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Synthesis, stereochemical determination and biochemical characterization of the enantiomeric phosphate esters of the novel immunosuppressive agent FTY720

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Abstract—The novel immunosuppressive agent FTY720 (**1**) is phosphorylated in vivo in a variety of species yielding an active metabolite that is an agonist of four of the five known G-protein-coupled sphingosine-1-phosphate (S1P) receptors. A synthesis amenable to producing gram quantities of the stereoisomeric phosphate esters, a determination of their absolute stereochemistry via an enantioselective synthesis and their characterization as S1P receptor agonists and antagonists is reported.

1. Introduction

Current immunosuppressive agents are in part responsible for the recent significant advances in prolonging allograft survival after organ transplantation.[1] The potential to extend many of these therapies to the treatment of chronic autoimmune disorders and inflammatory diseases, such as rheumatoid arthritis and multiple sclerosis, has been realized, but suboptimal therapeutic indices and a lack of immunosuppressive selectivity currently limits applicability. Calcineurin inhibitors such as cyclosporin A (CsA, Sandimmune®, Neoral®) and FK506 (Prograf®) are widely used in transplantation therapy, but both exhibit a mechanism-based nephrotoxicity at or near therapeutic doses. The efficacy of rapamycin (Rapamune®) arises from its disruption of the cytokine signaling that promotes the growth and proliferation of lymphocytes, but it also has demonstrated toxicities (thrombocytopenia, hyperlipidemia). Antimetabolites such as mycophenolate mofetil (MMF, Cellcept®) and leflunomide (Arava®) have long been known to be potent immunosuppressants, but exhibit myelotoxicity and, in the case of MMF, gastrointestinal toxicity.[2] There exists a clear need for immunosuppressive therapies with improved safety profiles arising from the enhanced inherent potency and selectivity of new agents and/or synergisms between them with existing drugs.

2-Amino-2-(4-octylphenyl)propane-1,3-diol (FTY720, **1**) is a novel, orally active immunosuppressive agent first identified by researchers at Yoshitomi Pharma.[3] The unique mode of action of **1** results in immunosuppression by promoting a lowering of circulating T lymphocytes, which prevents their infiltration into transplanted or antigen-bearing nonlymphoid tissues.[4] Preclinical studies have demonstrated the efficacy of **1** in prolonging allograft survival in rodents, dogs and cynomolgus monkeys; synergy has been demonstrated when CsA, FK506 or rapamycin was co-administered.[5] The ability of **1** to suppress adjuvant- and collagenase-induced arthritis,[6] experimental autoimmune encephalitis,[7] acute viral myocarditis[8] autoimmune diabetes,[9] and systemic lupus erythematosus[10] in rodents has been demonstrated. The mechanism of action of **1** appears to confer advantages regarding host defense in that it does not impair myelomonocytic cell function; responses to bacterial and fungal challenge in preclinical models appear

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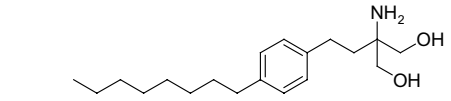
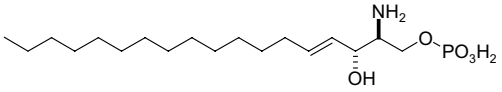
to be spared.[11] In Phase I clinical trials with stable renal transplant patients, **1** was reported to induce the lowering of circulating lymphocytes that was observed preclinically while being well-tolerated. A transient, asymptomatic bradycardia was the most commonly encountered adverse event.[12] Twelve month preliminary results from phase II clinical trials in de novo kidney transplant patients indicate that **1**, in combination with low dose CsA and steroids, appears to be at least equally efficacious as the currently recognized standard of care (full dose CsA, steroids, MMF).[13]



1, FTY720

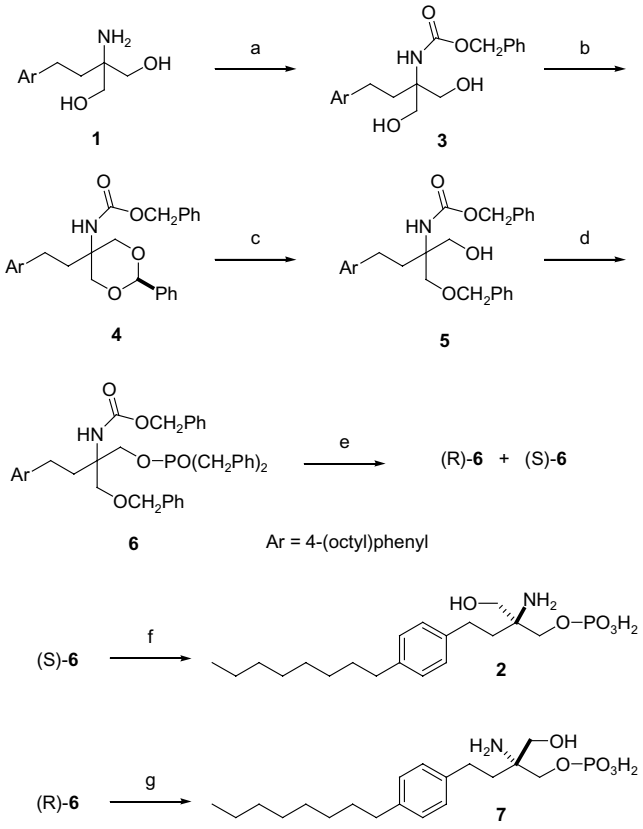


Sphingosine-1-phosphate (S1P)

It was only recently that a molecular target was connected to the immunosuppressive actions of **1**.[14,15] Compound **1** was shown to be rapidly metabolized in the blood of a variety of species to the corresponding phosphate ester, which is an agonist of four of the five known G-protein-coupled sphingosine-1-phosphate receptors ($S1P_{1\text{-}5}$).[16] Agonism of S1P receptors by the phosphate ester of **1** appears to inhibit lymphocyte migration into lymphatic sinuses; this alteration in lymphocyte trafficking has been postulated to lead to the observed peripheral lymphopenia and immunosuppressive efficacy. In this paper, we wish to describe our efforts to synthesize and determine the absolute stereochemistries of the enantiomeric phosphate esters of **1** and to characterize further the biochemical properties of these compounds.

2. Chemistry

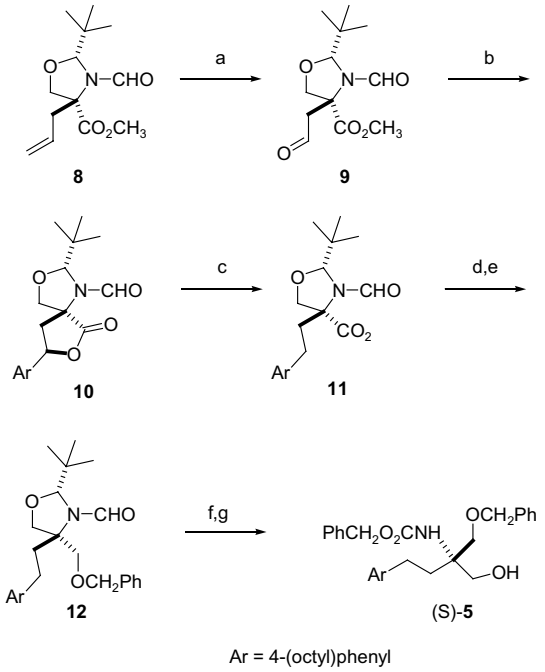
Two factors influenced the route chosen to synthesize the enantiomeric phosphate esters of **1** (Scheme 1). First, it was envisioned that these compounds could be obtained via resolution of a late-stage synthetic intermediate by chiral HPLC and that converting **1** to an unsymmetric derivative with multiple aromatic functional groups would aid in accomplishing this. Second, it was desired that a final global deprotection with a low potential for phosphate ester scrambling be employed to give the target compounds. In practice, the tertiary amino group of **1** was selectively acylated on treatment with benzyl chloroformate under Schotten–Baumann conditions to give **3**. Crude **3** was converted to the corresponding benzylidene acetal **4**; this was followed by selective reduction with borane dimethylamine complex in the presence of boron trifluoride etherate[17] to give the unsymmetric intermediate **5**. The free hydroxy group of **5** was dibenzylphosphorylated using the protocol described by Frasier-Reid[18] to give **6**. The



Scheme 1. (a) $PhCH_2OCOCl$, $KHCO_3$, EtOAc/H_2O; (b) PhCHO, cat. *p*-TSA, toluene, 100 °C (57%, 2 steps); (c) $BH_3{\cdot}NHMe_2$, $BF_3{\cdot}OEt_2$, CH_2Cl_2, −78 °C → −5 °C (92%); (d) $iPr_2N{-}P(OCH_2Ph)_2$, 1*H*-tetrazole, CH_2Cl_2, 0 °C, then MCPBA −78 °C → rt (99%); (e) enantiomer resolution (chiral HPLC); (f) TMS-I, $CHCl_3$ (63%); (g) Na, NH_3, THF, −33 °C (81%).

enantiomers of both **5** and **6** were found to be readily separable on a Chiralcel® OD HPLC column. It was initially thought that the global deprotection of **6** could be readily accomplished using catalytic hydrogenation, but this was found not to be the case. Reactions using various catalysts and solvents were found to require forcing conditions to proceed and the mixtures obtained were often difficult to purify. Reduction of **6** with an excess of sodium metal in ammonia gave much better results affording product that was determined to be 95% pure by HPLC. Global deprotection of (*S*)-**6** with trimethylsilyliodide proved to be optimal; analytically pure **2** was obtained after HPLC purification.

An enantioselective route to **5** was executed in order to establish the absolute configurations of **2** and **7**. Oxidative cleavage of the double bond of the known L-serine-derived oxazolidine **8**[19] afforded aldehyde **9** in excellent yield. Treatment of **9** with 4-(octyl)phenylmagnesium bromide resulted in the formation of lactone **10**, which was obtained as a 1:1 mixture of diastereomers. The stereochemical outcome of the Grignard addition was without consequence as catalytic reduction of the mixture of lactones afforded a single compound, carboxylic acid **11**. Reduction of **11** to the corresponding alcohol followed by *O*-benzylation gave **12**. Acid hydrolysis of the oxazoline ring of **12** and subsequent *N*-acylation of



Scheme 2. (a) cat. OsO_4, $NaIO_4$, 3:1 v/v dioxane/H_2O, rt (85%); (b) 4-(octyl)phenylmagnesium bromide, THF, −78 °C to rt (67%, *ds* = 1:1); (c) H_2, 10% Pd/C, MeOH, rt (90%); (d) CH_3CH_2OCOCl, TEA, THF, 0 °C, then $NaBH_4$, THF/H_2O, 0 °C to rt (69%); (e) $PhCH_2Br$, NaH, $Bu_4N^+I^-$, DMF, 65 °C (93%); (f) 6.0 N HCl, MeOH, reflux; (g) $PhCH_2OCOCl$, $KHCO_3$, EtOAc/H_2O, rt (73%, 2 steps).

the resulting amino alcohol afforded (*S*)-**5**. Elaboration of this material (as described for the racemate in the preceding paragraph) ultimately gave **7**, which established that it is the (*R*)-enantiomer and that **2** is the (*S*)-enantiomer (Scheme 2).

Attempts to develop analytical chiral HPLC methods suitable to measure the enantiomeric excesses of **2** and **7** have been unsuccessful. Phosphate esters **2** and **7** were prepared using both of the deprotection methods described (Na/NH_3, TMS-I) and the receptor profiles (see below) were found to be the same regardless of the manner of preparation. Based on this, it appears unlikely that significant racemization occurs during the deprotection reactions. However, while the enantiomeric excesses of **2** and **7** in all likelihood are similar those of (*S*)-**6** and (*R*)-**6**, respectively, they have not yet been accurately quantitated.

3. In vitro biochemistry

Ligand competition studies with [^{33}P]-S1P were carried out with S1P and compounds **1**, **2** and **7** for each of the five human S1P receptors stably expressed in Chinese Hamster Ovary (CHO) cell membranes.[14] Human S1P receptor agonism by the test compounds was determined by measurement of ligand-induced [^{35}*S*]-5′-*O*-3-thiotriphosphate (GTPγS) binding for $S1P_1$, $S1P_3$ and $S1P_5$ in CHO cells membranes[14] and ERK activation for $S1P_4$. (*S*)-Phosphate ester **2** was found to be the enantiomer with the greater affinity for all five S1P receptors. Additionally, GTPγS functional data indicated that **2** was a full agonist of $S1P_1$ and an agonist of $S1P_3$, $S1P_4$ and $S1P_5$ with 70–90% maximal efficacy compared to the endogenous ligand, S1P. Calculated EC_{50} values agreed well with the IC_{50} values obtained from the ligand competition experiments. (*R*)-Phosphate ester **7** was found to be a weaker full agonist of $S1P_1$ and $S1P_4$, but a weak antagonist of $S1P_3$ and $S1P_5$ based on the observations that **7** competed with S1P binding to $S1P_3$ and $S1P_5$, but failed to activate GTPγS binding to these receptors. The antagonist activity was confirmed with further functional experiments; **7** was found to antagonize GTPγS binding to $S1P_3$ and $S1P_5$ stimulated by **2** (data not shown) and to block Ca^{++} flux induced by either S1P or **2** in $S1P_3$-bearing CHO cells with an IC_{50} = 80 nM (Table 1).

4. Conclusions

The novel immunosuppressive agent FTY720 (**1**) is phosphorylated in vivo in a variety of species yielding an active metabolite that is an agonist of four of the five known G-protein-coupled S1P receptors. Despite the advanced clinical development of **1**, little has appeared in the literature regarding the synthesis and characterization of **2** and/or **7**. The fact that **2** and **7** have different S1P receptor profiles indicates that the efficient access to both of these compounds will be of value for both the study and understanding of the pharmacology arising

Table 1. Ligand competition[a] and functional activity[b]

Compd		$S1P_1$	$S1P_2$	$S1P_3$	$S1P_4$	$S1P_5$
S1P	IC_{50} (nM)	0.67	0.35	0.26	34	0.55
	EC_{50} (nM)	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]
1	IC_{50} (nM)	840	>10,000	>10,000	>10,000	>10,000
	EC_{50} (nM)	145[d]	nd[c]	>10,000	>10,000	>10,000
2	IC_{50} (nM)	0.28	1100	6.3	15	0.77
	EC_{50} (nM)	0.28	nd[c]	3.1	12	2.5
7	IC_{50} (nM)	25	>10,000	120	380	49
	EC_{50} (nM)	32	nd[c]	>10,000	93	300[e]

[a] Inhibition of [^{33}P]-S1P binding to stably expressed human $S1P_{1-5}$ in CHO cell membranes.
[b] EC_{50} for ligand-induced GTPγS binding ($S1P_1$, $S1P_3$, $S1P_5$) or ERK activation ($S1P_4$).
[c] Not determined.
[d] 44% maximal efficacy compared to **2**.
[e] 39% maximal efficacy compared to **2**. IC_{50} and EC_{50} data are the averages from multiple ($n \geqslant 3$) assays; standard deviations were generally ±50% of the average values.

from the systemic administration of **1** and the design and synthesis of future S1P agonists and antagonists.

5. Experimental

Flash chromatography was carried out using a Biotage Flash Chromatography apparatus (Dyax Corp.) on silica gel (32–63 mM, 60 Å pore size) in pre-packed cartridges of the size noted. NMR spectra were obtained in $CDCl_3$ solution unless otherwise noted. Coupling constants (*J*) are in hertz (Hz). Analytical HPLC conditions: HPLC A: Analytical Sales and Service Armor C8, 5 Å, 4.6 mm × 50 mm column, gradient 10:90 → 90:10 v/v CH_3CN:H_2O + 0.05% TFA over 4 min, then hold at 90:10 v/v CH_3CN:H_2O + 0.05% TFA for 4 min; 2.5 mL/min, 210 nm. HPLC B: YMC ODS A, 5 Å, 4.6 × 50 mm column, gradient 10:90 → 95:5 v/v CH_3CN:H_2O + 0.05% TFA over 4.5 min, then hold at 95:5 v/v CH_3CN:H_2O + 0.05% TFA for 1.5 min; 2.5 mL/min, 210 nm.

5.1. 2-Benzyloxycarbonylamino-2-hydroxymethyl-4-(4-(octyl)phenyl)butanol (3)

A mixture of **1**[20] (3.07 g, 10.0 mmol) and $KHCO_3$ (3.00 g, 30.0 mmol) in EtOAc (200 mL) and H_2O (150 mL) was treated with benzyl chloroformate (1.50 mL, 10.0 mmol), then stirred at room temperature for 2 h. The organic layer of the reaction mixture was separated, dried over $MgSO_4$ and concentrated to afford 5.29 g of crude **3**. This material was used without further purification: ^{1}H NMR (500 MHz) δ 0.88 (t, J = 6.5, 3H), 1.22–1.34 (12H), 1.55–1.60 (m, 2H), 1.87–1.91 (m, 2H), 2.53–2.59 (4H), 3.23 (br s, 2H), 3.67 (dd, J = 6.5, 11.5, 1H), 3.90 (dd, J = 6.5, 11.5, 1H), 5.08 (s, 2H), 5.30 (s, 1H), 7.05–7.09 (4H), 7.32–7.36 (5H); HPLC A 5.34 min.

5.2. 2-Phenyl-4-(benzyloxycarbonylamino)-4-(4-(octyl)-phenyl)-1,3-dioxane (4)

A mixture of **3** (5.29 g, ~10.0 mmol), benzaldehyde (1.10 mL, 11.0 mmol) and p-TSA · H_2O (95 mg, 0.05 mmol) in toluene (50 mL) was stirred at 100 °C for 1 h. The reaction mixture was treated with additional benzaldehyde (2 mL) and stirring was continued at 100 °C for 2 h. The mixture cooled to room temperature, then partitioned between ether (400 mL) and 1.0 N NaOH (150 mL). The organic layer was separated, dried over $MgSO_4$ and concentrated. Chromatography on a Biotage Flash 75S cartridge using 20:1 v/v heptane/EtOAc (3 L), then 10:1 v/v heptane/EtOAc (3 L) as the eluant afforded 3.02 g of **4** (57%, 2 steps): ^{1}H NMR (500 MHz) δ 0.88 (t, J = 6.5, 3H), 1.22–1.36 (12H), 1.55–1.60 (m, 2H), 1.94–2.06 (m, 2H), 2.54–2.58 (4H), 3.69 (d, J = 11.5, 2H), 4.31 (d, J = 11.5, 2H), 5.13 (s, 2H), 5.41 (br s, 1H), 5.45 (s, 1H), 7.06–7.10 (4H), 7.29–7.40 (8H), 7.46–7.48 (2H); HPLC A 6.08 min.

5.3. (±)-2-Benzyloxycarbonylamino-2-benzyloxymethyl-4-(4-(octyl)phenyl) butanol (5)

A solution of **4** (3.90 g, 7.4 mmol) and BH_3·$NHMe_2$ (2.18 g, 37.0 mmol) in CH_2Cl_2 (150 mL) at −78 °C was treated with BF_3·Et_2O (4.70 mL, 37.0 mmol). The resulting mixture was allowed to warm to −5 °C and was stirred for 2 h. The reaction mixture was poured into 1.0 N NaOH (25 mL) and the resulting mixture was extracted with CH_2Cl_2 (100 mL). The extract was separated and dried over $MgSO_4$. The aqueous layer was extracted with ether (200 mL). The ether extract was dried and the two organic extracts were combined and concentrated. Chromatography on a Biotage 75S cartridge using 6:1 v/v heptane/acetone as the eluant afforded 3.63 g (92%) of **5**: ^{1}H NMR (500 MHz) δ 0.88 (t, J = 6.5, 3H), 1.26–1.31 (10H), 1.54–1.60 (m, 2H), 1.85–1.89 (m, 1H), 2.05–2.08 (m, 1H), 2.44–2.61 (m, 2H), 2.55 (t, J = 7.5, 2H), 3.58 (AB q, J = 130, 2H), 3.72–3.78 (m, 2H), 4.50 (app s, 2H), 5.08 (s, 2H), 5.45 (s, 1H), 7.04–7.08 (4H), 7.30–7.38 (10H); ESI-MS 532 ($M+H^+$); HPLC A: 5.98 min; HPLC B: 5.32 min.

The enantiomers of **5** were found to be separable by chiral HPLC. Conditions: Chiral Technologies Chiralcel™ OD 4.6 × 250 mm column, 65:35 v/v hexanes/*i*PrOH, 1.0 mL/min, 210 nm. For the faster eluting enantiomer, t = 5.5 min. For the slower eluting (*S*)-enantiomer, t = 9.0 min. Elaboration of L-serine-derived oxazoline **8** as described in the text afforded the enantiomer of **5**, which corresponded to the slower eluting peak thus establishing that it was the (*S*)-enantiomer.

5.4. (±)-1-Dibenzyloxyphosphoryloxy-2-benzyloxycarbonylamino-2-benzyloxymethyl-4-(4-(octyl)phenyl)butane (6)

A solution of **5** (2.90 g, 5.4 mmol) and dibenzyl diisopropylphosphoramidite (2.00 mL, 6.0 mmol) in CH_2Cl_2 (40 mL) at 0 °C was treated with 1*H*-tetrazole (117 mg, 8.2 mmol). The resulting mixture was stirred at room temperature for 1.25 h, then cooled to −78 °C. MCPBA (2.0 g, ~8.2 mmol) was added, the cooling bath was removed and the reaction was stirred at ambient temperature for 45 min. The reaction mixture was quenched with satd $NaHCO_3$ (50 mL), then extracted with CH_2Cl_2 (200 mL). The extract was separated and dried over $MgSO_4$. The aqueous layer was extracted with ether (200 mL). The ether extract was dried and the two organic extracts were combined and concentrated. Chromatography on a Biotage 75S cartridge using 8:1 v/v heptane/acetone (4.5 L), then 4:1 v/v heptane/acetone (2 L) as the eluant afforded 4.25 g (99%) of **6**: ^{1}H NMR (500 MHz) δ 0.88 (t, J = 6.5, 3H), 1.22–1.36 (12H), 1.55–1.61 (m, 2H), 1.94–2.04 (m, 1H), 2.08–2.18 (m, 1H), 2.47 (app t, J = 8.5, 1H), 2.55 (app t, J = 7.5, 1H), 3.54 (AB q, J = 26.0, 2H), 4.18–4.25 (m, 2H), 4.44 (s, 2H), 4.98 (s, 2H), 5.00 (s, 2H), 5.03 (s, 2H), 5.11 (br s, 1H), 6.96–7.06 (8H), 7.24–7.36 (36H); HPLC A: 6.31 min.

The enantiomers of **6** were resolved using preparative chiral HPLC. Conditions: Chiral Technologies Chiralcel™ OD 20 mm × 250 mm column, 60:40 v/v hexanes/*i*PrOH, 9.0 mL/min, 210 nm, ~25 mg of **6** per injection. For the faster eluting enantiomer, t = 16.7 min. For the slower eluting enantiomer, t = 24.0 min. Subjecting (*S*)-**5** to the reaction conditions described above afforded

the enantiomer of **6** that corresponded to the faster eluting peak thus establishing that it was the (*R*)-enantiomer.

5.5. (*S*)-2-Amino-2-phosphoryloxymethyl-4-(4-(octyl)phenyl)butanol (2)

A solution of (*S*)-**6** (4.20 g, 5.3 mmol) in $CHCl_3$ (50 mL) at 0 °C was treated with iodotrimethylsilane (4.60 mL, 32 mmol). The cooling was removed and the reaction mixture was stirred at room temperature for 16 h. The reaction mixture was treated with MeOH (50 mL) and stirred for 1 h, then concentrated. The crude product was purified by preparative reverse-phase HPLC. Conditions: Kromasil® KR100-10-C8 30 mm × 100 mm column, gradient 10/90 → 90:10 v/v CH_3CN/H_2O + 0.05% TFA over 12 min, then hold at 90:10 v/v CH_3CN/H_2O + 0.05% TFA for 4 min, 20 mL/min, 220 nM, ~20 mg of crude **2** per injection. Fractions containing **2** were pooled and concentrated to remove the CH_3CN. The solid that precipitated was filtered, washed with water and dried to obtain 1.30 g (63%) of pure **2**: ^1H NMR (500 MHz, CD_3OD) δ 0.88 (t, $J = 7.0$, 3H), 1.22–1.36 (12H), 1.54–1.64 (m, 2H), 1.86–2.04 (m, 2H), 2.55 (app t, $J = 7.5$, 2H), 2.63–2.68 (m, 2H), 3.66 (app q, $J = 5.5$, 1H), 3.95–4.05 (m, 2H), 7.07 (d, $J = 8.0$, 2H), 7.13 (d, $J = 8.0$, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 14.4, 23.7, 29.6, 30.3, 30.4, 30.6, 32.8, 33.0, 34.9, 36.5, 61.4 (d, $J = 6.8$), 62.5, 65.8 (d, $J = 3.9$), 129.2, 129.6, 139.5, 141.9; ESI-MS 388 ($M+H^+$); HPLC A: 3.96 min; HPLC B: 3.83 min; Anal. Calcd for $C_{19}H_{34}NO_5P$: C, 58.90; H, 8.84; N, 3.62. Found: C, 58.90; H, 9.20; N, 3.57.

5.6. (*R*)-2-Amino-2-phosphoryloxymethyl-4-(4-(octyl)-phenyl)butanol (7)

Sodium metal (500 mg, 20.8 mmol) was added in portions to ammonia (~10 mL) that had been condensed with dry ice cooling. A solution of (*R*)-**6** (320 mg, 0.4 mmol) in THF (3 mL) was added and the resulting mixture was stirred cold for 30 min. The reaction mixture was stirred at ambient temperature for 1 h, then carefully quenched with H_2O (25 mL). The aqueous mixture was extracted with ether (25 mL), then filtered to remove residual solids. The pH of the filtrate was adjusted to 7 with 1.0 N HCl. The solid that precipitated was filtered, rinsed with H_2O and dried to afford 127 mg (81%) of the title compound.

5.7. $S1P_4$ ERK activation assay

HEK cells stably transfected with both human $S1P_4$ and Gqi5 were grown in normal growth medium (IMDM supplemented with HT, sodium pyruvate, Geneticin, zeomycin, hygromycin B and 10% charcoal-stripped FBS) to ~75% confluence in 24 well plates. Cells were subsequently placed in serum-free medium 18–24 h prior to ERK activation. ERK activation was induced by treatment of cells with various mediators or the vehicle control for 5 min at 37 °C. Treated cells were washed with ice-cold PBS and lysed with lysis buffer. Approximately 1/10 the total lysate volume was then analyzed by SDS-PAGE. Compound-induced phosphorylation of ERK 1/2 was measured by quantitative immunoblotting with a phospho-ERK antibody. HRP-linked anti-rabbit IgG was used as the secondary antibody with the ECL Plus chemiluminescent detection kit. Images were captured using the Storm 860 phosphorimager and phosphorylated ERK bands were quantitated using the Imagequant program.

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