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Synthesis, stability, and implications of phosphothioate agonists of sphingosine-1-phosphate receptors

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Abstract—Phosphothioates may provide metabolic stability when compared to their phosphate counterparts, while retaining the potency and efficacy as agonists at sphingosine-1-phosphate (S1P) G-protein coupled receptors. Unlike their phosphate precursors, phosphothioate compounds with S1P-receptor profiles similar to that of FTY720, an emerging immunomodulator, were shown to evoke prolonged lymphopenia in vivo. Analysis of mouse plasma concentrations for a series of related alcohol/phosphate/phosphothioate compounds showed the conversion of the phosphate to alcohol. These preliminary data highlight the importance of metabolic regulation of S1P receptor ligands.

Known as an intracellular messenger molecule,[1] sphingosine-1-phosphate (S1P, Table 1) is a lysophospholipid mediator that has been receiving increasing attention due to its extracellular signaling at five G-protein coupled receptors ($S1P_{1-5}$).[2] Activation of these receptors is reportedly responsible for many of S1P's myriad cellular physiologies,[1,3,4] including cell growth, proliferation, survival and migration.[5,6] Specifically, S1P receptors have been implicated in controlling blood vessel development, cardiac rate, blood pressure, and immune regulation.[7–10]

FTY720 (Fig. 1) is a novel immunomodulator that, when activated by one or more kinases to FTY720-phosphate (FTY720-P), acts at four of S1P's five receptors.[11] Unlike conventional immunosuppressants, this drug evokes lymphopenia by inhibiting the egress of lymphocytes from secondary lymphoid tissues into the peripheral circulation. $S1P_{1,4}$ were discovered to be abundantly expressed on peripheral blood T-lymphocytes.[12] Several studies suggest that this non-cytotoxic lymphopenia occurs through FTY720-P's agonism specifically at the $S1P_1$ receptor.[12–14] Therefore, the discovery of subtype selective $S1P_1$ agonists may be desirable as potential therapeutics.

We previously reported the synthesis of potent, subtype selective S1P analogues with receptor profiles similar to that of FTY720-P.[15] However, initial in vivo studies on these and other S1P/FTY720-P phosphate analogues, such as compound **12** (Table 1), revealed that they evoked lymphopenia transiently (<8 h), even when administered intraperitoneally (data unpublished). To account for this observation, we hypothesized that perhaps the dynamic equilibrium that regulates sphingolipids may be of consequence.

One or more kinases are crucial for the activation of FTY720 to FTY720-P.[8,16] While sphingosine is phosphorylated by both human sphingosine kinases (SphK1 and SphK2), recent studies point to SphK2 as the kinase more likely to be responsible for phosphorylation of FTY720 to FTY720-P.[17,18] Numerous lipid phosphatases may play a role in the dephosphorylation of FTY720-P, but, to date, no study has indicated a specific enzyme.

Considering this enzymatic regulation and FTY720's activity in vivo, we hypothesized that our previously synthesized compounds were deactivated by phospha-

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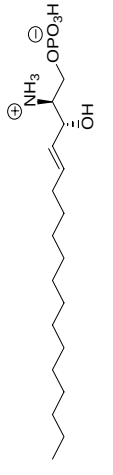
Table 1. GTP [γ-^{35}S] binding at individual S1P receptors[a]

Structure	Compound	Head group	Receptor				
			$S1P_1$	$S1P_2$	$S1P_3$	$S1P_4$	$S1P_5$
	**S1P**	Phosphate	4.50E-09	8.30E-09	8.70E-09	2.70E-07	9.20E-09
	FTY720-P	Phosphate	1.26E-09	NA	1.00E-10 (PA)	3.98E-09	3.98E-08 (PA)
	5	Alcohol	9.10E-07(PA)	NA	2.80E-06(PA)	8.70E-06(PA)	NA
	6	Phosphate	6.30E-09	NA	1.40E-07(PA)	1.60E-07	1.40E-08(PA)
	7	Phosphothioate	4.90E-08	NA	6.00E-07(PA)	8.60E-07	1.30E-07(PA)
	11	Alcohol	1.70E-06	NA	NA	4.90E-07	NA
	12	Phosphate	5.10E-09	NA	3.40E-07(PA)	3.00E-08	2.00E-08(PA)
	13	Phosphothioate	5.10E-09	NA	1.20E-07(PA)	8.60E-08	2.40E-08

[a] Values are EC_{50}s (M) determined by the means of at least three experiments (NA = no activation, PA = partial agonism).

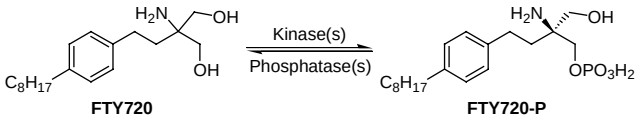


Figure 1. Enzymatic control of FTY720:FTY720-P. Kinases convert inactive FTY720 to active immunomodulator FTY720-P. Conversely, FTY720-P is dephosphorylated by one or more phosphatases.

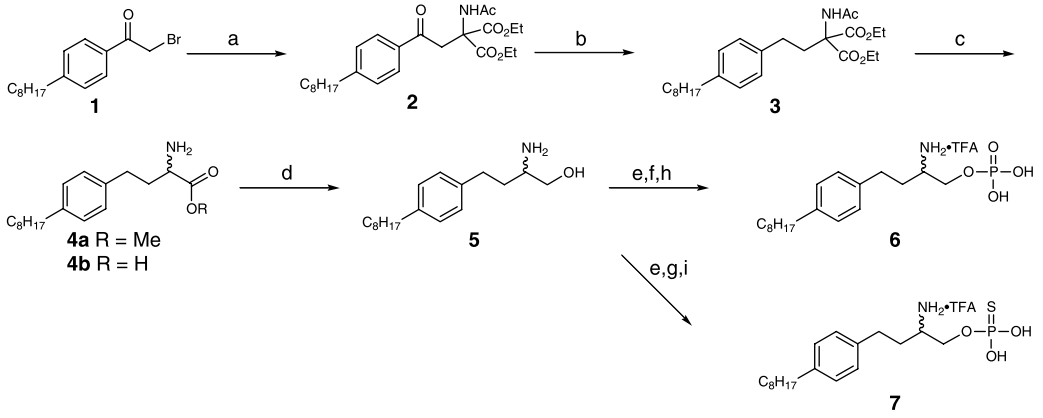
tase activity. Furthermore, we investigated two apparent strategies to deal with the subject of bio-activation. Both methods take advantage of the equilibrium mentioned above. One approach, based on early structure activity relationship studies of FTY720,[19] pertains to the synthesis of sterically hindered alcohols that contain or resemble the 2-amino-1,3-propanediol moiety of FTY720. The second tactic, also investigated by other groups,[20] uses the synthesis of more biologically stable phosphate isosteres, such as the phosphothioates displayed in this work.

While both avenues are being examined in our laboratories, this paper describes the preparation of phosphothioate analogues of S1P that: (i) are readily synthesized in parallel with their phosphate counterparts; (ii) are comparatively subtype-selective at S1P receptors; and (iii) markedly induce sustainable lymphopenia in vivo.

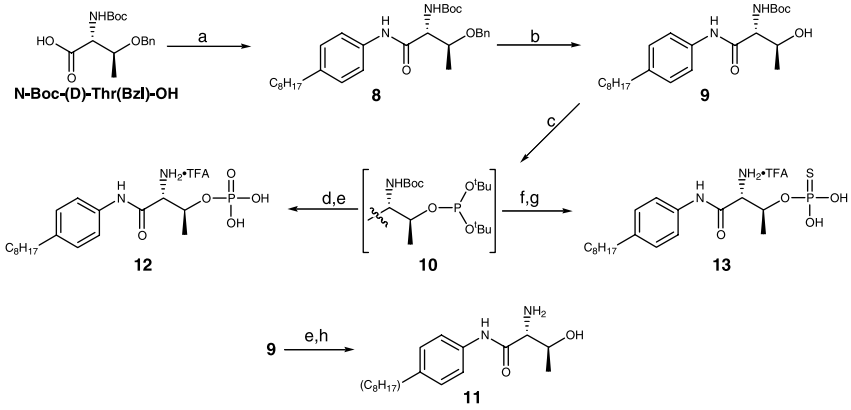
Specifically, a series of 2-demethylenehydroxy-FTY720 analogues (Scheme 1, **5**–**7**) was synthesized. These compounds were chosen with the knowledge that alcohol **5** does not induce lymphopenia.[20] Also, synthesized was phosphothioate **13** (Scheme 2): This compound was chosen to complement phosphate **12**, one of our initial lead candidates.

Initial steps in the synthesis of compounds **5**–**7** (Scheme 1) were adapted from the work of Durand et al.[21] Compound **1** was obtained by the Friedel–Crafts acylation of commercially available materials, 1-phenyloctane in 2-bromoacetyl bromide, with the help of $AlCl_3$. Notably, this reaction proceeded with higher yields in the absence of solvent as compared to the addition of 1,2-dichloroethane. α-Bromo-ketone **1** was then converted to intermediate **3**, as previously described.[21]

Hydrolysis-decarboxylation of **3** was achieved in one step under harsh acidic conditions. Problems related to initial solubility, under standard aqueous conditions, were best overcome by the addition of methanol. This technique yielded a surprisingly small percent (~20% by ^{1}H NMR) of the anticipated methyl ester **4a**, as well as desired compound **4b**. Conveniently, the acid/ester mixture was carried onto a lithium aluminum hydride reduction to provide the racemic amino alcohol **5** in sufficient yield. After standard N-Boc protection, compound **5** was efficiently transformed to a common phosphite intermediate (see **10**, Scheme 2), with di-*tert*-butyl-*N*,*N*-diisopropylphosphoramidite. This intermediate was not isolated, but rather oxidized, in situ, by H_2O_2 or elemental sulfur (S_8), to form the protected phosphate or phosphothioate tri-esters, respectively. Acid mediated



Scheme 1. Reagents and conditions: (a) NaOEt, EtOH, 60 °C, 1 h, then *N*-acetamido-diethylmalonate, 60 °C, 1 h, 90%; (b) Et_3SiH, $TiCl_4$, CH_2Cl_2, 12 h, rt, 83%; (c) 12 M HCl, MeOH, reflux, 2 h, 78%; (d) $LiAlH_4$, THF, reflux, 10–16 h, 74%; (e) Boc_2O Et_3N, CH_2Cl_2, 0 °C to rt, 4 h, 91%; (f) di-*tert*-butyl-*N*,*N*-diisopropylphosphoramidite, 1*H*-tetrazole, CH_3CN, CH_2Cl_2, THF, rt, 12 h, then H_2O_2, rt, 1 h, 36%; (g) di-*tert*-butyl-*N*,*N*-diisopropylphosphoramidite, 1*H*-tetrazole, CH_3CN, CH_2Cl_2, THF, rt, 12 h, then S_8, rt, 1 h, 61%; (h) trifluoroacetic acid, CH_2Cl_2, rt, 4 h, 70%; (i) trifluoroacetic acid, bromotrimethylsilane, thiophenol, CH_2Cl_2, 4 h, 0 °C to rt, 95%.



Scheme 2. Reagents and conditions: (a) 4-octylaniline, PyBOP, DIEA, CH_2Cl_2, rt, 4 h, 96%; (b) H_2, 10% Pd/C, EtOH, rt, 12 h, 96%; (c) 1*H*-tetrazole, di-*tert*-butyl-*N*,*N*-diisopropylphosphoramidite, 1:1 CH_2Cl_2/THF, rt, 12 h; (d) H_2O_2, rt, 4 h, 40% (two steps); (e) 1:1 TFA/CH_2Cl_2, rt, 4 h, 98%; (f) S_8, rt, 3 h, 37% (two steps); (g) thiophenol, TMSBr, 1:1 TFA/CH_2Cl_2, rt, 4 h, 94%; (h) Na_2CO_3(aq)/EtOAc.

deprotection was achieved on completion of the synthesis of both compounds **6** and **7**. An excess of cation scavengers was employed in the formation of **7** to avoid an O- to S- transfer of a *t*-butyl group, which could not be deprotected under numerous acidic conditions.

The synthesis of compounds **11**, **12**, and **13** (Scheme 2) began with, commercially available protected threonine. A PyBOP condensation with 4-octylaniline led to amide **8**. The alcohol moiety was unmasked by hydrogenolysis to compound **9** and subsequently phosphorylated, selectively oxidized, and deprotected by the methods described above to provide both the phosphate **12** and phosphothioate **13**. The alcohol **11** was accomplished by a similar deprotection and neutralization of intermediate **9**.

Receptor activities of S1P; FTY720-P; and compounds **5**–**7** and **11**–**13** are given as EC_{50} values (Table 1). The results were determined by ligand dependent binding of GTP[γ^{35}S] to individual S1P-receptor-G-protein complexes that were overexpressed in HEK293T cells.[15] Efficacy levels were also determined as a percentage of S1P's maximal effect at each individual receptor. Maxima determined to be lower than 80% of S1P's maximum are noted as partial agonists (PA).

Binding assay data show that phosphates **6** and **12** have EC_{50} values similar to that of FTY720-P at $S1P_1$. While phosphothioate **7** was found to be less potent at $S1P_1$ than phosphate **6**, phosphothioate **13** was equipotent to phosphate **12**. Desirably, the two phosphothioates retained a similar subtype selectivity between $S1P_1$ and $S1P_3$ receptors when compared to their phosphate precursors.

Compounds **6**, **7**, **12**, and **13** were tested further for their ability to induce lymphopenia in mice (see Fig. 2). Mice were rendered lymphopenic 4 h after the injection of any of the compounds, but this response was markedly diminished at 19 h post injection in mice treated with phosphate-containing compounds. In contrast, phosphothioates **7** and **13** evoked lymphopenia at the 19 h interval.

While phosphates **6** and **12** are slightly less potent than FTY720-P, neither caused extensive lymphopenia at 19 h. This inactivity was described for compound **6**;[18] however, its comparatively higher potency at $S1P_1$,

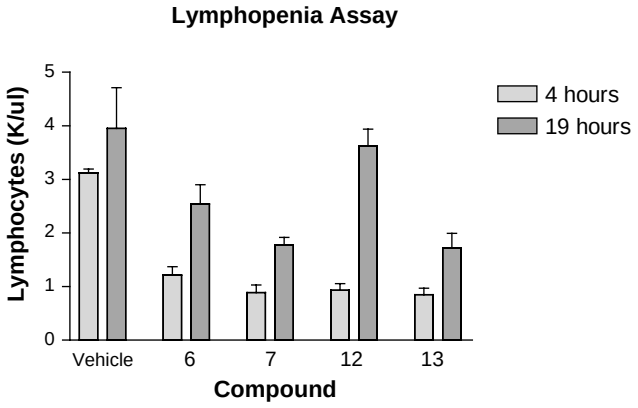


Figure 2. Lymphopenia induced by compounds **6**, **7**, **12**, and **13**. Compounds were dissolved in 3% fatty acid free-BSA and injected ip at 8 mg/kg. Blood was drawn from the orbital sinus at 4 and 19 h; lymphocyte counts were determined using a Hemavet blood analyzer. Results are mean of three measurements.

while suspected, was not previously known. The data suggest that potency of compounds **6** and **12**, as was hypothesized, is of less consequence when enzymatic regulation is taken into account.

To support the hypothesis that kinases and phosphatases were specifically involved in regulating our phosphate compounds, plasma concentrations of compounds **11**, **12**, and **13** were investigated and compared in vivo (see Fig. 3).[22,23] The compounds were administered independently by intraperitoneal injection and blood was drawn at 4 and 24 h. The data show that **11** is present in plasma from 4 to 24 h in detectable concentrations, 2.3 and 0.6 μM, respectively. No phosphate is detected in mice treated with compound **11**. In comparison, the phosphate was detected at concentrations below those of the alcohol at both time points. As hypothesized, the alcohol **11** was present in larger concentrations than phosphate **12** for mice treated with **12**.

Plasma concentrations of animals treated with **11** or **12** support the hypothesis that our phosphate analogues undergo dephosphorylation. However, the eluent (containing H_2O, CH_3CN, and TFA) used to analyze these

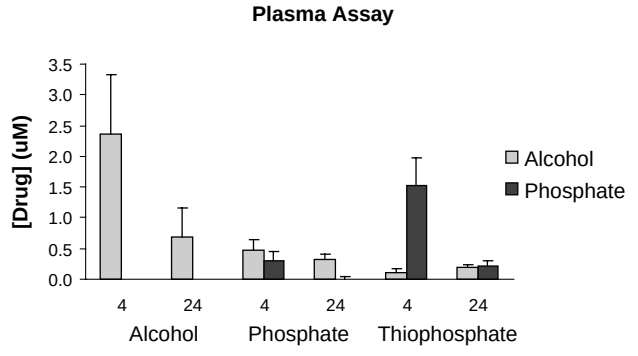


Figure 3. Plasma concentrations of compounds **11**, **12**, and **13**. Compounds were dissolved in 3% fatty acid free-BSA and injected ip at 8 mg/kg. Blood was drawn from the orbital sinus at 4 and 24 h; plasma concentrations were determined using a LCQ LC–MS. Results are means of five measurements.

compounds by LC–MS understandably catalyzed the hydrolysis of phosphothioate **13** to phosphate **12**.[24] For this reason, it was difficult to quantify the concentration of phosphothioate in plasma. The large amount of detected phosphate in animals treated with phosphothioate **13** is likely explained by this acid-catalyzed hydrolysis. Successfully, a lower concentration of alcohol was present in animals treated with phosphothioate **13** than in animals treated with phosphate **12**.

That compound **6** induces brief lymphopenia in animal models, unlike less potent phosphothioate **7**, suggests that **6**'s equilibrium lies far to the left (dephosphorylated) in the relationship described in Figure 1. Without knowledge of kinase or phosphatase specificity, it is premature to state whether this lack of activity is due to alcohol **5** being a relatively poor substrate for SphK2 or **6** being a reasonably good substrate for one or more phosphatases.

A greater understanding of SphK2 and other sphingosine related enzymes may allow for the development of $S1P_1$ agonists with increased bioavailability as phosphates. Reports in this area, and the synthesis of various phosphate mimetics, are to follow.

Acknowledgments

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22. Plasma levels of compounds **11**, **12**, and **13** were determined by high performance liquid chromatography with detection by electrospray ionization mass spectrometry (LCQ-Classic, ThermoFinnigan), similar to a previously published procedure.[23] In summary, plasma samples were purified using a Strata X solid-phase extraction cartridge (Phenomenex) that had been pre-equilibrated with methanol and ultrapure water (Barnsted). The plasma samples and internal standards were loaded and washed with ultrapure water. The analytes of interest were eluted with 50:50 methanol/acetonitrile under vacuum and concentrated by lyophilization (Savant). Partially purified samples were reconstituted in 30 μL acetonitrile, injected into a Phenomenex phenyl-hexyl column (5 μm, 50× 2.00 mm) using a Water 2695 HPLC system, and separated using a gradient method of 0.02% trifluoroacetic acid in water and acetonitrile. Initial composition consisted of 0.02% trifluoroacetic acid in water and the concentration of acetonitrile was increased to 100% over 25 min. Samples were analyzed in positive ion mode, with cations monitored by their appropriate mass to charge ratios.
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24. This conversion was confirmed by the comparison of MS data obtained by direct injection of **13** with the MS data obtained post-LC elution of the pure compound.