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Synthesis of *Para*-Alkyl Aryl Amide Analogues of Sphingosine-1-phosphate: Discovery of Potent S1P 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. This report describes the synthesis of several potent S1P receptor agonists. For instance, compound **9c** displayed an $EC_{50}=8.6$ nM at the $S1P_1$ receptor using a [γ-^{35}S]GTP binding assay as compared to an $EC_{50}=4.5$ nM for the endogenous ligand. We also report the effects associated with introduction of a phenyl ring between the 'linker' and 'lipophilic tail' regions of the analogues, for example total loss of activity at $S1P_2$ and increased agonism at $S1P_5$.

Sphingosine-1-phosphate (S1P) has been demonstrated to act as both an intercellular signaling molecule and an intracellular second messenger (Fig. 1).[1] Extracellular S1P binds a specific subset of seven trans-membrane G-protein coupled receptors, namely the $S1P_1$ (formerly Edg-1), $S1P_2$ (Edg-5), $S1P_3$ (Edg-3), $S1P_4$ (Edg-6), and $S1P_5$ (Edg-8) receptors.[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]

The ability of S1P to mediate biological processes such as immune modulation make S1P receptor agonists and antagonists attractive candidates for use as therapeutic agents.[4] For example, the sphingosine analogue, FTY720, is a novel immunosuppressive agent that acts by reversibly sequestering lymphocytes to secondary lymphoid tissues.[5] FTY720 is a pro-drug that, after *O*-phosphorylation by a yet to be defined kinase, elicits lymphopenia via its interaction with one or more of the S1P receptors.[5,6] Phosphorylated FTY720 (phospho-FTY720) shares several structural similarities with S1P including a lipophilic tail region, a 2-amino group, and a phosphate head group.

To understand better the processes associated with S1P binding to its receptors, it is vital to establish a structure–activity relationship (SAR) for S1P with respect to each individual receptor. The generation of S1P analogues with different binding profiles across the S1P receptors proves useful in elucidating this SAR. More specifically, the synthesis of S1P receptor specific agonists and antagonists could prove useful in the discovery of novel drugs. To supplement our previously reported $S1P_1/S1P_3$ receptor agonist,[7] we now wish to report the synthesis of potent $S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$ receptor agonists along with their activity profiles across all five recombinant human S1P receptors. We also report several interesting effects in activity with the incorporation

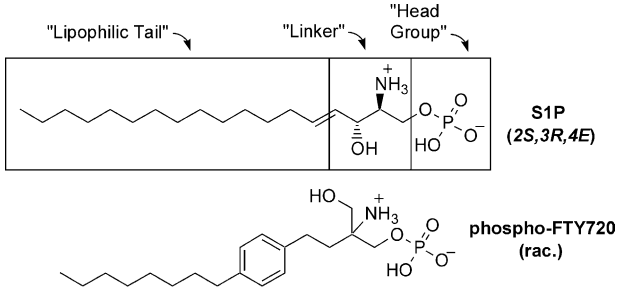

Figure 1. Structures and regions of S1P and phospho-FTY720.

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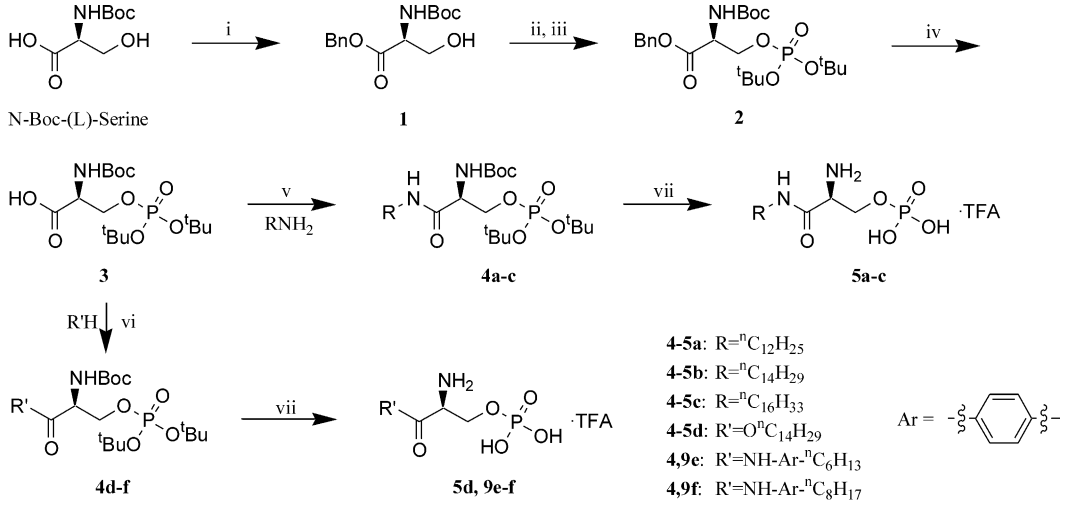
of a phenyl ring between the 'linker' and 'lipophilic tail' regions of the S1P analogues, as is the case in phospho-FTY720. These effects include a total loss of activity at $S1P_2$, increase in agonism at $S1P_5$, and a loss of C2-stereospecificity at the $S1P_1$ and $S1P_3$ receptors.

Our preparation of S1P analogues began with the synthesis of the (2*S*)-*N*-alkyl amide, (2*S*)-*O*-alkyl ester, and (2*S*)-*N*-aryl amide compounds, **5a–c**, **5d**, and **9e–f**, respectively (Scheme 1).[7] N-Boc-(L)-Serine was protected as the benzyl ester using benzyl bromide under standard conditions. The protected amino acid **1** was then phosphorylated using di-*tert*-butyl diisopropylphosphoramidite with subsequent hydrogen peroxide oxidation of the phosphite to afford **2** which underwent hydrogenolysis to give acid **3**. Coupling of **3** to the appropriate alkyl amine using DCC afforded the protected *N*-alkyl amides **4a–c**. Coupling of **3** to 1-tetradecanol using the PyBOP reagent gave the protected *O*-alkyl ester **4d**. Coupling of **3** to the appropriate *p*-alkyl aniline using the PyBOP reagent afforded the protected *N*-aryl amides **4e–f**. Deprotection of the amides **4a–c** and **4e–f** and ester **4d** using 1:1 TFA/CH_2Cl_2 gave the final products **5a–d** and **9e–f** as the TFA salts.

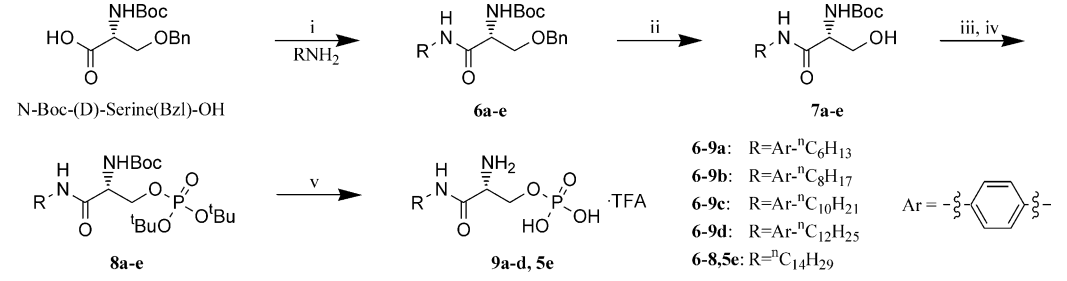
The synthetic route to the (2*R*)-*N*-alkyl amide and (2*R*)-*N*-aryl amide compounds, **5e** and **9a–d**, respectively, commenced with PyBOP mediated coupling of *N*-Boc-(D)-Serine(Bzl)–OH with the appropriate alkyl amine or *p*-alkyl aniline (Scheme 2).[7] The resulting amides **6a–e** were then subjected to hydrogenolysis to afford the deprotected alcohols **7a–e**. These alcohols were then phosphorylated using di-*tert*-butyl diisopropylphosphoramidite with subsequent oxidation of the phosphite by hydrogen peroxide to give the protected phosphates **8a–e**. Deprotection of **8a–e** using 1:1 TFA/CH_2Cl_2 gave the final products **5e** and **9a–d** as the TFA salts.

Receptor activation by S1P and the synthetic analogues was determined in vitro by measuring the ligand dependant binding of [γ-^{35}S]GTP to membranes containing each of the five human S1P receptors expressed in HEK293T cells.[8] 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). As was previously reported, *R*-configuration at the C2 position of the *N*-alkyl amide analogues appears to result in increased binding at $S1P_1$ and $S1P_3$, but not at the other S1P receptors (Table 1).[7]

Although the *N*-alkyl amide and *O*-alkyl ester compounds were poor agonists at $S1P_2$, introduction of a phenyl ring between the 'linker' and 'lipophilic tail'



Scheme 1. Reagents and conditions: (i) Cs_2CO_3, BnBr, DMF, rt, 12 h, quant.; (ii) tetrazole, di-*tert*-butyl diisopropylphosphoramidite, 1:1 CH_2Cl_2/THF, rt, 12 h; (iii) H_2O_2, rt, 4 h, quant. (2 steps); (iv) H_2, 10% Pd/C, EtOH, rt, 12 h, 91%; (v) DCC, DMAP, HOBT, CH_2Cl_2, rt, 12 h, 26–41%; (vi) PyBOP, DIEA, CH_2Cl_2, rt, 6 h, 43–60% (15% for **4d**); (vii) 1:1 TFA/CH_2Cl_2, rt, 4 h, 96–100%.



Scheme 2. Reagents and conditions: (i) PyBOP, DIEA, CH_2Cl_2, rt, 6 h, 65–77%; (ii) H_2, 10% Pd/C, EtOH, rt, 12 h, 84–96%; (iii) tetrazole, di-*tert*-butyl diisopropylphosphoramidite, 1:1 CH_2Cl_2/THF, rt, 12 h; (iv) H_2O_2, rt, 4 h, 60–100% (2 steps, 9% for **9d**); (v) 1:1 TFA/CH_2Cl_2, rt, 4 h, 56–100%.

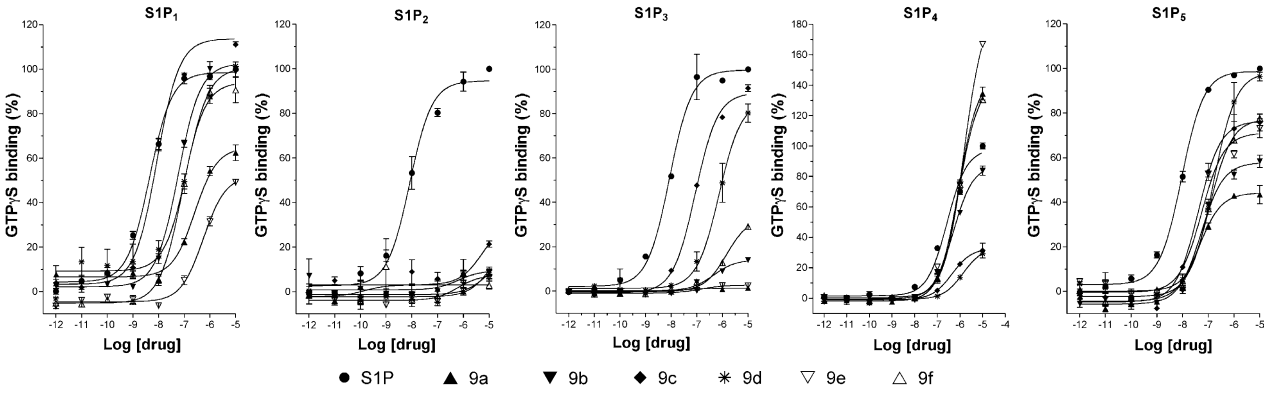


Figure 2. [γ-^{35}S]GTP binding to HEK293T cell membranes in response to S1P and *N*-aryl amide S1P analogues. Each data point represents the mean of three determinations.

regions of the analogues, as in compounds **9a–f**, resulted in a complete loss of activity at that receptor type (Table 1, Fig. 2). This structural modification also resulted in increased binding affinity towards $S1P_5$. In contrast to the *N*-alkyl amide compounds, stereochemical configuration at the C2 position of the *N*-aryl amide analogues does not appear to have an effect on activity at any of the S1P receptors. This finding also differs from data reported by Lynch, et al. for similar compounds containing 2-methyl groups, (*S*)-AFD and (*R*)-AFD, in which only the 2*R* enantiomer acted as a potent agonist at the S1P receptors (Fig. 3).[6]

Table 1. EC_{50} Values (nM) for S1P and *N*/*O*-alkyl amide/ester and *N*-aryl amide analogues at S1P receptors determined by a [γ-^{35}S]GTP binding assay[a,b]

N/*O*-Alkyl Compd	$S1P_1$	$S1P_2$	$S1P_3$	$S1P_4$	$S1P_5$
S1P	0.9	2.9	1.1	nd	43.9
5a	598.4	973.2	845.4	nd	645.5
5b	397.0	2685.0	862.4	nd	1606.0
5c	1805.0	>5000	878.6	nd	1220.0
5d	322.1	2760.0	601.9	nd	>5000
5e	12.7	2107.0	50.8	nd	>5000
N-Aryl Compd	$S1P_1$	$S1P_2$	$S1P_3$	$S1P_4$	$S1P_5$
S1P	4.5	8.3	8.7	270	9.2
9a	260	na	na	880	43
9b	58	na	450	500	52
9c	8.6	na	89	450	41
9d	130	na	740	1400	160
9e	550	na	na	1500	55
9f	82	na	1500	910	110

[a]Values are means of three experiments (na = not active, nd = no data).
[b]Data for *N*/*O*-alkyl compounds are taken from ref 7.

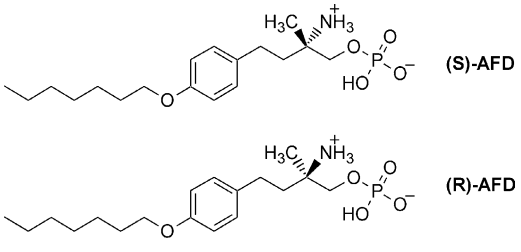


Figure 3. Structures of (*S*)-AFD and (*R*)-AFD (see ref 6).

Regarding the $S1P_1$ and $S1P_3$ receptors, the length of the alkyl chain of the *N*-aryl amide compounds was optimized at 10 carbons, compound **9c** displayed EC_{50}s = 8.6 nM and 89 nM, respectively, on those receptors. With respect to the $S1P_4$ receptor, shorter chain lengths of six and eight carbons on the *N*-aryl amide compounds exhibited higher potency and efficacy than the longer chain lengths of 10 and 12 carbons. At the $S1P_5$ receptor, the greatest efficacy of the *N*-aryl amide compounds was achieved with the longest chain length, 12 carbons. The potency, however, of the *N*-aryl amide compounds on $S1P_5$ was approximately equal, about one log order less than that of S1P itself.

To summarize, we have synthesized a second generation of S1P analogues that incorporate a phenyl ring between the 'linker' and 'lipophilic tail' regions of the pharmacophore, as is the case in phospho-FTY720. This structural modification has resulted in the generation of highly potent, fully efficacious agonists on four of the S1P receptors; $S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$. In particular, introduction of the phenyl ring between the 'linker' and 'lipophilic tail' regions of the S1P analogues described here results in complete loss of activity at $S1P_2$, increased activity at $S1P_5$, and a loss of C2-stereospecificity at the $S1P_1$ and $S1P_3$ receptors. These findings have helped to decipher further the SAR of S1P on the five S1P receptors and will serve as the basis for additional studies along this route.

Acknowledgements

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8. 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.