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Synthesis of (S)-FTY720 vinylphosphonate analogues and evaluation of their potential as sphingosine kinase 1 inhibitors and activators

Zheng Liu^a, Neil MacRitchie^b, Susan Pyne^b, Nigel J. Pyne^b, and Robert Bittman^{a,*}

^aDepartment of Chemistry and Biochemistry, Queens College, The City University of New York, Flushing, New York 11367-1597, USA

^bCell Biology Group, Strathclyde Institute of Pharmacy and Biomedical Science, University of Strathclyde, Glasgow G4 0RE, UK

Abstract

Sphingosine kinase 1 (SK1) is over-expressed in many cancers where it provides a selective growth and survival advantage to these cells. SK1 is thus a target for anti-cancer agents that can promote apoptosis of cancer cells. In previous work, we synthesized a novel allosteric SK1 inhibitor, (S)-FTY720 vinylphosphonate. We now report a more expeditious route to this inhibitor which features B-alkyl Suzuki coupling as a key step and show that replacement of the amino group in (S)-FTY720 vinylphosphonate with an azido group converts the vinylphosphonate from an allosteric inhibitor to an activator of SK1 at low micromolar concentrations. Our results demonstrate the feasibility of using the (S)-FTY720 vinylphosphonate scaffold to define structure-activity relationships in the allosteric site of SK1.

Keywords

Sphingosine 1-phosphate; B-alkyl Suzuki cross-coupling; FTY720; Epoxide opening; Allosterism

1. Introduction

Sphingosine kinase 1 (SK1) is over-expressed in various human cancers and therefore inhibitors of SK1 are potential anticancer agents.¹ FTY720 (**1**, also known as fingolimod and by the trademark name Gilenya, Fig. 1) is a structural analogue of sphingosine that affects the activities of many sphingolipid-metabolizing enzymes in its non-phosphorylated form.² On phosphorylation by sphingosine kinase 2 (SK2), FTY720 is converted into the immunomodulator (S)-FTY720-phosphate, which is the first compound in a new class of small molecule sphingosine 1-phosphate (S1P) receptor agonists that alters lymphocyte trafficking.³ In 2010, **1** was approved as an orally deliverable drug for the treatment of

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*Corresponding author. Tel.: (+1)718-997-3279; fax: (+1)718-997-3349; robert.bittman@qc.cuny.edu.

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Supplementary data

Supplementary data (¹H, ¹³C, ³¹P, and ¹⁹F NMR spectral data for all compounds) associated with this article can be found, in the online version, at doi:.

multiple sclerosis.⁴ The finding that non-phosphorylated **1** inhibited SK1 and SK2 led to interest in synthetic analogues of **1** as putative antitumor agents.⁵ Indeed, we reported that (*S*)-FTY720 vinylphosphonate (**2**),⁶ a metabolically stabilized derivative of **1**, is an uncompetitive inhibitor (with sphingosine) and a mixed inhibitor (with ATP) of SK1.^{7,8} This mode of inhibition is indicative of allosterism contingent on formation of the sphingosine-SK1 complex, which offers new drug discovery opportunities and also permits further assessment of the role of SK1 in cancer.¹

To gain further insight into the structure-activity relationships in **2** that result in binding at allosteric site(s) in SK1 we have prepared analogues **3-7** and evaluated their ability to modulate SK1 activity. We focused on modifying the polar head group while retaining the lipid tail of **2** as follows: (a) changing the hydroxyl group to a fluorine atom, (b) converting the amino group to an azide group, (c) replacing the vinyl-phosphonate moiety with a vinyl-carboxylic acid, and (d) limiting the conformational freedom in the polar head group by installing a lactone ring. The synthesis of chiral analogues **3-7** involves a B-alkyl Suzuki cross-coupling reaction as the key step. The route reported here is more efficient and practical than the previous synthetic route to **2**.⁵

2. Chemistry

The challenge in the synthesis of **2** and analogues **3-7** lies in the construction of the sole quaternary stereocenter. Given the high yield and stereoselectivity in our previous synthesis,⁵ we decided to continue to employ Sharpless asymmetric epoxidation (SAE)⁹ of allylic alcohol **12** to introduce the stereocenter. Our new synthetic route to allylic alcohol **12** started with Wittig methylenation of the commercially available aldehyde **8** (Scheme 1). The resulting terminal alkene **9** was subjected to a B-alkyl Suzuki cross-coupling reaction¹⁰ with (2-iodoallyloxy)(*tert*-butyl)dimethylsilane (**10**),¹¹ followed by removal of the TBS group in **11**. Thus **12** was prepared in 66% yield (over four steps), with only one purification step (by column chromatography).

The scale used in this four-step procedure was up to 20 mmol. It is noteworthy that after the hydroboration of **9** with 9-BBN, the reaction mixture must be mixed with aqueous basic solution for at least 30 min prior to the addition of the palladium catalyst. The yield of **11** was markedly decreased when an aqueous NaOH solution and Pd(dppf)Cl₂·CH₂Cl₂ were added simultaneously. In addition, ethanolamine¹² did not completely precipitate the boron complex; we found that the rest of the complex was removed with H₂O₂/NaOH solution.¹³

For SAE of **12** on a scale of 13 mmol, a substoichiometric amount of catalyst [0.55 equiv. of Ti(O-*i*-Pr)₄] was used, leading to epoxy alcohol **13** in 74% yield and 11% of a side product¹⁴ formed via epoxide opening by isopropoxide. It was reported by Sharpless and coworkers¹⁵ that the *catalytic* procedure [10 mol % of Ti(O-*i*-Pr)₄] can prevent the epoxide-opening reaction for unsymmetrical-disubstituted allylic alcohols. The *er* value of the resulting epoxide was >20:1, as determined by analysis of the ¹H NMR spectra of the (*R*)- and (*S*)-MTPA derivatives of **13**. Dess-Martin oxidation of epoxy alcohol **13** proceeded in 90% yield.

Horner-Wadsworth-Emmons (HWE) olefination of aldehyde **14** with tetramethyl methylenediphosphonate afforded exclusively *E*-alkene **15** in 94% yield (Scheme 2). In contrast, poor *E* selectivity (**16a**: *E/Z* 3:1)¹⁶ was observed when **14** was treated with triethyl phosphonoacetate in H₂O/*i*PrOH (1:1).¹⁷ However, the use of the sterically larger diisopropyl (ethoxycarbonylmethyl)phosphonate in the HWE reaction, as expected,¹⁸ provided excellent *E* selectivity (**16**: *E/Z* 25:1).

Since opening of a vinyl epoxide with an azide generally generates an allylic azide when no additional stabilizing group is present, a [3,3]-sigmatropic equilibration process takes place to create a mixture of regioisomers. Therefore, this reaction is of little value,¹⁹ and only a few systematic studies on ring-opening reactions of vinyl epoxides by azide ion have been published.¹⁹⁻²³ We achieved regioselective epoxide ring openings of γ,δ -epoxy- α,β -unsaturated esters **15** and **16** with $\text{Ti}(\text{O}-i\text{-Pr})_2(\text{N}_3)_2$ and $\text{NaN}_3/\text{NH}_4\text{Cl}$, which permitted structural diversification of **2**. With γ,δ -epoxy- α,β -unsaturated esters, nucleophilic attack at the γ position has been shown to predominate.²⁰⁻²⁴ As shown in Scheme 3, we employed two different azide sources. Surprisingly, as shown in Scheme 3, we found that azidolysis of **15** and **16** with $\text{NaN}_3/\text{NH}_4\text{Cl}$ in EtOH ²⁵ afforded the highly δ -selective regioisomers **17** (68% yield) and **19** (72% yield), respectively. The ratio of regioisomers was determined by analysis of the ^{31}P NMR spectra. On the other hand, the desired and expected regioisomer **17** (γ -attack) was obtained by treatment of **15** with $\text{Ti}(\text{O}-i\text{-Pr})_2(\text{N}_3)_2$ (which was prepared from $\text{Ti}(\text{O}-i\text{-Pr})_4$ and TMSN_3).²⁶

To our surprise, the reaction of **16** and $\text{Ti}(\text{O}-i\text{-Pr})_2(\text{N}_3)_2$ (3.9 equiv.) in toluene afforded **20** (26% yield), accompanied by lactone **21** (45% yield). The regioisomer **19** was not detected. The *Z* configuration of alkene **21** was confirmed by the ^1H NMR spectrum, which shows correlated two doublets (δ 6.20 ppm, $J = 9.8$ Hz, $=\text{CHCO}_2\text{Et}$; δ 6.75 ppm, $J = 9.8$ Hz, RCHCH=). Since δ -lactone formation from δ -hydroxy-*trans*- α,β -unsaturated carboxylic acids can be achieved via a pyridine-assisted *trans-cis* isomerization,²⁷⁻³⁰ it appears that azide anion might play a similar role as pyridine in our reaction.

Reduction of an azide to an amine in the presence of a double bond is not trivial. Both Staudinger reduction (Ph_3P , $\text{THF}/\text{H}_2\text{O}$) and 1,3-propanedithiol/ Et_3N ³¹ failed to produce satisfactory results. Reduction using Lindlar's catalyst (H_2 , Pd/CaCO_3 , EtOH)³² resulted in saturation of the double bond. Fortunately, as illustrated in Scheme 4, we found that simultaneous reduction of the azide and demethylation of methyl ester **17** was accomplished by using SnCl_2 in 95% MeOH ,³³ providing **2** in 69% yield, together with **22** (17% yield). Methyl ester **22** was transformed to **2** by treatment with TMSBr in quantitative yield. Our new synthetic route to **2** consists of nine steps from commercially available aldehyde **8** in 19% overall yield. The azide analogue **5** was formed by demethylation of **17** with TMSBr , followed by aqueous MeOH , in a quantitative yield. The stereochemistry of **22** was confirmed by its specific rotation: $[\alpha]^{25}_{\text{D}} +20.0$ (c 0.18, CHCl_3) [lit.⁵ $[\alpha]^{25}_{\text{D}} +18.8$ (c 1.52, CHCl_3)].

Fluorination of **17** with DAST ³⁴ (-78 °C, overnight, and then at rt for 3 h) produced **23** in 75% yield (Scheme 4). Termination of the reaction at low temperature led to incomplete conversion. In contrast to **17**, reduction of **23** using Lindlar's catalyst (H_2 , Pd/CaCO_3 , EtOH)³² did not reduce the double bond, providing **24** in 51% yield. Demethylation of methyl esters **23** and **24** with TMSBr followed by 95% MeOH afforded the target fluorine-containing analogues **4** and **3**, respectively, in quantitative yields. The unsaturated carboxylic acid analogue **6** was prepared by reduction of **20** (SnCl_2 in MeOH), followed by hydrolysis of ester **25** with LiOH in $\text{THF}/\text{MeOH}/\text{H}_2\text{O}$. Catalytic hydrogenation of **21** (H_2 , Pd/C) provided lactone analogue **7** in 46% yield.

3. Biological evaluation

We have previously shown that (*S*)-vinylphosphonate **2** (50 μM) inhibits SK1 activity by 62.7 $\pm$ 0.9 % ($n = 3$) using 3 μM sphingosine (Sph) as the substrate. (*S*)-FTY720 vinylphosphonate (**2**) is an uncompetitive inhibitor (with sphingosine) of SK1 with a $K_{\text{iu}} = 14.5 \pm 4.4$ μM .⁷ (*S*)-Vinylphosphonate **2** was not a substrate for SK1 (data not shown). We now show that analogues **3-7** have markedly different effects on SK1 activity (Fig. 2).

We found that compounds **3-7** are not substrates, since no phosphorylated product was formed when Sph was omitted from the assay. In the presence of Sph, fluoride **3** is a weak inhibitor of SK1 (20% inhibition), while vinyl-carboxylate **6** is ineffective (Fig. 2), indicating that replacement of the $-P(O)(OH)_2$ group with $-CO_2H$ (which retains a negative charge at this position of the molecule) eliminates inhibition. Indeed, we have shown that (*S*)-vinylphosphonate **2** is a mixed inhibitor (with ATP) of SK1, contingent on formation of the ATP-SK1 complex.⁸ The enzyme kinetics data for binding of **2** to the allosteric site, after ATP binds first to the active site, are uncompetitive with ATP, $K_{iu} = 65.0 \pm 5.0 \mu M$, and competitive with ATP, $K_{ic} = 10.0 \pm 4.6 \mu M$. We proposed a 'see-saw' model for the binding of **2** to the ATP binding site and to the allosteric site.⁸ In this model, compound **2** competes with ATP for the ATP-binding site, but flips to bind to an allosteric site when ATP binds to the catalytic site.⁸ Therefore, the $-P(O)(OH)_2$ group appears to be essential for binding of compound **2** to both the catalytic and allosteric sites. Amino lactone **7** is also an inhibitor of SK1 (35% inhibition) (Fig. 2). Replacement of the amino group with an azide group in compound **2** produced compounds **4** and **5**; surprisingly, azido-fluoride **4** and azido-alcohol **5** are activators of SK1 at low micromolar concentrations (and they could not replace sphingosine as a substrate) (Fig. 2). Compounds **4** and **5** both induced a 30-60% stimulation of SK1 activity at low concentrations. The difference in these azide-containing compounds concern fluorine in compound **4**, which can only accept hydrogen bonds, and a hydroxyl group in compound **5**, which can both accept and donate hydrogen bonds.

At concentrations below $50 \mu M$, compound **5** stimulated SK1 activity with an EC_{50} of $8.3 \pm 3.0 \mu M$, $n = 3$ (Fig. 3), indicative of binding to an allosteric site. At concentrations above $50 \mu M$, the % activation in response to compound **5** diminished markedly (Fig. 3). This biphasic response suggests that compound **5** might bind to the catalytic site (or alter its conformation) to inhibit SK1 activity at these higher concentrations. Similar results were obtained with compound **4** (EC_{50} of $5.7 \pm 5.5 \mu M$, $n = 3$, data not shown). The biphasic curves found with both **4** and **5** indicate that the azido group and not the fluoro or hydroxyl group is responsible for this phenomenon.

4. Conclusion

We have previously demonstrated that SK1 contains an allosteric site which binds both inhibitors and activators. This is based on three observations.⁷ First, compound **2** inhibited SK1 activity with kinetics indicative of an allosteric interaction. Second, two novel compounds, Bodipy-sphingosine and a regioisomer of FTY720, activated SK1 activity. Third, titration of an inactive SK1 mutant into cells expressing wild-type SK1 inhibited wild-type SK1 activity in a concentration-dependent manner. These findings are explained by a model in which SK1 is a minimal dimer, and that formation of the wild-type SK1-inactive SK1 mutant dimer produces an enzyme in which the number of allosteric sites exceed the competent catalytic site. Suppression of catalytic activity by an excess of allosteric sites suggests that this site might constitute an inhibitory domain. We proposed that SK1 contains an auto-inhibitory domain which exists in an 'on' and 'off' state in equilibrium.⁷ The 'on' state is stabilized by allosteric inhibitors to further suppress catalytic activity. Conversely, the 'off' state is stabilized by allosteric activators to remove the effect of the inhibitory domain on catalytic activity.⁷ These findings can be adapted to an induced-fit model. In the current study we show that the replacement of the amino group with an azido group in (*S*)-FTY720 vinylphosphonate (**2**) remarkably changed the compound from an inhibitor to an activator of SK1. That such a relatively minor chemical modification in compound **2** affects the nature of allosterism is entirely consistent with the notion that small changes in the chemical nature of a compound can be translated into subtle changes in the conformational state of the allosteric site. These conformational changes result in profound changes, as evidenced by turning an allosteric inhibitor into a compound that enhances

enzymatic activity at low micromolar concentrations of compound. Our results also clearly demonstrate the feasibility of using the (*S*)-FTY720 vinylphosphonate scaffold to define the structure-activity relationships of the allosteric site in SK1.

5. Experimental section

5.1. Chemistry

5.1.1. General experimental information—All reactions were carried out under a dry N₂ atmosphere using oven-dried glassware and magnetic stirring. The solvents were dried before use as follows: THF and Et₂O were heated at reflux over sodium benzophenone ketyl; toluene was heated at reflux over sodium; CH₂Cl₂ was dried over CaH₂. Anhydrous *N,N*-diisopropylethylamine, acetonitrile, triethylamine, and benzene were used directly as purchased. Aluminum TLC sheets (silica gel 60 F₂₅₄) of 0.2-mm thickness were used to monitor the reactions. The spots were visualized with short wavelength UV light or by charring after spraying with a solution prepared from one of the following solutions: phosphomolybdic acid (5.0 g) in 95% EtOH (100 mL); *p*-anisaldehyde solution (2.5 mL of *p*-anisaldehyde, 2 mL of AcOH, and 3.5 mL of conc. H₂SO₄ in 100 mL of 95% EtOH); or KMnO₄ solution (1.5 g of KMnO₄ and 1.5 g of K₂CO₃ in 100 mL of 0.1% NaOH). Flash chromatography was carried out with silica gel 60 (230-400 ASTM mesh). NMR spectra were obtained on a 400-MHz or 500-MHz spectrometer. Chemical shifts were referenced on residual solvent peaks: CDCl₃ (δ = 7.26 ppm for ¹H NMR and 77.00 ppm for ¹³C NMR); external 85% H₃PO₄ (δ 0.00) was used for ³¹P NMR and CFC₃ (δ 0.00) for ¹⁹F NMR. Optical rotations were measured at rt in a 1.0-dm cell. High-resolution mass spectra were acquired by electrospray ionization.

5.1.2. Preparation of 1-octyl-4-vinylbenzene(9)—To a stirred suspension of Ph₃PCH₃Br (22.3 g, 62.4 mmol) in anhydrous THF (350 mL) was added *n*-BuLi (25 mL, 2.5 M in hexane, 62.5 mmol) dropwise at −78 °C. The mixture was stirred for 3 h at −78 °C. A solution of aldehyde **8** (6.80 g, 31.2 mmol) in 20 mL of THF was added slowly. The cooling bath was removed after the addition, and the reaction mixture was stirred for 2 h, during which time the temperature was increased slowly from −78 °C to rt. After 700 mL of hexane was added, the mixture was passed through a pressed pad of silica gel, which was rinsed with hexane/Et₂O (10:1). The filtrate was concentrated under reduced pressure to provide the pure alkene **9** (6.34 g, 94%). ¹H NMR (500 MHz, CDCl₃) δ 0.83-0.92 (m, 3H), 1.22-1.41 (m, 10H), 1.56-1.67 (m, 2H), 2.55-2.65 (m, 2H), 5.18-5.23 (m, 1H), 5.69-5.76 (m, 1H), 6.67-6.76 (m, 1H), 7.14-7.19 (m, 2H), 7.32-7.38 (m, 2H); ¹³C NMR (125 MHz, CDCl₃) δ 14.1, 22.7, 29.26, 29.30, 29.5, 31.5, 31.9, 35.7, 112.7, 126.1, 128.5, 135.0, 136.7, 142.7.

5.1.3. Preparation of 2-methylene-4-(4-octylphenyl)butan-1-ol (12)—A solution of alkene **9** (4.14 g, 19.1 mmol) in THF (15 mL) was added dropwise to a solution of 9-BBN (200 mL, 0.5 M in THF, 100 mmol) at 0 °C. The reaction mixture was allowed to warm to rt. After 1 h at rt, the mixture was cooled to 0 °C and 3 M aqueous NaOH (210 mmol, 70 mL) was added. The mixture was stirred vigorously for 1 h at rt, and then was added to a suspension of (2-iodoallyloxy)(*tert*-butyl)dimethylsilane¹⁰ (6.35 g, 21.3 mmol) and Pd(dppf)Cl₂·CH₂Cl₂ (570 mg, 0.70 mmol) in THF (50 mL). The reaction mixture was stirred at 60 °C for 3 h. After cooling to rt, the mixture was added to ethanolamine (12 mL) to precipitate the 9-BBN/ethanolamine complex. The mixture was stirred overnight at rt, poured into saturated aqueous NH₄Cl solution, and extracted with Et₂O. The combined organic extracts were concentrated under reduced pressure and suspended in hexane/Et₂O (100 mL, 10:1). The mixture was filtered through a short pad of silica gel, which was

washed with hexane/Et₂O (10:1). The filtrate was concentrated under reduced pressure to provide the crude coupling product.

To a solution of crude coupling product in MeOH (200 mL) was added 6 M HCl (100 μ L, 0.6 mmol). After being stirred at rt for 1 h, the reaction mixture was quenched by slowly adding saturated aqueous NaHCO₃ solution. MeOH was removed under reduced pressure, and the aqueous phase was extracted with Et₂O. In order to completely remove the boron complex, the combined organic extracts were treated with 100 mL of 15% NaOH, followed by dropwise addition of 30% H₂O₂ (50 mL). The mixture was stirred at rt for 0.5 h. The aqueous phase was extracted with Et₂O, and the combined organic phases were dried (MgSO₄) and concentrated under reduced pressure. The residue was purified by flash chromatography (hexane/EtOAc 8:1, 6:1, to 4:1) to afford 3.67 g (70%) of **12**. The ¹H and ¹³C NMR spectra of **12** matched the reported data.⁵ ESI-HRMS (M+Na)⁺ calcd for C₁₉H₃₀NaO⁺ 297.2189, found 297.2193.

5.1.4. Preparation of (S)-2-(4-octylphenethyl)oxirane-2-carbaldehyde(**14**)—

Dess-Martin periodinane (3.18 g, 7.50 mmol) was added portionwise to a solution of alcohol **13** (1.45 g, 85% purity, 4.24 mmol,) in 4 mL of pyridine and 50 mL of CH₂Cl₂. After 2 h, the reaction mixture was diluted with Et₂O (100 mL), and saturated aqueous NaHCO₃ solution (20 mL) and saturated aqueous Na₂S₂O₃ (20 mL) were added to the reaction mixture. After vigorous stirring for 10 min, the mixture was extracted with Et₂O. The combined organic layers were washed with saturated aqueous NaHCO₃ solution, dried (MgSO₄), and filtered. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography on silica gel (EtOAc/hexane 1:10, 1:8, to 1:6) to afford 1.1 g (90%) of aldehyde **14**. ¹H NMR (500 MHz, CDCl₃) δ 0.83-0.92 (m, 3H), 1.22-1.36 (m, 10H), 1.56-1.64 (m, 2H), 2.01-2.08 (m, 1H), 2.20-2.28 (m, 1H), 2.58 (t, *J* = 7.8 Hz, 2H), 2.72 (t, *J* = 8.2 Hz, 2H), 2.99 (d, *J* = 4.6 Hz, 1H), 3.04 (d, *J* = 4.6 Hz, 1H), 7.10-7.13 (m, 4H), 8.89 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 14.1, 22.6, 29.2, 29.3, 29.5, 29.9, 30.2, 31.5, 31.9, 35.5, 49.8, 60.9, 128.1, 128.5, 138.0, 140.8, 198.8; ESI-HRMS (M+Na)⁺ calcd for C₁₉H₂₈NaO₂⁺ 311.1982, found 311.1986.

5.1.5. Preparation of (E)-dimethyl 2-[(R)-2-(4-octylphenethyl)oxiran-2-yl]vinylphosphonate (**15**)—

To a mixture of NaH (57-63% oil dispersion, 240 mg, 6.00 mmol) and 50 mL of THF was added a solution of 1.41 g (6.08 mmol) of CH₂[(P(O)(OMe)₂] in 10 mL of THF at 0 °C. After the mixture was stirred for 30 min, a solution of **14** (585 mg, 2.03 mmol) in 10 mL of THF was added. The reaction mixture was stirred for 1 h at 0 °C, quenched with saturated aqueous NH₄Cl solution, and extracted with EtOAc. The organic layer was dried (MgSO₄) and concentrated under reduced pressure. The residue was purified by chromatography (elution with EtOAc) to give 750 mg (94%) of **15**. ¹H NMR (400 MHz, CDCl₃) δ 0.83-0.92 (m, 3H), 1.20-1.38 (m, 10H), 1.53-1.64 (m, 2H), 1.90-2.01 (m, 1H), 2.08-2.18 (m, 1H), 2.53-2.59 (m, 2H), 2.62-2.77 (m, 3H), 2.87 (t, *J* = 5.4 Hz, 1H), 3.72 (d, *J* = 5.5 Hz, 3H), 3.74 (d, *J* = 5.5 Hz, 3H), 5.95 (dd, *J* = 17.2, 19.4 Hz, 1H), 6.83 (dd, *J* = 17.2, 22.2 Hz, 1H), 7.05-7.13 (m, 4H); ¹³C NMR (100 MHz, CDCl₃) δ 14.1, 22.6, 29.2, 29.3, 29.4, 30.6, 31.5, 31.8, 35.2, 35.5, 52.38 (d, *J* = 5.4 Hz), 52.41 (d, *J* = 5.4 Hz), 55.9, 58.2 (d, *J* = 24.0 Hz), 116.5 (d, *J* = 189.6 Hz), 128.0, 128.5, 137.9, 140.8, 151.6 (d, *J* = 6.5 Hz); ³¹P NMR (162 MHz, CDCl₃) δ 20.6; ESI-HRMS (M+H)⁺ calcd for C₂₂H₃₆O₄P⁺ 395.2346, found 395.2346.

5.1.6. Preparation of (E)-ethyl 3-[(R)-2-(4-octylphenethyl)oxiran-2-yl]acrylate (16**)—**K₂CO₃ (4.51 g, 32.6 mmol) was dissolved in water (10 mL), and the solution was cooled to rt. This aqueous solution was then added to a solution of aldehyde **14** (350 mg, 1.21 mmol) and (*i*-PrO)₂P(O)CH₂CO₂Et (866 μ L, 3.64 mmol) in 2-propanol (10 mL) at 0

°C. The reaction mixture was allowed to warm to rt and was stirred overnight at rt. The reaction mixture was separated and the lower aqueous layer was extracted with EtOAc. The combined organic layers were dried (Na₂SO₄), filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (hexane/EtOAc 3:1) to give 409 mg (94%) of **16**. ¹H NMR (500 MHz, CDCl₃) δ 0.83-0.92 (m, 3H), 1.20-1.36 (m, 13H), 1.54-1.63 (m, 2H), 1.93-2.01 (m, 1H), 2.09-2.16 (m, 1H), 2.57 (t, *J* = 7.7 Hz, 2H), 2.65-2.75 (m, 3H), 2.88 (d, *J* = 5.4 Hz, 1H), 4.21 (q, *J* = 7.1 Hz, 2H), 6.10 (d, *J* = 15.7 Hz, 1H), 6.91 (d, *J* = 15.7 Hz, 1H), 7.06-7.12 (m, 4H); ¹³C NMR (100 MHz, CDCl₃) δ 14.1, 14.2, 22.6, 29.2, 29.3, 29.5, 30.7, 31.5, 31.9, 35.45, 35.52, 55.8, 57.6, 60.6, 122.2, 128.1, 128.5, 138.1, 140.8, 146.6, 166.0; ESI-HRMS (M+Na)⁺ calcd for C₂₃H₃₄NaO₃⁺ 381.2400, found 381.2401.

5.1.7. Preparation of (S,E)-dimethyl 3-azido-3-(hydroxymethyl)-5-(4-octylphenyl)pent-1-enylphosphonate (17)—Ti(O-*i*-Pr)₄ (2.4 mL, 8.02 mmol) and TMSN₃ (2.2 mL, 16.6 mmol) were added to anhydrous toluene (50 mL), and the mixture was heated at reflux (85 °C) under N₂ for at least 5 h. A solution of epoxide **15** (670 mg, 1.70 mmol) in anhydrous toluene (10 mL) was added to the above solution in one portion. The mixture was stirred for 15 min at 85 °C and was then cooled to rt. The solvent was removed under reduced pressure. Et₂O (20 mL) was added, followed by 10% HCl (40 mL). The solution was stirred at rt until two clear phases appeared. The aqueous phase was extracted with Et₂O. The organic phase was dried over MgSO₄, filtered, and concentrated under reduced pressure. Purification by flash chromatography (elution with EtOAc) afforded **17** (500 mg, 68%) as a yellow oil. ¹H NMR (500 MHz, CDCl₃) δ 0.83-0.92 (m, 3H), 1.20-1.36 (m, 10H), 1.54-1.62 (m, 2H), 1.90-1.99 (m, 1H), 2.03-2.11 (m, 1H), 2.51-2.67 (m, 4H), 3.01 (s, 1H, OH), 3.70-3.80 (m, 8H), 6.03 (dd, *J* = 17.1, 19.3 Hz, 1H), 6.72 (dd, *J* = 17.2, 22.7 Hz, 1H), 7.06-7.12 (m, 4H); ¹³C NMR (100 MHz, CDCl₃) δ 14.1, 22.6, 29.2, 29.3, 29.46, 29.50, 31.6, 31.9, 35.5, 36.0, 52.53 (d, *J* = 5.5 Hz), 52.55 (d, *J* = 5.5 Hz), 67.4, 69.0 (d, *J* = 19.4 Hz), 118.1 (d, *J* = 186.9 Hz), 128.1, 128.6, 137.9, 140.9, 151.0 (d, *J* = 6.3 Hz); ³¹P NMR (162 MHz, CDCl₃) δ 20.5; ESI-HRMS (M+H)⁺ calcd for C₂₂H₃₆N₃O₄P⁺ 438.2516, found 438.2519.

5.1.8. Preparation of (R,E)-dimethyl 3-(azidomethyl)-3-hydroxy-5-(4-octylphenyl)pent-1-enylphosphonate (18)—A mixture of **15** (45 mg, 0.114 mmol), NaN₃ (37 mg, 0.569 mmol), and NH₄Cl (49 mg, 0.916 mmol) in EtOH (2 mL) was heated at 100 °C via microwave radiation for 20 min. The reaction mixture was allowed to cool to rt and was then filtered through a thin pad of Celite (eluting with EtOAc), and the filtrate was concentrated under reduced pressure. The crude material was purified by flash chromatography (elution with EtOAc) on silica gel to provide azide **18** (36 mg, 72%). ¹H NMR (400 MHz, CDCl₃) δ 0.83-0.92 (m, 3H), 1.20-1.36 (m, 10H), 1.52-1.62 (m, 2H), 1.80-1.90 (m, 1H), 1.92-2.02 (m, 1H), 2.48-2.59 (m, 3H), 2.62-2.72 (m, 1H), 3.13 (s, 1H, OH), 3.39 (s, 2H), 3.74 (dd, *J* = 2.4, 11.1 Hz, 6H), 6.10 (dd, *J* = 17.1, 20.3 Hz, 1H), 6.78 (dd, *J* = 17.1, 22.3 Hz, 1H), 7.04-7.12 (m, 4H); ¹³C NMR (100 MHz, CDCl₃) δ 14.1, 22.6, 29.0, 29.2, 29.3, 29.4, 31.5, 31.8, 35.5, 39.3, 52.46 (d, *J* = 5.6 Hz), 52.49 (d, *J* = 5.6 Hz), 59.1, 76.0 (d, *J* = 19.2 Hz), 116.6 (d, *J* = 187.8 Hz), 128.1, 128.5, 138.2, 140.8, 154.9 (d, *J* = 5.5 Hz); ³¹P NMR (162 MHz, CDCl₃) δ 20.9; ESI-HRMS (M+Na)⁺ calcd for C₂₂H₃₆N₃NaO₄P⁺ 460.2336, found 460.2334.

5.1.9. Preparation of (R,E)-ethyl 4-(azidomethyl)-4-hydroxy-6-(4-octylphenyl)hex-2-enoate (19)—A mixture of **16** (47 mg, 0.131 mmol), NaN₃ (26 mg, 0.400 mmol), and NH₄Cl (21 mg, 0.40 mmol) in EtOH (2 mL) was heated at 100 °C via microwave radiation for 1 h. The reaction mixture was allowed to cool to rt and was then filtered through a thin pad of Celite (eluting with EtOAc), and the filtrate was concentrated

under reduced pressure. The crude material was purified by flash chromatography (hexane/EtOAc 20:1, 10:1, to 8:1) on silica gel to provide azide **19** (34 mg, 65%). ^1H NMR (400 MHz, CDCl_3) δ 0.83-0.92 (m, 3H), 1.20-1.36 (m, 13H), 1.52-1.63 (m, 2H), 1.82-1.92 (m, 1H), 1.93-2.03 (m, 1H), 2.50-2.60 (m, 3H), 2.61-2.71 (m, 1H), 3.37-3.45 (m, 2H), 4.23 (q, $J = 7.1$ Hz, 2H), 6.19 (d, $J = 15.6$ Hz, 1H), 6.90 (d, $J = 15.6$ Hz, 1H), 7.04-7.12 (m, 4H); ^{13}C NMR (100 MHz, CDCl_3) δ 14.1, 14.2, 22.6, 29.1, 29.2, 29.3, 29.4, 31.5, 31.9, 35.5, 39.5, 59.5, 60.7, 75.4, 122.3, 128.1, 128.6, 138.2, 140.8, 149.1, 166.1; ESI-HRMS ($\text{M}+\text{Na}$) $^+$ calcd for $\text{C}_{23}\text{H}_{35}\text{N}_3\text{NaO}_3^+$ 424.2571, found 424.2573.

5.1.10. Preparation of (S,E)-ethyl 4-azido-4-(hydroxymethyl)-6-(4-octylphenyl)hex-2-enoate (20) and (S)-5-(4-octylphenethyl)-5-azido-5,6-dihydropyran-2-one (21)—

$\text{Ti}(\text{O}-i\text{Pr})_4$ (400 mg, 1.40 mmol) and TMSN_3 (394 μL , 3.00 mmol) were added to anhydrous toluene (10 mL), and the mixture was heated at reflux at 75 $^\circ\text{C}$ under N_2 overnight. A solution of epoxide **16** (129 mg, 0.36 mmol) in anhydrous toluene (5 mL) was added to the above solution in one portion. The mixture was stirred for 15 min at 85 $^\circ\text{C}$ and was cooled to rt. The solvent was removed under reduced pressure. Et_2O (20 mL) was added, followed by 10% HCl (10 mL). The solution was stirred at rt until two clear phases appeared. The aqueous phase was extracted with Et_2O . The organic phase was dried over MgSO_4 , filtered, and concentrated under reduced pressure. Purification of the residue by flash chromatography (elution with hexane/EtOAc, 10:1, 6:1, 5:1, to 4:1) afforded **21** (58 mg, 45%) and **20** (37 mg, 26%). Data of **20**: ^1H NMR (400 MHz, CDCl_3) δ 0.83-0.92 (m, 3H), 1.20-1.36 (m, 13H), 1.52-1.63 (m, 2H), 1.92-2.01 (m, 1H), 2.03-2.13 (m, 1H), 2.53-2.70 (m, 4H), 3.69-3.78 (m, 2H), 4.22 (q, $J = 7.1$ Hz, 2H), 6.16 (d, $J = 15.7$ Hz, 1H), 6.84 (d, $J = 15.7$ Hz, 1H), 7.04-7.12 (m, 4H); ^{13}C NMR (100 MHz, CDCl_3) δ 14.1, 14.2, 22.6, 29.2, 29.3, 29.4, 29.5, 31.5, 31.9, 35.5, 36.3, 60.9, 67.5, 68.0, 123.6, 128.1, 128.6, 137.9, 140.9, 145.1, 165.9; ESI-HRMS ($\text{M}+\text{Na}$) $^+$ calcd for $\text{C}_{23}\text{H}_{35}\text{N}_3\text{NaO}_3^+$ 424.2571, found 424.2570. Data of **21**: ^1H NMR (400 MHz, CDCl_3) δ 0.83-0.92 (m, 3H), 1.20-1.36 (m, 10H), 1.53-1.63 (m, 2H), 1.86-2.04 (m, 2H), 2.53-2.59 (m, 2H), 2.62-2.78 (m, 2H), 4.34-4.46 (m, 2H), 6.20 (d, $J = 9.8$ Hz, 1H), 6.75 (d, $J = 9.8$ Hz, 1H), 7.04-7.13 (m, 4H); ^{13}C NMR (100 MHz, CDCl_3) δ 14.1, 22.6, 29.0, 29.2, 29.3, 29.4, 31.5, 31.8, 35.5, 37.6, 58.7, 73.6, 122.6, 128.0, 128.7, 137.0, 141.2, 144.7, 161.6; ESI-HRMS ($\text{M}+\text{Na}$) $^+$ calcd for $\text{C}_{21}\text{H}_{29}\text{N}_3\text{NaO}_2^+$ 378.2152, found 378.2151.

5.1.11. Preparation of (S,E)-dimethyl 3-amino-3-(hydroxymethyl)-5-(4-octylphenyl)pent-1-enylphosphonate (22) and (S)-FTY720 vinylphosphonate (2)—

To a solution of azide **17** (50 mg, 0.114 mmol) in 95% MeOH (3 mL) was added SnCl_2 (85 mmol, 0.448 mmol) at rt. After the reaction mixture was stirred at rt overnight, the solvent was removed under reduced pressure. The residue was diluted with Et_2O , and the mixture was neutralized with aqueous saturated NaHCO_3 solution, followed by aqueous NaOH solution (15%). The aqueous layer was extracted with Et_2O , and the combined organic layers were dried (K_2CO_3), filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography ($\text{CHCl}_3/\text{MeOH}$ 20:1, $\text{CHCl}_3/\text{MeOH}/\text{NH}_4\text{OH}$ 130:25:4, $\text{CHCl}_3/\text{MeOH}/\text{AcOH}$ 65:25:4) to give 8 mg (17%) of **22** and 30 mg (69%) of (S)-FTY720 vinylphosphonate (**2**). Data of **22**: The ^1H , ^{13}C , and ^{31}P NMR spectra of **22** matched the reported data;⁵ see the Supporting Information; $[\alpha]_D^{25} +20.0$ (c 0.18, CHCl_3) [lit.⁵ $[\alpha]_D^{25} +18.8$ (c 1.52, CHCl_3)]; ESI-HRMS ($\text{M}+\text{H}$) $^+$ calcd for $\text{C}_{22}\text{H}_{40}\text{NO}_4\text{P}^+$ 414.2758, found 414.2769. Data of **2**: ^1H , ^{13}C , and ^{31}P NMR spectra of **2** matched the reported data;⁵ see the Supporting Information.

5.1.12. Preparation of (S,E)-3-azido-3-(hydroxymethyl)-5-(4-octylphenyl)pent-1-enylphosphonic acid (5)—To a solution of **17** (10 mg, 0.023 mmol) in 2 mL of dry CH_2Cl_2 at rt was added 30 μL (0.23 mmol) of TMSBr. After the reaction mixture had been

stirred for 6 h, the solvent was removed, and the residue was dried and dissolved in 2 mL of 95% MeOH with stirring for 1 h. Removal of the solvent afforded 9 mg (100%) of **5**. ^1H NMR (500 MHz, CDCl_3) δ 0.83-0.92 (m, 3H), 1.20-1.36 (m, 10H), 1.52-1.63 (m, 2H), 1.85-1.94 (m, 1H), 1.97-2.07 (m, 1H), 2.52-2.68 (m, 4H), 3.67-3.74 (m, 2H), 6.09-6.22 (m, 1H), 6.45-6.59 (m, 1H), 7.07-7.13 (m, 4H); ^{13}C NMR (125 MHz, CDCl_3) δ 13.7, 22.4, 28.98, 29.04, 29.1, 29.2, 29.4, 31.3, 31.6, 35.2, 36.0, 68.4 (d, J = 18.4 Hz), 127.8, 128.4, 138.1, 140.5, 146.7; ^{31}P NMR (202 MHz, CDCl_3) δ 15.5; ESI-HRMS ($\text{M}+\text{H}$) $^+$ calcd for $\text{C}_{22}\text{H}_{33}\text{N}_3\text{O}_4\text{P}^+$ 410.2203, found 410.2206.

5.1.13. Preparation of (S,E)-dimethyl 3-azido-3-(fluoromethyl)-5-(4-octylphenyl)pent-1-enylphosphonate (23)—To a solution of alcohol **17** (75 mg, 0.171 mmol) in anhydrous CH_2Cl_2 (1.0 mL) was added diethylaminosulfur trifluoride (DAST) (68 μL , 0.515 mmol) at -78°C . The reaction mixture was stored at -78°C overnight, and then was stirred at rt for 3 h. The mixture was poured into aqueous saturated NaHCO_3 solution, the aqueous phase was extracted with CH_2Cl_2 , and the combined CH_2Cl_2 layers were dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Purification by flash chromatography (elution with PhMe/EtOAc, 1:1 to 100% EtOAc) afforded **23** (49 mg, 65%), along with 10 mg (13%) of recovered alcohol **17**. ^1H NMR (500 MHz, CDCl_3) δ 0.83-0.92 (m, 3H), 1.20-1.36 (m, 10H), 1.53-1.63 (m, 2H), 1.88-2.02 (m, 1H), 2.12-2.22 (m, 1H), 2.51-2.60 (m, 3H), 2.64-2.72 (m, 1H), 3.34-3.53 (m, 2H), 3.77 (d, J = 11.1 Hz, 6H), 6.12 (dd, J = 17.1, 18.8, 1H), 6.73 (ddd, J = 17.1, 21.4, 22.8 Hz, 1H), 7.03-7.13 (m, 4H); ^{13}C NMR (100 MHz, CDCl_3) δ 14.1, 22.6, 28.6, 28.7, 29.2, 29.3, 29.4, 31.5, 31.9, 35.5, 37.4 (d, J = 21.9 Hz), 52.5 (t, J = 5.5 Hz), 56.5 (d, J = 23.9 Hz), 97.5 (dd, J = 19.6, 184.9 Hz), 118.0 (dd, J = 9.6, 187.8 Hz), 128.0, 128.6, 137.5, 141.0, 149.5 (dd, J = 5.8, 20.4 Hz); ^{31}P NMR (162 MHz, CDCl_3) δ 19.6; ^{19}F NMR (376 MHz, CDCl_3) δ -164.5; ESI-HRMS ($\text{M}+\text{H}$) $^+$ calcd for $\text{C}_{22}\text{H}_{36}\text{FN}_3\text{O}_3\text{P}^+$ 440.2473, found 440.2472.

5.1.14. Preparation of (S,E)-dimethyl 3-amino-3-(fluoromethyl)-5-(4-octylphenyl)pent-1-enylphosphonate (24)—A solution of azide **23** (40 mg, 0.091 mmol) in ethanol (2 mL) was treated with Lindlar's catalyst (46 mg). The suspension was purged with H_2 for approximately 10 min and then was stirred for 2 h under a balloon filled with H_2 . The crude reaction mixture was filtered through a short pad of Celite, washed with EtOAc, and concentrated. The residue was purified by flash chromatography ($\text{CHCl}_3/\text{MeOH}$ 20:1) to give 19 mg (51%) of **24**. ^1H NMR (500 MHz, CDCl_3) δ 0.83-0.92 (m, 3H), 1.20-1.36 (m, 10H), 1.53-1.63 (m, 2H), 1.84-1.98 (m, 1H), 2.03-2.13 (m, 1H), 2.51-2.59 (m, 3H), 2.64-2.71 (m, 1H), 2.81-3.00 (m, 2H), 3.76 (dd, J = 3.5, 11.2 Hz, 6H), 6.05 (dd, J = 17.2, 19.9, 1H), 6.72 (ddd, J = 17.2, 22.0, 23.1 Hz, 1H), 7.04-7.12 (m, 4H); ^{13}C NMR (100 MHz, CDCl_3) δ 14.1, 22.6, 28.8, 28.9, 29.2, 29.3, 29.4, 31.5, 31.8, 35.5, 37.6 (d, J = 21.8 Hz), 48.9 (d, J = 25.0 Hz), 52.5 (d, J = 5.5 Hz), 98.6 (dd, J = 18.8, 180.9 Hz), 117.3 (dd, J = 9.6, 188.2 Hz), 128.0, 128.5, 138.0, 140.8, 151.5 (dd, J = 5.7, 20.9 Hz); ^{31}P NMR (162 MHz, CDCl_3) δ 20.3; ^{19}F NMR (376 MHz, CDCl_3) δ -170.3.

5.1.15. Preparation of (S,E)-3-amino-3-(fluoromethyl)-5-(4-octylphenyl)pent-1-enylphosphonic acid (3)—To a solution of **24** (19 mg, 0.046 mmol) in 2 mL of dry CH_2Cl_2 at rt was added 120 μL (0.92 mmol) of TMSBr. After the reaction mixture had been stirred for 6 h, the solvent was removed, and the residue was dried and dissolved in 2 mL of 95% MeOH with stirring for 1 h. Removal of the solvent afforded 18 mg (100%) of **3** as a white solid. ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}/\text{CD}_3\text{CO}_2\text{D}$ 80:20:1) δ 0.83-0.92 (m, 3H), 1.20-1.36 (m, 10H), 1.50-1.63 (m, 2H), 1.97-2.16 (m, 2H), 2.50-2.73 (m, 4H), 3.25-3.48 (m, 2H), 6.26-6.43 (m, 1H), 6.46-6.68 (m, 1H), 7.03-7.12 (m, 4H); ^{13}C NMR (125 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}/\text{CD}_3\text{CO}_2\text{D}$ 80:20:1) δ 13.7, 22.3, 27.93, 27.96, 28.96, 29.03, 29.2, 31.3, 31.6, 35.2, 37.9 (d, J = 21.0 Hz), 45.1 (d, J = 22.4 Hz), 95.0 (dd, J = 183.4, 20.2 Hz), 125.8

(d, $J = 180.6$ Hz), 127.7, 128.3, 137.1, 140.7, 142.4 (d, $J = 20.5$ Hz); ^{31}P NMR (162 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}/\text{CD}_3\text{CO}_2\text{D}$ 80:20:1) δ 13.3; ^{19}F NMR (376 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}/\text{CD}_3\text{CO}_2\text{D}$ 80:20:1) δ -167.7; ESI-HRMS ($\text{M}+\text{H}$) $^+$ calcd for $\text{C}_{20}\text{H}_{34}\text{FNO}_3\text{P}^+$ 386.2255, found 386.2256.

5.1.16. Preparation of (S,E)-3-azido-3-(fluoromethyl)-5-(4-octylphenyl)pent-1-enylphosphonic acid (4)—To a solution of **23** (10 mg, 0.023 mmol) in 2 mL of dry CH_2Cl_2 at rt was added 30 μL (0.23 mmol) of TMSBr. After the reaction mixture had been stirred for 6 h, the solvent was removed, and the residue was dried and dissolved in 2 mL of 95% MeOH with stirring for 1 h. Removal of the solvent afforded 10 mg (100%) of **4** as a white solid. ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}/\text{CD}_3\text{CO}_2\text{D}$ 80:20:1) δ 0.83-0.92 (m, 3H), 1.20-1.36 (m, 10H), 1.52-1.63 (m, 2H), 1.87-2.05 (m, 1H), 2.10-2.24 (m, 1H), 2.50-2.69 (m, 4H), 3.40-3.50 (m, 2H), 6.16-6.28 (m, 1H), 6.49-6.68 (m, 1H), 7.04-7.14 (m, 4H); ^{13}C NMR (125 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}/\text{CD}_3\text{CO}_2\text{D}$ 80:20:1) δ 13.7, 22.3, 28.3, 29.0, 29.2, 31.3, 31.6, 35.2, 37.1, 37.3, 56.3 (d, $J = 24.4$ Hz), 97.2 (d, $J = 184.2$ Hz), 127.8, 128.3, 137.7, 140.6, 145.2; ^{31}P NMR (162 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}/\text{CD}_3\text{CO}_2\text{D}$ 80:20:1) δ 15.2; ESI-HRMS ($\text{M}+\text{H}$) $^+$ calcd for $\text{C}_{20}\text{H}_{32}\text{FNO}_3\text{P}^+$ 386.2255, found 386.2256.

5.1.17. Preparation of (S,E)-ethyl 4-amino-4-(hydroxymethyl)-6-(4-octylphenyl)hex-2-enoate (25)—To the solution of azide **20** (12 mg, 0.03 mmol) in anhydrous MeOH (1 mL) was added SnCl_2 (27 mmol, 0.142 mmol) at rt. After the reaction mixture was stirred at rt for 24 h, the solvent was removed under reduced pressure. The residue was diluted with Et_2O , and the mixture was neutralized with aqueous saturated NaHCO_3 solution, followed by aqueous NaOH solution (15%). The aqueous layer was extracted with Et_2O , and the combined organic layers were dried (MgSO_4), filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography ($\text{CHCl}_3/\text{MeOH}$ 40:1 to 20:1) to give 9 mg (80%) of **25**. ^1H NMR (500 MHz, CDCl_3) δ 0.83-0.92 (m, 3H), 1.20-1.36 (m, 13H), 1.52-1.63 (m, 2H), 1.73-1.89 (m, 2H), 2.47-2.64 (m, 4H), 3.48-3.54 (m, 2H), 4.22 (q, $J = 7.1$ Hz, 2H), 6.04 (d, $J = 15.9$ Hz, 1H), 6.98 (d, $J = 15.9$ Hz, 1H), 7.05-7.11 (m, 4H); ^{13}C NMR (100 MHz, CDCl_3) δ 14.1, 14.2, 21.9, 22.7, 29.2, 29.3, 29.4, 29.5, 29.7, 31.5, 31.9, 35.5, 39.7, 58.2, 60.6, 69.0, 121.3, 128.1, 128.5, 138.6, 140.7, 152.2, 166.3; ESI-HRMS ($\text{M}+\text{H}$) $^+$ calcd for $\text{C}_{23}\text{H}_{37}\text{NO}_3^+$ 376.2846, found 376.2849.

5.1.18. Preparation of (S,E)-4-amino-4-(hydroxymethyl)-6-(4-octylphenyl)hex-2-enoic acid (6)—To a solution of ester **25** (9 mg, 0.024 mmol) in THF/MeOH/ H_2O (1 mL/1 mL/0.5 mL) was added $\text{LiOH}\cdot\text{H}_2\text{O}$ (20 mg, 0.48 mmol) at rt. The reaction mixture was stirred overnight at rt, and then was neutralized with 1 M HCl (5 mL). The mixture was separated and the aqueous layer was extracted with CH_2Cl_2 . The combined organic layers were dried (Na_2SO_4), filtered, and concentrated under reduced pressure to provide **6** (8 mg, 96%) as a white solid. ^1H NMR (500 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}/\text{CD}_3\text{CO}_2\text{D}$ 80:20:1) δ 0.83-0.92 (m, 3H), 1.20-1.36 (m, 10H), 1.54-1.63 (m, 2H), 2.01-2.18 (m, 2H), 2.53-2.69 (m, 4H), 3.76-3.82 (m, 2H), 6.15 (d, $J = 16.3$ Hz, 1H), 6.89 (d, $J = 16.3$ Hz, 1H), 7.09-7.12 (m, 4H); ^{13}C NMR (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}/\text{CD}_3\text{CO}_2\text{D}$ 80:20:1) δ 13.7, 22.4, 28.6, 29.0, 29.1, 29.2, 29.5, 31.3, 31.7, 35.3, 36.1, 61.1, 64.0, 124.0, 127.8, 128.4, 136.9, 140.9, 143.4, 167.3; ESI-HRMS ($\text{M}+\text{H}$) $^+$ calcd for $\text{C}_{21}\text{H}_{34}\text{NO}_3^+$ 348.2533, found 348.2536.

5.1.19. Preparation of (S)-5-(4-octylphenethyl)-5-amino-tetrahydropyran-2-one (7)—A solution of **21** (14 mg, 0.039 mmol) in EtOH (5 mL) was treated with Pd/C (14 mg, 10% w/w). The suspension was purged with H_2 for approximately 10 min and then was stirred overnight at rt under a balloon filled with H_2 . The crude reaction mixture was filtered through a short pad of Celite, washed with CH_2Cl_2 , and concentrated. The residue was purified by flash chromatography ($\text{CHCl}_3/\text{MeOH}$ 20:1 to 10:1) to give 6 mg (46%) of **7**. ^1H

NMR (400 MHz, CDCl_3) δ 0.83-0.92 (m, 3H), 1.20-1.36 (m, 10H), 1.53-1.63 (m, 2H), 1.80-1.90 (m, 2H), 1.90-2.09 (m, 2H), 2.34-2.51 (m, 2H), 2.51-2.64 (m, 4H), 3.50 (d, J = 11.3 Hz, 1H), 3.61 (d, J = 11.3 Hz, 1H), 7.04-7.13 (m, 4H); ^{13}C NMR (100 MHz, CDCl_3) δ 14.1, 22.6, 27.8, 29.3, 29.4, 29.5, 29.6, 30.8, 31.6, 31.9, 35.5, 38.8, 63.5, 68.3, 128.1, 128.5, 138.4, 140.7, 178.5; ESI-HRMS ($\text{M}+\text{Na}$) $^+$ calcd for $\text{C}_{21}\text{H}_{31}\text{NNaO}_2^+$ 354.2404, found 354.2404.

5.2. Biology

5.2.1. Cell culture—HEK 293 cells stably over-expressing GFP-SK1 (30-fold increase in SK1 activity versus vector-transfected cells) were cultured in DMEM supplemented with 10% European fetal calf serum, 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, 1% non-essential amino acids, and 0.8% geneticin at 37 °C in 5% CO_2 .

5.2.2. Sphingosine kinase 1 assay—Sphingosine was solubilized in Triton X-100 (final concentration, 0.063% w/v) and combined with 20 mM Tris (pH 7.4), 1 mM EDTA, 1 mM Na_3VO_4 , 40 mM β -glycerophosphate, 1 mM NaF, 0.007% (v/v) β -mercaptoethanol, 20% (v/v) glycerol, 10 $\mu\text{g}/\text{mL}$ aprotinin, 10 $\mu\text{g}/\text{mL}$ soybean trypsin inhibitor, 1 mM PMSF, and 0.5 mM 4-deoxypyridoxine. SK1 activity was determined by incubating 30 μg of recombinant SK1 in lysates from HEK 293 cells for 30 min at 30 °C, in the presence of 3 μM sphingosine and 250 μM [γ - ^{32}P]ATP (4.4×10^4 cpm/nmol) in 10 mM MgCl_2 with or without inhibitor dissolved in DMSO or control (5% v/v DMSO). SK1 reactions were terminated by the addition of 500 μL of 1-butanol and mixed with 1 mL of 2 M KCl. The organic phase containing [^{32}P]-S1P was then extracted by washing twice with 1 mL of 2 M KCl before quantification by Cerenkov counting. The presence or absence of [^{32}P]-labeled products

5.2.3. Testing compounds for ability to act as SK1 substrates in assays in which sphingosine was omitted—The presence or absence of [^{32}P]-labeled products in the butanol phase was assessed by liquid-scintillation counting. No [^{32}P]-labeled products were formed from compounds **3-7**. We have previously used this method to establish that other sphingosine-based compounds may serve as substrates for SK1.³⁶

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
FTY720 and analogues **2-7**.

Figure 2. Effects of compounds 3-7 on SK1 activity

Substrates were 3 μ M sphingosine (which corresponds to the K_m of SK1)⁷ and 250 μ M ATP (n = 3, results expressed as mean inhibition $\pm$ S.E.). Compounds 2-7 were not substrates for SK1 and were each used at 50 μ M. The SK1-selective inhibitor BML-258³⁵ (50 μ M) inhibited SK1 activity by 74.5 $\pm$ 3.3 % (n = 3). Sph denotes sphingosine. The control is set at 100% and represents the SK1 activity against sphingosine alone.

Figure 3.

Concentration response of compound **5** on SK1 activity. Substrates were 3 μ M sphingosine and 250 μ M ATP ($n = 3$, results expressed as mean inhibition $\pm$ S.E.). The control is set at 100% and represents the SK1 activity against sphingosine alone.

Scheme 1.
Synthesis of **14**.

Scheme 2.
Synthesis of **15** and **16** via HWE reaction.

Scheme 3.
Opening of epoxide **15** and **16**.

Scheme 4.
Synthesis of **2** and **5**.

Scheme 5.
Synthesis of **3**, **4**, **6**, and **7**.