

# Asymmetric Synthesis of Conformationally Constrained Fingolimod Analogues—Discovery of an Orally Active Sphingosine 1-Phosphate Receptor Type-1 Agonist and Receptor Type-3 Antagonist

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Compound **1** (FTY720, Fingolimod) represents a new generation of immunosuppressant that modulates lymphocyte trafficking by interacting with the S1P<sub>1</sub> receptor. Compound **1** also provides a template molecule for studying the molecular biology of S1P receptors and related enzymes (kinases and phosphatases). In this study, two conformationally constrained analogues of **1** (**3a** and **3c**) were asymmetrically synthesized in high optical purity. *In vitro* assessment documented that both analogues are Sphk2 substrates, their phosphorylated species are potent S1P<sub>1</sub> receptor agonists, and **3a-P** is a potent S1P<sub>3</sub> antagonist. After oral administration in mice, both compounds evoked lymphopenia, but their duration of action differed markedly.

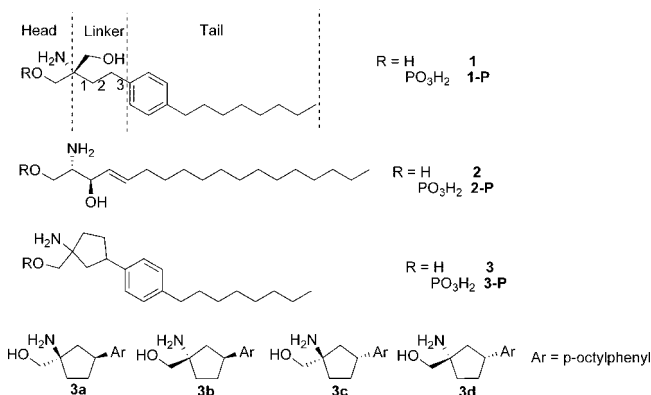
## Introduction

Drug-induced modulation of the immune system is a cornerstone of the treatment of autoimmune diseases and allows organ allografting. In addition to biological (e.g., monoclonal antibody) immunosuppressants, the small-molecule drugs used commonly are cytotoxins (e.g., cyclophosphamide), corticosteroids, purine synthesis inhibitors, and immunophilin-binding compounds [cyclosporine A (CsA), rapamycin,<sup>1</sup> and tacrolimus (FK506)<sup>2</sup>].<sup>3</sup> All of these chemical agents function by either depleting lymphocytes or preventing lymphocyte activation. Although these small-molecule drugs have enabled widespread organ transplantation, they also have significant adverse effects as consequences of both the therapeutic target and often alternative, “off” targets. Thus, additional drugs are needed to modulate the immune system.

A new modality for the treatment of immunological disorders arose from the discovery of **1** (FTY720, Fingolimod).<sup>4</sup> This compound, which is a sphingosine (**2**) analogue, modulates the immune system by a unique mechanism, i.e., disruption of normal lymphocyte trafficking. Specifically, **1** inhibits egress of lymphocytes from secondary lymphoid tissues, such as lymph nodes;<sup>5</sup> the biomarker of **1** is thus lymphopenia (abnormally low numbers of circulating lymphocytes). Preventing effector lymphocytes from traveling to sites of inflammation might underlie the efficacy of **1** in prolonging allograft survival and in autoimmune disease models. Compound **1** has completed a phase-III human renal transplantation trial, and phase-III trials for relapsing-remitting multiple sclerosis (MS) are underway currently.

Compound **1** is a prodrug; it enters cells and invades the sphingolipid catabolic pathway, proceeding through cycles of phosphorylation and dephosphorylation.<sup>6</sup> *In vivo* phosphoryla-

**Chart 1.** Structures of **1** and **1-P** *S*-Phosphate, **2** Sphingosine and **2-P**, and **3** and its four stereoisomers



tion of **1** by Sphk2<sup>a</sup> produces the *S*-phosphate species **1-P** specifically,<sup>7</sup> which mimics much of S1P's (**2-P** in Chart 1 is an important extracellular lipid mediator) biological behavior. Compound **2-P** signals cells via five GPCRs: S1P<sub>1-5</sub> (formerly, EDG1, EDG5, EDG3, EDG6, and EDG8).<sup>8</sup> Compound **1-P** is an agonist at four of the five receptors: S1P<sub>1</sub>, S1P<sub>3</sub>, S1P<sub>4</sub>, and S1P<sub>5</sub>. The physiological activities of the S1P family of receptors are currently under intense investigation through both genetic and chemical biological approaches. Current evidence documents that *in vivo* activation of the S1P<sub>1</sub> receptor by agonists (e.g., **2-P** and **1-P**) induces lymphopenia, but the precise mechanism is disputed.<sup>9–11</sup> Thus, **1** occupies an interesting chemical space; it is a substrate for phosphorylation by Sphk2, avoids the phosphatase activities relative to **2-P**, and interacts with a subset of S1P receptors, including those necessary for lymphopenia.

An adverse event associated with **1-P** administration is bradycardia; in rodents and perhaps other mammalian species,

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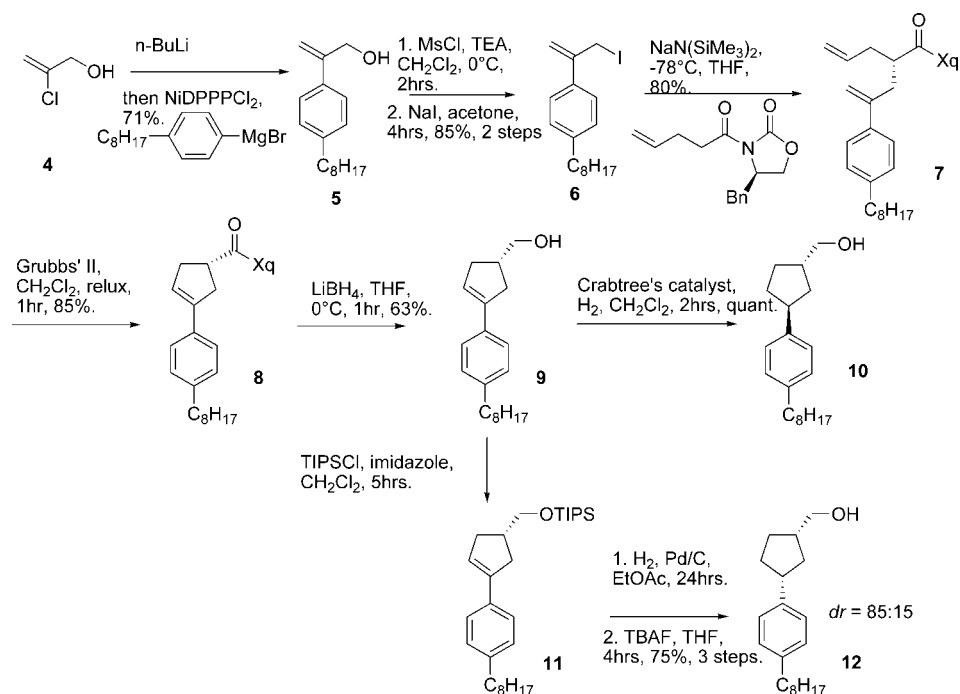
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<sup>a</sup> Abbreviations: S1P, sphingosine 1-phosphate; Sphk1, sphingosine 1-phosphate kinase type I; Sphk2, sphingosine 1-phosphate kinase type II; S1P<sub>n</sub>, sphingosine 1-phosphate receptor subtype *n* (*n* = 1–5); GPCR, G-protein coupled receptor; EDG, endothelial differentiation gene; SAR, structure–activity relationship; PPA, pyrophosphoric acid.

## Scheme 1



this response is mapped to the  $S1P_3$  receptor.<sup>12</sup> Activation of the  $S1P_3$  receptor is also suspected to cause pulmonary leakage.<sup>13</sup> Thus,  $S1P_3$  receptor activity may limit the usefulness of **1** as a therapeutic agent.

The study reported herein was undertaken in efforts to define further the molecular pharmacology of the  $S1P$  receptor family and **2-P**'s activating (Sphks) and inactivating (phosphatases) enzymes. Using **1** as a template, we sought to restrict the conformations of the three bonds that "link" the (hydroxymethyl)amino-substituted carbon "head group" and the octylphenyl "tail group" (see **1** in Chart 1). Our initial efforts focused on two-dimensional or planar "linker" groups to restrict conformations.<sup>14</sup> Subsequent studies have restricted the  $sp^3$  hybridized "linker" backbone of the parent molecule through ring formation. These studies led to the identification of **3** (VPC01091<sup>15</sup>), which evokes a profound, long lasting lymphopenia in rodents without a reduction in heart rate (data not shown). However, our lead compound **3** was a mixture of four stereoisomers. To elucidate the structure–activity relationships (SARs) of the stereoisomers, it was necessary to isolate and identify the active isomer(s) from the 4-component mixture (**3a–3d**). This work presents the activities of each of the four stereoisomers and selective asymmetric syntheses of the isomers active *in vivo*.

## Results

**Chemistry.** Asymmetric synthesis (Scheme 1) began with a modified Kumada cross-coupling of 2-chloropropenol **4** to (*p*-octyl)phenyl magnesium bromide to produce hydroxymethyl styrene **5**,<sup>16</sup> which was subsequently transformed into the corresponding iodide **6** in two steps. The C-1 chiral center was generated by alkylation of 4-pentenoic oxazolidinone using the Evans' chiral auxiliary technology.<sup>17</sup> Cyclization using a second-generation Grubbs' catalyst furnished the cyclopentene ring with the retention of stereochemistry at the hydroxymethyl-bearing carbon, and reductive removal of the chiral auxiliary gave the first key intermediate, 1-(4'-Octyl)phenyl-4(*S*)-hydromethyl cyclopent-1-ene (**9**).

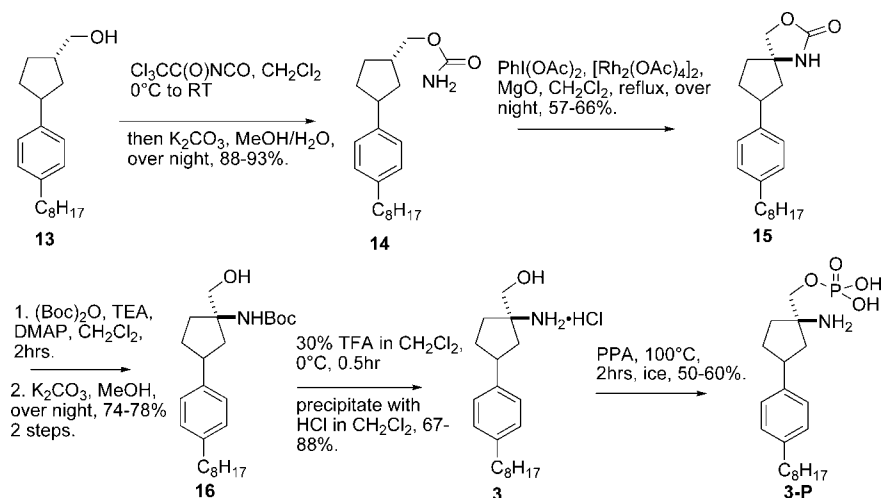
We intended to use the hydroxymethyl group to direct reduction selectively to each face of the cyclopentene, thereby inducing chirality at the phenyl-bearing carbon. The Crabtree catalyst efficiently hydrogenated the alkene from the same face of alcohol, producing the *S* stereochemistry of the octylphenyl group (see **10**). However, generation of the *R* stereochemistry was more challenging. Little stereoselectivity was obtained with most common heterogeneous catalysts, and many homogeneous catalysts were unreactive, for example, Pfaltz's catalyst.<sup>18</sup> Only after installation of the bulky TIPS group on the alcohol could hydrogenation on the opposite face of the hydroxymethyl group be effective with acceptable selectivity (see **9–12**).

The critical transformation in the syntheses was the rhodium-catalyzed C–H bond amination developed by Du Bois (Scheme 2).<sup>19</sup> After transforming the 1(*S*)-hydroxymethyl-3-octylphenyl alcohol **13** (**13** represents either **10** or **12**) to carbamate **14**, the carbamate amine inserted into the tertiary C–H bond on the cyclopentyl ring via a rhodium-mediated nitrene mechanism with retained stereochemistry to give oxazolidinone **15**. Introduction of the Boc group on the oxazolidinone nitrogen facilitated the hydrolysis of carbamate to the N-Boc diastereomers **16**.<sup>20</sup> After removal of the N-Boc group with trifluoroacetic acid, the final products **3a** and **3c** could be obtained as their hydrochloride salts. For *in vitro* receptor assays, we treated **3** with PPA to transform the alcohol into phosphates **3-P**. Ultimately, this was found to work equally well on both diastereomers **10** and **12**.

To confirm the validity of this synthesis, we compared the stereoselectively synthetic isomers with racemic **3** by chiral chromatography (Figure 1) and nuclear magnetic resonance (NMR). The asymmetrically synthesized **3a** and **3c** proved to be the first and third eluent, respectively.

**Biology.** We phosphorylated the four isomers of **3** obtained either by chiral chromatography or by our asymmetric synthetic routes. Because of the greater degree of purity of the synthetic material, we present the data obtained from that set of phosphorylated compounds. In a broken-cell assay of ligand efficacy and potency, both **3a-P** and **3c-P** proved to be partial

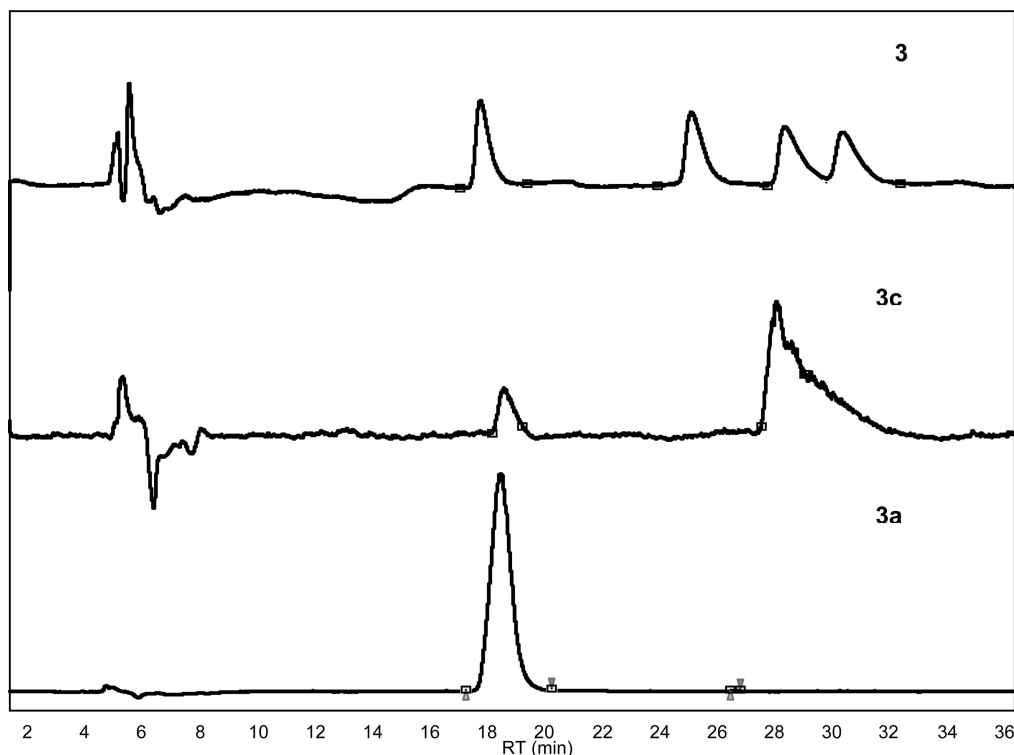
## Scheme 2



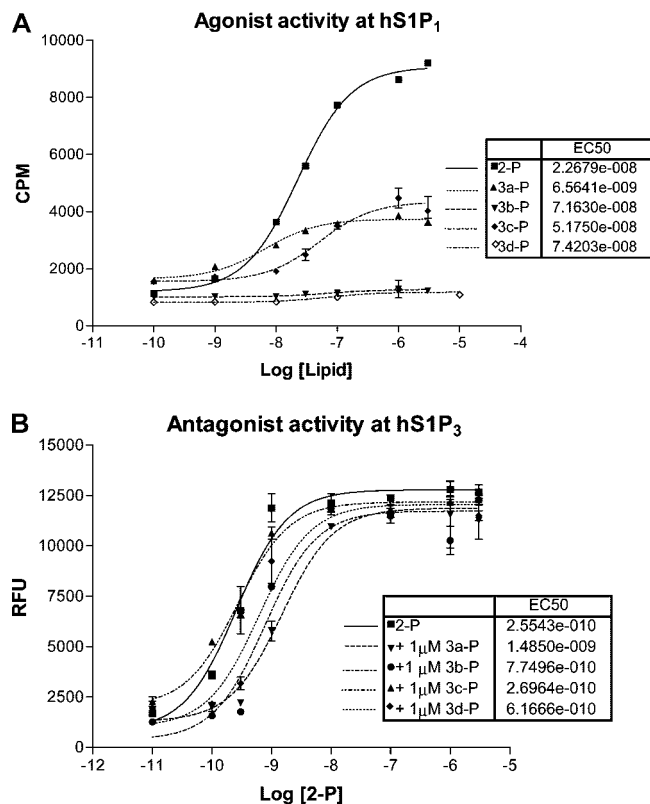
agonists at the SIP<sub>1</sub> receptor, with potency comparable to **2-P** (Figure 2a). Interestingly, their respective enantiomers, **3d-P** and **3b-P** exhibited minimal, if any, agonist activity at the SIP<sub>1</sub> receptor. Conversely, all four isomers lack agonist activity at the SIP<sub>3</sub> receptor. Because **3a-P** (derived from the parent alcohol-isolated chiral chromatography) showed inverse agonism (i.e., negative efficacy) at the SIP<sub>3</sub> receptor (not shown), we collided each of the phosphorylated synthetic isomers with **2-P** at the SIP<sub>3</sub> receptor. As documented in Figure 2b, **3a-P**, **3b-P**, and **3d-P** but not **3c-P** behaved as surmountable antagonists at the SIP<sub>3</sub> receptor. We did not test the individual phosphorylated synthetic isomers at the SIP<sub>2</sub>, SIP<sub>4</sub>, or SIP<sub>5</sub> receptors, but the racemate, **3-P**, is inactive at SIP<sub>2</sub>, a full agonist at SIP<sub>4</sub>, and a partial agonist at SIP<sub>5</sub> (not shown).

Compounds **3a** and **3c** but not **3b** or **3d** were substrates of recombinant Sphk2 (Figure 3). None of the isomers is either a substrate for or an inhibitor of Sphk1.

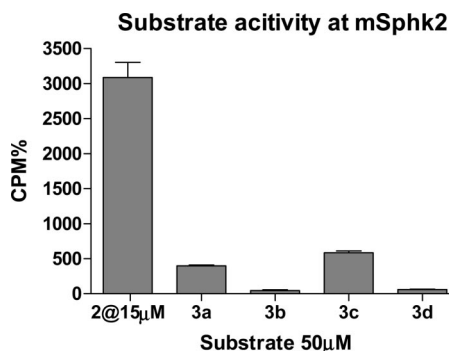
From reference to **1**, we expect that **3** is also a prodrug, i.e., requiring phosphorylation to achieve activity (indeed, **3** is not active in a Sphk2 null mouse<sup>22</sup>). To test the hypothesis that only the Sphk2 substrates **3a** and **3c** evoke lymphopenia, each of the isomers was introduced into mice by oral gavage. After a single dose (1 mg/kg body weight), both **3a** and **3c** evoked lymphopenia and maintained a low level of circulating lymphocytes for at least 48 h after dosing (Figure 4). Interestingly, 8 days after dosing, the mice receiving **3c** remained lymphopenic, while the blood lymphocyte counts in the **3a**-treated mice had returned to normal. As expected from the *in vitro*



**Figure 1.** Chiral chromatographic separation of racemic **3** (top), **3c** (middle), and **3a** (bottom) synthesized according to Schemes 1 and 2. Separation conditions: Chiralpak AD 4.6 mm i.d. × 250 mm at 35 °C, isocratic mobile phase of 95% A and 5% B at 0.8 mL/min, detection by UV absorption (254 nm). Mobile phase A was heptane with a 0.2% diethylamine modifier. Mobile phase B was 50:50 methanol/ethanol with a 0.2% diethylamine modifier.



**Figure 2.** *In vitro* evaluation of **3a-P–3d-P**. (a) Concentration effect curves of **2-P** and **3a-P–3d-P** at the recombinant human S1P<sub>1</sub> receptor. The parameter measured is membrane-bound GTP[γ-<sup>35</sup>S] (see methods for details).<sup>21</sup> (b) Concentration effect curve for **2-P** and **2-P** in the presence of 1 μM **3a-P–3d-P**. The parameter measured is calcium mobilization (see methods for details).<sup>21</sup> The images shown are representative of three independent experiments.

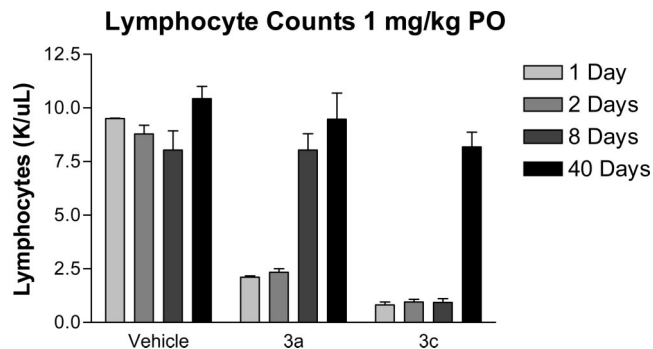


**Figure 3.** **2** and **3a–3d** were incubated with homogenates of HEK293T cells, wherein mouse Sphk2 expression had been forced.  $n = 2$  (see methods for details).<sup>22</sup>

activity profiles at both the kinase and the S1P<sub>1</sub> receptor, neither **3b** nor **3d** evoked lymphopenia (not shown).

## Discussion

Understanding the molecular pharmacology of the S1P receptors is evolving rapidly. Although an increasing number of compounds have been developed to study these receptors,<sup>14,23</sup> additional molecules of varying behavior will be necessary to fully elucidate the pharmacology of these new targets. Here, we report the asymmetric syntheses of two compounds that are S1P<sub>1</sub> agonists, but one is an S1P<sub>3</sub> antagonist; a profile heretofore unreported. Although both diastereomers are prodrugs, are analo-



**Figure 4.** Blood lymphocyte counts following administration of **3a** and **3c** to C57Bl/6j mice (female,  $n = 3$  per treatment group). Compounds were dissolved in vehicle (2% hydroxypropyl β-cyclodextrin) and administered once by oral gavage (1 mg/kg) on day 0. On the indicated days, mice were lightly anesthetized and blood was drawn from the orbital sinus. Lymphocyte counts were determined with a Hemavet model 950 blood analyzer.<sup>21</sup>

gous to **1**, and have similar kinase and receptor profiles, they exhibit very different duration *in vivo*. Thus, these compounds may have significant utility in further parsing out the roles that these receptors play in normal and pathologic physiology.

These compounds (**3a** and **3c**) were designed as conformationally restricted analogues of **1**, and analysis of the limited conformational space of each diastereomer is facilitating the definition of the binding site for the S1P<sub>1</sub> and S1P<sub>3</sub> receptors. The compounds also have three preferred properties of 1-like prodrugs: first, the potency and efficacy at S1P<sub>1</sub> is retained; second, the new analogues are substrates for either Sphk1 or Sphk2 (or possibly other lipid kinases); and third, the activity at the S1P<sub>3</sub> receptor is minimized.

Because of their highly hydrophobic structures, it was challenging to isolate the four isomers in quantities sufficient for broad biological evaluation. Milligram scale pure isomers could be isolated through high-performance liquid chromatography (HPLC) on chiral columns, and a preliminary assay of these four isomers suggested that **3a** and **3c** are the two active species *in vivo* (named in order of the chiral HPLC eluting sequence). These two compounds proved to be diastereomers (at the C-3 position) instead of enantiomers (as assessed by optical rotation and NMR spectroscopy).

To establish definitively the stereochemistry of **3a** and **3c**, we chemically synthesized the two isomers through a short sequence involving an intramolecular nitrene C–H insertion. It was suspected that the active species would have the *R* stereocenter at the quaternary amino-bearing carbon,<sup>7,14a,24</sup> and thus, our synthesis was designed with that stereochemical outcome anticipated. The stereochemical configuration of the benzylic carbon remained undefined until this work was done. Using several established asymmetric synthesis methods, we constructed the two stereocenters predicted for **3a** with high selectivity and **3c** with >85% optical purity (with ~15% **3a**).

The pharmacological profiles of the isomers are different in important ways, particularly *in vivo*. As determined using the GTP[γ-<sup>35</sup>S] binding assay, compound **3c** was slightly more efficacious than **3a**. The greater efficacy of **3c** is perhaps reflected in the lower lymphocyte counts in **3c**- versus **3a**-treated mice at 24 and 48 h. However, **3c** is also a somewhat better substrate at mouse Sphk2; this may further enhance the lymphopenic effect of **3c**. In contrast, there was no indication from *in vitro* analysis that the **3a/3c** enantiomers would differ greatly in their duration of action. It will be interesting to learn

whether the pharmacokinetic profile of these compounds parallels this pharmacodynamic property.

Perhaps the most salient property of compound **3a-P** is the antagonism at the S1P<sub>3</sub> receptor. The lead compound in this class, **1**, is a potent S1P<sub>3</sub> agonist. In rodents, agonist activity at the S1P<sub>3</sub> receptor is associated with toxicities.<sup>12,13</sup> If this is true in humans also, the more selective **3**-type compounds might be superior because of reduced toxicity. Finally, **3a-P** is 1 log order more potent than previously reported S1P<sub>3</sub> antagonists.<sup>25</sup> However, we do not know at present whether antagonism at the S1P<sub>3</sub> receptor would have any therapeutic benefit.

## Conclusion

We asymmetrically synthesized all of the four stereoisomers of a 4-component immunomodulatory S1P prodrug. Two of the isomers, **3a** and **3c**, are substrates for Sphk2 and, following phosphorylation, are potent partial agonists at the S1P<sub>1</sub> receptor. Further, one isomer is the most potent S1P<sub>3</sub> antagonist reported to date. The unique biological properties of **3** make it a useful chemical tool for the study of S1P signaling.

## Experimental Section

**General Procedure.** All chemicals and solvents were purchased from Aldrich or Fisher unless specified; anhydrous solvents and amine bases were used without further distillation. All reactions were performed under inert gas protection in oven-dried reaction vessels unless specified. Analytical thin-layer chromatography (TLC) was carried out on a Merck silica gel 60F<sub>254</sub> aluminum-based plate, and preparative TLC separation was performed on Analtech 02013 (1000 μm) or a similar plate with a varied thickness of coating. UV absorption was detected at 254 nm. All NMR raw data were collected on a Varian Mercury Plus or Unity Inova 300 MHz (75 MHz carbon) and processed by MestReC 4.9.9.6 with the default setting. Optical rotation was obtained on a Perkin-Elmer 343 plus polarimeter. Low-resolution mass spectrometry (MS) [electrospray ionization (ESI)] were recorded on ThermoFinnigan LCQ, and high-resolution MS (ESI-TOF) analysis was performed by the University of Illinois MS laboratory. Elemental analysis was performed by the Atlantic Micro Laboratory. Chiral HPLC separations were obtained from a Chiralpak AD 4.6 mm i.d. × 250 mm column using isocratic conditions with 95% A and 5% B at a flow rate of 0.8 mL/min and monitoring at a wavelength of 254 nm or with an evaporative light scattering detector (ELSD). The column was maintained at 35 °C using a column heater. Mobile phase A was heptane with a 0.2% diethylamine modifier. Mobile phase B was 50:50 methanol/ethanol with a 0.2% diethylamine modifier.

**Chemistry. 2-(4-Octylphenyl)prop-2-en-1-ol (5).** In a 250 mL round-bottom flask, 24 mmol (0.576 g) of magnesium turnings and 20 mmol (5.36 g) of *p*-octylbromobenzene (TCI Chemicals) in 80 mL of dry tetrahydrofuran (THF) were refluxed for 1 h. In another 100 mL flask, 16 mmol (1.472 g) of 2-chloro-2-propen-1-ol (**4**, 90% tech grade) in 40 mL of dry THF was cooled to 0 °C and 17 mmol (6.8 mL) of 2.5 M *n*-butyl lithium (in hexane) was added dropwise. After 10 min, the deprotonated chloride was syringed into the flask containing the Grignard reagent. After the addition of 1 mmol (0.542 g) NiDPPPCl<sub>2</sub>, the reaction mixture was refluxed for 3 h. After removal of the solvent, the mixture was partitioned between half-saturated NH<sub>4</sub>Cl solution (25 mL) and CH<sub>2</sub>Cl<sub>2</sub> (25 mL × 2). The combined organic solution was dried over NaSO<sub>4</sub>, concentrated, and columned with CH<sub>2</sub>Cl<sub>2</sub> (*R*<sub>f</sub> = 0.25). A total of 2.51 g (10.2 mmol, 71%) of white solid was collected. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ: 7.50–7.08 (m, 4H), 5.46 (s, 1H), 5.31 (m, 1H), 4.54 (d, *J* = 5.66 Hz, 1H), 2.62 (app t, *J* = 8.00 Hz 2H), 1.61 (m, 1H), 1.31 (m, 10H), 0.90 (t, *J* = 6.69 Hz, 3H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ: 147.3, 143.2, 135.9, 128.8, 126.1, 112.1, 65.3, 35.9, 32.2, 31.7, 29.8, 29.6, 29.5, 22.9, 14.4.

**1-(3-Iodoprop-1-en-2-yl)-4-octylbenzene (6).** A total of 9.55 mmol (2.35 g) of **5** in 40 mL of dry CH<sub>2</sub>Cl<sub>2</sub> was reacted with 10

mmol (1.34 g) of MsCl and 11 mmol (1.11 g) of TEA at 0 °C for 1 h. After removal of the solvent and excess reagents, the mixture was suspended in 30 mL of acetone and filtered through a Celite pad. After another two washes, 10 mmol (1.50 g) of NaI was added to the combined filtrate and stirred overnight. Removal of the solvent was followed by hexane washes (30 mL × 3). When the filtrate was concentrated, 3.00 g (85%) of light brown oil was collected and used for the next step without further purification. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ: 7.52–7.20 (m, 4H), 5.50 (s, 1H), 5.48 (s, 1H), 4.33 (s, 2H), 2.63 (app t, *J* = 7.52 Hz 2H), 1.64 (m, 2H), 1.36 (m, 10H), 0.90 (t, *J* = 6.71 Hz, 3H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ: 145.5, 143.5, 135.2, 128.8, 126.1, 115.1, 35.9, 32.1, 31.6, 29.7, 29.6, 29.5, 22.9, 14.4, 7.6.

**(R)-4-Benzyl-3-((S)-2-(2-(4-octylphenyl)allyl)pent-4-enoyl)-oxazolidin-2-one (7).** A total of 9 mmol (2.33 g) of (*R*)-4-benzyl-3-pent-4-enoyloxazolidin-2-one in 60 mL of dry THF was cooled to –78 °C, and 9 mmol (9 mL) of 1 N NaHMDS (in THF) was added slowly. After 30 min, 9 mmol (3.18 g) of **6** was added dropwisely through a syringe. The reaction was maintained at –78 °C for 1.5 h, then warmed up to 0 °C, and quenched with half-saturated NH<sub>4</sub>Cl solution. After removal of THF, the residue was partitioned between NH<sub>4</sub>Cl solution (25 mL) and CH<sub>2</sub>Cl<sub>2</sub> (25 mL × 2). The combined organic layer was dried over NaSO<sub>4</sub> and concentrated. Flash column eluting with 10% EtOAc in hexanes (*R*<sub>f</sub> = 0.35) gave 3.5 g (7.2 mmol, 80%) of clear oil. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ: 7.50–6.97 (m, 9H), 5.91–5.62 (m, 1H), 5.33 (d, *J* = 1.04 Hz, 1H), 5.20–4.92 (m, 3H), 4.61 (m, 1H), 4.13 (m, 3H), 3.25–2.90 (m, 2H), 2.73 (m, 1H), 2.57 (m, 3H), 2.41 (m, 1H), 2.33 (m, 1H), 1.58 (m, 1H), 1.27 (s, 10H), 0.89 (t, *J* = 6.69 Hz, 3H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ: 175.6, 153.2, 145.9, 142.7, 138.2, 135.7, 135.4, 129.6, 129.1, 128.6, 127.5, 126.4, 117.5, 114.0, 66.1, 55.6, 41.7, 38.1, 37.2, 37.0, 35.8, 32.1, 31.6, 29.7, 29.6, 29.5, 22.9, 14.3.

**(R)-4-Benzyl-3-((S)-3-(4-octylphenyl)cyclopent-3-enecarbonyl)oxazolidin-2-one (8).** A total of 3.5 g (11.2 mmol) of **7** was refluxed in 40 mL of CH<sub>2</sub>Cl<sub>2</sub> with 20 mg of second-generation Grubbs catalyst for 2 h. Flash column with 10% EtOAc in hexanes (*R*<sub>f</sub> = 0.3) gave 2.70 g (9.5 mmol, 85%) of clear oil. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ: 7.55–6.93 (m, 9H), 6.08 (m, 1H), 4.72 (m, 1H), 4.41 (m, 1H), 4.33–4.10 (m, 2H), 3.32 (dd, *J* = 13.35, 3.12 Hz, 1H), 3.23 (m, 1H), 3.16–3.03 (m, 1H), 2.98–2.89 (m, 2H), 2.82 (dd, *J* = 13.34, 9.61 Hz, 1H), 2.62 (t, *J* = 7.66 Hz, 2H), 1.62 (m, 2H), 1.32 (m, 10H), 0.91 (t, *J* = 6.67 Hz, 3H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ: 175.7, 153.4, 142.4, 140.4, 135.6, 133.5, 129.7, 129.2, 128.6, 127.6, 125.8, 122.8, 66.4, 55.7, 41.8, 38.1, 37.3, 36.6, 35.9, 32.2, 31.7, 29.8, 29.6, 29.5, 22.9, 14.4.

**(S)-3-(4-Octylphenyl)cyclopent-3-enylmethanol (9).** A total of 3.4 mmol (1.55 g) of **8** and 3.5 mmol (0.11 g) of MeOH were dissolved in 40 mL of THF, and 7 mmol (3.5 mL) of 2 M LiBH<sub>4</sub> in THF was added at 0 °C. After 1 h of the reaction at 0 °C, the mixture was concentrated and partitioned between water and CH<sub>2</sub>Cl<sub>2</sub> (25 mL/25 mL × 2). The combined organic layer was dried over NaSO<sub>4</sub> and concentrated. Flash column eluting with CH<sub>2</sub>Cl<sub>2</sub> (*R*<sub>f</sub> = 0.2) gave 0.61 g (2.1 mmol, 63%) of white flakes (recrystallized from hexanes). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ: 7.36 (d, *J* = 8.13 Hz, 2H), 7.14 (d, *J* = 8.13 Hz, 2H), 6.17–5.99 (m, 1H), 3.64 (d, *J* = 6.63 Hz, 2H), 2.96–2.81 (m, 1H), 2.69 (m, 2H), 2.63–2.57 (m, 2H), 2.52 (m, 1H), 2.32 (m, 1H), 1.62 (m, 3H), 1.43–1.17 (m, 10H), 0.90 (t, *J* = 6.71 Hz, 3H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ: 142.1, 141.3, 134.1, 128.6, 125.6, 123.8, 67.5, 39.9, 36.5, 35.9, 32.1, 31.7, 29.7, 29.6, 29.5, 22.9, 14.4.

**(1S,3S)-3-(4-Octylphenyl)cyclopentylmethanol (10).** A total of 0.20 g of **9** and 5 mg of Crabtree catalyst (Strem) were dissolved in 50 mL of dry CH<sub>2</sub>Cl<sub>2</sub> in an oven-dried 100 mL flask under nitrogen protection. The mixture reacted under a hydrogen balloon for 1 day. After removal of the solvent, the product was used for the next step without purification. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ: 7.15 (q, *J* = 8.17 Hz, 4H), 3.58 (d, *J* = 7.02 Hz, 2H), 3.17–2.98 (m, 1H), 2.66–2.52 (app t, *J* = 7.55 Hz, 2H), 2.48–2.30 (m, 1H), 2.21–1.54 (m, 8H), 1.47–1.19 (m, 10H), 0.91 (t, *J* = 6.66 Hz, 3H).

$^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$ : 143.2, 140.7, 128.5, 127.1, 67.7, 44.5, 41.5, 37.1, 35.8, 35.0, 32.2, 31.9, 29.8, 29.7, 29.6, 29.6, 23.0, 14.4.

**((1*S*,3*R*)-3-(4-Octylphenyl)cyclopentyl)methanol (12).** A total of 1.33 mmol (0.380 g) of **9**, 1.6 mmol (0.307 g) of  $\text{TIPSCl}$ , and 2.0 mmol (0.136 g) of imidazole were dissolved in 15 mL of  $\text{CH}_2\text{Cl}_2$  and reacted overnight. Upon removal of the solvent, the mixture was suspended in 20 mL of hexane and filtered through a Celite pad. The filtrate was concentrated and redissolved in 50 mL of EtOAc; this solution was hydrogenated under a  $\text{H}_2$  balloon overnight with Pd/C (10%, wt). When strong UV absorption disappeared on TLC, the Pd/C was filtered off followed by removal of EtOAc. The mixture was deprotected with 4 mmol (1.046 g) of TBAF in 40 mL of THF overnight. After removal of most of the solvent, the mixture was separated on a 1000  $\mu\text{m}$  preparative TLC (or a small column) with  $\text{CH}_2\text{Cl}_2$  ( $R_f = 0.2$ ). A total of 0.285 g (9.9 mmol, 75%) of clear oil was collected.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$ : 7.24–7.08 (m, 4H), 3.61 (dd,  $J = 6.58, 1.51$  Hz, 2H), 3.06 (m, 1H), 2.59 (app t,  $J = 7.58$  Hz, 3H), 2.41–1.81 (m, 6H), 1.75–1.51 (m, 4H), 1.45–1.21 (m, 10H), 0.91 (t,  $J = 6.85$  Hz, 3H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$ : 142.8, 140.7, 128.5, 127.1, 67.7, 45.8, 42.2, 38.5, 35.8, 33.6, 32.2, 31.9, 29.8, 29.7, 29.6, 28.6, 23.0, 14.4.

**((1*S*,3*S*)-3-(4-Octylphenyl)cyclopentyl)methyl Carbamate (14a).** A total of 0.42 mmol (0.120 g) of **10** was dissolved in 15 mL of dry  $\text{CH}_2\text{Cl}_2$ , and 0.50 mmol (0.094 g) of  $\text{Cl}_3\text{CC}(\text{O})\text{NCO}$  was added at 0 °C. After about 0.5–1 h (track by TLC in  $\text{CH}_2\text{Cl}_2$ ), the solvent was removed and the mixture was redissolved in 10 mL of MeOH and 1 mL of  $\text{H}_2\text{O}$ . A total of 0.5 g of  $\text{K}_2\text{CO}_3$  was added and reacted for 5 h. Upon removal of most solvents, the residue was loaded on preparative TLC and developed in  $\text{CH}_2\text{Cl}_2$  (just above the starting point, weak UV absorption). A total of 0.130 g (3.9 mmol, 93%) of white solid was collected.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$ : 7.22–7.04 (m, 4H), 5.05 (b, 2H), 4.08–3.96 (m, 2H), 3.18–3.00 (m, 1H), 2.59 (app t,  $J = 7.76$  Hz, 2H), 2.54–2.42 (m, 1H), 2.19–1.54 (m, 8H), 1.48–1.16 (m, 10H), 0.91 (t,  $J = 7.47$  Hz, 3H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$ : 157.8, 143.0, 140.7, 128.6, 127.1, 69.4, 44.3, 38.2, 37.3, 35.8, 34.9, 32.2, 31.9, 29.8, 29.7, 29.6, 23.0, 14.4.

**((1*S*,3*R*)-3-(4-Octylphenyl)cyclopentyl)methyl Carbamate (14c).** This compound was prepared in a manner similar to **14a** (88%).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$ : 7.13 (q,  $J = 8.20$  Hz, 4H), 4.87 (b, 1H), 4.03 (m, 2H), 3.14–2.96 (m, 1H), 2.58 (app t,  $J = 7.75$  Hz, 2H), 2.40 (m, 1H), 2.24 (m, 1H), 2.15–2.02 (m, 1H), 1.98–1.51 (m, 6H), 1.46–1.15 (m, 10H), 0.89 (t,  $J = 6.63$  Hz, 3H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$ : 157.6, 142.6, 140.8, 128.5, 127.1, 69.5, 45.7, 38.8, 38.6, 35.8, 33.5, 32.2, 31.9, 29.8, 29.7, 29.5, 28.7, 22.9, 14.4.

**15a.** A total of 0.78 mmol (0.26 g) of **14a**, 1.10 mmol (0.354 g) of BAIB, 1.79 mmol (0.072 g) of  $\text{MgO}$ , and 0.039 mmol (0.017 g) of  $[\text{Rh}(\text{CH}_3\text{COO})_2]_2 \cdot 2\text{H}_2\text{O}$  were refluxed in 10 mL of  $\text{CH}_2\text{Cl}_2$  for 14 h. The reaction mixture was TLC-separated in acetone/ $\text{CHCl}_3$  (5:95, v/v,  $R_f = 0.3$ , weak UV). A total of 0.14 g (0.44 mmol, 57%) of white solid was collected.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$ : 7.13 (m, 1H), 6.64 (s, 1H), 4.29 (dd,  $J = 17.60, 8.40$  Hz, 2H), 3.01 (m, 1H), 2.62–2.47 (app t,  $J = 7.75$  Hz, 2H), 2.29 (dd,  $J = 13.23, 6.98$  Hz, 1H), 2.20–2.03 (m, 3H), 2.03–1.73 (m, 3H), 1.59 (m, 2H), 1.40–1.16 (m, 10H), 0.88 (t,  $J = 6.70$  Hz, 3H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$ : 159.6, 141.4, 140.8, 128.8, 126., 65.0, 47.2, 43.3, 38.7, 35.8, 32.4, 32.1, 31.8, 29.7, 29.6, 29.5, 22.9, 14.4.

**15c.** This compound was prepared in a manner similar to **15a** (66%).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$ : 7.19–7.06 (m, 4H), 4.32 (q,  $J = 8.50$  Hz, 2H), 3.38–3.15 (m, 0.86H), 3.11–2.92 (m, 0.14H), 2.63–2.50 (app t,  $J = 7.75$  Hz, 2H), 2.38 (dd,  $J = 13.51, 7.49$  Hz, 1H), 2.31–1.94 (m, 4H), 1.88–1.65 (m, 2H), 1.59 (m, 2H), 1.28 (m, 10H), 0.88 (t,  $J = 6.62$  Hz, 3H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$ : 159.9, 141.3, 141.2, 128.7, 126.9, 65.4, 47.3, 43.5, 39.7, 35.8, 32.6, 32.1, 31.8, 29.7, 29.6, 29.5, 22.9, 14.4.

**Tert-butyl (1*R*,3*S*)-1-(Hydroxymethyl)-3-(4-octylphenyl)cyclopentylcarbamate (16a).** A total of 0.14 mmol (46 mg) of **15a**, 0.2 mmol (44 mg) of  $\text{Boc}_2\text{O}$ , 0.3 mmol (30 mg) of triethylamine, and a catalytic amount of DMAP were reacted in 5 mL of  $\text{CH}_2\text{Cl}_2$  for 4 h. After removal of the solvent, the mixture was redissolved in

5 mL of MeOH and 2 drops of water and 0.1 g of  $\text{K}_2\text{CO}_3$  was added and reacted overnight. The reaction mixture was then TLC-purified in acetone/ $\text{CHCl}_3$  (5:95, v/v,  $R_f = 0.3$ , weak UV). A total of 42 mg (0.1 mmol, 74%) of product was collected.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$ : 7.18–7.07 (m, 4H), 4.93 (s, 1H), 3.72 (q,  $J = 11.27$  Hz, 1H), 3.08 (m, 1H), 2.57 (app t,  $J = 7.75$  Hz, 1H), 2.46 (dd,  $J = 13.29, 7.55$  Hz, 1H), 2.16–2.03 (m, 1H), 2.00–1.80 (m, 3H), 1.73 (dd,  $J = 13.16, 11.13$  Hz, 1H), 1.59 (m, 2H), 1.45 (s, 9H), 1.38–1.18 (m, 10H), 0.88 (t,  $J = 6.74$  Hz, 3H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$ : 156.6, 141.8, 141.0, 128.6, 127.1, 80.2, 69.4, 65.0, 44.4, 44.0, 43.3, 35.9, 35.8, 32.9, 32.1, 31.8, 29.7, 29.6, 29.5, 22.9, 14.4.

**Tert-butyl (1*R*,3*R*)-1-(Hydroxymethyl)-3-(4-octylphenyl)cyclopentylcarbamate (16c).** This compound was prepared in a manner similar to **16a** (78%).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$ : 7.13 (m, 4H), 4.89 (s, 1H), 3.73 (dd,  $J = 23.07, 11.08$  Hz, 2H), 3.30 (m, 0.86H), 3.13–3.01 (m, trace amount), 2.57 (app t,  $J = 7.77$  Hz, 1H), 2.29 (m, 1H), 2.23–2.03 (m, 2H), 1.78 (m, 3H), 1.58 (m, 2H), 1.53–1.38 (m, 9H), 1.28 (m, 10H), 0.88 (t,  $J = 6.69$  Hz, 3H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$ : 156.5, 141.7, 141.0, 128.6, 127.1, 80.1, 69.9, 65.1, 43.8, 39.7, 36.4, 35.8, 33.3, 32.1, 31.8, 29.7, 29.6, 29.5, 28.7, 22.9, 14.4.

**((1*R*,3*S*)-1-Amino-3-(4-octylphenyl)cyclopentyl)methanol Hydrochloride (3a).** A total of 100 mg (0.25 mmol) of **16a** was stirred in 5 mL of  $\text{CH}_2\text{Cl}_2$  and 1.5 mL of TFA at 0 °C for 1 h; after quenching with 5 mL of methanol and evaporation of excess reagents, the product was partitioned between  $\text{CH}_2\text{Cl}_2$  and 1 N NaOH (10 mL/10 mL  $\times$  3). The combined organic layer was dried over  $\text{Na}_2\text{SO}_4$  and concentrated to 15 mL; this solution was treated with 1 drop of concentrated hydrochloric acid, and a fine needle product precipitated within minutes. A total of 73 mg (0.22 mmol, 88%) of HCl salt was collected. NMR, elemental analysis, and rotation was performed on the free amine form of the product.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$ : 7.14 (dd,  $J = 17.60, 8.04$  Hz, 4H), 3.46 (m, 2H), 3.06 (m, 1H), 2.65–2.49 (m, 2H), 2.28 (m, 4H), 2.14–2.00 (m, 1H), 1.90 (m, 1H), 1.70 (m, 2H), 1.65–1.55 (m, 2H), 1.50 (m, 1H), 1.29 (m, 10H), 0.88 (t,  $J = 6.33$  Hz, 3H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$ : 142.2, 140.9, 128.6, 127.0, 70.2, 62.3, 46.5, 44.8, 38.6, 35.8, 33.3, 32.1, 31.8, 29.7, 29.5, 22.9, 14.4. HRMS ( $\text{ESI}^+$ ) ( $m/z$ ): calcd for  $\text{C}_{20}\text{H}_{34}\text{NO}$  ( $\text{MH}^+$ ), 304.2640; found, 304.2641. Anal. Calcd for  $\text{C}_{20}\text{H}_{33}\text{NO}$ : C, 79.15; H, 10.96; N, 4.62. Found: C, 78.86; H, 11.01; N, 4.60.  $[\alpha]_D^{20} = -2.8$  (c 1.05,  $\text{CHCl}_3$ ).

**((1*R*,3*R*)-1-Amino-3-(4-octylphenyl)cyclopentyl)methanol Hydrochloride (3c).** This compound was prepared in a manner similar to **3a** (67%).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$ : 7.13 (m, 4H), 3.46 (s, 2H), 3.40–3.21 (m, 0.85H), 3.09–2.97 (m, 0.15H), 2.54 (m, 2H), 2.11 (m, 4H), 2.04–1.83 (m, 2H), 1.78–1.64 (m, 2H), 1.58 (m, 4H), 1.27 (m, 10H), 0.88 (t,  $J = 8.27$  Hz, 3H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$ : 142.2, 141.0, 128.6, 127.0, 70.9, 62.4, 46.9, 43.8, 37.9, 35.8, 33.7, 32.1, 31.8, 29.7, 29.6, 29.5, 22.9, 14.4. HRMS ( $\text{ESI}^+$ ) ( $m/z$ ): calcd for  $\text{C}_{20}\text{H}_{34}\text{NO}$  ( $\text{MH}^+$ ), 304.2640; found, 304.2641.

**((1*R*,3*S*)-1-Amino-3-(4-octylphenyl)cyclopentyl)methyl Dihydrogen Phosphate (3a-P).** A total of 10.0 mg (0.03 mmol) of **3a** was vigorously stirred in  $\sim$ 2 mL of freshly prepared pyrophosphoric acid (PPA) at 100 °C for 2 h. Upon cooling,  $\sim$ 9 mL of ice was added to the reaction mixture and the product precipitated as a white solid. The product was collected on a Buchner funnel, further washed by water (8 mL  $\times$  2), and then vacuum-dried. A total of 6.3 mg (0.016 mmol, 56%) of white solid was collected.  $^1\text{H}$  NMR (300 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$ : 7.27–7.12 (m, 4H), 4.04–3.86 (app q,  $J = 5.62$  Hz, 1H), 2.94–2.72 (b, 2H), 2.66–2.57 (m, 2H), 2.57–2.48 (m, 1H), 2.23–2.13 (m, 1H), 2.13–1.89 (m, 2H), 1.82–1.69 (m, 1H), 1.68–1.55 (m, 2H), 1.33 (m, 10H), 0.93 (t,  $J = 6.60$  Hz, 1H). HRMS ( $\text{ESI}^+$ ) ( $m/z$ ): calcd for  $\text{C}_{20}\text{H}_{35}\text{NO}_4\text{P}$  ( $\text{MH}^+$ ), 384.2304; found, 384.2305.

**((1*R*,3*R*)-1-Amino-3-(4-octylphenyl)cyclopentyl)methyl Dihydrogen Phosphate (3c-P).** A total of 10.0 mg (0.03 mmol) of **3c** was treated similarly to PPA as **3a**. After hydrolysis, the gummy product was briefly centrifuged; after removal of phosphoric acid, the product was further washed and centrifuged in water (8 mL  $\times$  2). A total of 5.5 mg (0.014 mmol, 50%) of white solid was

collected.  $^1\text{H NMR}$  (300 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$ : 7.18 (m, 4H), 4.21–4.07 (m, 1H), 4.05–3.92 (m, 1H), 2.60 (app t,  $J = 7.65$  Hz, 2H), 2.39–2.14 (m, 3H), 2.07–1.75 (m, 3H), 1.62 (m, 2H), 1.43–1.26 (m, 10H), 0.93 (t,  $J = 6.65$  Hz, 3H). HRMS (ESI $^+$ ) ( $m/z$ ): calcd for  $\text{C}_{20}\text{H}_{35}\text{NO}_4\text{P}$  ( $\text{MH}^+$ ), 384.2304; found, 384.2311.

**Biology. [ $\gamma$ - $^{35}\text{S}$ ]GTP Binding Assay.**<sup>21</sup> A plasmid encoding the human  $\text{SIP}_1$  receptor was introduced into CHO K1 cells by DNA-mediated transfection, and a clonal line expressing high levels of the receptor was isolated. Crude membrane fractions from these cells containing ca. 5  $\mu\text{g}$  of protein were incubated in 0.1 mL of binding buffer [50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 100 mM NaCl, and 10 mM  $\text{MgCl}_2$  at pH 7.5] containing 5  $\mu\text{g}$  of saponin, 0.10 mM GDP, 0.1 nM [ $\gamma$ - $^{35}\text{S}$ ]GTP (1200 Ci/mmol), and the indicated compounds for 30 min at 30  $^\circ\text{C}$ . Membranes were collected on GF/C filters using a Brandell Cell Harvester (Gaithersburg, MD), and samples were analyzed for bound radionuclide using a TopCount  $\beta$  scintillation counter. Each of the eight points on a concentration effect curve were determined in triplicate and are presented as standard errors of the mean.

**Calcium Mobilization.**<sup>21</sup> A plasmid encoding the human  $\text{SIP}_3$  receptor containing an amino-terminal HA epitope tag was introduced into CHO K1 cells by DNA-mediated transfection, and a clonal line expressing high levels of the receptor was isolated using a fluorescence-activated cell sorter. Cells were plated on a clear bottom, black wall 96-well plate; the cell monolayers were grown to confluence, washed, and loaded with a calcium-sensitive fluorescent dye; and calcium transients in response to compound addition were monitored using a Molecular Devices FLEXStation. Each of the eight points on a concentration effect curve were determined in triplicate and are presented as standard errors of the mean.

**Sphk Assay.**<sup>22</sup> Mouse Sphk2 was expressed by the introduction of plasmid DNA into HEK293T cells. Sphk2 activity was measured in a solution that consisted of 20 mM Tris-Cl (pH 7.4), 1 mM 2-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM sodium orthovanadate, 40 mM  $\beta$ -glycerophosphate, 15 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 mM  $\text{MgCl}_2$ , 0.5 mM 4-deoxyribose, 10% glycerol, and 0.01 mg/mL each of leupeptin, aprotinin, and soybean trypsin inhibitor. To determine the fractional activity of Sphk1 versus Sphk2, the buffer was supplemented with either 0.5% Triton X-100 or 1 M KCl, respectively. The buffer was supplemented with substrate (*D*-erythro-sphingosine, 15  $\mu\text{M}$ ; **3a–3d**, 50  $\mu\text{M}$ ), [ $\gamma$ - $^{32}\text{P}$ ]ATP (10  $\mu\text{M}$ , specific activity = 8.3 Ci/mmol), and cell extract (0.02–0.03 mg of total protein). After 30 min at 37  $^\circ\text{C}$ , the reaction mixture was extracted with 2 volumes of chloroform/methanol/HCl (100:200:1), and the components in the organic phase were separated by normal-phase TLC using a 1-butanol/acetic acid/water (3:1:1) solvent system. Radiolabeled enzyme products were detected by autoradiography and identified by migration, relative to authentic standards. For quantification, the silica gel containing radiolabeled lipid was scraped into a scintillation vial and counted.

**Lymphopenia Assay.** Mice were dosed by oral gavage with isomers of **3** (1 mg/kg), dissolved in 2% hydroxypropyl  $\beta$ -cyclodextrin. Peripheral blood was collected from the orbital sinus of lightly anesthetized mice, and total lymphocytes were counted using a Hemavet 950 blood analyzer (Drew Scientific, Oxford, CT).

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**Supporting Information Available:** Proton and carbon NMR of new compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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