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Synthesis of benzimidazole based analogues of sphingosine-1-phosphate: discovery of potent, subtype-selective $S1P_4$ receptor agonists

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Abstract—Sphingosine-1-phosphate (S1P) is a biologically active lysophospholipid with the capacity to induce a broad range of cellular responses via its interaction with the S1P family of G-protein coupled receptors. A member of this receptor family, $S1P_4$, is highly and almost exclusively expressed in the lymphoid system and has been implicated in regulation of cell shape and motility. This report describes the synthesis of several potent benzimidazole based $S1P_4$ receptor selective agonists. For instance, compound **9b** displayed an $EC_{50} = 36$ nM at the $S1P_4$ receptor using a [γ-^{35}S]GTP binding assay as compared to an $EC_{50} = 37$ nM for the endogenous ligand. We also report the effects of altering stereochemistry at the C2 position, methylation at the C1 and C2 position, and activity differences between the alcohol and phosphate head groups of the analogues.

Sphingosine-1-phosphate (S1P) acts as both an intercellular mediator and an intracellular second messenger (Fig. 1).[1] Although the molecular target of intracellular S1P remains to be defined, extracellular S1P binds a specific subset of seven *trans*-membrane G-protein coupled receptors, namely $S1P_{1-5}$.[2] Through its interaction with these receptors, S1P has been shown to induce a wide variety of cellular effects including differentiation, motility, and escape from apoptosis.[3] Recently, S1P receptor activation has been implicated in regulation of immunosuppression through studies on the novel immunomodulator FTY720 and several analogues.[4] Agonism at $S1P_3$ has been shown to produce acute toxicity and regulate cardiovascular function whereas agonism at $S1P_1$ has been implicated in the regulation of lymphocytes via sequestration from the blood to secondary lymphoid tissues. Nevertheless, understanding of individual S1P receptors remains limited by the lack of selective agonists and antagonists for these receptors.

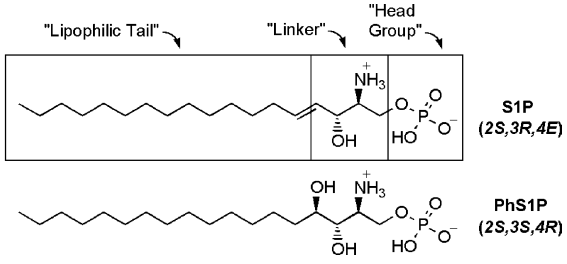

Figure 1. Structures and regions of S1P and PhS1P.

The G-protein coupled receptor $S1P_4$, unlike the other S1P receptors, has been shown to be predominately expressed in the lymphoid system.[5] Recently $S1P_4$ was implicated in regulation of cell shape and motility via coupling to $G\alpha_i$ and $G\alpha_{12/13}$ subunits of heterotrimeric G-proteins.[6] To date, no selective agonist for $S1P_4$ has been reported, although phytosphingosine-1-phosphate (PhS1P), a naturally occurring minor component of S1P species, has been established as a high affinity agonist at $S1P_4$.[7] To supplement our previously reported S1P receptor agonists,[8] we report now the synthesis of potent, benzimidazole based $S1P_4$ selective agonists along with their activity profiles across all five

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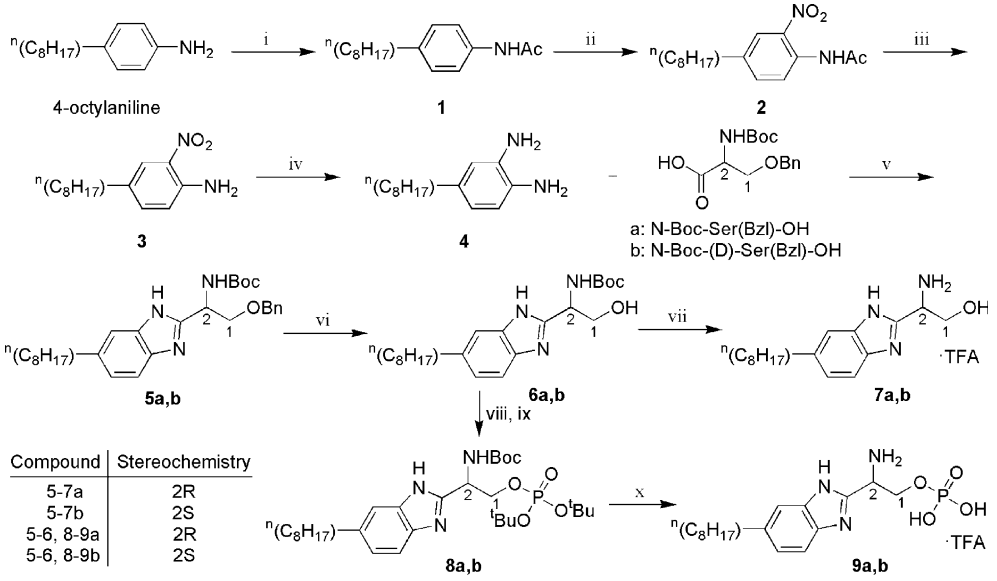
recombinant human S1P receptors. We report also the effects of alternating stereochemistry of the C2 amino group, differences in agonism between the alcohol and phosphate head groups, and the effects of methylation at the C1 and C2 position of the analogues.

Our preparation of S1P analogues initiated with the synthesis of the benzimidazole based **7a**, **7b**, **9a**, and **9b** compounds (Scheme 1). 4-Octylaniline was protected as the mono-acetate affording the desired electronic conditions for mono *ortho*-nitration of the ring to give **2**. The protected *ortho*-nitro aniline **2** was then deprotected in strong base and subsequently reduced to the diamine **4**, which was used rapidly in ensuing steps after isolation. Coupling of **4** to the stereochemically appropriate amino acid using the PyBOP reagent afforded, after consequential cyclization, the protected benzimidazole compounds **5a** and **5b**. To the best of our knowledge, this is the first example of the PyBOP reagent promoting a condensation–cyclization–dehydration event to give a benzimidazole, albeit in unoptimized yield. Hydrogenolysis of **5a** and **5b** afforded the *N*-Boc protected alcohols **6a** and **6b**, respectively. The final alcohol compounds **7a** and **7b** were obtained by deprotection of **6a** and **6b**, respectively, in strong acid. The route to the phosphate compounds continued with phosphorylation of the alcohols **6a** and **6b** followed by oxidation utilizing hydrogen peroxide and finally global deprotection in strong acid to yield **9a** and **9b**, respectively, as the TFA salts.

The synthetic route to the 1-methyl benzimidazole analogues **12** and **14** commenced with coupling of the intermediate **4** (synthesis described in Scheme 1) with *N*-Boc-Thr(Bzl)-OH using the PyBOP reagent affording a chromatographically inseparable mixture of cyclized benzimidazole **10** and the mono-coupled amide compound, which did not undergo cyclization–dehydration (Scheme 2). Treatment of this crude mixture with Lawesson's reagent effectively completed the conversion to **10**. Birch reduction of the benzyl protected alcohol **10** supplied the free alcohol **11**, which was converted to the final benzimidazole based 1-methyl alcohol **12** by treatment with strong acid. The route to the benzimidazole based 1-methyl phosphate compound continued with phosphorylation of the secondary alcohol **11** followed by oxidation employing hydrogen peroxide and ensuing global deprotection to afford **14** as the TFA salt.

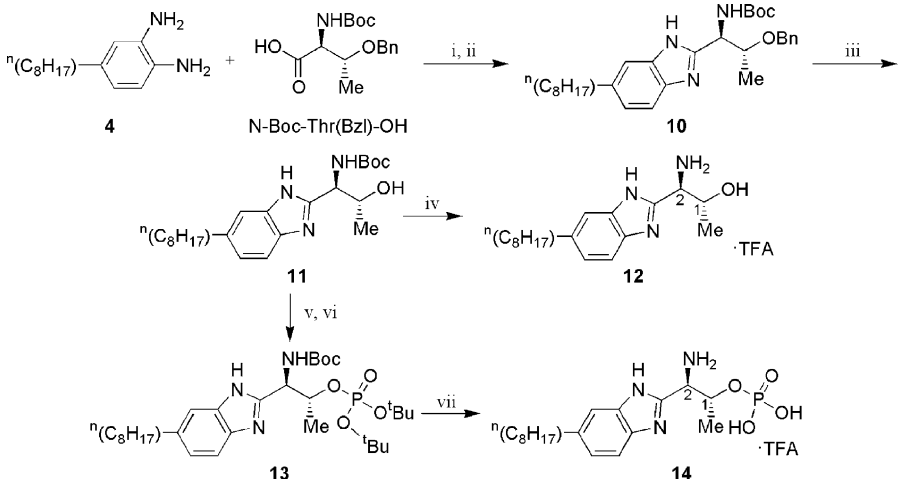
Synthesis of the racemic 2-methyl benzimidazole analogue **18** initiated with Fisher esterification of α-methyl-(DL)-serine followed by *N*-Boc protection to give alcohol **15** (Scheme 3). Phosphorylation of **15** with subsequent oxidation by hydrogen peroxide followed by saponification of the crude protected phosphate gave the suitably protected acid **16**. Coupling of compound **4** (synthesis described in Scheme 1) to acid **16** using the PyBOP reagent gave, after resulting cyclization, the protected benzimidazole compound **17**, which was then wholly deprotected in strong acid to provide the benzimidazole based 2-methyl phosphate **18** as the TFA salt.

Receptor activation by S1P and the synthetic analogues was determined in vitro by measuring the ligand dependent binding of [γ-^{35}S]GTP to membranes containing each of the five human S1P receptors expressed in HEK293T cells.[9] None of the compounds in these series showed any activity at the related lysophosphatidic acid receptors (LPA_{1-3}) at concentrations up to 10 μM in this assay (data not shown). In comparison to our previously reported *N*-alkyl amide, *O*-alkyl ester, and *N*-aryl amide S1P analogues, agonistic activity at $S1P_1$, $S1P_3$, and $S1P_5$ has been drastically reduced with incor-

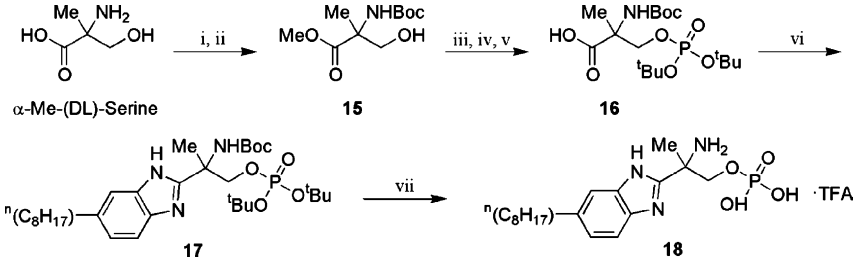


Compound	Stereochemistry
5-7a	2R
5-7b	2S
5-6, 8-9a	2R
5-6, 8-9b	2S

Scheme 1. Reagents and conditions: (i) Ac_2O, rt, 1 h, quant.; (ii) HNO_3, HOAc, Ac_2O, −15 → 0 °C, 3 h, quant.; (iii) 40% KOH, EtOH, reflux, 1 h, 84%; (iv) Zn dust, HOAc, rt, 2 h, 92%; (v) PyBOP, DIEA, CH_2Cl_2, rt, 6 h, 17–33%; (vi) H_2, 10% Pd/C, cat. HCOOH, EtOH, rt, 12 h, 47–57%; (vii) 1:1 TFA/CH_2Cl_2, rt, 4 h, 71–90%; (viii) tetrazole, di-*tert*-butyl diisopropylphosphoramidite, 1:1 CH_2Cl_2/THF, rt, 12 h; (ix) H_2O_2, rt, 4 h, 32–52% (two steps); (x) 1:1 TFA/CH_2Cl_2, rt, 4 h, 62–74%.



Scheme 2. Reagents and conditions: (i) PyBOP, DIEA, CH_2Cl_2, rt, 6 h; (ii) [2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane-2,4-disulfide], THF, reflux, 5 h, 30% (two steps); (iii) Na^0, NH_3, THF, −78 °C, 1 h, 63%; (iv) 1:1 TFA/CH_2Cl_2, rt, 4 h, 86%; (v) tetrazole, di-*tert*-butyl diisopropylphosphoramidite, 1:1 CH_2Cl_2/THF, rt, 12 h; (vi) H_2O_2, rt, 4 h, 16% (two steps); (vii) 1:1 TFA/CH_2Cl_2, rt, 4 h, quant.



Scheme 3. Reagents and conditions: (i) $SOCl_2$, MeOH, rt, 6 h; (ii) Boc_2O, satd aq $NaHCO_3$, *p*-dioxane, rt, 3 h, 33% (two steps); (iii) tetrazole, di-*tert*-butyl diisopropylphosphoramidite, 1:1 CH_2Cl_2/THF, rt, 12 h; (iv) H_2O_2, rt, 4 h; (v) 2 M NaOH, MeOH, rt, 8 h, 48% (three steps); (vi) **4**, PyBOP, DIEA, CH_2Cl_2, rt, 6 h, 15%; (vii) 1:1 TFA/CH_2Cl_2, rt, 4 h, 77%.

poration of a benzimidazole group in the 'linker region' of the analogues (Table 1, Figs. 2 and 3). Agonism at the $S1P_4$ receptor type, however, has been increased by one to two log orders in the transition of *N*-aryl amide to benzimidazole functionality.

Although relatively potent EC_{50} values were obtained for the benzimidazole compounds on $S1P_1$ and $S1P_5$, the compounds are very poorly efficacious at these receptor types. With regard to stereochemistry, *S*-configuration at the C2 position resulted in slightly increased potency at $S1P_4$ for both of the alcohol compounds **7a** and **7b** and the phosphate compounds **9a** and **9b**. Compounds with free alcohol head groups were determined to be approximately two to three log orders less potent on $S1P_4$ than their phosphate counterparts for compounds **7a**, **7b**, **9a**, **9b**, **12**, and **14**.

In a preliminary attempt to uncover metabolically stable S1P analogues, with regard to phosphatase activity, the benzimidazole compounds were methylated at the 1 and 2 positions. The racemic 2-methyl phosphate **18** lost potency at $S1P_4$ by approximately one to two log orders, as compared to **9a** and **9b**. The (1*R*,2*R*)-1-methyl

Table 1. EC_{50} values (nM) for S1P and benzimidazole analogues at S1P receptors determined by a [γ-^{35}S]GTP binding assay[a]

Compound	$S1P_1$	$S1P_2$	$S1P_3$	$S1P_4$	$S1P_5$
S1P	20	2.2	1.2	37	1.7
7a	2100 (pa)	na	na	4200	2400 (pa)
7b	330 (pa)	na	na	1600	390 (pa)
9a	37 (pa)	na	na	48	6.6 (pa)
9b	21 (pa)	na	na	36	8.2 (pa)
12	2900 (pa)	na	na	33,000	na
14	240 (pa)	na	na	94	77 (pa)
18	1000 (pa)	na	na	1200	370 (pa)

[a] Values are means of three experiments (pa = partial agonist, na = not active).

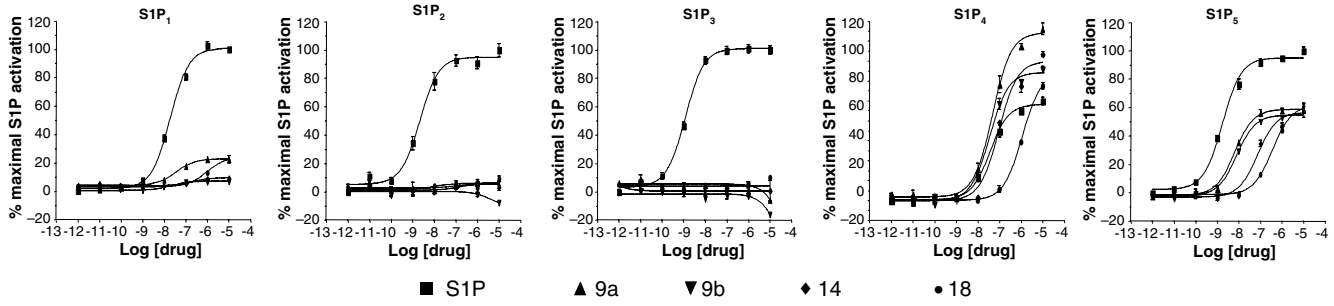


Figure 2. [γ-^{35}S]GTP binding to HEK293T cell membranes in response to S1P and benzimidazole based S1P analogues. Each data point represents the mean of three determinations.

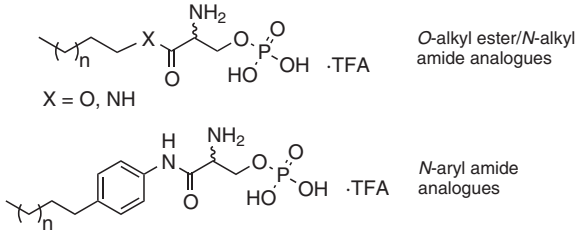


Figure 3. Structures of previously reported S1P analogues (see Ref. 8).

phosphate **14**, derived from L-threonine, retained approximately half of the potency (94 nM) on $S1P_4$ as compared to the nonmethylated counterpart **9a** (48 nM).

To summarize, we have synthesized a series of S1P analogues that incorporate a benzimidazole ring system in the 'linker' region of the pharmacophore. This structural modification has resulted in the generation of highly potent and fully efficacious $S1P_4$ selective agonists. We have determined a slight preference in potency for 2*S*-configuration as well as a necessity for the phosphate head group to obtain significant potency. We have also demonstrated, with regard to potency, a reasonable toleration of 1-methylation of the benzimidazole analogues whereas 2-methylation furnished a drastic loss in agonism. Our findings have helped to develop the SAR of S1P on the $S1P_4$ receptor and will serve as the basis for additional studies along this route.

Acknowledgements

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9. Brief assay protocol: S1P was purchased from Biomol (Plymouth Meeting, PA). [γ-^{35}S]GTP was purchased from New England Nuclear (Boston, MA). HEK293T cells were a gift from Dr. Judy White (Department of Cell Biology, University of Virginia). First, 5 μg of membranes from the appropriate receptor and G-protein expressing HEK293T cells were incubated in 0.1 mL of GTP-binding buffer (in mM: HEPES 50, NaCl 100, $MgCl_2$ 5), pH 7.5 containing 5 μg saponin, 10 μM GDP, 0.1 nM [γ-^{35}S]GTP (1200 Ci/mmol) and test lipid. After incubating for 30 min at 30 °C, bound radionuclide was separated from free by filtration through Whatman GF/C paper using a Brandel Cell Harvester (Gaithersburg, MD). Bound radionuclide was detected with a Packard Top Count liquid scintillation counter.