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Brief Article

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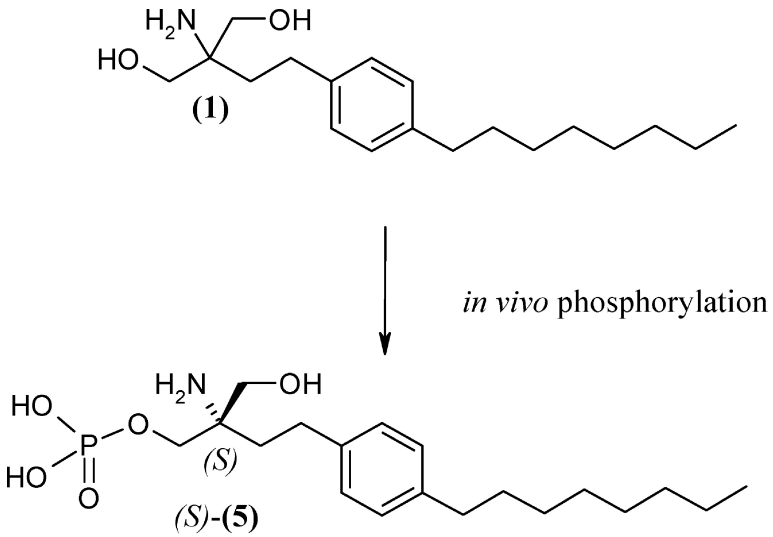

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Novel Immunomodulator FTY720 Is Phosphorylated in Rats and Humans To Form a Single Stereoisomer. Identification, Chemical Proof, and Biological Characterization of the Biologically Active Species and Its Enantiomer

Rainer Albert,* Klaus Hinterding, Volker Brinkmann, Danilo Guerini, Constanze Müller-Hartwieg, Helmut Knecht, Corinne Simeon, Markus Streiff, Trixie Wagner, Karl Welzenbach, Frédéric Zécri, Markus Zollinger, Nigel Cooke, and Eric Francotte

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In vivo phosphorylation of FTY720 (**1**) in rats and humans resulted exclusively in the biologically active (*S*)-configured enantiomer, which was proven by an ex vivo *o*-phthaldialdehyde derivatization protocol especially elaborated for phosphates of **1**. Starting from the prochiral amino alcohol **1**, racemic and enantiomerically pure phosphates of **1** were synthesized. Pure enantiomers were obtained after purification of a partially protected key intermediate on an enantioselective support. The absolute stereochemistry was determined by X-ray diffraction.

Introduction

FTY720 (**1**) is a novel immunomodulator that is highly effective in animal models of transplantation and autoimmunity.[1] The drug has recently been shown to be active in preventing kidney allograft rejection in humans (phase III clinical trials) and may represent a novel modality for immunosuppressive therapy.[2] Unlike other agents used in transplantation to prevent acute rejection, **1** does not inhibit proliferation or activation of lymphocytes. Instead, it leads to their sequestration from the periphery into secondary lymphoid organs and inhibits recirculation of effector T cells to the transplanted graft, thereby preventing its rejection.[3]

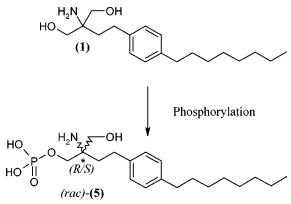
The amino alcohol **1** is phosphorylated in vitro by sphingosine kinases,[4] and only the phosphorylated molecule (*S*)-**5**, signals as an agonist through four of five S1P receptors (formerly known as EDG receptors),[5,6] whereas the second enantiomer (*R*)-**5** shows weak or no agonistic activity on these receptors. It has been convincingly demonstrated that S1P 1-receptor agonism is responsible for the observed pharmacological action of the drug.[7] While **1** itself is prochiral (Scheme 1), in vivo phosphorylation generates a chiral phosphate of **1** with hitherto unknown stereoisomeric composition.[8]

We report herein proof that the amino alcohol **1** is phosphorylated in rats and humans to form a single stereoisomer, namely, the biologically active (*S*)-enantiomer (*S*)-**5**. To this end, enantiomers (*S*)-**5** and (*R*)-**5** were synthesized with high optical purity and characterized in biological assays. An ex vivo derivatization of the phosphate (*S*)-**5** was used to determine the enantiomeric excess of the in vivo generated phosphorylated analogue of **1** by enantioselective HPLC. The absolute configuration was determined by X-ray diffraction.

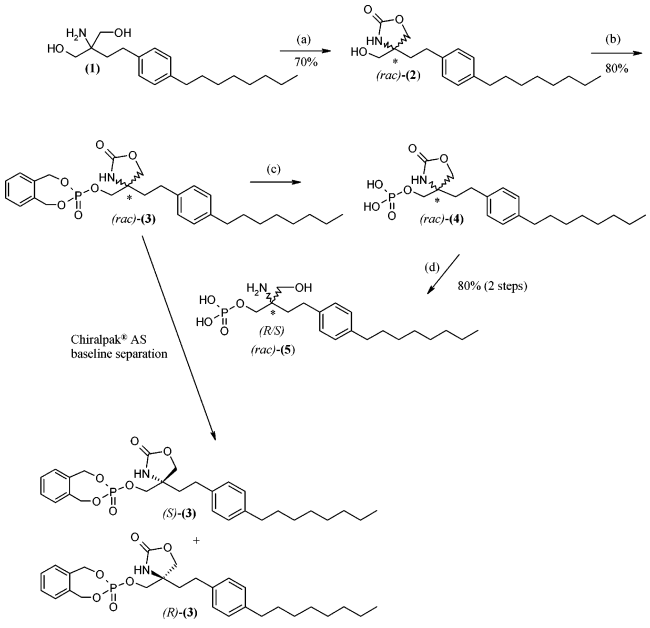
Chemistry

The synthesis of (*rac*)-**5** and the synthesis of (*S*)-**5** and (*R*)-**5** in optically pure form are outlined in Schemes 2 and 3. After protection of the prochiral **1** as an oxazolidinone[9] to give (*rac*)-**2**, phosphorylation of the remaining free hydroxyl group was performed with 3-(diethylamino)-1,5-dihydro-2,4,3-benzodioxaphosphepin[10] and subsequent oxidation gave 56% overall yield. From this

Scheme 1. General Phosphorylation Pathway of **1**



Scheme 2 Synthesis of (*S*)-**3**, (*R*)-**3**, and (*rac*)-**5**[a]



[a] (a) Benzyl chloroformate, NaOH, rt, 48 h; (b) 3-(diethylamino)-1,5-dihydro-2,4,3-benzodioxaphosphepintriphenyl phosphite, tetrazole, CH_2Cl_2/THF, rt, 18 h; then H_2O_2, rt, 90 min; (c) $Pd/C_{10\%}$, H_2, MeOH, rt, 90 min; (d) LiOH (10% in water), EtOH, reflux, 18 h.

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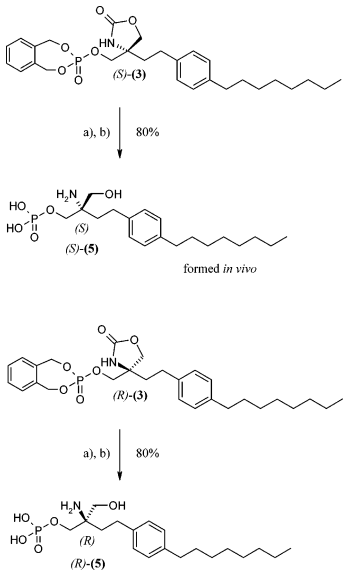
Scheme 3. Synthesis of Enantiomerically Pure (*S*)-**5** and (*R*)-**5**[a]



[a] (a) Pd/$C_{10\%}$, H_2, MeOH, rt, 90 min; (b) LiOH (10% in water), EtOH, reflux, 18 h.

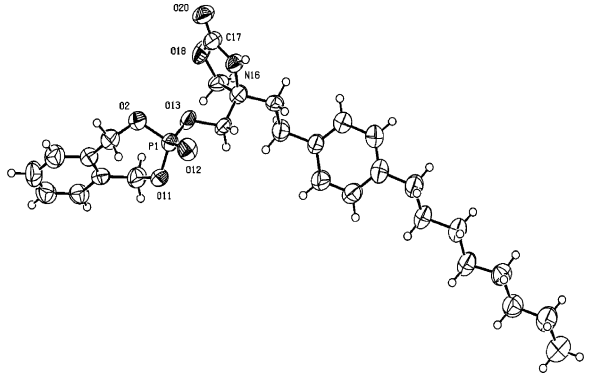


Figure 1. X-ray structure of (*R*)-**3**.

key intermediate (*rac*)-**3**, (*rac*)-**5** was obtained by removal of phosphate protecting group by hydrogenation in MeOH over 10% palladium on carbon, and subsequent hydrolysis of the oxazolidinone with 10% aqueous lithium hydroxide solution under reflux and final neutralization with solid carbon dioxide gave 80% overall yield. Pure enantiomers of **3** were accessible after separation of intermediate (*rac*)-**3** using Chiralpak AS[11] as an enantioselective support using *n*-hexane/ethanol, 40:60 (volume), as the mobile phase. Deprotection of (*S*)-**3** and (*R*)-**3** was performed under the same conditions as applied for the key intermediate (*rac*)-**3**, which was proven not to cause racemization by phosphate migration, yielding enantiomers (*S*)-**5** and (*R*)-**5** in pure form.

Absolute stereochemical assignment was performed on the basis of the crystal structure of intermediate (*R*)-**3**. Figure 1 shows the X-ray structure of (*R*)-**3** in the crystal.[12] Atomic displacement ellipsoids are drawn at the 50% probability level, and hydrogen atoms are drawn as spheres of arbitrary radius. The Flack *x* parameter for (*R*)-**3** is refined to 0.06(4).[13]

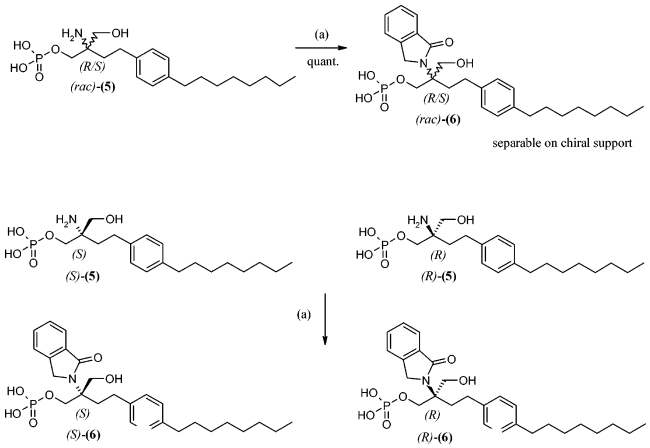
Biological Activity

The biological activity of all analogues of **1** was assessed in a S1P (hEDG) dependent SPA based GTPγS binding assays for all five known S1P receptors. This scintillation proximity assay measures agonist-induced GTP[γ-^{35}S] binding as functional assay for human S1P receptors (EDG receptors). For this assay, membrane protein of CHO cells expressing S1P receptor N-terminal c-myc was prepared at large scale and stored frozen, and 10 μg/well of the protein was immobilized onto 1 mg/well SPA beads. GTP[γ-^{35}S] binding was conducted with 200 pM GTP[γ-^{35}S] in the presence of 50 mM Hepes, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4, 10 μM GDP, 20 μg/mL saponin for 90−120 min. After a centrifugation step at 1500 rpm, plates were read with a TOPcount. The reference compound for all measurements was the natural ligand S1P.

Table 1. Agonistic Activities of Selected Compounds on S1P Receptors **1** and **5**

	EC_{50} (nM)				
compd	S1P-1	S1P-3	S1P-2	S1P-4	S1P-5
1	>10000	>10000	>10000	>10000	>10000
(*rac*)-**5**	1.2	1.4	>10000	2.4	4.9
(*S*)-**5**	0.3	3.1	>10000	0.6	0.3
(*R*)-**5**	218	28.9	>10000	80.1	>10000

Scheme 4. OPA Derivatization Protocol for (*rac*)-**5**, (*S*)-**5**, and (*R*)-**5**[a]



[a] (a) EDTA (10 mM in water), boric acid (3% in water), KOH (10% in water), *o*-phthaldialdehyde, rt, 1 h.

Results in Table 1 indicate a striking difference in the biological activities of (*S*)-**5** and (*R*)-**5** and provided a first biological evidence for the optical purity of the synthesized samples. Only (*S*)-**5** acts as an agonist on S1P receptors at low nanomolar concentrations, and these results are in agreement with published data of closely related structures.[8,14]

Derivatization and Chiral Separation

Since our efforts to separate the enantiomers of **5** directly on enantioselective support were met with limited success,[15] the optical purity of both enantiomers was determined by analyzing the corresponding derivatives that were prepared according to an OPA derivatization protocol.[16] Derivatization (Scheme 4) resulted in the formation of the lactams (*S*)-**6** and (*R*)-**6** by heterocycle condensation.

HPLC separation of the enantiomeric OPA derivatives (*S*)-**6** and (*R*)-**6** could be successfully achieved on an ion exchange quinine-based column (Prontosil AXQN 1) and revealed that (*S*)-**5** and (*R*)-**5** were formed with >99%

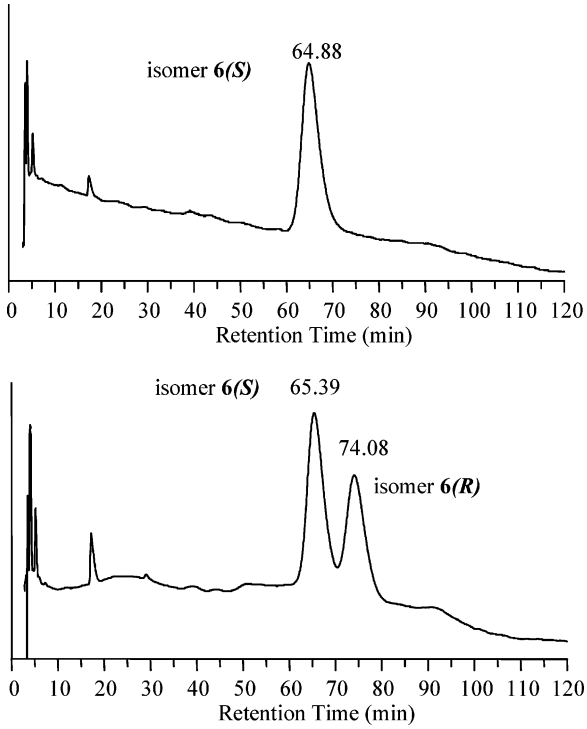


Figure 2. Chiral separation of lactams (*S*)-**6** and (*R*)-**6**.

optical purity (Figure 2), providing additional evidence that no racemization occurs during the deprotection protocol (Scheme 2).

Ex Vivo Analysis

We used the OPA derivatization protocol to investigate the stereoselectivity of the biological phosphorylation of **1** in rats and humans. The enantiomeric composition of the ^{14}C-labeled phosphate of **1** formed in vivo after single oral administration of ^{14}C-labeled **1** to rats or humans was determined according to the protocols described in refs 17 and 18, respectively. ^{14}C-labeled **1** was synthesized according to a published route using ^{14}C-labeled diethyl acetamidomalonate.[19]

Briefly, ^{14}C-labeled phosphate of **1** was isolated from blood taken from rats and humans at different times postdose by extraction with methanol, followed by nonenantioselective HPLC purification. The isolated metabolite was derivatized with OPA and subjected to enantioselective HPLC with radioactivity detection. In rats and humans, only the OPA derivative of the pharmacologically active (*S*)-**5** was detected in blood. The OPA derivative of the pharmacologically inactive (*R*)-**5** was below the detection limit (estimated to be <3% of total FTY720-P). Figure 3 shows the results of the enantioselective HPLC analysis of ^{14}C-labeled phosphate of **1** isolated from human blood at 12 h postdose. The results demonstrate that a single stereoisomer of phosphorylated **1** is formed in vivo and suggest a very high stereoselectivity of the rat and human kinase(s) involved in the formation of phosphorylated **1**.

Conclusion

We have demonstrated that FTY720 is phosphorylated in vivo in rats and humans to form a single stereoisomer, namely, the biologically active (*S*)-enantiomer. For this purpose we developed an efficient

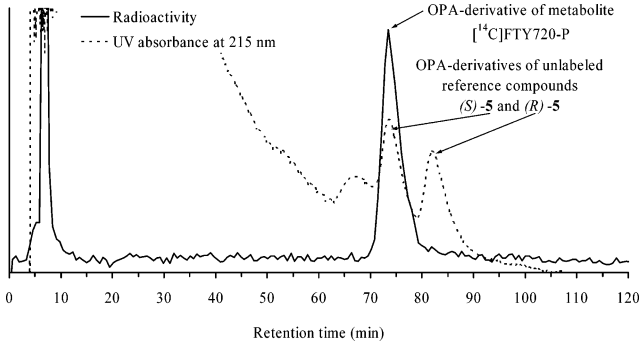


Figure 3. Enantioselective HPLC analysis of ^{14}C-labeled phosphate of 1 from human blood (12 h) following OPA derivatization.

synthesis of both enantiomers of phosphorylated **1** in their optically pure form and determined their biological activity. We defined the absolute configuration of both enantiomers based on the crystal structure of a protected intermediate (*R*)-**3** and successfully used a chemical derivatization of phosphorylated **1** ex vivo followed by enantioselective HPLC to determine the enantiomeric excess of the biologically formed species in rats and humans.

Experimental Section

1. Chemistry. General Methods. All reactions were carried out at room temperature if not otherwise stated. Commercial reagents and absolute solvents were used. The ^{1}H, ^{13}C, and ^{31}P NMR spectra were determined with a Bruker DRX 400 MHz spectrometer and referenced to the solvent. Chemical shifts are expressed in ppm. HRMS results were obtained on a 9.4 T APEX-III FT-MS (Bruker Daltonics), and ESI$^+$ and ESI$^-$ mass spectra were obtained on a Micromass Platform LCZ LC−MS. Preparative flash column chromatographies were performed using silica gel (Merck) G60 230−240 mesh. Analytical thin layer chromatography was performed on precoated silica gel 60 F_{254} GLP glass plates (Merck 1.05702).

2 Synthesis. (*R/S*)-4-Hydroxymethyl-4-[2-(4-octylphenyl)ethyl]oxazolidin-2-one ((*rac*)-2). Benzyl chloroformate (0.45 mL, 3.2 mmol) was added to a suspension of **1** (1.03 g, 3 mmol) in 2 N NaOH (20 mL). The mixture was kept at room temperature overnight, and to complete the reaction, more benzyl chloroformate (0.9 mL, 6.4 mmol) was added. After 2 days at room temperature the mixture was acidified with 1 N HCl, extracted with methylene chloride, and purified on a silica gel column using methylene chloride/methanol/acetic $acid_{50\%}$, 9:1:0.125 (volume), as the mobile phase. ESI-MS (ESI$^+$), *m/z*: 334 (M + H)$^+$. HRMS (FT), *m/z*: 334.2377 (M + H)$^+$, 356.2196 (M + Na)$^+$. Anal. ($C_{20}H_{31}NO_3$) C, H, N, O.

(*R/S*)-4-[2-(4-Octylphenyl)ethyl]-4-(3-oxo-1,5-dihydro-3λ^5-benzo[*e*][1,3,2]dioxaphosphepin-3-yloxymethyl)oxazolidin-2-one ((*rac*)-3). To a solution of (*rac*)-**2** (2.4 g, 7.2 mmol) in methylene chloride/THF, 1:1 (100 mL), at 0 °C was added tetrazole (recrystallized, 2.52 g, 36 mmol) and 3-(diethylamino)-1,5-dihydro-2,4,3-benzodioxaphosphepintriphenyl phosphite (5.17 g, 21.6 mmol). After 18 h at room temperature, H_2O_2 (8.2 mL [30% in water], 72 mmol) was added (cooling) to the solution and kept at room temperature for an additional 90 min. After the reaction was quenched with saturated $Na_2S_2O_3$ (100 mL), the mixture was extracted with ethyl acetate (3×). The organic layer was dried over Na_2SO_4, and the compound was purified on silica gel using cyclohexane/ethyl acetate, 1:1 (volume), as the mobile phase. HRMS (FT), *m/z*: 538.2331 (M + Na)$^+$, 547.2934 (M + $MeNH_3$)$^+$, 554.2070 (M + K)$^+$. Anal. ($C_{28}H_{38}NO_6P$) C, H, N, O, P.

Phosphoric Acid Mono[(*R/S*)-2-amino-2-hydroxymethyl-4-(4-octylphenyl)butyl] Ester ((*rac*)-5). Step 1. (*rac*)-**3** (1.03 g, 2 mmol) was hydrogenated at normal pressure ($Pd/C_{10\%}$, 50

mg) over 90 min. After filtration, the solution was concentrated and used in step 2 without further purification. ESI-MS (ESI^-), *m/z*: 412 $(M - H)^-$.

Step 2. To a solution of the end product of step 1 in ethanol (20 mL), LiOH (20 mL, 10% solution in water) was added. After 24 h at reflux, the mixture was neutralized with HCl (1 N in water) and concentrated. The residue was treated with glacial acetic acid (5 mL), and precipitation of the end product occurred after addition of water (50 mL). After the mixture was filtered, washed (water), and dried, pure (*rac*)-**5** was obtained as a white amorphous powder in an overall yield of 80%. ESI-MS (ESI^-), *m/z*: 386 $(M - H)^-$.

(*S*)-4-[2-(4-Octylphenyl)ethyl]-4-(3-oxo-1,5-dihydro-3λ^5-benzo[*e*][1,3,2]dioxaphosphepin-3-yloxymethyl)oxazolidin-2-one and (*R*)-4-[2-(4-Octylphenyl)ethyl]-4-(3-oxo-1,5-dihydro-3λ^5-benzo[*e*][1,3,2]dioxaphosphepin-3-yloxymethyl)oxazolidin-2-one ((*S*)-3 and (*R*)-3). Both enantiomers were isolated in optically pure form after preparative enantiselective HPLC. Preparative separation of (*S*)-**3** and (*R*)-**3** was performed by chromatography on a Chiralpak AS-V (20 μm) column, 5 cm × 20 cm, with *n*-hexane/ethanol, 60:40 (volume), as the mobile phase at a flow rate of 50 mL/min. Analytical HPLC: Chiralpak AS (4.6 mm × 250 mm), *n*-hexane/ethanol, 40:60, 1.5 mL/min. UV: 210 nm. For (*S*)-**3**: >99% ee, $[\alpha]^{20}_D$ −2.1° (*c* 1.75, $CHCl_3$); t_R = 13.01 min; HRMS, *m/z* 516.2512 $(M + H)^+$, 538.2331 $(M + Na)^+$, 547.2934 $(M + MeNH_3)^+$. For (*R*)-**3**: >99% ee; $[\alpha]^{20}_D$ +1.9° (*c* 1.75, $CHCl_3$); t_R = 6.31 min; HRMS, *m/z* 516.2512 $(M + H)^+$, 538.2331 $(M + Na)^+$, 547.2934 $(M + MeNH_3)^+$.

Phosphoric Acid Mono[(*S*)-2-amino-2-hydroxymethyl-4-(4-octylphenyl)butyl] Ester ((*S*)-5). The compound was synthesized according to the procedure given for (*rac*)-**5** starting with (*S*)-**3** as intermediate. HRMS, *m/z*: 386.2103 $(M - H)^-$. Anal. ($C_{19}H_{34}NO_5P$) C, H, N, O, P.

Phosphoric Acid Mono[(*R*)-2-amino-2-hydroxymethyl-4-(4-octylphenyl)butyl] Ester, (*R*)-FTY720-phosphate ((*R*)-5). The compound was synthesized according to the procedure given for (*rac*)-**5** starting with (*R*)-**3** as intermediate. HRMS, *m/z*: 386.2103 $(M - H)^-$. Anal. ($C_{19}H_{34}NO_5P$) C, H, N, O, P.

Phosphoric Acid Mono[(*R/S*)-2-hydroxymethyl-4-(4-octylphenyl)-2-(1-oxo-1,3-dihydroisoindol-2-yl)butyl] Ester (OPA Derivatization) ((*rac*)-6). (*rac*)-**5** (50 mg, 0.125 mmol) was suspended in a solution of EDTA (0.5 mL, 10 mM in water) and aqueous boric acid (0.5 mL, 3% in water, adjusted to pH 10.5 with aqueous 10% KOH). After addition of OPA (33 mg, 0.25 mmol) dissolved in ethanol (0.5 mL), the mixture was kept at room temperature for 1 h (ultrasound). After that the pH was adjusted to 3.5 (aqueous 1 N HCl) and the mixture was extracted with ethyl acetate (3×). The organic layer was dried over Na_2SO_4, and the compound was purified on silica gel using methylene chloride/methanol (95:5 → 0:100) as the mobile phase. ESI-MS (ESI^-), *m/z*: 502.5 $(M - H)^-$. Enantioselective HPLC: Prontosil AXQN 1 (150 mm × 4.0 mm), NaH_2PO_4−Na_2HPO_4 (0.05 M, pH 5.0)/2-propanol, 40:60 → 60:40, 0.4 mL/min, rt, DAD, t_R = 65.39 and 74.08 min.

Phosphoric Acid Mono[(*S*)-2-hydroxymethyl-4-(4-octylphenyl)-2-(1-oxo-1,3-dihydroisoindol-2-yl)butyl] Ester ((*S*)-6). The compound was prepared similarly to (*rac*)-**6** with (*S*)-**5** as starting material. HRMS, *m/z*: 502.2368 $(M - H)^-$. Enantioselective HPLC: Prontosil AXQN 1 (150 mm × 4.0 mm) (Bischoff Analysentechnik, Leonberg, Germany), NaH_2PO_4−Na_2HPO_4 (0.05 M, pH 5.0)/2-propanol, 40:60 → 60:40, 0.4 mL/min, rt, DAD, t_R = 64.88 min, >99% ee, $[\alpha]^{20}_D$ +4.6° (*c* 0.48, $CHCl_3$).

Phosphoric Acid Mono[(*R*)-2-hydroxymethyl-4-(4-octylphenyl)-2-(1-oxo-1,3-dihydroisoindol-2-yl)butyl] Ester ((*R*)-6). The compound was prepared similarly to (*rac*)-**6** with (*R*)-**5** as starting material. HRMS, *m/z*: 502.2367 $(M - H)^-$. Enantioselective HPLC: Prontosil AXQN 1, (Bischoff Analysentechnik, Leonberg, Germany) (150 mm × 4.0 mm), NaH_2PO_4−Na_2HPO_4 (0.05 M, pH 5.0)/2-propanol, 40:60 → 60:40, 0.4 mL/min, rt, DAD, t_R = 73.52 min, >97.7% ee; $[\alpha]^{20}_D$ −4.9° (*c* 0.45, $CHCl_3$).

Protocol for Rats. Blood samples from male Wistar rats were taken after single oral (7.5 mg/kg, 8 and 72 h) administration of [^{14}C]FTY720 and stored at −20 °C until analysis. Each blood sample (pool of three animals) was extracted with methanol, and [^{14}C]FTY720-P was isolated by nonenantioselective HPLC. The isolated [^{14}C]FTY720-P was derivatized with OPA. The derivative was spiked with unlabeled (*S*)-**6** and (*R*)-**6** as retention time markers and subjected to enantioselective HPLC separation on a 150 mm × 4.0 mm Prontosil AXQN 1 column packed with 5 μm particles (Bischoff Analysentechnik, Leonberg, Germany). The components were eluted isocratically with a 4:6 (v/v) mixture of 50 mM aqueous sodium phosphate buffer, pH 5.0, and 2-propanol at a total flow rate of 0.4 mL/min. Derivatized [^{14}C]FTY720-P was monitored by radioactivity detection. The unlabeled reference compounds (*S*)-**6** and (*R*)-**6** were monitored by UV detection at 215 nm.

Protocol for Humans. Blood samples from healthy male humans were taken after single oral administration of 4.5 mg of [^{14}C]FTY720 (12 and 72 h) and stored at −80 °C until analysis. Each blood sample (pool of four subjects) was extracted with methanol, [^{14}C]FTY720-P was isolated by nonenantioselective HPLC, derivatized with OPA, spiked with unlabeled (*S*)-**6** and (*R*)-**6**, and analyzed by enantioselective HPLC as described in ref 17.

Acknowledgment. We gratefully acknowledge Jean-Pierre Baldeck for performing the enantioselective analysis of the phosphate of **1** in blood, Hans-Peter Gschwind for organizing the human ADME study in vivo, Hansrudolf Walter for the preparation of crystals for the X-ray studies, and Caroline Radoch and Gabrielle Lecis for the enantioselective separation of the OPA analogues of (*rac*)-**5**, (*S*)-**5**, and (*R*)-**5**.

Appendix

Abbreviation. FTY720, 2-amino-2-[2-(4-octylphenyl)-ethyl]propane-1,3-diol; FTY720-P, phosphoric acid mono-[2-amino-2-hydroxymethyl-4-(4-octylphenyl)butyl] ester; S1P, sphingosine-1-phosphate; hEDG, human endothelial cell differentiation gene; OPA, *o*-phthalaldehyde; HPLC, high-performance liquid chromatography; GTPγS, guanosine 5′-(γ-thio)triphosphate; CHO, Chinese hamster ovary; GDP, guanosine diphosphate; SPA, scintillation proximity assays; NMR, nuclear magnetic resonance; HRMS, high-resolution mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; rt, room temperature; MeOH, methanol; THF, tetrahydrofuran; DMSO, dimethyl sulfoxide; TMS, tetramethylsilane; EDTA, ethylenediaminetetraacetic acid).

Supporting Information Available: ^{1}H NMR, HRMS, elemental analysis, and HPLC results of selected coumpounds described in the Experimental Section and a crystallographic file in CIF format. This material is available free of charge via the Internet at http://pubs.acs.org.

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