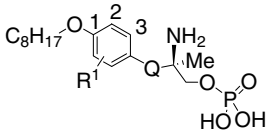
The lead compounds **4b** (PPI-4621) and **10a** (PPI-4691) were further investigated for in vivo dose response when orally administered at doses between 0.3 and 10 mg/kg. Both compounds showed excellent lymphopenia at 10 and 3 mg/kg (Fig. 3). At 0.3 mg/kg both compounds showed little or no response while at 1 mg/kg both compounds showed good to moderate response with

**Table 2**
Percent lymphopenia obtained upon 10 mg/kg oral (PO) administration of the alcohol at 6 h post-dosing in mice[9]



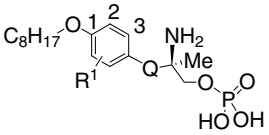

| Alcohol | $R^1$ | $R^2$ | Percent lymphopenia |
|---|---|---|---|
| **10a** | H | $C_8H_{17}$ | 83 |
| **10b** | H | $C_7H_{15}$ | 74 |
| **10c** | 2-F | $C_8H_{17}$ | 70 |
| **10d** | 3-F | $C_8H_{17}$ | 81 |

**Table 4**
[$^{35}$S]GTPγS binding activity on human $S1P_{1,3-5}$ receptor subtypes




| Agonist | Q | $R^1$ | $hS1P_1$ $EC_{50}$ (nM) | $hS1P_3$ $EC_{50}$ (nM) | $S1P_3/S1P_1$ |
|---|---|---|---|---|---|
| **S1P** | — | — | 7.9 | 3 | 0.4 |
| **12a** | Amide | H | 2.4 | 16.3 | 6.9 |
| **12b** | Amide | 2-F | 1.1 | 10.1 | 9.2 |
| **12c** | Amide | 3-F | 2.1 | 9.8 | 4.7 |
| **12d** | Imidazole | H | 0.55 | 14.8 | 26.9 |
| **12e** | Imidazole | 2-F | 0.45 | 10.8 | 24 |

**Table 3**
[$^{33}$P]S1P binding activity on human $S1P_{1,3-5}$ receptor subtypes




| Agonist | Q | $R^1$ | $hS1P_1$ $IC_{50}$ (nM) | $hS1P_3$ $IC_{50}$ (nM) | $hS1P_4$ $IC_{50}$ (nM) | $hS1P_5$ $IC_{50}$ (nM) | $S1P_3/S1P_1$ |
|---|---|---|---|---|---|---|---|
| **S1P** | — | — | 0.47 | 0.66 | 2.4 | 1.1 | — |
| **12a** | Amide | H | 0.73 | 5.9 | 4.0 | 8.8 | 8.1 |
| **12b** | Amide | 2-F | 0.14 | 5.9 | 3.5 | 6.9 | 42 |
| **12c** | Amide | 3-F | 0.35 | 3.6 | 1.8 | 3.2 | 10.2 |
| **12d** | Imidazole | H | 0.26 | 5.3 | 1.1 | 1.4 | 20.3 |
| **12e** | Imidazole | 2-F | 0.35 | 5.8 | 1.9 | 1.1 | 16.5 |

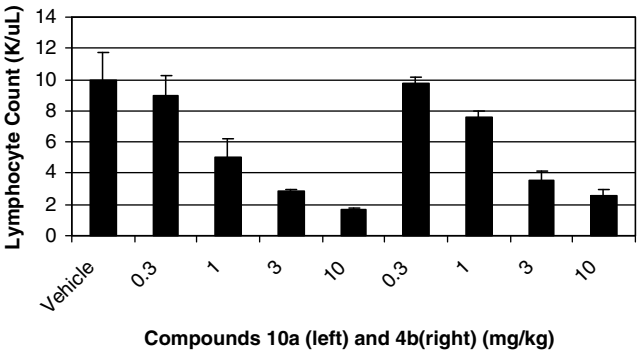



**Figure 3.** Dose responsive lymphopenia for lead compounds **10a** (PPI-4691) and **4b** (PPI-4621) relative to the vehicle.

compound **10a** showing better in vivo activity. Overall, both compounds **4b** and **10a** demonstrated excellent dose responsiveness when administered orally at doses between 0.3 and 10 mg/kg.

In summary, we have generated a robust SAR around two different scaffolds of $S1P_1$ receptor agonists. We have demonstrated excellent $S1P_1$ receptor binding potency with moderate to good selectivity in both series of molecules, with the phenyl-imidazole series of molecules giving further improvement in receptor selectivity and in vivo potency. Our lead molecules from these series, **4b** (PPI-4621) and **10a** (PPI-4691), demonstrated that both alkoxy-phenylamide and alkoxy-phenylimidazole analogs have excellent in vivo oral activity. These new chemical entities provide a solid foundation for further structural modifications and exploration to enhance agonist selectivity.

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8. In order to induce in vivo activity, that is, lymphopenia, mice were treated with the indicated compounds described herein as follow: Male C57Bl/6 mice were divided into groups of three. A control group received the 3% BSA vehicle only. The other groups received a specified dose of test compound in vehicle administered orally (PO). After 6 h, the mice were anesthetized with isoflurane and approximately 250 μl of blood was removed from the retroorbital sinus and collected in an EDTA microtainer, mixed with an anticoagulant and placed on a tilt table until complete blood count (CBC) analysis.
9. FTY-720 was used as the control in these experiments. Upon 1.0 mg/kg oral (PO) administration of FTY-720 generally 80% lymphopenia was observed.
10. The $IC_{50}$ of a compound was determined using a modified version of the [$^{33}$P]sphingosine-1-phosphate binding assay described by Davis, M. D.; Clemens, J. J.; Macdonald, T. L.; Lynch, R. K. *J. Biol. Chem.* **2005**, *280,* 9833' as follows: For the binding assay, [$^{33}$P]sphingosine-1-phosphate (obtained from American Radiolabeled Chemicals, Inc) was added to membranes in 200 μl in 96-well plates with assay concentrations of 2.5 pM [$^{33}$P]sphingosine-1-phosphate, 4 mg/ml BSA, 50 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, and 5 μg of protein. Binding was performed for 60 min at room temperature with gentle mixing and terminated by collecting the membranes onto GF/B filter plates. After drying the filter plates for 10 min, 50 μl of Microscint 40 was added to each well, and filter-bound radionuclide was measured on a Packard Top Count. Non-specific binding was defined as the amount of radioactivity remaining in the presence of excess of unlabeled S1P.
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