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Potent S1P receptor agonists replicate the pharmacologic actions of the novel immune modulator FTY720

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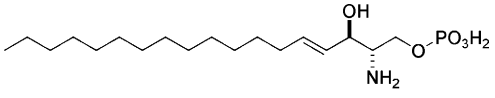
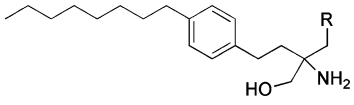
Abstract—Alteration in lymphocyte trafficking and prevention of graft rejection in rodents observed on exposure to FTY720 (**1**) or its corresponding phosphate ester **2** can be induced by the systemic administration of potent sphingosine-1-phosphate receptor agonists exemplified by **19**. The similar S1P receptor profiles of **2** and **19** coupled with their comparable potency in vivo supports a connection between S1P receptor agonism and immunosuppressive efficacy.

FTY720 (**1**) is a novel, orally active immunosuppressive agent that is currently in Phase II clinical trials for the prevention of allograft rejection.[1] While the pharmacodynamic effects that follow the systemic administration of **1** have been described,[2,3] entry into the clinic apparently preceded an understanding of the mechanism(s) by which this compound worked. Compound **1** has since been reported to be rapidly metabolized in the blood of a variety of species (including man) to the corresponding phosphate ester **2**, which is a potent agonist of four of the five known sphingosine-1-phosphate (S1P) receptors.[4,5] Agonism of S1P receptors appears to alter the trafficking of circulating lymphocytes and this has been postulated to contribute to the immunosuppressive efficacy of **1**. With a molecular target connected to the actions of **1**, we sought to obtain S1P receptor agonists without the complication of the observed in vivo equilibrium between **1** and **2** so that we could directly study the pharmacologic effects of the agonism of S1P receptors. Our efforts to obtain such compounds and the details of their characterization are the subject of this report.



Sphingosine-1-phosphate (**S1P**)



1, R = -OH (FTY720)
2, R = $-OPO_3H_2$
3, R = $-CH_2PO_3H_2$

Our initial medicinal chemistry efforts were focused on obtaining nonhydrolyzable phosphonate analogs of **2** that had comparable S1P receptor affinities and pharmacokinetic profiles making them suitable for use as molecular tools. An obvious target was phosphonate **3**;[4] this compound was found to lower circulating lymphocytes in rodents similarly to **1**. Relatively higher doses were required to elicit the lymphopenic response and

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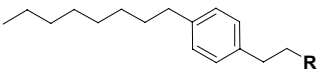
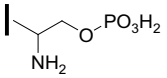
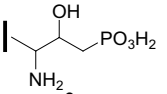
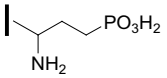
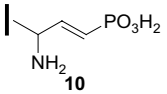
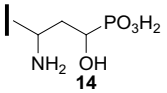
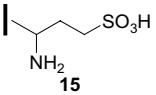
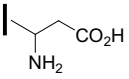
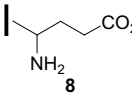
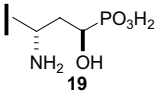
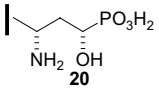
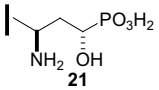
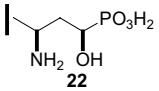
this could be attributed in part to its significantly lower affinity for S1P receptors as compared to **2** (Table 1). With this data in hand, we initiated efforts to further refine the structure of **3** with the goal of obtaining analogs with enhanced potency in vitro and in vivo.

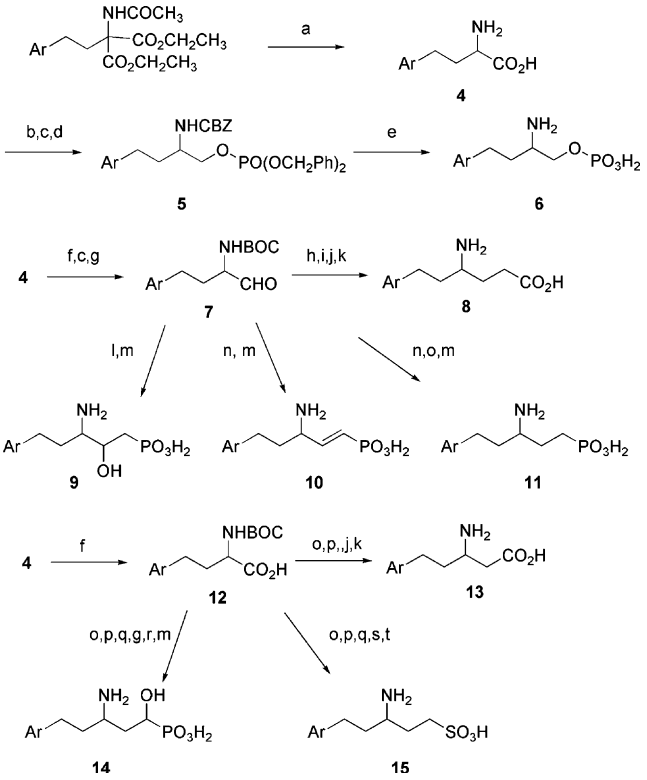
Table 1. Inhibition (IC_{50}, nM) of [^{33}P]-S1P binding to S1P receptors[a]



R (Compd)	$S1P_1$	$S1P_3$	$S1P_4$	$S1P_5$
S1P	0.16	0.04	23	0.23
2	0.28	6.3	15	0.77
3	8.7	510	210	230
 **6**	0.76	0.71	1.7	1.8
 **9**	14	68	140	240
 **11**	8.4	280	240	100
 **10**	14	720	140	76
 **14**	0.16	62	23	9.8
 **15**	1700	7600	>10,000	4400
 **13**	1900	>10,000	>10,000	3300
 **8**	710	6600	>10,000	1000
 **19**	0.10	18	16	4.5
 **20**	4.8	85	280	33
 **21**	17	320	800	73
 **22**	15	320	500	27

[a] Displacement of [^{33}P]-labeled sphingosine-1-phosphate (S1P) by test compounds from human S1P receptors expressed on CHO cell membranes. All new compounds had $S1P_2$ $IC_{50} > 10\,\mu M$. Data are reported as mean for $n = 3$ determinations. SD were generally ±20% of the average. See Ref. 3 for assay protocol.

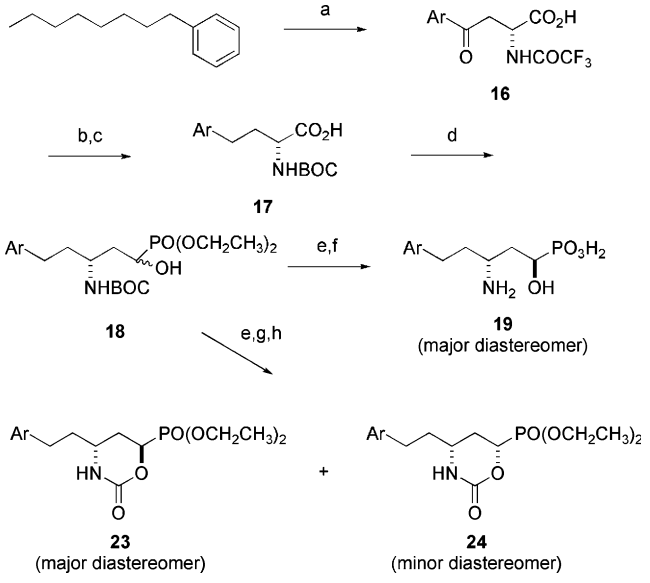
The syntheses of some of the various test compounds are shown in Scheme 1. Hydrolysis/decarboxylation of diethyl 2-(acetylamino)-2-(2-(4-octylphenyl)ethyl) malonate[6] afforded amino acid **4** which was a key intermediate required to prepare many of the desired targets. N-Protection of **4** as the benzyl carbamate followed by carboxyl reduction and dibenzylphosphorylation gave **5**. Global deprotection with sodium/ammonia afforded phosphate ester **6**. Amino acid **4** was readily elaborated to N-Boc aldehyde **7** which could be subjected to various homologation sequences to give analogs such as γ-amino acid **8**, α,β-unsaturated phosphonate **10**, or phosphonate **11**. Addition of dibenzyl methylphosphonate to **7** followed by deprotection afforded β-hydroxy phosphonate analog **9**. Conversion of amino acid **4** to N-Boc carboxylate **12** followed by elaboration to β-amino acid **13** featured an Arndt–Eisert rearrangement.



Scheme 1. Reagents and conditions: (a) conc HCl, 100 °C (100%); (b) benzyl chloroformate, dioxane/aq NaOH, rt (70%); (c) isobutyl chloroformate, TEA, THF, 0 °C, then $NaBH_4$, THF/H_2O; (d) dibenzyl (*N*,*N*-diisopropyl)phosphoramidite, 1*H*-tetrazole, CH_2Cl_2, 0 °C, then MCPBA (58%, two steps); (e) Na/NH_3, THF, −33 °C (73%); (f) di-*t*-butyldicarbonate, dioxane/aq NaOH (100%); (g) $(COCl)_2$, DMSO, DIEA, −78 to 0 °C (80%, two steps); (h) trimethyl phosphonoacetate, NaHMDS, THF, 0 °C to rt (72%); (i) H_2, 10% Pd/C, MeOH; (j) TFA, CH_2Cl_2; (k) NaOH, aq MeOH (**8**: 80%, three steps, **13**: 79%, two steps); (l) dibenzyl methylphosphonate, LDA, THF, −78 °C; (m) iodotrimethylsilane, CH_2Cl_2 (**9**: 10%, **10**: 73%, two steps, **11**: 56%, two steps, **14**: 81%); (n) tetraethyl methylenediphosphonate, NaHMDS, THF; (o) isobutyl chloroformate, NMM, CH_2Cl_2/ether, then CH_2N_2 (86%); (p) silver benzoate, TEA, MeOH (87%); (q) DIBALH, CH_2Cl_2, −78 °C to rt (91%); (r) diethyl phosphite, NaHMDS, THF, 0 °C (63%, ds = 3:1); (s) I_2, PPh_3, imidazole, CH_2Cl_2 (76%); (t) Na_2SO_3, aq EtOH, 70 °C (42%). All compounds are racemic or equal mixtures of diastereomers.

Compound **12** could also be carried through straightforward sequences to give either α-hydroxy phosphonate analog **14** or sulfonic acid **15**. The individual diastereomers of **14** were synthesized as shown in Scheme 2. The stereocenter of the carbon bearing the amino group was set during a Friedel–Crafts reaction between octylbenzene and enantiopure (−)-2-trifluoroacetamidosuccinic anhydride[7] to give **16**. Ketone reduction followed by switching of N-protecting groups afforded the chiral N-Boc amino acid **17**. A sequence of reactions analogous to those used in the preparation of **14** afforded **18** as a mixture of diastereomers which were separated using silica gel chromatography. Global deprotection of the individual diastereomers with iodotrimethylsilane afforded **19** and **20**. Diastereomers **21** and **22** were obtained by employing the (*R*)-anhydride in the Friedel–Crafts reaction. The configuration of the carbon bearing the hydroxy group in these compounds was determined by carrying out NOE experiments on the cyclic carbamates **23** and **24** which were prepared as shown.[8]

Ligand competition studies between [^{33}P]-S1P and **2**, and **3** and all of the new compounds were carried out for each of the five human S1P receptors stably expressed in Chinese Hamster Ovary (CHO) cell membranes.[4] S1P receptor agonism by the test compounds was also determined by measurement of ligand-induced [^{35}S]-5′-O-3-thiotriphosphate (GTPγS) binding; all of the compounds tested were found to be agonists of S1P receptors (data not shown).[9] Several things were readily observed of the S1P receptor data (Table 1). Deletion of the hydroxymethyl group of either **2** or **3** was found to have a minimal effect on S1P receptor affinity. Phosphate **6** had only 3-fold lower affinity for $S1P_1$ and approximately 10-fold higher affinity for both $S1P_3$ and $S1P_4$ as compared to **2** while the profile of *des*-hydroxymethyl phosphonate **11** was almost identical to that of **3**. The structural requirements of a bioisosteric replacement for the phosphate ester of either **2** or **6** appear to be fairly restricted as only α-hydroxy phosphonate analog **14** was found to have affinities for the various S1P receptors approaching those of **2** and **6**. The data for the individual α-hydroxy phosphonate diastereomers **19**–**22** indicate that the 2-(*R*), 4-(*S*)-stereochemistry of **19** is preferred for maximal S1P receptor affinity and the configuration of these centers has little effect on selecting either for or against the different S1P receptor sub-types. Additionally, aside from a 6-fold lower affinity for $S1P_5$, the S1P receptor profile of diastereomer **19** was found to be quite comparable to that of **2**.



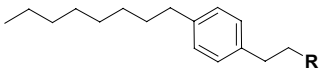
Scheme 2. Reagents and conditions: (a) 2-(*R*)-Trifluoroacetamidosuccinic anhydride, $AlCl_3$, nitromethane, CH_2Cl_2 (70%); (b) H_2, 10% Pd/C, HOAc; (c) NaOH, aq dioxane, then di-*t*-butyl dicarbonate; (d) see Scheme 1; (e) separate diastereomers; (f) TMS-I, CH_2Cl_2 (77%); (g) HCl, EtOH; (h) $COCl_2$, DIEA, MeCN, rt (**23**: 74%, two steps, **24**: 53%, two steps).

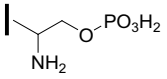
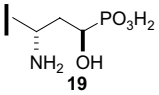
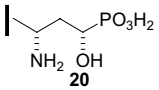
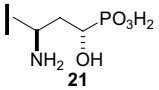
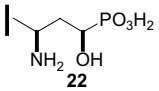
Recent data demonstrate that hematopoietic cells genetically deficient in $S1P_1$ receptors have defects in thymic emigration and recirculation similar to lymphocytes from normal mice treated with **1** which indicates that an agonist-driven functional antagonism of $S1P_1$ is an important component in the efficacy of **1**.[10,11] We have found that cell surface expression of FLAG-tagged $S1P_1$ on HEK293 cells is down-regulated for sustained periods in response to treatment with **2** and **19**. $S1P_1$ is also desensitized by **1** as reported,[12] but with delayed kinetics that are consistent with the requirement for phosphorylation to the active form.

The immunosuppressive efficacy of **1** has been proposed to arise from its ability to promote the sequestration of $CD4^+$ and $CD8^+$ T cells and B cells in secondary lymphoid organs which prevents their infiltration into transplanted or antigen-bearing nonlymphoid tissues.[3] Measurement of blood lymphocyte counts in rodents after the administration of test compounds provides a convenient surrogate marker for efficacy that is amenable to the screening of multiple analogs in vivo.[13] Intravenous administration of either **1** or **2** to mice or rats has been previously found to lead to a rapid lowering of circulating lymphocytes with the nadir being reached in 2–3 h.[4] Each of the α-hydroxy phosphonate analogs **19**–**22** was found to maximally lower circulating lymphocytes in a manner similar to **2** at a three hour time point after iv administration to mice. Pharmacodynamic dose-titration data for **19**–**22** (Table 2) indicate that in vivo potency tracks well with S1P receptor affinity. The comparable rat pharmacokinetic profiles of **2**, **19**, and **20** are also consistent with this observation.

Circulating lymphocytes were found to be minimally lowered 3 h after administration of phosphate ester **6** to mice at a dose of 10 mpk iv (Table 2); this result appears to be in agreement with the lack of immunosuppressive efficacy in rodents that has been reported for the corresponding amino alcohol.[2] It should be noted that the attenuated pharmacodynamic activity of **6** as compared to analogs with comparable S1P receptor affinities (e.g., **2** or **19**) can not be explained by species differences as all of the compounds described here have very similar affinities for the mouse, rat, and human S1P receptors. Pharmacokinetic factors, such as enhanced metabolism

Table 2. Mouse peripheral lymphocyte lowering[a] (PLL) and rat pharmacokinetic[b] data for selected S1P receptor agonists



R (Compd)	Murine $PLLED_{50}$ (mpk iv)	Rat PK (1.0 mpk iv)
2	0.06	$Cl_p = 17.8$ mL/min/kg $Vd_{ss} = 5.9$ L/kg, $t_{1/2} = 7.6$ h
 **6**	35% @ 10 mpk[c]	$Cl_p = 29.8$ mL/min/kg $Vd_{ss} = 35.1$ L/kg, $t_{1/2} = 4.2$ h
 **19**	0.15	$Cl_p = 5.2$ mL/min/kg $Vd_{ss} = 1.4$ L/kg, $t_{1/2} = 5.7$ h
 **20**	0.6	$Cl_p = 4.3$ mL/min/kg $Vd_{ss} = 0.7$ L/kg, $t_{1/2} = 3.6$ h
 **21**	3.0	nd[d]
 **22**	2.5	nd[d]

[a] Individual data points for dose titrations were the average percentage decrease of peripheral blood lymphocyte counts in $n = 3$ animals versus control ($n = 3$) 3 h after iv administration of the test compound. SD were generally ±20% of the average. See Ref. 13.
[b] See Ref. 14.
[c] Percentage decrease of peripheral blood lymphocyte counts in test animals ($n = 3$) versus control.
[d] Not determined.

of **6** in the mouse or the large steady state volume of distribution observed for this compound in the rat, or as yet undetermined subtleties between S1P receptor selectivity and alterations in lymphocyte trafficking are potential sources that could require further investigation.

Diastereomer **19** and FTY720 (**1**) were further characterized in rodent models of immunosuppressive efficacy. In an in vivo mixed lymphocyte reaction,[15] **1** and **19** reduced the alloantigen stimulated increase in the draining popliteal lymph node following footpad injection of Fisher splenocytes into Lewis rats. Mean lymph node weight ratios of intra-subject allogeneic to syngeneic splenocyte challenges for **1** and **19** were 1.0 (0.3 mpk/day sc) and 1.6 (0.5 mpk/day sc), respectively, compared to 3.4 for vehicle controls. In a mouse thyroid allograft model,[16] **1** and **19** dose dependently prolonged graft function. Similar maximal efficacy was achieved with both compounds at 3 mpk/day, and **1** appeared to be 3- to 10-fold more potent than **19**.

In conclusion, nonhydrolyzable phosphonate analogs of FTY720 phosphate (**2**) have been identified as potent agonists of S1P receptors. While one report describing S1P receptor agonists has appeared,[17] no in vivo characterization of those compounds was disclosed. Systemic administration of the compounds described herein results in a replication of the primary pharmacodynamic effect of **1** in rodents, which is a dose-responsive lowering of circulating lymphocytes. An analog (**19**) with an S1P receptor profile comparable to bioactive metabolite **2** also behaves similarly in two rodent models for the prevention of allograft rejection. These data support the connection between S1P receptor agonism and the immunosuppressive efficacy of FTY720. Investigations into the relationships between S1P receptor subtype selectivity and the immunomodulatory effects of S1P receptor agonists are underway.

Acknowledgements

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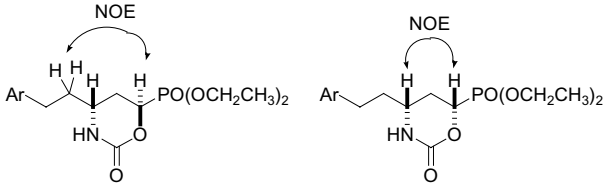
8. NOE data were collected at 600 MHz in $CDCl_3$ with a mixing time of 0.3 s. The observed NOEs indicated below were used to assign the structures of **23** and **24** and are consistent with those reported for related compounds. See Shi, Z.; Harrison, B. A.; Verdine, G. L. *Org. Lett.* **2003**, *5*, 633–636.



23 **24**

9. All test compounds were found to be agonists of human $S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$ receptors as evidenced by their ability to induce levels of GTPγS binding comparable to S1P. The magnitudes of the calculated EC_{50} values from these assays were generally ±5-fold of the IC_{50} values.
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13. Mice were dosed intravenously into the tail vein with 0.1 mL of test compound dissolved in vehicle (2% (w/v) hydroxypropyl-β-cyclodextrin (Cerestar, USA), 0.12 M NaCl). For screening purposes, lymphocyte lowering was assessed at 3 h post dose. After rendering each mouse unconscious by CO_2 to effect, the chest was opened, 0.5 mL of blood was withdrawn via direct cardiac puncture, and the blood was immediately stabilized with EDTA and hematology evaluated using a clinical hematology autoanalyzer calibrated for performing murine differential counts (H2000, CARESIDE, Culver City, CA). Reduction in lymphocytes by test compound treatment was established by comparison of hematological parameters of three mice versus three vehicle treated mice.
14. Plasma compound concentration measurements used to calculate pharmacokinetic parameters were obtained after iv administration of test compounds via a cannula that had been previously implanted in the femoral vein of male Sprague-Dawley rats ($n = 2$). Compounds **2** and **6** were formulated at 1.0 mg/mL in 2% hydroxymethyl-β-cyclodextrin/10 nM Na_2CO_3. Compounds **19** and **20** were formulated at 1.0 mg/mL in ethanol/PEG-400/water (20:30:50, v/v/v).
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