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BZM055, an Iodinated Radiotracer Candidate for PET and SPECT Imaging of Myelin and FTY720 Brain Distribution

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FTY720 (fingolimod, Gilenya®) is a sphingosine 1-phosphate (S1P) receptor modulator that shows significant therapeutic efficacy after oral administration to patients of multiple sclerosis. Because FTY720 does not contain any atom whose PET or SPECT radioisotope would have a half-life compatible with its pharmacokinetic properties, it cannot be used directly for imaging. Instead, we propose BZM055 as a surrogate tracer to study its pharmacokinetics and organ distribution in patients and, given that FTY720 accumulates in myelin sheaths, for myelin imaging. BZM055 (**2a**, 2-iodo-FTY720) can be easily radiolabeled with ^{123}I (for SPECT) or ^{124}I (for PET). Not only does it closely mimic the pharmacokinetics and organ distribution of FTY720, but also its affinity, selectivity for S1P receptors, phosphorylation kinetics, and overall physicochemical properties. [^{123}I]BZM055 is currently under development for clinical imaging.

Introduction

FTY720 (fingolimod, Gilenya®, Scheme 1 below) is a sphingosine 1-phosphate (S1P) receptor modulator that has shown significant improvement in relapse rate and MRI outcomes after oral administration to patients of multiple sclerosis (MS).[1] FTY720 prevents lymphocyte egress from lymph nodes,[2] leading to decreased infiltration of lymphocytes into the central nervous system (CNS).[3] Preclinical evidence also suggests that it may provide additional neuroprotection to the CNS by modulation of cerebral S1P receptors.[4,5]

To further study the therapeutic action of FTY720, it would be useful to quantify its pharmacokinetics and organ distribution in patients. As it has already been shown in rodents that FTY720 accumulates in myelin sheaths,[6] we reasoned that an imaging agent based on this drug might also allow myelin imaging and the study of disease progression, in addition to following the pharmacokinetics of FTY720 in the brain tissues of MS patients.

FTY720 does not contain any atom whose positron emission tomography (PET) or single photon emission computed tomography (SPECT) radioisotope would have a half-life compatible with its pharmacokinetic properties. It is therefore not possible to study this drug with such imaging techniques without modifying it slightly. With this in mind, we defined the criteria that would make a radiolabeled derivative of FTY720 an adequate surrogate of the drug, mimicking its pharmacokinetics and organ distribution, while retaining affinity and selectivity for S1P receptors, as well as having similar overall physicochemical properties.

FTY720 itself is inactive at S1P receptors, but is phosphorylated in vivo by sphingosine kinase-2 (SphK2) to (*S*)-FTY720 phosphate (AML629), which is the active moiety that binds to S1P receptors.[2b,7] As a consequence, any derivative that mimics FTY720 should be a substrate of SphK2 and phosphorylated in a similar manner. Besides measuring phosphorylation rates of tracer candidates, we also synthesized their phosphate analogues to enable comparison of their affinity for S1P receptors, and to adequately compare their overall properties with AML629. After all candidate radiotracers were profiled according to these criteria, we selected BZM055 (**2a**) as the closest mimic of the drug for imaging studies.

Results and Discussion

Chemistry

The most common radioisotopes for PET imaging are ^{18}F and ^{11}C. However, their short half-lives (109.8 and 20.3 min, respectively) are not compatible with the distribution kinetics of FTY720 in humans. The alternative approach, using the side chain of FTY720 to attach a chelating group for ^{64}Cu was not considered, as this would have modified its properties too much. Instead, we elected to prepare iodinated derivatives of FTY720. Iodine-123, with a radioactivity half-life of 13.2 h, is widely used for SPECT. In contrast, iodine-124 has a radioactive half-life of 4.2 days and is used for PET. This makes iodine derivatives well-suited candidates for imaging FTY720, the plasma half-life of which in human subjects is six days.[8] Alter-

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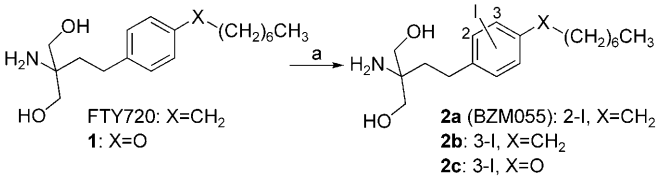
natively, ^{76}Br, which decays with a half-life of 16.1 h, would be a possible alternative for PET imaging.

Based on available structure–activity information, we focused on two options to introduce an iodine atom on the FTY720 scaffold.[9] The first and more straightforward approach is by aromatic iodination (Scheme 1), which we expected to maintain affinity to S1P receptors based on closely related derivatives.[10] This iodination was also performed on **1**,[9] an equipotent alkyl ether analogue of FTY720.



Scheme 1. Iodinating FTY720 and its ether analogue **2**: a) I_2, Ag_2SO_4, CF_3SO_3Ag, RT, 18 h in CH_2Cl_2/CH_3OH: **2a**, **2b** 60% or wet CH_2Cl_2: **2c** 64%.

FTY720 was iodinated by using iodine and silver sulfate in the presence of a catalytic amount of silver trifluoromethanesulfonate[11] to give a 2:1 mixture of **2a** and **2b** in 60% yield. The two regioisomers were separated by supercritical fluid chromatography. In contrast, compound **2c** was obtained as a single regioisomer in 64% yield, starting from aryl alkyl ether **1**.

The synthesis of the corresponding chiral phosphates **5a**–**5c** is outlined in Scheme 2: After protection of **2a**–**2c** as oxazolidinones **3a**–**c**, phosphorylation with di-*tert*-butyl-*N*,*N*-diethylphosphoramidite, oxidation with *t*BuOOH, and subsequent enantiomeric separation using Chiralpak AS yielded the protected phosphate esters **4a**–**c**. Hydrolysis with lithium hydroxide and hydrochloric acid gave the desired phosphates **5a**–**c**.

The second approach to iodinated FTY720 derivatives introduces a halogen atom on the aliphatic side chain (Scheme 3). It is known that the latter can be modified relatively broadly while maintaining affinity for S1P receptors.[12] To prevent deiodination and enhance chemical stability, these derivatives were prepared as vinyl iodides.[13]

It was not possible to directly convert the terminal acetylenic group of an FTY720 analogue to an iodovinylic derivative, as an incomplete and unselective iodination led to inseparable mixtures. Instead, we started from isomerically pure iodovinylic building blocks as depicted in Scheme 3. Compounds **6a**, **6b**, and **6c** were prepared by following modified published procedures,[14,15] followed by treatment under high vacuum to remove contaminating hex-6-yn-1-ol and hex-6-en-1-ol. Mitsunobu condensation with **7**[16] yielded compounds **8a**–**c**, which were then deprotected under acidic conditions to avoid iodolysis, yielding FTY720 iodovinyl analogues **9a**–**c**.

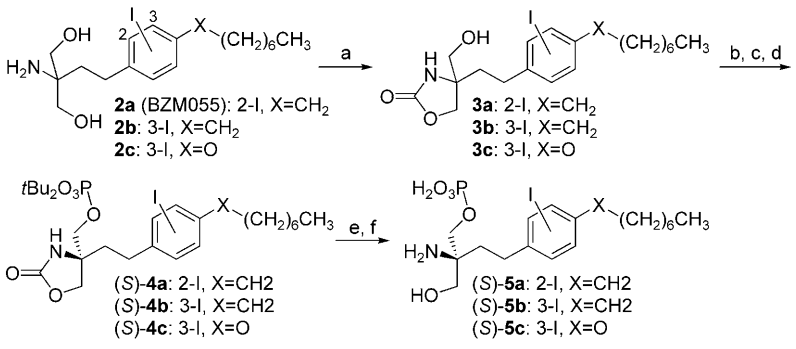
Phosphorylation of **8a**–**c** was carried out under standard conditions, with racemic material rather than after optical resolution, as racemization of the pure enantiomers of **8a**–**c** had been observed at room temperature. Compounds **10a**–**c** were isolated as mixtures with hydroxylated methyl oxazoles as byproducts. They were used in the next step without further purification. Deprotection under acidic conditions yielded iodovinylic phosphonates **11a**–**c** as off-white powders. Because it was not possible to separate the enantiomers of **11a**–**c** using classical Chiralpack AS, those were further characterized as racemic mixtures.

Profiling

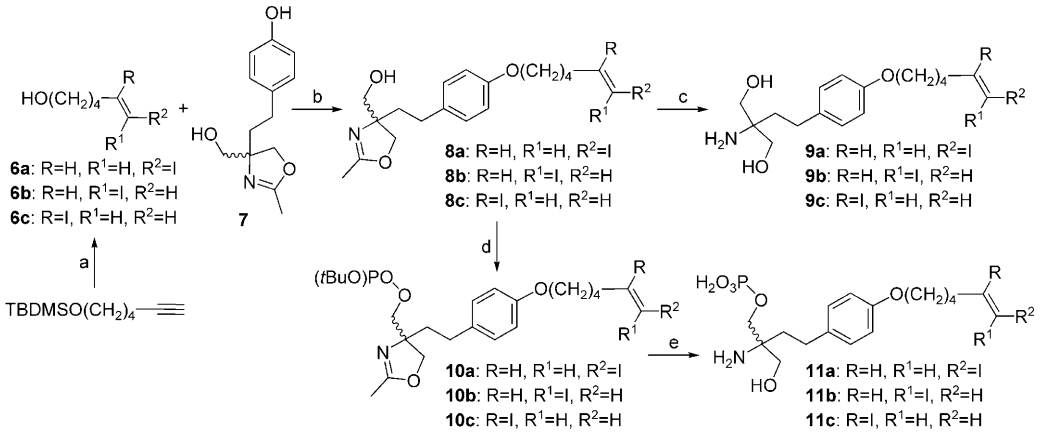
The addition of an iodine atom to FTY720 has a significant effect on its molecular weight, corresponding to a mass increase of 41% (33% for the phosphate). It also significantly increases lipophilicity, as indicated by the log*D* (pH 7.4) values of **2a** and **2b**, relative to FTY720 (Table 1). Other compounds listed in this table have lower log*D* values, resulting from an oxygen atom inserted in the side chain. Interestingly, despite these changes the affinity for S1P receptors (Table 2) was hardly altered, except for a decrease in S1P3 affinity.

Relative to the natural substrate D-sphingosine, FTY720 is phosphorylated by human recombinant SphK2 at a rate of 14.4% relative to D-sphingosine.[7] The rates for the iodinated derivatives are similarly low, ranging from 2.4 to 9% (Table 1). Overall, this conversion remains rapid relative to the distribution kinetics of both species in vivo,[8] and minor differences are not expected to have an influence on imaging properties.

FTY720 is mainly metabolized in the liver by hydroxylation and oxidation of the alkyl chain,[17] and it was previously shown that a rat PBPK model can predict human PK.[6b] We therefore used in vitro clearance measurements (CL'_{int}[18]) in rat microsomes (Table 1) to compare the anticipated metabolic rates of **2a**–**c** and **9a**–**c**. These results show that iodophenyl deriva-



Scheme 2. Phosphorylation of iodinated FTY720 derivatives: a) benzylchloroformate, NaOH, RT, 12 h: **3a** 75%, **3b** 77%, **3c** 78%; b) $(tBuO)_2PN(Et)_2$, 1*H*-tetrazole, CH_2Cl_2/THF, RT, 18 h; c) *t*BuOOH, RT, 90 min; d) Chiralpak separation; e) $LiOH{\cdot}H_2O$, 60 °C, 20 h; f) HCl, RT, 1 h: (*S*)-**5a** 32%, (*S*)-**5b** 63%, (*S*)-**5c** 57%.



Scheme 3. Synthesis of iodovinylic FTY720 analogues **11a–c**: a) **6a**: 1) Cp_2ZrCl_2/$LiEt_3BH$, THF, 2) *N*-iodosuccinimide, 3) AcOH/TEA (overall yield 19%); **6b**: 1) ref. [14], 2) AcOH/TEA; **6c**: ref. [15]; b) DIAD, PPh_3, THF, RT: **8a** 59%, **8b** 53%, **8c** 55%; c) 33% $HCl_{(aq)}$, EtOH, 85 °C, 2.5 h: **9a** 78%, **9b** 33% or 33% $HCl_{(aq)}$, dioxane, 50 °C, 20 h, then 70 °C, 4 h: **9c** 54%; d) 1) 1*H*-tetrazole, $(tBuO)_2P(O)O$, CH_2Cl_2, 6 h, RT, 2) 30% wt H_2O_2, 90 min, RT: **10a** 32% (50% purity), **10b** 98% (70% purity), **10c** 80% (70% purity); e) 33% $HCl_{(aq)}$, EtOH, 85 °C, 2.5 h: **11a** 84%, **11b** 42% or 33% $HCl_{(aq)}$, dioxane, 50 °C, 4 h: **11c** 30%.

Table 1. Profile of iodinated derivatives of FTY720 and their phosphorylated analogues.

Parent	log *D*[a]	M_r [Da]	PSA [Å^2]	Phos. Rate [%][b]	CL'_{int} [mL min^{-1} kg^{-1}][c]
FTY720	1.9	307	66.5	14.4 ± 1.2	13.7
2a (BZM055)	2.9	433	66.5	9.0 ± 0.5	30.4
2b	2.8	433	66.5	5.6 ± 0.3	5.5
2c	2.0	435	75.7	2.7 ± 0.15	< 5.5
9a	0.9	419	75.7	2.4 ± 0.2	34.3
9b	1.0	419	75.7	2.7 ± 0.2	48.8
9c	0.7	419	76	6.0 ± 0.4	38.5

[a] Measured by HPLC. [b] Phosphorylation rate relative to S1P; $n = 3$. [c] In vitro clearance measured with rat liver microsomes.

tives **2b** and especially **2c** have lower clearance than FTY720, whereas clearance is higher for **2a** and iodovinyl derivatives **9a–c**.

Compound **2c** has properties that clearly differ from FTY720, especially with regards to its very low clearance. In contrast, the iodovinyl derivatives **9a–c** are metabolized faster than FTY720. They are also the compounds with the greatest structural differences from the drug, and possibly the most challenging radiochemistry. As a consequence, they do not seem to offer any advantage over other derivatives. We hence selected **2a** and **2b**, which have the closest profile to FTY720, for further evaluation.

As a next step, and because PET tracers are usually given intravenously, we compared the rat PK/PD profiles of these two tracer candidates with those of FTY720, after i.v. administration. Compound **2a** (BZM055) shows a concentration profile similar to that of FTY720 in blood. In the brain, its profile also follows the same kinetics, although with a 2.8-fold lower exposure. Phosphate **5a** has similar kinetics to AML629 in blood and brain, equally with a lower exposure, possibly due to a slightly larger distribution volume (Figure 1).

Compound **2b** has similar properties to FTY720 as well; however, the blood AUC of its phosphate **5b** is proportionally lower. Furthermore, **5b** only penetrates the brain to a limited extent: With a brain-to-blood AUC ratio of 0.2, it differs significantly from AML629 and **5a**, which have ratios of 1.4 and 1.2, respectively (Table 3). This indicates that although **2b** might be a valuable tool to quantify the contribution of brain penetra-

Table 2. Affinity and maximal efficacy values for S1P receptors .

		S1P1		S1P3		S1P4		S1P5	
Phosphates	M_r [Da]	EC_{50} [nM][a]	E_{max} [%][b]	EC_{50} [nM][a]	E_{max} [%][b]	EC_{50} [nM][a]	E_{max} [%][b]	EC_{50} [nM][a]	E_{max} [%][b]
AML629	387	0.17 ± 0.067 (10)	96	2.5 ± 0.9 (6)	97	0.9 ± 0.33 (4)	68	0.58 ± 0.32 (15)	64
5a	513	0.6 ± 0.37 (3)	104	47 ± 25 (2)	44	7.1 ± 3 (2)	98	1.6 ± 0.35 (2)	72
5b	513	0.2 ± 0.12 (3)	107	85 ± 78 (2)	28	5 ± 2.1 (2)	118	2.1 ± 0.5 (2)	87
5c	515	0.8 ± 0.2 (3)	96	33 ± 16 (2)	31	2.5 ± 1.1 (3)	136	1.1 ± 0.5 (4)	66
11a	499	1.7 ± 1.4 (2)	93	7.5 ± 2.5 (4)	60	1.7 ± 1 (4)	127	0.6 ± 0 (3)	62
11b	499	3.2 ± 1.6 (3)	89	11 ± 11 (3)	67	3.8 ± 2.6 (2)	114	13 ± 14 (3)	68
11c	499	3.5 ± 2.6 (2)	88	11 ± 2.2 (2)	66	2.9 ± 0.5 (3)	118	3.5 ± 1.8 (3)	59

[a] Affinity values were estimated with a [γ-^{35}S]GTP binding assay; values represent the mean ± SEM (*n*). [b] E_{max}: maximal efficacy of analogue of S1P at S1P1, S1P3, and S1P5 and AFD(*R*)[19] at S1P4.

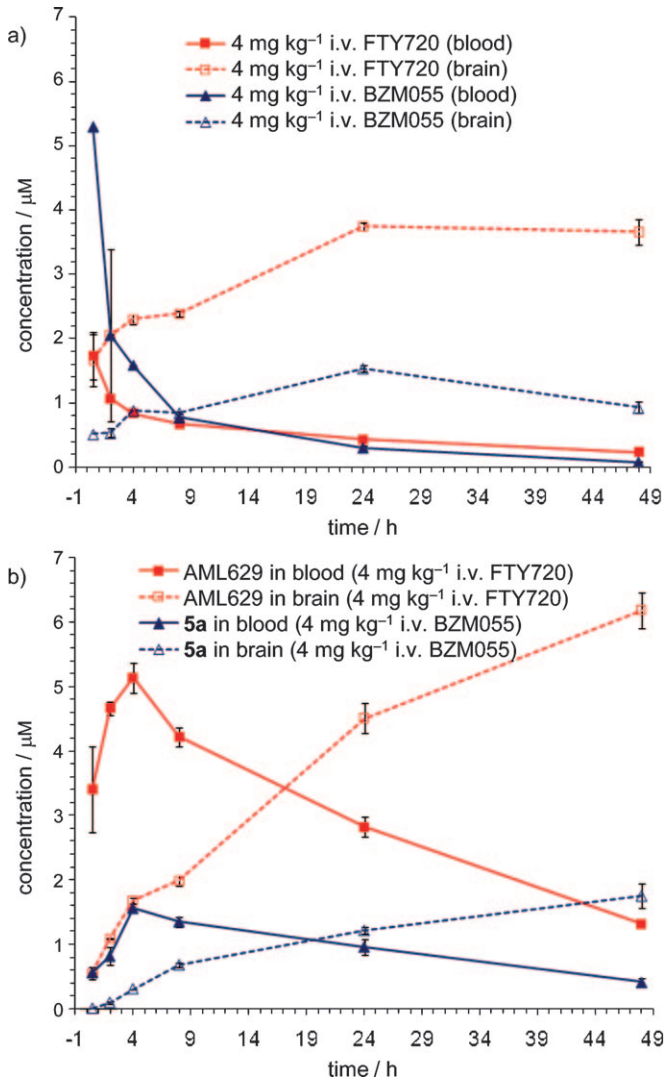


Figure 1. Blood–brain distribution of a) amino alcohols and b) phosphates: comparison of the time–concentration curves for BZM055 and **5a** relative to FTY720 and its phosphate AML629 after intravenous administration. For technical reasons $n=1$ for the 0.5 and 4 h time points, for all others $n=3$; error bars represent the mean ±SEM.

tion to the therapeutic efficacy of S1P agonists, it would not be an adequate surrogate of FTY720 for in vivo imaging.

Pharmacodynamic effects on lymphocyte depletion were measured in the same experiment as the PK properties of parent and phosphates, providing additional information on the similarity of these molecules. As an example, when administered to rats at 4 mg kg^{-1} i.v., BZM055 and **2b** induced a similar decrease in circulating lymphocytes as did FTY720, with similar kinetics and amplitudes. Their blood exposures induced full efficacy on peripheral lymphocyte reduction over 48 h. In contrast, the effect of **9c**, an iodovinyl derivative tested for comparison, started to subside toward the end of the observation period (Figure 2).

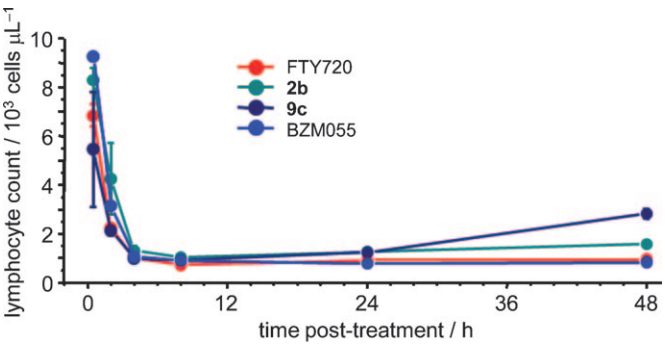


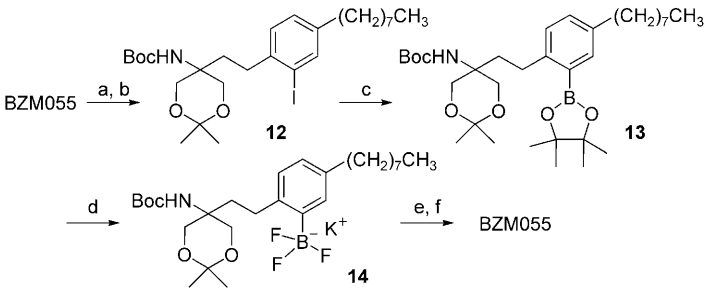
Figure 2. Kinetics of decreasing lymphocyte count in Lewis rats treated with BZM055, **2b**, and **9c** versus FTY720 (each at 4 mg kg^{-1} i.v., $n=3$).

Based on a similar PK profile to that of FTY720, relatively good brain penetration, and a similar rate of phosphorylation, BZM055 was selected for further development. The feasibility of radiosynthesis of BZM055, under conditions that are compatible with PET radiochemistry, was confirmed by using stable iodine (Scheme 4). The aim was to prepare the precursor that would be used for radiolabeling in the SPECT or PET center (compound **14**), and to demonstrate that it could be converted into BZM055 using reagents and conditions approaching those that would be used with radioactive iodine.

The precursor for radiolabeling was prepared from BZM055 itself, which was first protected with di-*tert*-butyldicarbonate and 2,2-dimethoxypropane, yielding **12**. This protected derivative was converted into pinacolato boronate **13**. However, direct iodination of **13** as well as of the corresponding neopentyl boronate, was unsuccessful. We therefore converted **13** into the more reactive potassium trifluoroborate **14** using aqueous potassium hydrogen fluoride.[20] In a cold run approaching PET radiolabeling conditions, **14** was converted back into BZM055 by reaction with sodium iodide in the presence of chloramine T in ~60% yield (10 mg scale). This

Table 3. PK parameters of FTY720, BZM055, **2b** and their phosphates.

Compd		$AUC_{0.5-48h}$ [µM·h]	C_{max} [µM]	t_{max} [h]	$t_{1/2}$ [h]	Brain/Blood Ratio
FTY720	Blood	23.8	1.7	0.5	26.3	
	Brain	*154.4*	*3.7*	*24*	*n.a.*	*6.5*
BZM055	Blood	26.9	5.3	0.5	12.2	
	Brain	*54.3*	*1.5*	*24*	*n.a.*	*2.0*
2b	Blood	29.8	1.2	2	22.5	
	Brain	*47.5*	*1.3*	*48*	*n.a.*	*1.6*
AML629	Blood	140.5	5.1	4	23.6	
	Brain	*191.6*	*6.2*	*48*	*n.a.*	*1.4*
5a	Blood	44.1	1.6	4	23.1	
	Brain	*53.2*	*1.8*	*48*	*n.a.*	*1.2*
5b	Blood	13.4	0.5	8	27.1	
	Brain	*2.1*	*0.05*	*48*	*n.a.*	*0.2*



Scheme 4. Synthesis of precursor **14** and iodination to BZM055 under conditions compatible with SPECT radiochemistry: a) Boc_2O, NaOH, dioxane, RT, 18 h, 83%; b) 2,2-dimethoxypropane, acetone, *p*TsOH, DMF, RT, 20 h, **12** 88%; c) bis(pinacolato)diboron, [$PdCl_2$(dppf)], KOAc, DMSO, 80 °C, 6 h, **13** 68%; d) aq. KHF_2, CH_3OH, RT, 15 min, **14** 74%; e) NaI (0.2 equiv), chloramine T, THF/H_2O (1 mL), 50 °C, 2 h; f) HCl (6 N), 50 °C, 1 h, **16** 60%.

reaction was later used for the production of [^{123}I]BZM055 for imaging studies.

Conclusions

A series of six iodinated FTY720 analogues were synthesized and profiled as potential surrogates of the drug, for imaging purposes. Iodovinyl and 3-substituted derivatives proved to differ from FTY720 in their properties, among others due to lower brain penetration of their phosphate, or faster metabolism. In contrast, 2-iodo-FTY720 (BZM055) is a promising tracer candidate for studying the pharmacokinetics and organ distribution of FTY720 in human subjects, as well as for imaging myelin sheaths. Despite having an additional iodine atom relative to FTY720, BZM055 demonstrates very similar properties, both in terms of the biological activity of its phosphate (affinity for S1P receptors, activity in the lymphocyte depletion assay) and pharmacokinetics properties of both the parent and phosphorylated analogue. Imaging studies with [^{123}I]BZM055 are ongoing, and the results will be reported in due course.

In contrast, and despite its resemblance to FTY720 and BZM055, compound **2b** is phosphorylated to a form that penetrates the brain to a much smaller extent. This property makes it unsuitable as a radiotracer to mimic the distribution of FTY720 in vivo. Nevertheless, **2b** remains an interesting tool, as it might allow study of the importance of central S1P receptor modulation on the pathogenesis of MS, for instance by comparing its efficacy in animal models of the disease to BZM055.

Experimental Section

Chemistry

All chemicals, reagents, and solvents for the synthesis of the compounds were analytical grade, purchased from commercial sources and used without purification, unless otherwise specified. ^{1}H NMR spectra were acquired on Bruker (360 MHz), Varian Mercury (400 MHz), or Bruker Advance (600 MHz) instruments. Chemical shift (δ) values are reported in parts per million (ppm) relative to the residual solvent peak. IR spectra were measured in transmission as a solid film on a Bruker Tensor 27 FTIR spectrometer coupled with a Bruker Hyperion 2000 microscope over a wavenumber range of 4000–600 cm^{-1}, with a resolution of 4 cm^{-1}. High-resolution and high-accuracy mass spectra were acquired on a LTQ Orbitrap XL mass spectrometer (Thermo Scientific) equipped with an electrospray ion source operated in positive ion mode.

Analytical LC–MS/HPLC conditions (% = percent by volume)

Method A (t_{RA} = retention time A): UPLC-ZQ2000, column: Acquity HSS-T3 1.8 μm, 2.1×50 mm; gradient: A, H_2O + 5% CH_3CN + 0.5–1.0% HCO_2H; B, CH_3CN + 0.5–1.0% HCO_2H; from 98:2 to 2:98 in 4.3 min + 0.7 min isocratic; flow rate: 1.0 mL min^{-1}.

Method B (t_{RB} = retention time B): Gilson 331 pumps coupled to a Gilson UV/Vis 152 detector and a Finnigan AQA spectrometer (ESI), a 50 mL loop injection valve, and a Waters XTerra MS C_{18} 3.5 mm 4.6×50 mm column running a gradient of H_2O + 0.05% TFA/CH_3CN + 0.05% TFA from 95:5 to 10:90 over 8 min with a flow rate of 1.5 mL min^{-1}.

Preparative HPLC

Method C: Gilson Trilution LC, column: SunFire C_{18} 5 μm, 30× 100 mm, eluent: H_2O (+0.1% TFA)/CH_3CN (+0.1% TFA) from 85:15 to 65:35 in 16 min; flow rate: 50 mL min^{-1}.

Compounds

2-Amino-2-[2-(2-iodo-4-octylphenyl)ethyl]-1,3-propandiol (2a) and 2-amino-2-[2-(3-iodo-4-octylphenyl)ethyl]-1,3-propandiol (2b): Ag_2SO_4 (5.07 g, 16.3 mmol) and I_2 (4.13 g, 16.3 mmol) were added to a stirred solution of FTY720 (5.0 g, 16.3 mmol) in CH_2Cl_2 (150 mL) and CH_3OH (7.5 mL) at RT, which was then treated with CF_3SO_3Ag (0.21 g, 0.8 mmol), and stirred for 18 h at 30 °C. The yellow solid AgI was filtered off, the organic phase washed with 15% aqueous $NaHCO_3$, dried over Na_2SO_4, filtered and evaporated to dryness. The residue was purified using SFC to yield, after evaporation, **2a** (2.1 g, 60%) and **2b** (2.1 g, 60%) as white solids: Thar SFC 200, Chiralpak IC, 30×250 mm, isocratic: CO_2/2-propanol/2-propylamine 75:25:0.25; flow rate: 90 g min^{-1}; back pressure: 15× 10^6 Pa.

2a: SFC t_R = 5.58 min; mp: 84 °C; ^{1}H NMR (600 MHz, [D_6]DMSO): δ = 7.61 (s, 1H), 7.19–7.06 (m, 2H), 4.54 (br s, 2H), 3.17–3.27 (m, 4H), 2.58–2.64 (m, 2H), 2.41–2.47 (m, 2H), 2.06 (br s, 2H), 1.39–1.42 (m, 4H), 1.20–1.31 (m, 10H), 0.85 ppm (t, J = 6.8 Hz, 3H); ^{13}C NMR (600 MHz, [D_6]DMSO): δ = 142.6, 142.1, 138.5, 129.0, 128.6, 100.5, 65.1, 55.7, 35.4, 33.9, 33.6, 31.2, 30.8, 28.7, 28.6, 28.5, 22.0, 13.9 ppm; IR (solid film): $\tilde{\nu}$ = 3373, 3344, 3235, 3055, 2953, 2920, 2851, 1578, 1463, 1454, 1060, 1045, 1035, 983 cm^{-1}; UPLC–MS[SQD] (ES+): *m/z* 434 [*M*+H]; FTMS-pESI *m/z* $[M+H]^+$; Anal. calcd for $C_{19}H_{33}O_2NI$: 434.15505, found: 434.15522.

2b: SFC t_R = 4.82 min; mp: 63 °C; ^{1}H NMR (600 MHz, [D_6]DMSO): δ = 7.63 (s, 1H), 7.09–7.27 (m, 2H), 4.50 (br s, 2H), 3.10–3.29 (m, 4H), 2.59 (t, J = 7.5 Hz, 2H), 2.55–2.36 (m, 2H), 1.65 (br s, 2H), 1.39–1.55 (m, 4H), 1.16–1.35 (m, 10H), 0.74–0.93 ppm (m, 3H); ^{13}C NMR (600 MHz, [D_6]DMSO): δ = 143.3, 141.5, 138.4, 129.2, 128.4, 100.5, 65.1, 55.6, 39.0, 36.5, 31.2, 29.9, 28.7, 28.7, 28.6, 28.4, 27.8, 22.1, 13.9 ppm; IR (solid film): $\tilde{\nu}$ = 3346, 3283, 3043, 2951, 2920, 2853, 2705, 1576, 1464, 1456, 1049, 1022, 961, 828 cm^{-1}; UPLC–MS[SQD]

(ES+): *m/z* 434 [*M*+H]; FTMS-pESI *m/z* [*M*+H]$^+$; Anal. calcd for $C_{19}H_{33}O_2NI$: 434.15505, found: 434.15519.

2-Amino-2-[2-[3-iodo-4-(heptyloxy)phenyl]ethyl]-1,3-propanediol (2c): Compound **2c** was obtained as a white solid (2.7 g, 64%) from **1** according to the procedure described for the synthesis of **2a** and **2b** by using wet CH_2Cl_2 as solvent. Purification of the residue by flash chromatography (CH_2Cl_2/CH_3OH/NH_4OH, 90:10:1) gave **2c** as white solid (2.7 g, 64%): t_{RA} = 2.62 min; mp: 71 °C; ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ = 7.57 (d, *J* = 6.7 Hz, 1H), 7.13 (dd, *J* = 8.4, 1.7 Hz, 1H), 6.87 (d, *J* = 8.5 Hz, 1H), 4.47 (brs, 2H), 3.96 (t, *J* = 6.3 Hz, 2H), 3.27–3.07 (m, 4H), 2.49–2.42 (m, 2H), 1.61–1.76 (m, 2H), 1.49–1.39 (brs, 2H), 1.36–1.18 (m, 10H), 0.86 ppm (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (600 MHz, [D$_6$]DMSO): δ = 155.0, 138.3, 137.6, 129.3, 112.4, 86.5, 68.5, 65.2, 55.5, 36.8, 31.3, 28.3, 25.5, 22.0, 13.4 ppm; IR (solid film): $\tilde{\nu}$ = 3351, 3322, 3289, 3123, 2923, 2856, 1598, 1492, 1471, 1280, 1250, 1078, 1039, 979, 811 cm^{-1}; UPLC–MS[SQD] (ES+): *m/z* 435 [*M*]; FTMS-pESI *m/z* [*M*+H]$^+$; Anal. calcd for $C_{18}H_{30}O_3NI$: 435.12703, found: 435.12698.

4-Hydroxymethyl-4-[2-(2-iodo-4-octylphenyl)ethyl]oxazolidin-2-one (3a): Benzyl chloroformate (0.25 mL, 1.7 mmol) was added to a suspension of **2b** (0.68 g, 1.6 mmol) in 2 N NaOH (10 mL), the mixture was stirred at RT overnight, then acidified with 1 N HCl and extracted with CH_2Cl_2. The combined organic phases were dried over Na_2SO_4, filtered and concentrated to give **3a** as a white powder (540 mg, 75%): t_{RA} = 3.77 min; ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ = 7.72 (s, 1H), 7.63 (s, 1H), 7.28–7.11 (m, 2H) 5.14 (t, *J* = 5.55 Hz, 1H) 4.19 (d, *J* = 8.68 Hz, 1H) 4.09 (d, *J* = 8.48 Hz, 1H) 3.42–3.34 (m, 2H) 2.70–2.56 (m, 2H) 2.50–2.45 (m, 2H) 1.64 (t, *J* = 8.68 Hz, 2H) 1.55–1.43 (m, 2H) 1.32–1.15 (m, 10H) 0.85 ppm (t, *J* = 6.96 Hz, 3H); ^{13}C NMR (600 MHz, [D$_6$]DMSO): δ = 158.4, 142.6, 141.1, 138.6, 129.2, 128.7, 100.4, 70.0, 65.2, 60.5, 36.1, 33.9, 31.2, 28.6, 22.1, 14.0 ppm; IR (solid film): $\tilde{\nu}$ = 3318, 3248, 3051, 3028, 2995, 2953, 2926, 2886, 2851, 1769, 1598, 1459, 1407, 1274, 1250, 1052 cm^{-1}; UPLC–MS[SQD] (ES+): *m/z* 460 [*M*+H].

4-Hydroxymethyl-4-[2-(3-iodo-4-octylphenyl)ethyl]oxazolidin-2-one (3b) (815 mg, 77%): Prepared in the same way as compound **3a**: t_{RA} = 3.79 min; ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ = 7.65 (s, 1H), 7.59 (s, 1H), 7.19–7.10 (m, 2H), 5.08 (t, *J* = 5.49 Hz, 1H), 4.16 (d, *J* = 8.46 Hz, 1H), 4.06 (d, *J* = 8.59 Hz, 1H), 3.41–3.30 (m, 2H), 2.69–2.55 (m, 2H), 2.47–2.42 (m, 2H), 1.63 (dd, *J* = 9.66, 7,64 Hz, 2H), 1.52–1.47 (m., 2H), 1.25–1.18 (m, 10H), 0.91–0.74 ppm (m, 3H); ^{13}C NMR (600 MHz, [D$_6$]DMSO): δ = 158.4, 142.0, 141.7, 138.5, 1293, 128.5, 100.6, 69.9, 65.3, 60.6, 37.0, 31.2, 29.9, 28.75, 28.7, 27.7, 22.1, 13.9 ppm; IR (solid film): $\tilde{\nu}$ = 3253, 3045, 3012, 2952, 2934, 2917, 851, 1721, 1471, 1409, 1042, 1019 cm^{-1}; UPLC–MS[SQD] (ES+): *m/z* 460 [*M*+H].

4-[2-(4-Heptyloxy-3-iodophenyl)ethyl]-4-hydroxymethyloxazolidin-2-one (3c) (660 mg, 78%): Prepared in the same way as **3a**: t_{RA} = 3.37 min; ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ = 7.71–7.54 (m, 2H), 7.15 (dd, *J* = 8.39, 1.68 Hz, 1H), 6.88 (d, *J* = 8.39 Hz, 1H), 5.09 (t, *J* = 5.57 Hz, 1H), 4.22–4.08 (m, 1H), 4.04 (d, *J* = 8.70 Hz, 1H), 3.96 (t, *J* = 6.26 Hz, 2H), 3.41–3.24 (m, 2H), 2.58–2.35 (m, 2H), 1.77–1.56 (m, 4H), 1.56–1.38 (m, 2H), 1.38–1.14 (m, 6H), 0.86 (t, *J* = 6.71 Hz, 3H); ^{13}C NMR (600 MHz, [D$_6$]DMSO): δ = 158.4, 155.3, 138.3, 136.0, 129.4, 112.4, 86.6, 69.9, 68.5, 65.3, 60.6, 37.2, 31.2, 28.7, 28.3, 28.0, 27.4, 25.5, 22.0, 14.0 ppm; IR (solid film): $\tilde{\nu}$ = 3422, 3314, 3028, 2930, 2870, 2857, 1727, 1599, 1493, 1400, 1282, 1260, 1043, 944 cm^{-1}; UPLC–MS[SQD] (ES+): *m/z* 462 [*M*+H].

Phosphoric acid di-*tert*-butyl ester (*S*)-4-[2-(2-iodo-4-octylphenyl)-ethyl]-2-oxo-oxazolidin-4-ylmethyl ester ((*S*)-4a): 1*H*-tetrazole (404 mg, 5.77 mmol) and di-*tert*-butyl diethylphosphoramidite (1.04 mL, 3.46 mmol) were added to a solution of **3a** (530 mg, 1.15 mmol) at 0 °C in CH_2Cl_2 (4 mL) and THF (4 mL). After 18 h at RT, 30% aqueous H_2O_2 (0.35 mL, 11.54 mmol) was added dropwise, and the mixture stirred at RT for an additional 90 min. The reaction mixture was quenched with saturated aqueous $Na_2S_2O_3$, and the aqueous phase extracted with CH_2Cl_2. The combined organic fractions were dried over Na_2SO_4, filtered and concentrated. The crude product was purified by flash chromatography on silica gel (MTBE, 100%) to give (*R*/*S*)-**4a** as a yellow oil (295 mg, 39%). Both enantiomers were isolated in optically pure form after preparative enantioselective HPLC (Chiralpak AS-PREP, heptane/EtOH/CH_3OH, 90:5:5, 1 mL min^{-1}, λ 210 nm).

(*S*)-**4a** (110.7 mg): t_R = 5.24 min; *ee* > 99%; $[\alpha]_D^{20}$ = −5.67 (*c* = 0.14 in CH_3Cl_3); ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ = 7.97 (s, 1H), 7.64 (s, 1H), 7.28–7.14 (m, 2H), 4.30–4.12 (m, 2H), 3.83 (d, *J* = 5.05 Hz, 2H), 2.76–2.56 (m, 2H), 2.50–2.38 (m, 2H), 1.81–1.61 (m, 2H), 1.61–1.42 (m, 2H), 1.42–1.34 (m, 18H), 1.34–1.17 (m, 10H), 0.85 ppm (t, *J* = 6.96 Hz, 3H); ^{13}C NMR (600 MHz, [D$_6$]DMSO): δ = 157.97, 142.69, 140.70, 138.68, 129.13, 128.74, 100.38, 82.02, 81.97, 69.60, 69.10, 59.35, 35.66, 33.87, 33.34, 31.26, 30.79, 29.42, 28.74, 28.63, 28.54, 22.08, 13.97 ppm; IR (solid film): $\tilde{\nu}$ = 3244, 2979, 2955, 2926, 2855, 1759, 1483, 1457, 1396, 1371, 1261, 1172, 1040, 1004 cm^{-1}; UPLC–MS[SQD] (ES+): *m/z* 696 [*M*+HCOO$^-$].

Phosphoric acid di-*tert*-butyl ester (*S*)-4-[2-(3-iodo-4-octylphenyl)ethyl]-2-oxo-oxazolidin-4-ylmethyl ester ((*S*)-4b): 1*H*-tetrazole (621 mg, 8.87 mmol) and di-*tert*-butyl diethylphosphoramidite (1.59 mL, 5.32 mmol) were added to a solution of **3b** (815 mg, 1.77 mmol) at 0 °C in CH_2Cl_2 (5 mL) and THF (5 mL). After 18 h at RT, 30% aqueous H_2O_2 (0.54 mL, 17.7 mmol) was added dropwise, and the mixture was stirred at RT for an additional 90 min. The reaction mixture was quenched with saturated aqueous $Na_2S_2O_3$, and the aqueous phase extracted with CH_2Cl_2. The combined organic fractions were dried over Na_2SO_4, filtered and concentrated. The crude product was purified by flash chromatography on silica gel (CH_2Cl_2/CH_3OH, 90:10) to give (*R*/*S*)-**4b** as a yellow oil (440 mg, 38%). Both enantiomers were isolated in optically pure form after preparative enantioselective HPLC (Chiralpak AS-PREP, heptane/EtOH/CH_3OH, 80:10:10, 1 mL min^{-1}, λ 210 nm).

(*S*)-**4b** (203 mg): t_R = 11.76 min; *ee* > 98.6%; $[\alpha]_D^{20}$ = −4.67 (*c* = 0.15 in CH_3Cl_3); ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ = 7.88 (s, 1H), 7.68 (s, 1H), 7.23–7.07 (m, 2H), 4.23–4.04 (m, 2H), 3.80–3.61 (m, 2H), 1.88–1.65 (m, 2H), 1.50–1.46 (m, 2H), 1.42–1.40 (m, 18H), 1.37–1.17 (m, 14H), 0.85 ppm (t, *J* = 6.96 Hz, 3H); ^{13}C NMR (600 MHz, [D$_6$]DMSO): δ = 158.03, 142.11, 141.32, 138.54, 129.37, 128.45, 100.67, 81.98, 81.93, 69.48, 69.26, 59.47, 40.02, 36.60, 31.25, 29.92, 29.41, 29.38, 28.77, 28.66, 28.63, 27.44, 22.09, 13.98 ppm; IR (solid film): $\tilde{\nu}$ = 3155, 2984, 2956, 2923, 2856, 1770, 1738, 1409, 1393, 1369, 1243, 1043, 1003 cm^{-1}; UPLC–MS[SQD] (ES+): *m/z* 696 [*M*+HCOO$^-$].

Phosphoric acid di-*tert*-butyl ester (*S*)-4-[2-(4-heptyloxy-3-iodophenyl)ethyl]-2-oxo-oxazolidin-4-ylmethyl ester ((*S*)-4c): 1*H*-tetrazole (501 mg, 7.15 mmol) and di-*tert*-butyl diethylphosphoramidite (1.28 mL, 4.29 mmol) were added to a solution of **3c** (660 mg, 1.43 mmol) at 0 °C in CH_2Cl_2 (5 mL) and THF (5 mL). After 18 h at RT, 30% aqueous H_2O_2 (0.44 mL, 14.3 mmol) was added dropwise and the mixture was stirred at RT for an additional 90 min. The reaction mixture was quenched with saturated aqueous $Na_2S_2O_3$, and the aqueous phase extracted with CH_2Cl_2. The combined organic fractions were dried over Na_2SO_4, filtered and concentrated. The crude product was purified by flash chromatography on silica gel (CH_2Cl_2/CH_3OH, 90:10) to give (*R*/*S*)-**4c** as a yellow oil (580 mg, 62%). Both enantiomers were isolated in optically pure form after

preparative enantioselective HPLC (Chiralpak AS-PREP, hexane/EtOH/CH_3OH, 70:15:15 + 0.1% DEA, 1 mL min^{-1}, λ 220 nm).

(*S*)-**4c** (180 mg): t_R = 8.19 min; *ee* > 99.9%; $[\alpha]_D^{20} = +4.68$ (*c* = 0.17 in CH_3Cl_3); ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ = 7.88 (s, 1H), 7.62 (d, *J* = 1.82 Hz, 1H), 7.17 (dd, *J* = 8.28, 1.82 Hz, 1H), 6.90 (d, *J* = 8.28 Hz, 1H), 4.17–4.11 (m, 2H), 3.97 (t, *J* = 6.16 Hz, 2H), 3.78 (d, *J* = 4.84 Hz, 2H), 2.56–2.44 (m, 2H), 1.78–1.63 (m, 4H), 1.47–1.37 (m, 20H), 1.35–1.23 (m, 6H), 0.86 ppm (t, *J* = 6.86 Hz, 3H); ^{13}C NMR (600 MHz, [D$_6$]DMSO): δ = 158.03, 155.40, 138.38, 135.53, 129.39, 112.44, 86.70, 81.97, 81.92, 69.51, 69.27, 68.55, 59.44, 36.87, 31.27, 29.41, 28.57, 23.33, 27.15, 25.53, 22.05, 13.98 ppm; IR (solid film): $\tilde{\nu}$ = 3159, 2980, 2953, 2928, 2871, 2857, 1769, 1738, 1599, 1490, 1469, 1406, 1398, 1369, 1281, 1252, 1241, 1043, 1002, 875, 813; UPLC–MS[SQD] (ES+): *m/z* 698 [*M*+HCOO$^-$].

Phosphoric acid mono-[(*S*)-2-amino-2-hydroxymethyl-4-(2-iodo-4-octylphenyl)butyl] ester ((*S*)-5a): 10% aqueous LiOH (0.5 mL, 2.09 mmol) was added to a solution of (*S*)-**4a** (20 mg, 0.031 mmol) in EtOH (0.5 mL). After 20 h at 60 °C, the reaction mixture was cooled to RT and stirred for an additional two days. Concentrated aqueous HCl (0.5 mL) was then added, and the solution was stirred at RT for 1 h, neutralized with 4 N aqueous NaOH, and concentrated. The residue was taken up in CH_2Cl_2 (2 mL), filtered on Hyflo, and the filtrate washed twice with CH_2Cl_2. The solution was concentrated to give (*S*)-**5a** as a white powder (5 mg, 32%); t_R = 135.8 min; *ee* > 92.3% (Sumichiral OA-7000, KH_2PO_4 (20 mM pH 3.0)/CH_3CN, 30:70, 1.5 mL min^{-1}, λ 228 nm); ^{1}H NMR (600 MHz, [D$_6$]DMSO, CF_3COOH 5%): δ = 8.08 (brs, 3H), 7.63 (s, 1H), 7.24–7.11 (m, 2H), 4.03–4.99 (m, 2H), 3.66–3.54 (m, 2H), 2.71–2.59 (m, 2H), 2.51–2.36 (m, 2H), 1.77 (dd, *J* = 10.19, 7.16 Hz, 2H), 1.52–1.48 (m, 2H), 1.29–1.10 (m, 10H), 0.83 ppm (t, *J* = 6.86 Hz, 3H); ^{13}C NMR (600 MHz, [D$_6$]DMSO, CF_3COOH 5%): δ = 142.84, 140.66, 138.69, 129.09, 128.72, 100.13, 62.01, 61.0, 58.80, 33.88, 32.88, 32.12, 32.21, 30.79, 28.69, 28.55, 22.00, 13.77 ppm; IR (zwitterion, solid film): $\tilde{\nu}$ = 3346, 3114, 3043, 2956, 2924, 2854, 2653, 2578, 1623, 1600, 1539, 1452, 1271, 1160, 1096, 1059, 1040, 834, 815 cm^{-1}; UPLC–MS[SQD] (ES+): *m/z* 514 [*M*+H]; FTMS-pESI *m/z* [*M*+H]$^+$; Anal. calcd for $C_{19}H_{34}O_5NIP$: 514.12138, found: 514.12167. (*S*)-**5b** and (*S*)-**5c** were obtained in a similar manner.

Phosphoric acid mono-[(*S*)-2-amino-2-hydroxymethyl-4-(3-iodo-4-octylphenyl)butyl] ester ((*S*)-5b): 15 mg, 63%; t_R = 22.09 min; *ee* > 90% (Sumichiral OA-7000, KH_2PO_4 (5 mM pH 3.0)/CH_3CN, 50:50, 1.0 mL min^{-1}, λ 224 nm); ^{1}H NMR (600 MHz, [D$_6$]DMSO, CF_3COOH 5%): δ = 8.05 (brs, 3H), 7.68 (s, 1H), 7.23–7.08 (m, 2H), 3.95 (d, *J* = 4.84 Hz, 2H), 3.57–3.53 (m, 2H), 2.60 (t, *J* = 7.57 Hz, 2H), 2.57–2.51 (m, 2H), 1.85–1.83 (m, 2H), 1.49–1.45 (m, 2H), 1.15–1.35 (m, 10H), 0.84 ppm (t, *J* = 6.56 Hz, 3H); ^{13}C NMR (600 MHz, [D$_6$]DMSO, CF_3COOH 5%): δ = 142.31, 141.13, 138.52, 129.35, 128.43, 100.54, 65.00, 60.95, 59.02, 33.16, 31.21, 29.92, 28.71, 28.65, 28.59, 29.96, 22.02, 13.79 ppm; IR (zwitterion, solid film): $\tilde{\nu}$ = 3154, 3042, 2954, 2924, 2853, 2574, 1732, 1623, 1599, 1537, 1465, 1158, 1057, 1043, 814 cm^{-1}; UPLC–MS[SQD] (ES+): *m/z* 514 [*M*+H]; FTMS-pESI *m/z* [*M*+H]$^+$; Anal. calcd for $C_{19}H_{34}O_5NIP$: 514.12138, found: 514.12150.

Phosphoric acid mono-[(*S*)-2-amino-4-(4-heptyloxy-3-iodophenyl)-2-hydroxymethylbutyl] ester ((*S*)-5c): 9 mg, 57%; t_R = 32.6 min; *ee* > 98.0% (Sumichiral OA-7000, KH_2PO_4 (20 mM pH 3.0)/CH_3CN, 40:60, 1.5 mL min^{-1}, λ 228 nm); ^{1}H NMR (600 MHz, [D$_6$]DMSO, CH_3COOH 5%): δ = 8.04 (brs, 3H), 7.62 (s, 1H), 7.15 (d, *J* = 6.86 Hz, 1H), 6.89 (d, *J* = 8.48 Hz, 1H), 3.99–3.93 (m, 4H), 3.55 (brs, 2H), 2.53–2.51 (m, 2H), 1.70 (d, *J* = 11.50 Hz, 2H), 1.73–1.64 (m, 2H), 1.50–1.33 (m, 2H), 1.35–1.17 (m, 6H), 0.85 ppm (t, *J* = 6.76 Hz, 3H); ^{13}C NMR (600 MHz, [D$_6$]DMSO, CH_3COOH 5%): δ = 155.51, 138.37, 135.39, 129.39, 112.37, 86.57, 68.55, 65.02, 60.95, 59.03, 33.45, 31.21, 28.29, 25.49, 21.98, 13.77 ppm; IR (solid film): $\tilde{\nu}$ = 3214, 2926, 2856, 1730, 1626, 1600, 1536, 1513, 1491.1468, 1281, 1251, 1155, 1074, 944, 810 cm^{-1}; UPLC–MS[SQD] (ES+): *m/z* 516 [*M*+H]; FTMS-pESI *m/z* [*M*+H]$^+$; Anal. calcd for $C_{18}H_{32}O_6NIP$: 516.10065, found: 516.10087.

(*E*)-6-Iodohex-5-en-1-ol (6a): In a flame-dried flask, $LiEt_3BH$ (1 M in THF, 7.7 mL, 1.1 equiv) was added to a solution of Cp_2ZrCl_2 (2.27 g, 1.1 equiv) in THF (20 mL). The resulting mixture was stirred for 30 min at RT, then treated with a solution of *tert*-butylhex-5-ynyloxydimethylsilane (1.5 g, 7.01 mmol) in THF (10 mL). The mixture turned into a clear yellow solution within a few seconds after the end of the addition. It was stirred for 30 min at RT prior to the addition of a solution of *N*-iodosuccinimide (1.75 g, 1.1 equiv) in THF, then stirred for an additional 3 h at RT. The mixture was poured into saturated $NaHCO_3$, and the aqueous phase was extracted with EtOAc. The organic phase was washed with 1 M $Na_2S_2O_3$ and brine, dried over Na_2SO_4 and concentrated in vacuo to afford a crude yellow liquid (2.1 g) which was purified by flash chromatography using *c*Hex/EtOAc (100:0→95:5). This yielded *tert*-butyl-((*E*)-6-iodohex-5-enyloxy)dimethylsilane as a colorless oil (1.45 g) with 70% purity. This oil was solubilized in Et_2O/CH_3OH (20 mL, 1:1), and CH_3COCl (1.5 mL) was added dropwise at 0 °C. The mixture was stirred at RT for 61 h. The reaction was quenched with TEA (2.85 mL). The salts were removed by filtration and washed with Et_2O. The filtrate was washed with saturated NH_4Cl, dried over Na_2SO_4, and the solvent was removed in vacuo to give a crude brown oil (1.0 g) which was purified by flash chromatography on silica gel using *c*Hex/EtOAc (100:0→50:50). From the purification, 440 mg of light-brown oil was collected containing **6a** plus 10% hexen-1-ol and 5% hexyn-1-ol. The volatile byproducts were removed under high vacuum to afford pure **6a** as a light-brown oil (330 mg, 58%): R_f = 0.28 (*c*Hex/EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 6.50 (m, 1H), 6.0 (d, *J* = 7.1 Hz, 1H), 3.63 (t, *J* = 7.1 Hz, 2H), 2.09 (m, 2H), 1.7–1.3 ppm (m, 4H).

(4-{2-[4-((*E*)-6-Iodohex-5-enyloxy)phenyl]ethyl}-2-methyl-4,5-dihydro-oxazol-4-yl)methanol (8a): DIAD (0.215 mL, 1.0 equiv) was added at 0 °C to **7** (260 mg, 1.1 mmol), **6a** (250 mg, 1.0 equiv), and PPh_3 (290 mg, 1.0 equiv) in THF (10 mL). The reaction mixture was stirred for 18 h at RT and 24 h at 50 °C, then treated with 0.8 equiv DIAD and PPh_3. The mixture was stirred for an additional 72 h at 50 °C, then partitioned between EtOAc and aqueous saturated NH_4Cl. The organic phase was separated, dried over Na_2SO_4, and concentrated in vacuo to afford a crude beige oil (1.54 g), which was purified by flash chromatography on silica gel using CH_2Cl_2/CH_3OH (100:0→90:10) as eluent. Compound **8a** was isolated as a colorless oil (290 mg, 59% yield): R_f = 0.45 (CH_2Cl_2/CH_3OH, 95:5); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.08 (d, *J* = 8.6 Hz, 2H), 6.79 (d, *J* = 8.6 Hz, 2H), 6.52 (m, 1H), 6.01 (d, *J* = 14 Hz, 1H), 4.18 (AB, *J* = 73, 8.5 Hz, 2H), 3.92 (t, *J* = 6.3 Hz, 2H), 3.58 (AB, *J* = 107, 11.4 Hz, 2H), 2.54 (m, 2H), 2.12 (m, 2H), 2.06 (s, 3H), 1.95–1.50 ppm (m, 6H); LC–MS: t_{RB} = 4.84 min; *m/z*: 444.0 [*M*+H].

(4-{2-[4-((*Z*)-6-Iodohex-5-enyloxy)phenyl]ethyl}-2-methyl-4,5-dihydro-oxazol-4-yl)methanol (8b) and (4-{2-[4-(5-iodohex-5-enyloxy)phenyl]ethyl}-2-methyl-4,5-dihydro-oxazol-4-yl)methanol (8c): Compounds **8b** and **8c** were prepared from **7** and **6b** (250 mg, 1.0 equiv) or **6c** (728 mg, 1.5 equiv) according to the procedure described for the synthesis of **8a**. Compounds **8b** (403 mg, 53.5% yield) and **8c** (520 mg, 54.6% yield) were isolated as clear oils.

8b: R_f = 0.28 (CH_2Cl_2/CH_3OH, 95:5); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.08 (d, J = 8.6 Hz, 2H), 6.81 (d, J = 8.6 Hz, 2H), 6.25–6.15 (m, 2H), 4.21 (AB, J = 74, 8.5 Hz, 2H), 3.94 (t, J = 6.3 Hz, 2H), 3.60 (AB, J = 111, 11.4 Hz, 2H), 2.54 (m, 2H), 2.22 (m, 2H), 2.10 (s, 3H), 1.95–1.55 ppm (m, 6H); LC–MS: t_{RB} = 4.76 min, m/z: 443.9 [M+H].

8c: R_f = 0.31 (CH_2Cl_2/CH_3OH, 95:5); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.08 (d, J = 8.6 Hz, 2H), 6.81 (d, J = 8.6 Hz, 2H), 6.04 (m, 1H), 5.71 (m, 1H), 4.18 (AB, J = 73, 8.5 Hz, 2H), 3.94 (t, J = 6.3 Hz, 2H), 3.58 (AB, J = 107, 11.4 Hz, 2H), 2.54 (m, 2H), 2.45 (m, 2H), 2.06 (s, 3H), 1.95–1.65 ppm (m, 6H); LC–MS: t_{RB} = 4.76 min, m/z: 444.0 [M+H].

2-Amino-2-{2-[4-((*E*)-6-iodohex-5-enyloxy)phenyl]ethyl}propane-1,3-diol·HCl (9a·HCl): Aqueous HCl (33%, 2.05 mL) was added to a solution of **8a** (60 mg, 0.135 mmol) in EtOH (2 mL), and the reaction mixture was stirred at 85 °C for 2.5 h. The solvents were removed in vacuo to afford a beige paste. After trituration in Et_2O, **9a**·HCl was isolated as a beige powder (48 mg, 78% yield). ^{1}H NMR (600 MHz, [D_6]DMSO): δ = 7.87 (br s, 3H), 7.10 (d, J = 8.7 Hz, 2H), 6.84 (d, J = 8.7 Hz, 2H), 6.53 (dt, J = 14.4 and 7.2 Hz, 1H), 6.24 (d, J = 14.4 Hz, 1H), 5.39 (t, J = 5.2 Hz, 2H), 3.91 (t, J = 6.5 Hz, 2H), 3.50 (m, 4H), 2.53 (m, 2H), 2.09 (m, 2H), 1.75 (m, 2H), 1.67 (m, 2H), 1.48 ppm (q, J = 8.3 Hz, 2H); ^{13}C NMR (150 MHz, [D_6]DMSO): δ = 24.3, 27.4, 28.0, 33.4, 35.0, 60.2, 60.9, 77.0, 114.3, 129.1, 133.4, 146.1, 156.8 ppm; IR (solid film): $\tilde{\nu}$ = 3365, 3278, 2938, 2866, 1611, 1582, 1513, 1246, 1179, 1069, 1046, 1027, 949, 829 cm^{-1}; LC–MS: t_{RB} = 4.62 min; m/z: 419.9 [M+H]; HRMS-pESI m/z [M+H]$^+$ calcd for $C_{17}H_{27}O_3NI$: 420.10301, found: 420.10287.

2-Amino-2-{2-[4-((*Z*)-6-iodohex-5-enyloxy)phenyl]ethyl}propane-1,3-diol·TFA (9b·TFA): **9b**·TFA was prepared from **8b** (75 mg, 0.169 mmol) according to the procedure described for the synthesis of **9a**. After an additional purification by reverse preparative HPLC (method C), **9b**·TFA was obtained as a white powder (30 mg, 33% yield). ^{1}H NMR (600 MHz, [D_6]DMSO): δ = 7.77 (br s, 3H), 7.09 (d, J = 8.7 Hz, 2H), 6.84 (d, J = 8.5 Hz, 2H), 6.40 (d, J = 7.3 Hz, 1H), 6.30 (q, J = 7.1 Hz, 1H), 5.39 (t, J = 5.0 Hz, 2H), 3.93 (t, J = 6.5 Hz, 2H), 3.50 (m, 4H), 2.52 (m, 2H), 2.14 (m, 2H), 1.72 (m, 4H), 1.53 ppm (q, J = 7.4 Hz, 2H); ^{13}C NMR (150 MHz, [D_6]DMSO): δ = 24.0, 27.4, 28.1, 33.4, 34.0, 60.1, 61.0, 67.1, 84.6, 114.3, 129.1, 133.3, 140.9, 156.8 ppm; IR (solid film): $\tilde{\nu}$ = 3404, 2983, 2934, 2866, 1596, 1613, 1514, 1246, 1185, 1073, 10489, 837 cm^{-1}; LC–MS: t_{RB} = 4.42 min; m/z: 420.0 [M+H]; HRMS-pESI m/z [M+H]$^+$ calcd for $C_{17}H_{27}O_3NI$: 420.10301, found: 420.10294.

2-Amino-2-{2-[4-(5-iodohex-5-enyloxy)phenyl]ethyl}propane-1,3-diol·HCl (9c·HCl): **9c**·HCl was prepared from **8c** (63 mg, 0.142 mmol) according to the procedure described for the synthesis of **9a**, except that the reaction was performed in dioxane instead of EtOH at 50 °C for 20 h and then 70 °C for 4 h. **9c**·HCl was obtained as a beige powder (35 mg, 54% yield). ^{1}H NMR (600 MHz, [D_6]DMSO): δ = 7.87 (br s, 3H), 7.10 (d, J = 8.5 Hz, 2H), 6.84 (d, J = 8.5 Hz, 2H), 6.19 (s, 1H), 5.72 (s, 1H), 5.39 (t, J = 5.0 Hz, 2H), 3.93 (t, J = 6.5 Hz, 2H), 3.51 (m, 4H), 2.53 (m, 2H), 2.43 (t, J = 7.1 Hz, 2H), 1.74 (m, 2H), 1.68 (m, 2H), 1.58 ppm (m, 2H); ^{13}C NMR (150 MHz, [D_6]DMSO): δ = 25.2, 27.2, 27.4, 33.4, 44.0, 60.2, 60.9, 66.9, 112.7, 114.3, 126.2, 129.1, 133.4, 156.8 ppm; IR (solid film): $\tilde{\nu}$ = 3355, 3274, 2937, 2866, 1613, 1582, 1513, 1246, 1178, 1069, 1046, 893, 831 cm^{-1}; LC–MS: t_{RB} = 4.64 min; m/z: 420.0 [M+H]; HRMS-pESI m/z [M+H]$^+$ calcd for $C_{17}H_{27}O_3NI$: 420.10301, found: 420.10287.

(*R*/*S*)-phosphoric acid di-*tert*-butyl ester 4-{2-[4-((*E*)-6-iodohex-5-enyloxy)phenyl]ethyl}-2-methyl-4,5-dihydro-oxazol-4-ylmethyl ester (10a): 1*H*-tetrazole (182 mg, 5.0 equiv) and di-*tert*-butyldiethylphosphoramidite (0.433 mL, 3.0 equiv) were added to a solution of **8a** (230 mg, 0.52 mmol) at 0 °C in 4 mL CH_2Cl_2/THF (1:1), then stirred at RT for 6 h. H_2O_2 (30% wt.% in H_2O, 0.159 mL, 10 equiv) was added, and the mixture was stirred for 1.5 h at RT. The reaction mixture was quenched by careful addition of a solution of 1 N $Na_2S_2O_3$ (10 mL), and the aqueous phase was extracted with CH_2Cl_2. The organic phases were combined, washed with brine, dried over Na_2SO_4, and concentrated in vacuo to afford a crude oil (500 mg), which was purified by flash chromatography on silica gel (eluent: CH_2Cl_2/CH_3OH 100:0→90:10). Compound **10a** (107 mg) was isolated as clear oil with a purity of ~50% and used as such in the next step. LC–MS: t_{RB} = 5.53 min, m/z: 636.1 [M+H].

(*R*/*S*)-phosphoric acid di-*tert*-butyl ester 4-{2-[4-((*Z*)-6-iodohex-5-enyloxy)phenyl]ethyl}-2-methyl-4,5-dihydro-oxazol-4-ylmethyl ester (10b) and (*R*/*S*)-phosphoric acid di-*tert*-butyl ester 4-{2-[4-(5-iodohex-5-enyloxy)phenyl]ethyl}-2-methyl-4,5-dihydro-oxazol-4-ylmethyl ester (10c): Compounds **10b** and **10c** were prepared from **8b** (330 mg, 0.744 mmol) or **8c** (200 mg, 0.45 mmol) according to the procedure described for the synthesis of **10a**. Compounds **10b** (665 mg, 98% yield, 70% purity) and **10c** (230 mg, 80% yield, 70% purity) were used as such in the next step.

(*R*/*S*)-phosphoric acid mono-{2-amino-2-hydroxymethyl-4-[4-((*E*)-6-iodohex-5-enyloxy)phenyl]butyl} ester (11a): **10a** (107 mg, 0.168 mmol) in EtOH (2.5 mL) was treated with 33% aqueous HCl (2.56 mL) and stirred at 85 °C for 2.5 h, then concentrated in vacuo to afford a beige paste. After trituration in Et_2O, **11a** was isolated as a beige powder (71 mg, 84% yield). ^{1}H NMR (600 MHz, [D_6]DMSO): δ = 8.27 (br s, 3H), 7.10 (d, J = 7.5 Hz, 2H), 6.81 (d, J = 8.1 Hz, 2H), 6.53 (dt, J = 14.4 and 7.0 Hz, 1H), 6.23 (d, J = 14.4 Hz, 1H), 3.9 (m, 4H), 3.4–3.6 (m, 2H), 2.52 (m, 2H), 2.09 (q, J = 7.1 Hz, 2H), 1.77 (m, 2H), 1.66 (m, 2H), 1.48 ppm (m, 2H); ^{13}C NMR (150 MHz, [D_6]DMSO): δ = 24.3, 27.4, 28.0, 33.7, 35.0, 59.3, 60.4, 64.2, 67.0, 77.0, 114.3, 129.1, 133.3, 146.1, 156.8 ppm; IR (solid film): $\tilde{\nu}$ = 2941, 2868, 1620, 1612, 1583, 1540, 1512, 1246, 1180, 1044, 1016, 952, 836 cm^{-1}; LC–MS: t_{RB} = 4.53 min; m/z: 500.0 [M+H]; HRMS-pESI m/z [M+H]$^+$ calcd for $C_{17}H_{28}O_6NIP$: 500.06935, found: 500.06949.

(*R*/*S*)-phosphoric acid mono-{2-amino-2-hydroxymethyl-4-[4-((*Z*)-6-iodohex-5-enyloxy)phenyl]butyl} ester (11b) and (*R*/*S*)-phosphoric acid mono-{2-amino-2-hydroxymethyl-4-[4-(5-iodohex-5-enyloxy)phenyl]butyl} ester (11c): Compounds **11b** and **11c** were prepared from **10b** (330 mg, 0.744 mmol, 70% purity) or **10c** (230 mg, 0.362 mmol, 70% purity) according to the procedure described for the synthesis of **11a**, except that for **11b**, the reaction was performed in dioxane at 50 °C for 4 h. **11b** was isolated as a white powder (152 mg, 42% yield), and after purification by preparative reversed-phase HPLC (method C), **11c** was isolated as a beige powder (54 mg, 30% yield).

11b: ^{1}H NMR (600 MHz, [D_6]DMSO): δ = 8.43 (br s, 3H), 7.10 (d, J = 7.5 Hz, 2H), 6.81 (d, J = 7.7 Hz, 2H), 6.40 (d, J = 7.7 Hz, 1H), 6.30 (q, J = 6.9 Hz, 1H), 3.8–4.0 (m, 4H), 3.4–3.7 (m, 2H), 2.53 (m, 2H), 2.13 (q, J = 7 Hz, 2H), 1.77 (m, 2H), 1.70 (m, 2H), 1.52 ppm (m, 2H); ^{13}C NMR (150 MHz, [D_6]DMSO): δ = 24.0, 27.5, 28.2, 33.8, 34.0, 59.2, 61.0, 64.2, 67.1, 84.6, 114.3, 129.1, 133.5, 140.9, 156.8 ppm; IR (solid film): $\tilde{\nu}$ = 2940, 2865, 1620, 1613, 1583, 1541, 1512, 1245, 1176, 1178, 1068, 948, 828 cm^{-1}; LC–MS. t_{RB} = 4.44 min; m/z: 500.1 [M+H]; HRMS-pESI m/z [M+H]$^+$ calcd for $C_{17}H_{28}O_6NIP$: 500.06935, found: 500.06934.

11c: ^{1}H NMR (600 MHz, [D_6]DMSO): δ = 8.26 (br s, 3H), 7.10 (d, J = 7.3 Hz, 2H), 6.83 (d, J = 7.9 Hz, 2H), 6.19 (s, 1H), 5.72 (s, 1H), 3.92 (m, 4H), 3.54 (m, 2H), 2.52 (m, 2H), 2.43 (t, J = 7.1 Hz, 2H), 1.79 (m, 2H), 1.68 (m, 2H), 1.58 ppm (m, 2H); ^{13}C NMR (150 MHz,

[D_6]DMSO): δ = 25.2, 27.2, 27.4, 33.6, 44.0, 59.2, 60.9, 66.9, 112.7, 114.3, 129.1, 133.3, 156.8 ppm; IR (solid film): $\tilde{\nu}$ = 2940, 2866, 1614, 1540, 1512, 1245, 1179, 1141, 1044, 934, 895, 835 cm^{-1}; LC–MS: t_{RB} = 4.45 min; *m/z*: 499.9 [*M*+H]; HRMS-pESI *m/z* [*M*+H]$^+$ calcd for $C_{17}H_{28}O_6NIP$: 500.06935, found: 500.06943.

{5-[2-(2-Iodo-4-octylphenyl)ethyl]-2,2-dimethyl[1,3]dioxan-5-yl}-carbamic acid *tert*-butyl ester (12): A mixture of **2a** (0.89 g, 2.05 mmol), $(Boc)_2O$ (0.67 g, 3.08 mmol) and 1 N NaOH (2.26 mL, 2.26 mmol) in dioxane (50 mL) was stirred at RT overnight. The reaction mixture was extracted with EtOAc, and the combined organic fractions were dried over Na_2SO_4, filtered, concentrated and purified by flash chromatography on silica gel (hexane/EtOAc, 60:40) to give Boc-**2a** as a colorless oil (905 mg, 83%). 2,2-dimethoxypropane (19.9 mL, 159 mmol), acetone (11.7 mL, 159 mmol), and *p*TsOH·H_2O (30 mg, 0.16 mmol) were added to a solution of Boc-**2a** (850 mg, 1.59 mmol) in DMF (15 mL) and stirred at RT for 1 h. The solution was quenched with saturated aqueous $NaHCO_3$, extracted with EtOAc, and the combined fractions dried over Na_2SO_4, filtered and concentrated. The crude product was purified by flash chromatography on silica gel (hexane/EtOAc, 90:10) to give 800 mg (88%) **12** as a colorless oil. t_{RA} = 4.51 min; ^{1}H NMR (600 MHz, [D_6]DMSO): δ = 7.62 (s, 1H) 7.19–7.08 (m, 2H) 6.69 (br s, 1H) 3.88–3.84 (m, 2H) 3.71 (d, *J* = 11.71 Hz, 2H) 2.49–2.64 (m, 2H) 2.30–2.47 (m, 2H) 1.94–1.77 (m, 2H) 1.46–1.44 (m, 2H) 1.43–1.37 (m, 9H) 1.36 (s, 3H) 1.34 (s, 3H) 1.16–1.30 (m, 10H) 0.85 ppm (t, *J* = 6.96 Hz, 3H); ^{13}C NMR (600 MHz, [D_6]DMSO): δ = 154.53, 142.40, 141.72, 138.60, 128.99, 128.73, 100.30, 97.72, 77.73, 64.86, 51.17, 33.88, 33.55, 32.63, 31.26, 30.81, 28.75, 28.62, 28.30, 23.63, 22.08, 13.98 ppm; IR (solid film): $\tilde{\nu}$ = 3342, 3352, 2956, 2927, 2856, 1718, 1600, 1496, 1454, 1368, 1248, 1200, 1167, 1073, 833 cm^{-1}; UPLC–MS[SQD] (ES+): *m/z* 574 [*M*+H]$^+$.

(2,2-Dimethyl-5-{2-[4-octyl-2-(4,4,5,5-tetramethyl[1,3,2]dioxaborolan-2-yl)phenyl]ethyl}[1,3]dioxan-5-yl)carbamic acid *tert*-butyl ester (13): A 20 mL vial was charged with 1,1′-bis(diphenylphosphino)ferrocene–Pd^{II} dichloride CH_2Cl_2 complex (8.54 mg, 10,46 μmol), KOAc (103 mg, 1046 mmol), bis(pinacolato)diboron (97 mg, 0.38 mmol), and flushed with argon. A solution of **12** (200 mg, 0.349 mmol) in DMSO (6 mL) was added, and the solution was stirred for 4 h at 80 °C. The reaction mixture was quenched with H_2O and extracted with EtOAc. The combined organic fractions were washed with brine and dried over Na_2SO_4, then filtered, concentrated under reduced pressure, and purified by flash chromatography on silica gel (hexane/EtOAc, 90:10) to give **13** as a white powder (137 mg, 68%). t_{RA} = 5.02 min; ^{1}H NMR (600 MHz, [D_6]DMSO): δ = 7.46–7.44 (m, 1H), 7.21 (d, 1H), 7.00 (d, 1H), 6.51 (br s, 1H), 3.92 (d, 2H), 3.69 (d, 2H) 2.72–2.64 (m, 2H), 2.56–2.52 (m, 2H), 1.85–1.78 (m, 2H), 1.58–1.49 (m, 2H), 1.46–1.41 (m, 9H), 1.36–1.34 (m, 6H), 1.32–1.30 (m, 12H), 1.29–1.25 (m, 10H), 0.90–0.84 ppm (m, 3H); ^{13}C NMR (600 MHz, [D_6]DMSO): δ = 154.54, 146.41, 138.71, 135.58, 131.20, 129.01, 127.25, 97.54, 83.26, 77.70, 64.71, 51.53, 35.85, 34.63, 31.26, 28.65, 28.24, 24.59, 22.08, 13.96 ppm; IR (solid film): $\tilde{\nu}$ = 3331, 3241, 3048, 3031, 2989, 2977, 2923, 2869, 2855, 1706, 1536, 1369, 1339, 1309, 1174, 1148, 1127, 1087, 1068, 858, 847, 831 cm^{-1}; UPLC–MS[SQD] (ES+): *m/z* 574 [*M*+H]$^+$.

Preparation of trifluoroboronate precursor: (2,2-dimethyl-5-{2-[4-octyl-2-(trifluoroborolan-2-yl)phenyl]ethyl}[1,3]dioxan-5-yl)-carbamic acid *tert*-butyl ester (14): Aqueous KHF_2 (0.44 mL, 1.97 mmol) was added to a solution of **13** (200 mg, 0.35 mmol) in CH_3OH (2 mL). The resulting slurry was stirred at RT for 15 min, concentrated in vacuo, and dissolved in hot acetone, filtered and concentrated again in vacuo. The residue was recrystallized from hot CH_3OH to afford **14** as a white solid (143 mg, 74%). t_{RA} = 4.35 min; ^{1}H NMR (600 MHz, [D_6]DMSO): δ = 7.13 (s, 1H), 6.753 (s, 2H), 6.42 (br s, 1H), 4.0–3.96 (m, 2H), 3.57 (d, *J* = 11.5 Hz, 2H), 2.56–2.52 (m, 2H), 2.40 (t, *J* = 7.17 Hz, *2H),* 1.82–1.75 (m, 2H), 1.56–1.48 (m, 2H), 1.43 (s, 9H), 1.34–1.31 (m, 6H), 1.30–1.23 (m, 10H), 0.84 ppm (t, *J* = 6.56 Hz, 3H); ^{13}C NMR (600 MHz, [D_6]DMSO): δ = 154.52, 142.58, 136.47, 132.22, 127.09, 125.06, 97.37, 77.40, 64.64, 51.72, 35.37, 31.52, 31.32, 28.96, 28.72, 28.27, 25.25, 22.10, 13.97 ppm; IR (solid film): $\tilde{\nu}$ = 3332, 2993, 2977, 2959, 2926, 2854, 1700, 1536, 1392, 1368, 1173 cm^{-1}; FTMS-pESI *m/z* [*M*−H]$^-$; Anal. calcd for $C_{27}H_{44}O_4NBF_3$: 514.305145, found: 514.33244.

[γ-^{35}S]GTP binding assay

Chinese hamster ovary (CHO) cells expressing the human S1P receptor were harvested in ice-cold buffer [10 mM HEPES (pH 7.5), 10 mM EDTA, and protease inhibitor cocktail (1:50 complete, Roche Applied Science)], centrifuged at 750 *g* for 10 min at 4 °C, and resuspended in 20 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM EDTA, and protease inhibitor cocktail. The cell suspension was homogenized on ice with a Polytron Homogenizer at 25 000 rpm, centrifuged at 26 900 *g* for 30 min at 4 °C, and resuspended in 20 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM EDTA, and protease inhibitor cocktail at 2–3 mg protein mL^{-1} and stored in aliquots at −80 °C. To characterize [γ-^{35}S]GTP binding, membrane proteins (10–25 μg mL^{-1}) were resuspended in assay buffer [20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, 20 μg mL^{-1} saponin, 10 mM GDP, and 0.1% fat-free BSA (pH 7.4) mixed with 5 mg mL^{-1} WGA-coated SPA beads (Amersham Biosciences)] and various concentrations of agonists and incubated for 10–15 min at RT. The [γ-^{35}S]GTP binding reaction was started by the addition of [γ-^{35}S]GTP (PerkinElmer, > 1000 Ci $mmol^{-1}$), final concentration: 200 pM. After 120 min incubation at RT, the plates were centrifuged for 10 min at 1200 rpm (300 *g*) and counted with a TopCountNXT instrument (Packard Instruments).

Animal studies

Animal work was performed according to the Swiss federal law for animal protection and approved by the Veterinary Office Basel (BS No. 1325), with an acclimation period of > 1 week before use, conventional hygienic conditions for housing (five animals per cage, temperature 20–24 °C, minimum relative humidity 40%, light/dark cycle 12 h), standard diet (KLIBA Nr. 3893.0.25) and drinking water ad libitum.

Lymphocyte depletion assay in Lewis rats

The rats (220–250 g males from Charles River, Germany) were treated i.v. via tail vein with test compound at 4 mg kg^{-1}. All compounds were dissolved in a solution of 0.9% NaCl at 2 mg mL^{-1} in order to get an injection volume of 2 mL kg^{-1}. For each treatment group, rats were anesthetized (5% *v/v* isoflurane; Forene®, Abbott, Baar, Switzerland) at 0.5, 2, 4, 8, 24 and 48 h post-treatment (n = 3 per time point) and blood samples (~200 μL) were collected by sublingual punctures in EDTA-coated Eppendorf tubes to assess the lymphocyte counts using an automated hematology analyzer (Technicon H1-E analyzer, Bayer Diagnostics, Zürich, Switzerland). Serum leftovers were kept frozen at −80 °C for latter processing to assess drug blood levels. The rats were then decapitated, brains collected, weighted and snap frozen in liquid nitrogen.

Quantitative determination in blood and brain

Sample preparation and analysis was based on a modified protein precipitation procedure followed by liquid chromatographic separation coupled with mass spectrometry for detection. PK parameters were evaluated with MS Excel 2007 and PK Solution 2.0 (Summit Research Sevices, Ashland, OH, USA).

Sample preparation

Prior sample preparation whole brains were homogenized in a Dispomix homogenizer (Milteniy Biotec, Bergisch Gladbach, Germany) by adding 50% CH_3OH to a final concentration of 0.25 g mL^{-1}. Blood samples were directly used for further preparation.

Calibration, quality control, and recovery control samples were prepared by spiking blank blood and blank brain homogenate with known quantities of the parent compound (between 1 and 10000 ng mL^{-1}) and the corresponding phosphate, respectively. For FTY720 and AML629 determination, the d4-labeled compounds were used as internal standards (IS), whereas for BZM055 (**2a**) and **5a**, structurally related compounds were used.

Aliquots of 100 μL calibration standard, quality control, recovery control, and unknown samples were transferred to 2 mL Eppendorf tubes (Vaudaux-Eppendorf, Schönenbuch, Switzerland) and 25 μL IS mixture (0.4 μg mL^{-1} in 50% CH_3CN) was added to each tube. For protein precipitation and extraction from the blood and brain matrix, 700 μL $CH_3CN/CH_3OH/CHCl_3$ 40:30:30 was added. After ultrasonication (Bandelin SONOREX RK126 at RT) for 10 min and vortexing for 15 min, the samples were centrifuged at 16000 *g* for 5 min at RT. The whole upper layer was transferred to 1.5 mL HPLC vials (Chromacol Gold, Infochroma AG, Zug, Switzerland) and reduced to dryness using a SpeedVac Plus concentrator (Thermo Scientific, Reinach, Switzerland). The residues were acetylated for 20 min at 40 °C in a water bath by adding 100 μL pyridine and 50 μL Ac_2O. All samples were evaporated to dryness in a SpeedVac concentrator using medium heater temperature. The residues were re-dissolved by adding 60 μL 0.2% formic acid in CH_3OH, then vortexing and ultrasonication (Bandelin SONOREX RK126 at RT) for 10 min each. After a short centrifugation step, samples were stored at 15 °C in a cooled autosampler until analysis.

LC–MS–MS analysis

For quantitative analysis, a 10 μL aliquot of each sample, including calibration, quality control, and recovery control samples were injected with a cooled PAL HTS autosampler (CTC Analytics, Ziefen Switzerland) on a 2.0×50 mm column filled with 1.8 μm Gold-Turbo Basic C_{18} stationary phase for blood samples and on a column of the same geometry filled with 2.5 μm Reprosil-Pur C_{18} stationary phase (Morvay Analytik GmbH, Basel, Switzerland) for brain samples. Columns were held at 40 °C (HotDog 5090, Prolab, Reinach, Switzerland), and for separation a two-step linear gradient from 5 to 60% B in 1 min and from 60 to 100% B in 5 min at a flow rate of 0.100 mL min^{-1} was applied using a Rheos Allegro pump (Thermo Scientific, Reinach, Switzerland). The total cycle time was 14 min. The mobile phase used was A: 5 mM ammonium formate with 0.2% formic acid in H_2O, and B: 5% CH_3CN in CH_3OH. To minimize carryover, the column and injector were washed extensively after each injection.

For detection the column effluent was directly guided in a API-ES source of a Thermo Scientific TSQ Quantum Ultra triple-quadrupole mass spectrometer (Thermo Scientific, Reinach, Switzerland). The detection was done in MRM on the triacetylated species in positive mode for the non-phosphorylated species, and in negative mode for the phosphorylated species with parameters optimized for the phosphorylated species. Quantification was based on the compound/IS ratio of the extracted ion chromatograms of the selected mass transitions 560 *m/z* → 500 *m/z* for BZM055 (**2a**) and 638 *m/z* → 536.2 + 596.3 *m/z* for **5a**. The unknown sample concentration was calculated using the polynomial fitted ($r^2 \geq 0.9962$) external calibration curves. The LLOQ of the method was 10 ng mL^{-1} for blood, and 50 ng mL^{-1} for brain samples, and the recovery from the matrix was >65%. All calculations were performed with Xcalibur 2.0.7 software (Thermo Scientific, Reinach, Switzerland), and PK calculation and data presentation were done with PK Solution 2.0 software (Summit Research Services, Ashland, OH, USA) and MS Excel 2007.

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