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Synthesis and SAR studies of benzyl ether derivatives as potent orally active $S1P_1$ agonists

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ABSTRACT

We report herein the synthesis and structure–activity relationships (SAR) of a series of benzyl ether compounds as an $S1P_1$ receptor modulator. From our SAR studies, the installation of substituents onto the central benzene ring of **2a** was revealed to potently influence the $S1P_1$ and $S1P_3$ agonistic activities, in particular, an ethyl group on the 2-position afforded satisfactory $S1P_1/S1P_3$ selectivity. These changes of the $S1P_1$ and $S1P_3$ agonistic activities caused by the alteration of substituents on the 2-position were reasonably explained by a docking study using an $S1P_1$ X-ray crystal structure and $S1P_3$ homology modeling. We found that compounds **2b** and **2e** had a potent in vivo immunosuppressive efficacy along with acceptable $S1P_1/S1P_3$ selectivity, and confirmed that these compounds had less in vivo bradycardia risk through the evaluation of heart rate change after oral administration of the compounds (30 mg/kg, p.o.) in rats.

1. Introduction

Sphingosine 1-phosphate (S1P), as a bioactive sphingolipid, interacts with the five membered S1P family ($S1P_1$-$S1P_5$) of G-protein coupled receptors and plays a role in a wide range of physiological processes such as cell differentiation, morphogenesis and motility.[1,2] Among the S1P family, $S1P_1$ modulators have been especially focused on as a suppressor of autoimmunity by affecting lymphocyte trafficking.[1–4] Indeed, FTY720 (Fingolimod, **1**), which was launched as an orally available therapeutic agent for multiple sclerosis in 2010, was known to readily be converted to its phosphate (FTY720-P, **1**-**P**)[5,6] as an active metabolite through phosphorylation by sphingosine kinase after systemic administration, and the phosphate acts as a pan S1P receptor agonist except for $S1P_2$ (Fig. 1). Recent rapid progress of the studies on both FTY720 (**1**) and the role of S1P receptors has also revealed that the agonism of the $S1P_1$ receptor alone is sufficient to control lymphocyte recirculation,[7] and $S1P_3$ is implicated in bradycardia in rodents as reported,[8] although some studies suggested that only removal of the $S1P_3$ agonism is insufficient to exclude the cardiovascular side effect.[9] Under these circumstances, numerous industrial and academia groups[10] have intensively conducted exploration research for selective $S1P_1$ modulators sparing the $S1P_3$ agonism in order to reduce potential side effects, such as bradycardia, which was demonstrated in the clinical trials of FTY720 (**1**).[11]

In our derivatization programs, we designed the benzyl ether scaffolds represented by **2a** based on the structure of FTY720 (**1**) (Fig. 1). Its metabolically labile structure, represented by several benzylic positions in the lipophilic side chain parts, seemed suitable to adjust the pharmacokinetic aspect of FTY720 (**1**), of which phosphate **1**-**P** was known to have quite a long half-life, thereby including a long duration of the lymphocyte trafficking effect. We also assumed that the ease of the synthesis of derivatives **2a**, which

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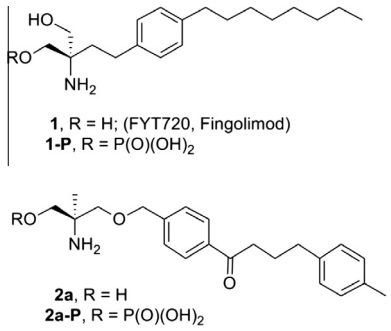


Figure 1. Structures of FTY720 (Fingolimod, **1**), newly designed templates **2a** and their phosphates (**1-P** and **2a-P**).

possesses benzyl ether group between chiral amino alcohol moiety and a lipophilic side chain part, enabled us to immediately collect necessary SAR. In this campaign, we envisaged the acquisition of promising compounds possessing both a shorter half-life and less potent agonistic activity to $S1P_3$ than FTY720-P (**1-P**) to provide a more manageable and safer immunosuppressive agent which will ensure wider clinical applications. To begin with a thorough derivatization, we attempted a preliminary evaluation of the in vitro and in vivo potencies of the scaffold **2a** and the results, compared with FTY720 (**1**), are summarized in Tables 1 and 2.

The agonistic activities of each compound were determined with their phosphates by measuring agonist-evoked [^{35}S]GTPγ-S binding activities to human $S1P_1$ and $S1P_3$ expressed in transfected CHO-K1 cells. While FTY720-P (**1-P**) showed no selective agonistic activity between $S1P_1$ and $S1P_3$ (EC_{50} = 7.0 and 2.0 nM, respectively), the activity directed toward the $S1P_1$ receptor of compound **2a-P** was serendipitously 29-fold higher than that toward $S1P_3$ (EC_{50} = 7.0 and 200 nM, respectively), suggesting the capability of compound **2a** to be a promising template for an $S1P_1$ agonist sparing $S1P_3$ agonism. Furthermore, to our delight, compound **2a** (0.1 mg/kg, i.v.) invoked a potent lymphocyte trafficking effect, which decreased the lymphocyte count to c.a. 39.5% of the vehicle treated control group at four hours after intravenous injection, and also demonstrated potent immunosuppressive efficacy (ID_{50} = 0.27 mg/kg) in the host versus a graft reaction (HvGR) model on rats[12] regardless of having a far shorter half-life than FTY720-P (**1-P**). Encouraged by these favorable profiles of compound **2a**, we initiated full-fledged SAR studies.

2. Chemistry

Concerning the synthesis of chiral derivatives, we initially prepared chiral protected amino alcohol **6** as a common intermediate by using enzymatic asymmetric desymmetrization (Scheme 1).

Compound **4** was readily prepared by the asymmetric desymmetrization of 2-*tert*-butoxycarbonylamino-2-methylpropane-1.3-diol **3** by using CHIRAZYME L-2 as an enzymatic catalyst in 66% yield (89%ee).[13a,b] The dimethylacetal protection of the amino alcohol **4** provided **5** without a loss of optical purity, and the subsequent saponification with aqueous NaOH gave the intermediate **6** in good yield. To improve the optical purity of compound **6**, tartaric acid salt **9** was prepared in three steps, including benzylation (to compound **7**), deprotection of amino alcohol moiety (to compound **8**) and salination with D-(−)-tartaric acid. Recrystallization of compound **9** from water could efficiently enhance the optical purity to afford enantiomerically pure **9** after only one manipulation.[14] Compound **9** was subjected to protection of both amine and alcohol groups followed by debenzylation with 10% Pd-C in MeOH under a hydrogen atmosphere to afford chiral alcohol **6** as a colorless oil.

Though the enzymatic desymmetrization method led us to obtain chiral alcohol unit **6** successfully, this procedure included cumbersome multi step conversions for recrystallization to enhance the optical purity. In order to prepare an equivalent of key alcohol intermediate **6** more efficiently, we synthesized the intermediate **13** by using Seebach's diastereoselective alkylation method starting from L-serine methyl ester hydrochloric acid, which was recently reported by our group.[13c] This second generation synthetic method enabled us to prepare chiral alcohol **13** more effectively compared to the enzymatic method (Scheme 2).

Installation of a lipophilic side chain part was implemented in the following manner, described in Scheme 3. Treatment of **13** with various 4-bromobenzyl bromide reagents in the presence of NaH in DMF provided compound **14**. A halogen–lithium exchange reaction of compound **14** with *n*-BuLi followed by exposure to *N*-methoxy-*N*-methyl-4-(4-methylphenyl)butanamide, which was prepared from the corresponding carboxylic acid, afforded acylated compound **15** in satisfactory yield. *tert*-Butyl acetal moiety of the amino alcohol group was deprotected by the treatment with *p*-TsOH monohydrate in MeOH to afford compound **16**, and the subsequent saponification of methylcarbamate with KOH in EtOH gave compound **2** in good yield. Finally, compound **2** was converted to a salt form by usage of either hydrochloride or oxalic acid. In the case of using compound **6** as a starting material, the final deprotection step of amino alcohol moiety was conducted by the same procedure as shown in Scheme 1 (from **7** to **8**).

The synthesis of phosphorylated compound **2-P** is presented in Scheme 4. After the protection of the amino group of **2** with allyl chloroformate (AllocCl), the primary hydroxyl group was phosphorylated by exposure with $(AllylO)_2PN(i\text{-}Pr)_2$ under the influence of tetrazole followed by an oxidation with *m*CPBA to provide **17** in good yield. Two allyl groups of a phosphate and an Alloc group were concurrently deprotected with pyrrolidine under the influence of a catalytic amount of $Pd(PPh_3)_4$ to give the phosphate of **2** (**2-P**).

3. Result and discussion

The SAR concerning the effect of substitutions on the central benzene ring toward $S1P_1$ and $S1P_3$ agonistic activity is summarized in Table 3.

In general, the introduction of substituents onto the central benzene ring of compound **2-P** largely influenced both the $S1P_1$ and $S1P_3$ agonistic activity. Namely, while installation of a methyl group at the 2-position slightly improved $S1P_1$ agonistic activity (**2a-P** vs **2b-P**), it did not affect $S1P_3$ agonistic activity. On the other hand, a methyl group attached at the 3-position deteriorated the activity toward $S1P_1$ (**2a-P** vs **2c-P**), although this functionalization could largely reduce $S1P_3$ agonistic activity. Considering the enhancing effect of $S1P_1$ agonistic activity, we focused on the various functionalities at the 2-position. It was revealed that all 2-substituted compounds generally increased $S1P_1$ agonistic activity, and disubstituted compounds such as 2,6-dimethyl (**2g-P**) and

Table 1
In vitro agonist-evoked GTPγS binding of phosphates of FTY720 (**1-P**) and benzyl compound (**2a-P**) to human $S1P_1$ and $S1P_3$

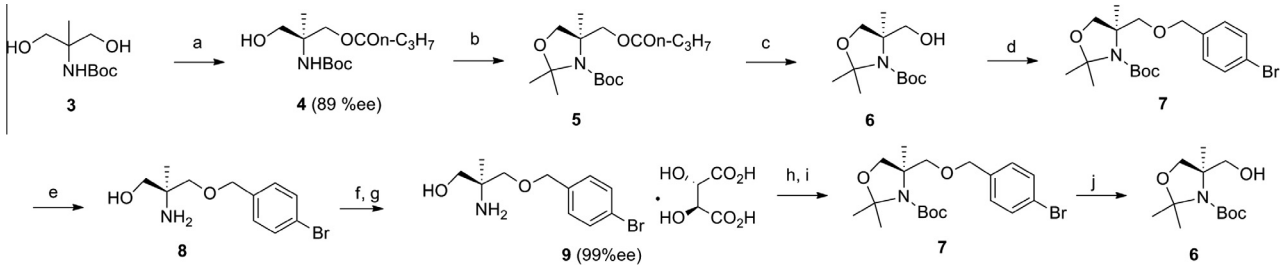
Compound	γGTP EC_{50}[a] (nM)		Selectivity
	$S1P_1$	$S1P_3$	($S1P_3$/$S1P_1$)
1-P	7.0	2.0	0.29
2a-P	7.0	200	29

[a] EC_{50} is defined as the midpoint between the binding or inhibitory ratio of the vehicle and the maximum response of the test compound.

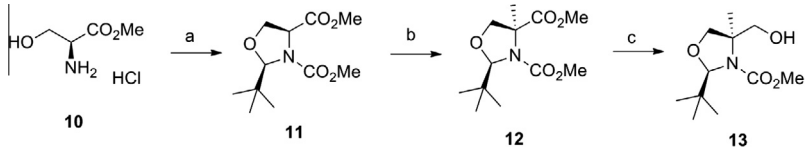
Table 2
In vivo profiles of FTY720 (**1**) and benzyl compound (**2a**)

Compound	Lymphopenia (% control at 0.1 mg/kg)	HvGR[a] (ID_{50}; mg/kg)	$T_{1/2}$[b] (h)
1	21.0	0.33	32.0
2a	39.5	0.27	7.8

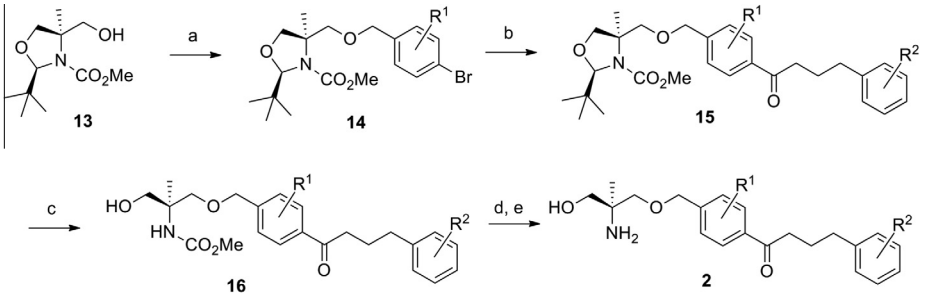
[a] ID_{50} is determined by results of at least three different doses.
[b] Half-life of each compound was determined by the concentration of phosphate **1-P** or **2a-P** in plasma after dosing of compound **1** or **2a** in rats (p.o., 1.0 mg/kg, $n = 2$), respectively.



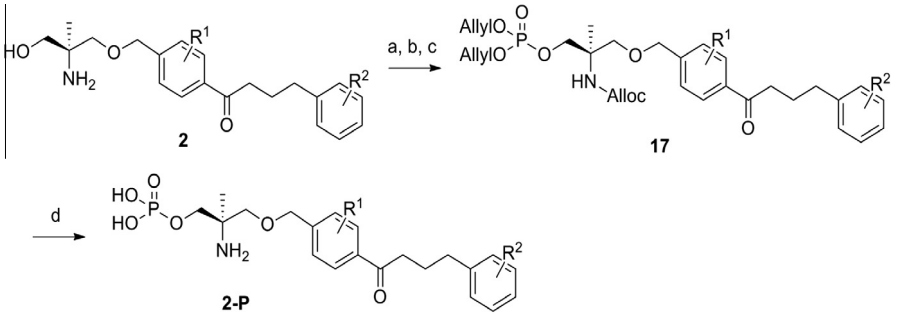
Scheme 1. Reagents and conditions: (a) CHIRAZYME L-2,carrier-fixed C3, Iyo, Vinyl *n*-butylate, *t*-BuOMe, 66% (89%ee); (b) acetone dimethylacetal, BF_3–OEt_2, CH_2Cl_2; (c) NaOH aq, THF, MeOH, 72% (2 steps); (d) 4-Bromobenzylbromide, NaH, DMF, 99%; (e) TFA, CH_2Cl_2, then H_2O, 88%; (f) D-(−)-Tartaric acid, EtOH, 70%; (g) Recrystallyzation from H_2O, 70%, 99%ee; (h) 1 N NaOH aq then Boc_2O, Et_3N, CH_2Cl_2, 90%; (i) Acetone dimethylacetal, BF_3–OEt_2, CH_2Cl_2, 94%; (j) H_2, 10% Pd-C, K_2CO_3, EtOH, 91%.



Scheme 2. Reagents and conditions: (a) *t*-BuCHO, Et_3N, toluene, then $ClCO_2Me$, Et_3N, toluene; (b) MeI, LiHMDS, DMPU, THF, 82% (2 steps); (c) $LiBH_4$, THF, 98%.



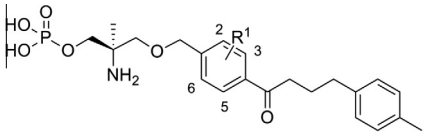
Scheme 3. Reagents and Conditions: (a) Benzylbromide, NaH, DMF, 64–95%; (b) *n*-BuLi, Weinreb amide, THF, 45–95%; (c) *p*-TsOH monohydrate, MeOH, 55–96%; (d) KOH, EtOH, 90–100%; (e) HCl or oxalic acid, EtOH, 86–99%.



Scheme 4. Reagents and conditions: (a) AllocCl, $KHCO_3$, AcOEt, H_2O; (b) $(AllylO)_2PN(i\text{-}Pr)_2$, tetrazole; (c) *m*CPBA, CH_2Cl_2, 45–88% (3 steps); (d) $Pd(PPh_3)_4$, pyrrolidine, CH_3CN, 40–73%.

2,5-dimethyl (**2h-P**) were also proved to be acceptable. Regarding the $S1P_3$, a relatively larger 2-ethyl (**2f-P**) group could decrease $S1P_3$ activity (EC_{50} = 12000 nM) drastically and 2,5-dimethyl (**2h-P**) functionalities also showed low $S1P_3$ activity (EC_{50} = >20,000 nM), whereas relatively small groups such as 2-methyl (**2b-P**), 2-fluoro (**2d-P**), and 2-chloro (**2e-P**) groups did

Table 3
The SAR information about the substitution of the central benzene ring



Compound	R^1	γGTP EC_{50}[a] (nM)		Selectivity ($S1P_3/S1P_1$)
		$S1P_1$	$S1P_3$	
1-P		7.0	2.0	0.29
2a-P	H	7.0	200	29
2b-P	2-Me	3.0	180	60
2c-P	3-Me	19.0	>20,000	>1052
2d-P	2-F	2.8	60	21
2e-P	2-Cl	4.0	220	55
2f-P	2-Et	2.3	12000	5217
2g-P	2,6-Me_2	2.2	340	155
2h-P	2,5-Me_2	6.5	>20,000	>3077

[a] EC_{50} is defined as the midpoint between the binding or inhibitory ratio of the vehicle and the maximum response of the test compound.

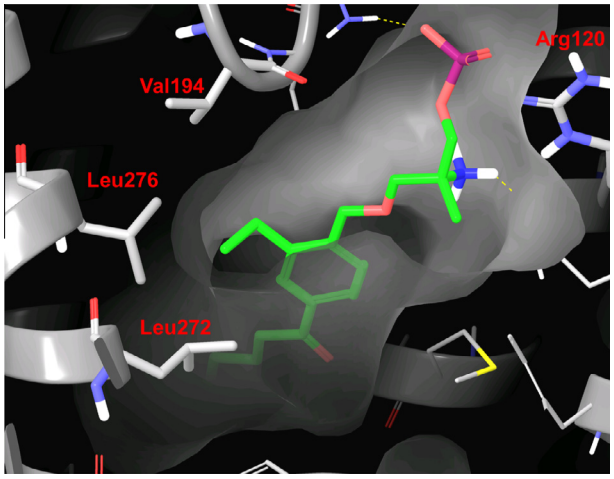


Figure 3. Hydrophobic pocket in the docking model of compound **2f** in the $S1P_1$ model.

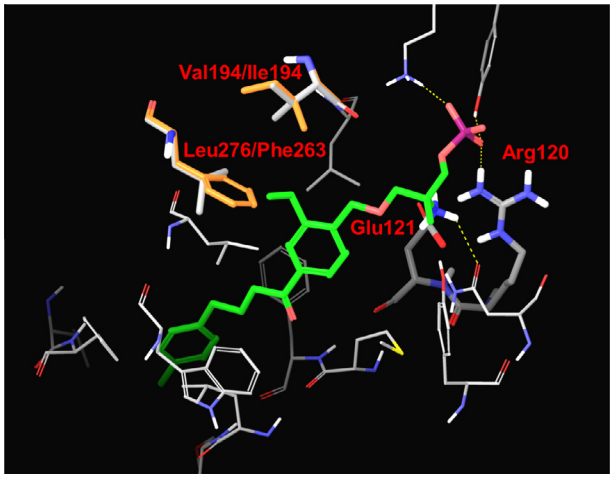


Figure 2. The docking model of compound **2f** into the $S1P_1$ and $S1P_3$ model.

not weaken $S1P_3$ agonistic activity. Consequently we could obtain promising compounds such as 2-ethyl (**2f-P**) and 2,5-dimethyl (**2h-P**), that showed good $S1P_1/S1P_3$ selectivity (5217 and >3077-fold, respectively).

A favorable effect of the 2-ethyl group on the central benzene ring was reasonably explained by an induced-fit docking study using the recently reported $S1P_1$ X-ray crystal structure,[15] and $S1P_3$ homology modeling based on the $S1P_1$–compound complex model. The docking model of compound **2f** into $S1P_1$ is illustrated in Figure 2. In our model, the phosphoric acid group and the amine group of **2f** reasonably interacted with Arg120 and Glu121 of $S1P_1$, respectively, which were almost the same aspects as the previously reported studies using the homology model of $S1P_1$ based on the X-ray crystal structure of bovine rhodopsin.[16a]

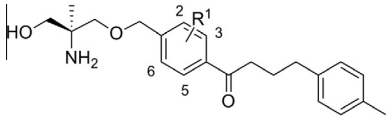
On the other hand, our model also suggested that whereas the ethyl group at the 2-position on the central benzene ring of **2f** was greatly interfered by Phe263 (orange stick model) in the $S1P_3$ model, it did not suffer the steric hindrance of Leu276 (white stick model) in the $S1P_1$ model. Furthermore, in the $S1P_1$ model, the ethyl group seemed to be favorably accommodated in a hydrophobic pocket consisting of several lipophilic residues such as Val194, Leu276 and Leu272 (Fig. 3). These results gave a reasonable explanation for the observed fact of the weakness of the activity of compound **2f** toward $S1P_3$, and the enhancement of the activity of 2-substituted compounds such as **2b**, **2d** and **2e** to $S1P_1$. A similar contribution by Phe263 and Leu276 for $S1P_1/S1P_3$ selectivity was reported by other groups.[16b,c]

Despite further derivatization focusing on the terminal benzene ring of **2a**, we unfortunately could not achieve any significant improvement in the selectivity between $S1P_1$ and $S1P_3$ (data not shown). Therefore, we selected the 4-methylphenyl group as the optimal structure in this moiety, and then changed our focus to the in vivo aspects of the selected compounds. We chose compounds **2a**, **2b**, **2e**, **2f** and **2h** for in vivo evaluation in rats, and their inhibitory activities in HvGR, the phosphorylation rate in whole blood and heart rate changes are summarized in Table 4.

Compounds **2a**, **2b** and **2e** showed quite high immunosuppressive efficacy on the rat HvGR model (0.27, 0.43 and 0.39 mg/kg, respectively), however, compounds **2f** and **2h**, of which phosphates showed high selectivity to $S1P_1$, unfortunately demonstrated almost no efficacy in rat HvGR, inhibiting only 10% and 11% of immunoreactions compared with the vehicle treated control group. To investigate the reason for this drastic deactivation, we measured the phosphorylation rate of each compound in rat whole blood. Through this analysis, it was revealed that efficacious compounds **2a**, **2b** and **2e** were properly converted to their phosphate after three hours of being added to rat whole blood, resulting in 39.5%, 83.8% and 38.4% conversion from parental alcohol to phosphate, respectively, while ineffective compounds **2f** and **2h** were transformed to their phosphate in only 13.9% and 6.0% of the total amount of compounds. With this result, it is reasonable to speculate that the bulkiness of the central benzene ring moiety of **2a** largely affected the substrate recognition of the responsible kinases which convert our compounds to their phosphates in whole blood, and thereby, the phosphorylation rate in the compounds possessing relatively bulky substituents such as **2f** and **2h** was too low for in vivo efficacy.

Ineffectiveness of the most $S1P_1$ selective compounds **2f** and **2h** in the rat HvGR model regrettably forced us to compromise on the point of $S1P_1$ selectivity, resulting in a focus on the compounds **2b** and **2e**, which exhibited robust HvGR efficacy and improved $S1P_1/S1P_3$ selectivity (60-fold and 55-fold, respectively, in Table 3) compared to our lead **2a**. We further evaluated these two compounds and compared them to FTY720 (**1**) in bradycardia risk in vivo by measuring the change in heart rate in rats after dosing at 30 mg/kg (p.o.). The maximum decrementation of the heart rate occurred during the 24 h period after dosing was denoted as an

Table 4
In vivo efficacy, the ratio of phosphate in plasma and heart rate change of selected compounds



Compound	R^1	Rat HvGR ID_{50}[a] (mg/kg) or % inhibition at 1 mg/kg	Phosphate in plasma[b] (%)	Heart rate decrease[c] (%)
1 (**FTY720**)		0.33	N.T.[e]	24.1[d]
2a	H	0.27	39.5	N.T.[e]
2b	2-Me	0.43	38.4	−4.4
2e	2-Cl	0.39	83.8	4.0
2f	2-Et	10%	13.9	N.T.[e]
2h	2,5-Me_2	11%	6.0	N.T.[e]

[a] ID_{50} is defined as the midpoint between the inhibitory ratio of the vehicle and the maximum response of the test compound.
[b] The ratio of phosphate was determined by the ratio of the HPLC peak area of the phosphorylated and the remaining test compound in plasma 3 h after the addition of a test compound to rat whole blood.
[c] Each value shows the percentage of the maximum heart rate decrementation that occurred during 48 h after 30 mg/kg oral administration in rats in comparison to the vehicle treated control group.
[d] p.o., 10 mg/kg.
[e] N.T. = not tested.

Table 5
The PK profiles of benzyl ether compounds in rats[a]

Compound	C_{max} (ng/mL)	AUC_{0-48} (ng h/mL)	$T_{1/2}$ (h)	Metabolic stability[b] (%)
1	N.D.[c]	N.D.[c]	N.D.[c]	97
1-P	111	5850	32.3	>100
2a	13	120	5.7	51
2a-P	72	799	7.8	92
2b	34	200	4.1	36
2b-P	104	806	4.3	92
2e	18	144	4.6	38
2e-P	97	658	4.6	86

[a] Each parameter was determined after oral dosing of each parental compound. (1.0 mg/kg, $n = 2$ or 3).
[b] Percentage of the remaining compounds after 30 min incubation at 37 °C with rat hepatic microsomes.
[c] N.D. = no data.

index of the potency of bradycardia. While FTY720 (**1**) showed transient bradycardia even at 10 mg/kg (p.o.), which decreased the heart rate by as much as 24% compared to the vehicle treated control group, our compounds **2b** and **2e** showed no serious change (only 4.4% increase and 4.0% decrease, respectively) in the same testing condition. Accordingly, **2b** and **2e** manifested a wider safety margin than FTY720 (**1**) in bradycardia risk because the ID_{50} values of **2b** and **2e** on rat HvGR were almost the same as FTY720 (**1**) and the exposure of compounds **2b** and **2e** increased linearly as the dose given increased between 1 and 30 mg/kg p.o. in rats (data not shown).

Finally, we examined the in vivo pharmacokinetic profile of **2b** and **2e** to confirm whether the phosphates of these promising compounds still maintained the favorably shortened half-life. PK parameters in Table 5 were measured in the phosphate after oral dosing in rats at 1.0 mg/kg of each parental compound. All parental compounds (**2a**, **2b** and **2e**) had lower plasma concentrations (C_{max} and AUC) and almost the same half-lives, compared with those parameters of corresponding phosphates (**2a-P**, **2b-P** and **2e-P**). It is noteworthy that both phosphates **2b-P** and **2e-P** demonstrated shorter, but appropriate, half-lives in rats (4.3 and 4.6 h, respectively) compared to FTY720-P (32.3 h) as initially intended. These shortenings of half-lives were reasonably explained by the lower metabolic stability of parental compound **2b** and **2e** in rat hepatic microsomes (36% and 38%, respectively) compared to FTY720 (97%).

4. Conclusions

We have reported the synthesis and SAR studies of benzyl ether compounds as an $S1P_1$ receptor modulator, which were inspired by the structure of FTY720 (Fingolimod, **1**). To differentiate FTY720 (**1**), our derivatization campaign especially focused on enhancing the S1P subtype selectivity between $S1P_1$ and $S1P_3$, and shortening the half-life of the active metabolite phosphate. It was revealed that the introduction of substituents onto the central benzene ring drastically increased $S1P_1/S1P_3$ selectivity while preserving potent $S1P_1$ agonistic activity, which was reasonably explained by the modeling study using both the X-ray structure of $S1P_1$ and homological model of $S1P_3$. However, the in vivo immunosuppressive potencies of those highly $S1P_1$ selective compounds **2f** and **2h** were drastically decreased due to the low conversion rate from parental alcohol to active phosphate, which was catalyzed by kinases in whole blood. Nevertheless, compounds **2b** and **2e** were found to be promising compounds showing almost the same in vivo activities along with fewer bradycardia risks and faster plasma disappearance compared to FTY720 (**1**). Continuous studies focusing on the replacement of the side chain to find a more promising compound will be reported in due course.

5. Experimental

5.1. Chemical synthesis

5.1.1. General Information

Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. ^1H NMR spectra were recorded on Unity Mercury Plus 400 or 500 spectrometer (Varian), and chemical shifts are given in ppm from tetramethylsilane (TMS) as an internal standard. Optical rotations were measured on a JASCO P-1030 or HORIBA SEPA-300 digital polarimeter. IR absorption spectra were recorded on a Jasco FT/IR-610 spectrophotometer. The mass spectra (Low- or High-resolution mass) spectroscopy was carried out with a JEOL GCmate or JEOL JMS-AX505H. Elemental analyses were performed by the analytical department of Daiichi Sankyo RD Novare Co., Ltd. Thin-layer chromatography (TLC) was performed on Merck precoated TLC glass sheets with silica gel 60F254. Separation of the compounds by column chromatography was carried out with silica gel 60 (Merck, 230–400 mesh ASTM).

5.1.2. [(2*S*)-2-(*tert*-Butoxycarbonylamino)-3-hydroxy-2-methyl-propyl] butanoate (4)

To a suspension of *tert*-butyl *N*-[2-hydroxy-1-(hydroxymethyl)-1-methyl-ethyl]carbamate (**3**) (22.2 g, 107 mmol) in *tert*-butylmethylether (550 mL) were added vinyl *n*-butanoate (40.8 mL, 322 mmol) and Lipase [CHIRAZYME L-2,carrier-fixed C3, Iyo] (5.4 g), and then the solution was stirred for 1.5 h at ambient temperature. The reaction mixture was filtrated and evaporated in vacuo. The residue was purified by silica gel column chromatography (hexane/AcOEt = 10:1 to 2:1) to provide **4** (19.4 g, 66% yield, 89%ee) as a colorless oil. The ee value of compound **4** was determined by a chiral HPLC; column [ChiralCel OF 4.6 φ × 250 mm]; eluent/*n*-hexane/2-propanol = 70:30; flow rate: 0.5 mL/min; t_R of (S)-isomer: 8.2 min; t_R of (R)-isomer: 10.5 min]. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.94 (t, 3H, J = 11.0 Hz), 1.23 (s, 3H), 1.41 (s, 9H), 1.65 (tq, 2H, J = 7.4 Hz, 7.4 Hz), 2.32 (t, 2H, J = 7.4 Hz), 3.56 (d, 1H, J = 11.7 Hz), 3.62 (d, 2H, J = 11.7 Hz), 4.18 (d, 1H, J = 11.1 Hz), 4.22 (d, 1H, J = 11.1 Hz), 4.85 (s, 1H); IR (film) 3415, 3380, 2961, 2935, 2874, 1721, 1505, 1458, 1392, 1368, 1293, 1248, 1168, 1076 cm^{-1}; MS(FAB) m/z: 276 (M+H)$^+$.

5.1.3. *tert*-Butyl (4*S*)-4-(butanoyloxymethyl)-2,2,4-trimethyl-1,3-oxazolidine-3-carboxylate (5) and *tert*-butyl (4*R*)-4-(hydroxymethyl)-2,2,4-trimethyl-1,3-oxazolidine-3-carboxylate (6)

To a solution of [(2*S*)-2-(*tert*-butoxycarbonylamino)-3-hydroxy-2-methyl-propyl] butanoate (**4**) (19.4 g, 70 mmol) in CH_2Cl_2 (200 mL) were added 2,2-dimethoxypropane (83 mL, 700 mmol) and BF_3–OEt_2 (0.4 mL, 3.4 mmol) at ambient temperature, and then the solution was stirred for 1 h. Et_3N (10 mL) was added to the reaction mixture, which was then evaporated in vacuo, giving a crude *tert*-butyl (4*S*)-4-(butanoyloxymethyl)-2,2,4-trimethyl-oxazolidine-3-carboxylate (**5**) as a yellow oil. The obtained crude compound (**5**) was diluted with THF (100 mL) and MeOH (160 mL). Aq 1 N NaOH (70 mL, 70 mmol) was added to the solution, which was then stirred for 20 min at ambient temperature. After removal of the solvent in vacuo, the residue was diluted with CH_2Cl_2 (100 mL) and poured into water. The resulting biphasic mixture was extracted with CH_2Cl_2 (100 mL × 2). The combined organic layer was washed with brine (50 mL), dried over Na_2SO_4, filtered and evaporated. Purification by silica gel column chromatography (hexane/AcOEt = 10:1 to 2:1) provided **6** (15.7 g, 91%) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.43 (s, 3H), 1.49 (s, 9H), 1.56 (s, 6H), 3.75–3.52 (m, 4H), 4.55 (br s, 1H), 4.57 (br s, 1H); IR (KBr) 3461, 2978, 1696, 1673, 1395, 1368, 1256, 1175, 1095 cm^{-1}; MS(FAB) m/z: 246 (M+H)$^+$.

5.1.4. *tert*-Butyl (4*R*)-4-[(4-bromophenyl)methoxymethyl]-2,2,4-trimethyl-1,3-oxazolidine-3-carboxylate (7)

Sodium hydride (60%, 0.6 g, 13 mmol) was added to a solution of *tert*-butyl (4*R*)-4-(hydroxymethyl)-2,2,4-trimethyl-1,3-oxazolidine-3-carboxylate (**6**) (2.2 g, 8.8 mmol) in DMF (50 mL) at 0 °C. The reaction mixture was stirred for 10 min at which time 4-bromobenzylbromide (3.3 g, 13 mmol) was added. The resulting mixture was warmed to ambient temperature and then stirred for 2 h. The reaction mixture was added to saturated aq NH_4Cl (50 mL). The resulting biphasic mixture was poured into water and extracted with AcOEt (50 mL × 2). The combined organic layer was washed with water (30 mL) and brine (30 mL), dried over Na_2SO_4, filtered and evaporated. Purification by silica gel column chromatography (hexane/AcOEt = 5:1 to 2:1) provided **7** (3.6 g, 99%) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.40–1.51 (m, 18H), 3.49 (dd, 1H, J = 14.1, 9.0 Hz), 3.61 (d, 1H, J = 9.0 Hz), 3.72 (d, 1H, J = 9.0 Hz), 4.08 (d, 1H, J = 9.0 Hz), 4.39–4.51 (m, 2H), 7.16 (d, 2H, J = 7.4 Hz), 7.45 (d, 2H, J = 7.4 Hz); IR (KBr) 2978, 2869, 1695, 1367, 1097, 1071, 804 cm^{-1}; MS(FAB) m/z: 414 (M+H)$^+$.

5.1.5. (2*S*)-2-Amino-3-[(4-bromophenyl)methoxy]-2-methyl-propan-1-ol (8)

To a solution of *tert*-butyl (4*R*)-4-[(4-bromophenyl)methoxymethyl]-2,2,4-trimethyl-1,3-oxazolidine-3-carboxylate (**7**) (3.6 g, 8.9 mmol) in CH_2Cl_2 (60 mL) was added TFA (30 mL) at ambient temperature. After stirring for 30 min, water (30 mL) was added to the reaction mixture, which was then stirred for 5 h. After removal of the solvent in vacuo, the residue was diluted with CH_2Cl_2 (50 mL) and basified to pH 14 with aq 4 N NaOH. The resulting biphasic mixture was extracted with CH_2Cl_2 (50 mL × 2). The combined organic layer was washed with water (30 mL) and brine (30 mL), dried over Na_2SO_4, filtered and evaporated. Purification by silica gel column chromatography (CH_2Cl_2/MeOH = 1:0 to 5:1) afforded **8** (2.2 g, 88%) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.02 (s, 3H), 3.29–3.42 (m, 4H), 4.43 (s, 2H), 7.13 (d, 2H, J = 8.6 Hz), 7.42 (d, 2H, J = 8.6 Hz); IR (KBr) 2924, 1464, 1095, 805 cm^{-1}; MS(FAB) m/z: 274 (M+H)$^+$.

5.1.6. (2*S*)-2-Amino-3-[(4-bromophenyl)methoxy]-2-methyl-propan-1-ol, D-(−)-tartaric acid (9)

D-(−)-Tartaric acid (1.1 g, 7.3 mmol) was added to a solution of (2*S*)-2-amino-3-[(4-bromophenyl)methoxy]-2-methyl-propan-1-ol (**8**) (2.1 g, 7.8 mmol) in EtOH (20 mL) and H_2O (4 mL), which was then stirred for 1 h. After removal of the solvent in vacuo, the resultant solid was filtered and washed with Et_2O, giving the crude compound **9** (2.1 g) as a colorless solid. The crude compound **9** was recrystallized from water (8 mL) to give **9** (1.5 g, 50% yield, 99%ee) as a colorless crystalline solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.13 (s, 3H), 3.38–3.47 (m, 4H), 4.52 (s, 2H), 7.34 (d, 2H, J = 8.0 Hz), 7.56 (d, 2H, J = 8.0 Hz); IR (KBr) 3320, 2918, 1905, 1729, 1575, 1404, 1304, 1263, 1214, 1134, 803, 680, 484 cm^{-1}; Anal. Calcd for $C_{11}H_{16}BrNO_2.C_4H_6O_6$. H_2O: C, 40.74; H, 5.47; N, 3.17; Br, 18.07. Found: C, 40.48; H, 5.49; N, 3.30; Br, 18.16.

5.1.7. A procedure of the determination of the enantio purity of (2*S*)-2-amino-3-[(4-bromophenyl)methoxy]-2-methyl-propan-1-ol, D-(−)-tartaric acid (9); synthesis of (4*R*)-4-[(4-bromophenyl)methoxymethyl]-4-methyl-1,3-oxazolidin-2-one (18)

To a suspension of **9** (10 mg, 0.024 mmol) in CH_2Cl_2 (3 mL) was added aq 1 N NaOH (0.2 mL), and then the suspension was stirred for 30 min at ambient temperature. The reaction mixture was poured into water and extracted with CH_2Cl_2 (5 mL × 2). The combined organic layer was dried over Na_2SO_4, filtered and evaporated, giving a free form of compound **9** (**8**) as colorless oil. To a solution of the crude compound **8** in CH_2Cl_2 (1 mL) was added 1,1′-carbonyldiimidazole (11 mg, 0.068 mmol), and then the solution was stirred for 2 h. After concentration in vacuo, the residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 20:1) provided **18** (6.5 mg, 90%) as a colorless solid. The ee value of compound **18** was determined by an chiral HPLC; column [column, CHIRALCEL OD-H 4.6 φ × 250 mm]; eluent/*n*-hexane/2-propanol = 70:30; flow rate: 1.0 mL/min; t_R of (*S*)-isomer, 5.98 min; t_R of (*R*)-isomer, 7.52 min]. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.35 (s, 3H), 3.30–3.37 (m, 2H), 4.01 (d, 1H, J = 8.8 Hz), 4.19 (d, 1H, J = 8.8 Hz), 4.45–4.51 (m, 2H), 5.22 (s, 1H), 7.15 (d, 2H, J = 8.0 Hz), 7.46 (d, 2H, J = 8.0 Hz); MS(FAB) m/z: 300 (M+H)$^+$.

5.1.8. *tert*-Butyl (4*R*)-4-[(4-bromophenyl)methoxymethyl]-2,2,4-trimethyl-1,3-oxazolidine-3-carboxylate (7) from 9

To a suspension of **9** (1.5 g, 3.9 mmol) in CH_2Cl_2 (30 mL) was added aq 1 N NaOH (10 mL), and then the suspension was stirred for 30 min at ambient temperature. The reaction mixture was poured into water and extracted with CH_2Cl_2 (30 mL × 2). The combined organic layer was dried over Na_2SO_4, filtered and evaporated, giving the crude compound **8** as colorless oil. To a solution of the crude compound **8** in CH_2Cl_2 (30 mL) were added Boc_2O

(0.9 g, 4.12 mmol) and Et_3N (0.7 mL, 4.8 mmol). The reaction mixture was stirred for 2 h, and concentrated in vacuo. The residue was diluted with CH_2Cl_2 (50 mL), and then 2,2-dimethoxypropane (5 mL, 34 mmol) and BF_3–OEt_2 (24 mg, 0.2 mmol) were added. After stirring for 1 h at room temperature, Et_3N (2 mL) was added to the reaction mixture, which was then concentrated in vacuo. Purification by silica gel column chromatography (hexane/AcOEt = 2:1) provided **7** (1.4 g, 94%) as a colorless oil.

5.1.9. *tert*-Butyl (4*R*)-4-(hydroxymethyl)-2,2,4-trimethyl-1,3-oxazolidine-3-carboxylate (6) from 7

To a solution of **7** (5.0 g, 12 mmol) in EtOH (100 mL) were added K_2CO_3 (2.5 g, 18 mmol) and 10% Pd-C (50% wet, 3.5 g). They were then stirred under a hydrogen atmosphere at room temperature for 24 h. After filtration, the filtrate was concentrated in vacuo. Purification by silica gel column chromatography (hexane/AcOEt = 10:1 to 4:1) provided **6** (3.0 g, 100%) as a colorless oil.

5.1.10. *N*-Methoxy-*N*-methyl-4-(*p*-tolyl)butanamide (19)

To a solution of 4-(*p*-tolyl)butanoic acid (5 g, 28 mmol) in CH_2Cl_2 (170 mL) were added *N*,*O*-dimethylhydroxylamine hydrochloride (3.3 g, 34 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (6.5 g, 34 mmol), and then the solution was stirred for 10 min at ambient temperature. The reaction mixture was cooled at 0 °C and *N*-methylmorpholine (9.3 mL, 84 mmol) was added dropwise to the solution. The reaction mixture was warmed to ambient temperature and then stirred for 2 h. After quenching with a saturated aq 1 N HCl (100 mL), the resulting mixture was poured into water and extracted with CH_2Cl_2 (100 mL × 2). The combined organic layer was washed with water (70 mL) and brine (70 mL), dried over Na_2SO_4, filtered and evaporated. Purification by silica gel column chromatography (hexane/AcOEt = 3:1 to 2:1) provided **19** (5.6 g, 46%) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.94–1.87 (m, 2H), 2.27 (s, 3H), 2.43–2.33 (m, 2H), 2.59 (t, 2H, *J* = 7.4 Hz), 3.12 (s, 3H), 3.58 (s, 3H), 7.02 (s, 4H); MS(FAB) *m*/*z*: 212 $(M+H)^+$.

5.1.11. *tert*-Butyl (4*R*)-2,2,4-trimethyl-4-[({4-[4-(4-methylphenyl)butanoyl]benzyl}oxy)methyl]-1,3-oxazolidine-3-carboxylate (15a)

To a solution of *tert*-butyl (4*R*)-4-[(4-bromophenyl)methoxymethyl]-2,2,4-trimethyl-1,3-oxazolidine-3-carboxylate (**7**) (1.0 g, 2.4 mmol) in THF (20 mL) was slowly added *n*-BuLi (1.57 M hexane solution, 2.3 mL, 3.6 mmol) at −78 °C under N_2 atmosphere, and then the solution was stirred for 30 min at the same temperature. A solution of *N*-methoxy-*N*-methyl-4-(*p*-tolyl)butanamide (**19**) (1.1 g, 4.8 mmol) in THF (5 mL) was added to the solution, and the reaction mixture was gradually warmed to ambient temperature and then stirred for 2 h. After quenching with a saturated aq NH_4Cl (15 mL), the resulting mixture was poured into water and extracted with AcOEt (40 mL × 2). The combined organic layer was washed with water (40 mL) and brine (40 mL), dried over Na_2SO_4, filtered and evaporated. Purification by silica gel column chromatography (hexane/AcOEt = 10:1 to 3:1) provided **15a** (0.51 g, 46%) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.61–1.32 (m, 18H), 2.05 (dt, 2H, *J* = 7.3, 7.3 Hz), 2.32 (s, 3H), 2.68 (t, 2H, *J* = 7.3 Hz), 2.95 (t, 2H, *J* = 7.3 Hz), 3.82–3.49 (m, 3H), 4.16–4.11 (m, 1H), 4.65–4.50 (m, 2H), 7.09 (s, 4H), 7.42–7.34 (m, 2H), 7.93–7.85 (m, 2H); IR (KBr) 2978, 2935, 1692, 1385, 1368, 1258, 1176, 1097, 1064 cm^{-1}; MS(FAB) *m*/*z*: 496 $(M+H)^+$.

5.1.12. 1-(4-{[(2*S*)-2-Amino-3-hydroxy-2-methylpropoxy]methyl}phenyl)-4-(4-methylphenyl)butan-1-one, hydrochloride (2a)

To a solution of (4*R*)-2,2,4-trimethyl-4-[({4-[4-(4-methylphenyl)butanoyl]benzyl}oxy)methyl]-1,3-oxazolidine-3-carboxylate (**15a**) (3.6 g, 8.9 mmol) in CH_2Cl_2 (60 mL) was added TFA (30 mL) at ambient temperature. After stirring for 30 min, water (30 mL) was added to the reaction mixture, which was then stirred for 5 h. After evaporation, the residue was diluted with CH_2Cl_2 (50 mL) and basified to pH 14 with aq 4 N NaOH. The resulting biphasic mixture was extracted with CH_2Cl_2 (50 mL × 2). The combined organic layer was washed with water (30 mL) and brine (30 mL), dried over Na_2SO_4, filtered and evaporated, giving a free form of **2a** as a pale yellow oil. To a solution of a free form of **2a** in EtOH (60 mL) was added 4 N HCl/dioxane solution (4.4 mL), and then the solution was stirred for 1 h. After removal of the solvent in vacuo, the resultant solid was filtered and washed with Et_2O, giving **2a** (2.2 g, 78%) as a colorless solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.18 (s, 3H), 1.89 (tt, 2H, *J* = 7.3, 7.3 Hz), 2.26 (s, 3H), 2.59 (t, 2H, *J* = 7.3 Hz), 3.02 (t, 2H, *J* = 7.3 Hz), 3.57–3.42 (m, 4H), 4.63 (s, 2H), 4.66 (t, 1H, *J* = 5.1 Hz), 7.09 (s, 4H), 7.51 (d, 2H, *J* = 8.0 Hz), 7.93 (d, 2H, *J* = 8.0 Hz), 8.02–7.79 (br s, 2H); IR (KBr) 3383, 3020, 2890, 1683, 1607, 1514, 1253, 1118, 979, 790 cm^{-1}; MS(FAB) *m*/*z*: 356 $(M+H)^+$; Anal. Calcd for $C_{22}H_{29}NO_3$.HCl: C, 67.42; H, 7.72; N, 3.57; Cl, 9.05. Found: C, 66.92; H, 7.75; N, 3.73; Cl, 9.04.

5.1.13. 1-(4-{[(2*S*)-2-Amino-3-hydroxy-2-methylpropoxy]methyl}-3-methylphenyl)-4-(4-methylphenyl)butan-1-one, hydrochloride (2b)

Compound **2b** was synthesized from compound **7** by using the same procedures of **4.1.4.**, **4.1.9**. and **4.1.10**.. Compound **2b** was obtained as a hydrochloride salt, and total yield was 18% over 4 steps. ^{1}H NMR (400 MHz, CD_3OD) δ 1.27 (s, 3H), 1.90–1.98 (m, 2H), 2.25 (s, 3H), 2.36 (s, 3H), 2.60 (t, 2H, *J* = 7.4 Hz), 2.94 (t, 2H, *J* = 7.4 Hz), 3.45–3.58 (m, 2H), 3.59–3.62 (m, 2H), 4.62 (s, 2H), 7.02 (br s, 4H), 7.43 (d, 1H, *J* = 7.4 Hz), 7.68 (s, 1H), 7.69 (d, 1H, *J* = 7.4 Hz); IR (KBr) 3387, 2944, 2558, 1680, 1606, 1513, 1257, 1101, 791 cm^{-1}; HRMS (ESI): calcd for $C_{23}H_{31}NO_3$ $[M+H]^+$: 370.2382, found 370.2395.

5.1.14. Methyl (2*R*,4*R*)-4-[(4-bromo-2-chloro-phenyl)methoxymethyl]-2-*tert*-butyl-4-methyl-1,3-oxazolidine-3-carboxylate (14e)

Sodium hydride (60%, 0.6 g, 13 mmol) was added to a solution of methyl (2*R*,4*R*)-2-*tert*-butyl-4-(hydroxymethyl)-4-methyl-1,3-oxazolidine-3-carboxylate (**13**) (3.0 g, 13 mmol) in DMF (30 mL) at 0 °C. The reaction mixture was stirred for 10 min, at which time 4-bromo-3-chlorobenzylbromide (5.5 g, 20 mmol) was added. The resulting mixture was warmed to ambient temperature and then stirred for 2 h. The reaction mixture was added to saturated aq NH_4Cl (50 mL). The resulting biphasic mixture was poured into water and extracted with AcOEt (50 mL × 2). The combined organic layer was washed with water (30 mL) and brine (30 mL), dried over Na_2SO_4, filtered and evaporated. Purification by silica gel column chromatography (hexane/AcOEt = 1:0 to 4:1) provided **14e** (3.6 g, 64%) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.96 (s, 9H), 1.46 (s, 3H), 3.71 (s, 3H), 3.75 (d, 1H, *J* = 8.8 Hz), 3.77 (d, 1H, *J* = 8.8 Hz), 3.98–3.93 (m, 1H), 4.21 (d, 1H, *J* = 8.8 Hz), 4.59 (s, 2H), 5.17 (s, 1H), 7.31 (d, 1H, *J* = 8.8 Hz), 7.42 (dd, 1H, *J* = 8.8, 1.5 Hz), 7.55 (d, 1H, *J* = 1.5 Hz); MS(FAB) *m*/*z*: 434 $(M+H)^+$.

5.1.15. Methyl (2*R*,4*R*)-2-*tert*-butyl-4-[({2-chloro-4-[4-(4-methylphenyl)butanoyl]benzyl}oxy)methyl]-4-methyl-1,3-oxazolidine-3-carboxylate (15e)

To a solution of methyl (2*R*,4*R*)-4-[(4-bromo-2-chloro-phenyl)methoxymethyl]-2-*tert*-butyl-4-methyl-1,3-oxazolidine-3-carboxylate (**14e**) (2.3 g, 5.4 mmol) in THF (40 mL) was slowly added *n*-BuLi (1.58 M *n*-hexane solution, 5.1 mL, 8.1 mmol) at −78 °C under N_2 atmosphere, and then the solution was stirred for 30 min at the same temperature. A solution of *N*-methoxy-*N*-methyl-4-(*p*-tolyl)butanamide (**19**) (2.4 g, 11 mmol) in THF

(5 mL) was added to the solution, and the reaction mixture was gradually warmed to ambient temperature and then stirred for 2 h. After quenching with a saturated aq NH_4Cl (50 mL), the resulting mixture was poured into water and extracted with AcOEt (80 mL × 2). The combined organic layer was washed with water (40 mL) and brine (40 mL), dried over Na_2SO_4, filtered and evaporated. Purification by silica gel column chromatography (hexane/AcOEt = 1:0 to 3:1) provided **15e** (1.3 g, 46%) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.97 (s, 9H), 1.49 (s, 3H), 2.12–2.07 (m, 2H), 2.36 (s, 3H), 2.71 (t, 2H, J = 7.3 Hz), 2.96 (t, 2H, J = 7.3 Hz), 3.72 (s, 3H), 3.83–3.77 (m, 2H), 4.03–3.97 (m, 1H), 4.26–4.23 (m, 1H), 4.68 (s, 2H), 5.19 (s, 1H), 7.13 (s, 4H), 7.55 (d, 1H, J = 7.8 Hz), 7.83 (dd, 1H, J = 7.8, 1.5 Hz), 7.91 (d, 1H, J = 1.5 Hz); MS(FAB) m/z: 516 $(M+H)^+$.

5.1.16. 1-(4-{[(2*S*)-2-Amino-3-hydroxy-2-methylpropoxy]methyl}-3-chlorophenyl)-4-(4-methylphenyl)butan-1-one, hydrochloride (2e)

To a solution of **15e** (1.0 g, 5.4 mmol) in MeOH (10 mL) was added *p*-TsOH monohydrate (0.76 g, 4.0 mmol) at ambient temperature, and then the solution was stirred for 2 h at 50 °C. The reaction mixture was evaporated in vacuo, giving methyl [(2*S*)-1-({2-chloro-4-[4-(4-methylphenyl)butanoyl]benzyl}oxy)-3-hydroxy-2-methylpropan-2-yl]carbamate (**16e**) as a yellow oil. To the solution of **16e** in EtOH (20 mL) was added aq 8 N KOH (2.5 mL, 20 mmol), and then the solution was refluxed for 6 h. After cooling to room temperature, the reaction mixture was concentrated in vacuo and diluted by water. The resulting mixture was extracted with CH_2Cl_2 (30 mL × 2) and the combined organic layer was washed with water (40 mL) and brine (40 mL), dried over Na_2SO_4, filtered and evaporated to give a salt free form of **2e** as a pale yellow oil. To a solution of a salt free form of **2e** in EtOH (15 mL) was added 4 N HCl/dioxane solution (1 mL), and then the solution was stirred for 1 h. After removal of the solvent in vacuo, the resultant solid was filtered and washed with Et_2O to give **2e** (0.58 g, 90%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.20 (s, 3H), 1.91–1.84 (m, 2H), 2.26 (s, 3H), 2.59 (t, 2H, J = 7.3 Hz), 3.04 (t, 2H, J = 7.3 Hz), 3.62–3.45 (m, 4H), 4.68 (s, 2H), 5.47 (t, 1H, J = 5.4 Hz), 7.09 (s, 4H), 7.78 (d, 1H, J = 8.3 Hz), 7.91 (d, 1H, J = 8.3 Hz), 7.94 (br s, 1H). 8.00 (br s, 3H); IR (KBr) 3384, 3018, 2935, 1692, 1198, 1129, 1044, 808 cm^{-1}; MS(FAB) m/z: 390 $(M+H)^+$; Anal. Calcd for $C_{22}H_{28}ClNO_3$.HCl: C, 59.46; H, 7.03; N, 3.15; Cl,15.96. Found: C, 59.59; H, 6.72; N, 3.20; Cl, 16.81.

5.1.17. 1-(4-{[(2*S*)-2-Amino-3-hydroxy-2-methylpropoxy]methyl}-3-ethylphenyl)-4-(4-methylphenyl)butan-1-one, 0.5 oxalic acid (2f)

Compound **2f** was synthesized from compound **6** by using the same procedure of **4.1.4.**, **4.1.9**. and **4.1.10**. Compound **2f** was obtained as a 0.5 oxalic acid salt in 18% yield over 4 steps. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.04 (s, 3H), 1.18 (t, 3H, J = 7.6 Hz), 1.91–1.85 (m, 2H), 2.26 (s, 3H), 2.59 (t, 2H, J = 7.6 Hz), 2.67 (q, 2H, J = 7.6 Hz), 3.01 (t, 2H, J = 7.2 Hz), 3.39–3.29 (m, 4H), 4.60 (s, 2H), 7.09 (s, 4H), 7.51 (d, 1H, J = 7.6 Hz), 7.74 (s, 1H), 7.75 (d, 1H, J = 7.6 Hz); IR (KBr) 3288, 2967, 2949, 2592, 1688, 1626, 1565, 1293, 1112, 1052, 796 cm^{-1}; HRMS (ESI): calcd for $C_{24}H_{34}NO_3$ $[M+H]^+$: 384.2539, found 384.2539.

5.1.18. 1-(4-{[(2*S*)-2-Amino-3-hydroxy-2-methylpropoxy]methyl}-2,5-dimethylphenyl)-4-(4-methylphenyl)butan-1-one, 0.5 oxalic acid (2h)

Compound **2h** was synthesized from compound **6** by using the same procedure of **4.1.4.**, **4.1.9**. and **4.1.10**. Compound **2h** was obtained as a 0.5 oxalic acid salt, and total yield was 21% over 4 steps. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.04 (s, 3H), 1.88–1.82 (m, 2H), 2.26 (s, 6H), 2.35 (s, 3H), 2.57 (t, 2H, J = 7.4 Hz), 2.90 (t, 2H, J = 7.4 Hz), 3.36–3.29 (m, 4H), 4.49 (s, 2H), 7.08 (s, 4H), 7.25 (s, 1H), 7.49 (s, 1H); IR (KBr) 2953, 2596, 1680, 1620, 1572, 1454, 1296, 1098, 1052, 797, 764 cm^{-1}; HRMS (ESI): calcd for $C_{24}H_{34}NO_3$ $[M+H]^+$: 384.2539, found 384.2542.

5.1.19. Allyl *N*-[(1*R*)-1-(diallyloxyphosphoryloxymethyl)-1-methyl-2-[[4-[4-(*p*-tolyl)butanoyl]phenyl]methoxy]ethyl]carbamate (17a)

To a suspension of 1-[4-[[(2*S*)-2-amino-3-hydroxy-2-methylpropoxy]methyl]-phenyl]-4-(*p*-tolyl)butan-1-one hydrochloride (**2a**) (0.15 g, 0.4 mmol) in AcOEt (4 mL) and water (4 mL) were added $KHCO_3$ (88 mg, 0.9 mmol) and allyl chloroformate (0.05 mL, 0.46 mmol) successively at ambient temperature. After stirring for 2 h, the reaction mixture was poured into water and extracted with AcOEt (10 mL × 2). The combined organic layer was washed with brine (10 mL), dried over Na_2SO_4, filtered and evaporated, giving a crude allyl *N*-[(1*S*)-1-(hydroxymethyl)-1-methyl-2-[[4-[4-(*p*-tolyl)butanoyl]phenyl]methoxy]ethyl]carbamate as a pale yellow oil. To a solution of *N*-[(1*S*)-1-(hydroxymethyl)-1-methyl-2-[[4-[4-(*p*-tolyl)butanoyl]phenyl]methoxy]ethyl]carbamate in CH_2Cl_2 (3 mL) were added diallyl *N*,*N*-diisopropylphosphoramidite (0.20 mL, 0.75 mmol) and tetrazole (0.18 g, 2.5 mmol) at 0 °C, and then the solution was stirred overnight at ambient temperature. The reaction mixture was cooled to 0 °C, and then 3-chloroperoxybenzoic acid (0.2 g, 0.8 mmol) was added. After stirring for 1 h at ambient temperature, the reaction mixture was quenched with 10% aq sodium thiosulfate (10 mL) and saturated aq $NaHCO_3$ (10 mL). The resulting biphasic mixture was extracted with CH_2Cl_2 (10 mL × 2) and the combined organic layer was washed with water (10 mL) and brine (10 mL), dried over Na_2SO_4, filtered and evaporated. Purification by silica gel column chromatography (hexane/AcOEt = 5:1 to 0:1) provided **17a** (0.23 g, 99%) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.37 (s, 3H), 2.03–2.00 (m, 3H), 2.28 (s, 3H), 2.64 (t, 2H, J = 7.2 Hz), 2.91 (t, 2H, J = 7.2 Hz), 3.48 (d, 1H, J = 9.0 Hz), 3.61 (d, 1H, J = 9.0 Hz), 4.17–4.11 (m, 2H), 4.57–4.45 (m, 6H), 5.31–5.19 (m, 6H), 5.93–5.83 (m, 3H), 7.04 (s, 4H), 7.32 (d, 2H, J = 8.2 Hz), 7.84 (d, 2H, J = 8.2 Hz); IR (KBr) 2925, 2854, 1724, 1684, 1463, 1262, 1028 cm^{-1}; MS(FAB) m/z: 600 $(M+H)^+$.

5.1.20. (2*R*)-2-Amino-2-methyl-3-({4-[4-(4-methylphenyl)butanoyl]benzyl}oxy)propyl dihydrogen phosphate (2a-P)

To a solution of allyl *N*-[(1*R*)-1-(diallyloxyphosphoryloxymethyl)-1-methyl-2-[[4-[4-(*p*-tolyl)butanoyl]phenyl]methoxy]ethyl]carbamate (**17a**) (0.23 g, 0.4 mmol) in CH_3CN (5 mL) were added $Pd(PPh_3)_4$ (24 mg, 0.02 mmol), PPh_3 (22 mg, 0.08 mmol) and pyrrolidine (0.22 mL, 2.6 mmol), which was then stirred overnight under N_2 atmosphere. The reaction mixture was evaporated in vacuo, and the residue was dissolved in 20% aq EtOH solution (6 mL) and treated with charcoal. After filtration, acetic acid (0.1 mL) was added to the filtrate. The precipitated crystals were collected by filtration and washed with EtOH (4 mL) to afford the crude product **2a-P**. The obtained crude product **2a-P** was dissolved in 2% aq pyrrolidine (4 mL) and EtOH (4 mL). After separation of insoluble materials by filtration, acetic acid (0.08 mL) was added to the filtrate. The precipitated crystals were collected by filtration, washed with EtOH (4 mL) and dried in vacuo at 50 °C to afford **2a-P** (74 mg, 46%) as a pale yellow solid. ^{1}H NMR (400 MHz, CD_3CO_2D) δ 1.48 (s, 3H), 1.98–2.07 (m, 2H), 2.28 (s, 3H), 2.65 (t, 2H, J = 7.3 Hz), 3.02 (t, 2H, J = 7.3 Hz), 3.73 (d, 1H, J = 8.8 Hz), 3.79 (d, 1H, J = 8.8 Hz), 4.22–4.34 (m, 2H), 4.69 (s, 2H), 7.08 (s, 4H), 7.49 (d, 2H, J = 8.0 Hz), 7.95 (d, 2H, J = 8.0 Hz); IR (KBr) 3192, 2955, 2594, 1681, 1609, 1517, 1282, 1105, 1033, 796 cm^{-1}; HRMS (ESI): calcd for $C_{22}H_{31}NO_6P$ $[M+H]^+$: 436.1889, found 436.1891.

5.1.21. (2*R*)-2-Amino-2-methyl-3-({2-methyl-4-[4-(4-methylphenyl)butanoyl]benzyl}oxy)propyl dihydrogen phosphate (2b-P)

Compound **2b-P** was synthesized from **2b** by using the same procedure of **4.1.18**. and **4.1.19**. The total yield from compound **2b** was 25% over 2 steps. ^{1}H NMR (400 MHz, CD_3CO_2D) δ 1.47 (s, 3H), 1.98–2.01 (m, 2H), 2.24 (s, 3H), 2.32 (s, 3H), 2.62 (t, 2H, J = 7.4 Hz), 2.97 (t, 2H, J = 7.4 Hz), 3.37 (br s, 2H), 4.20–4.36 (m, 4H), 4.60 (s, 2H), 7.03 (br s, 4H), 7.47 (d, 1H, J = 7.8 Hz), 7.69 (s, 1H), 7.70 (d, 1H, J = 7.8 Hz); IR (KBr) 2953, 2602, 1686, 1549, 1210, 1183, 1037, 939, 797 cm^{-1}; HRMS (ESI): calcd for $C_{23}H_{33}NO_6P$ $[M+H]^+$: 450.2045, found 450.2045.

5.1.22. (2*R*)-2-Amino-2-methyl-3-({3-methyl-4-[4-(4-methylphenyl)butanoyl]benzyl}oxy)propyl dihydrogen phosphate (2c-P)

Compound **2c-P** was synthesized from **2c** by using the same procedure of **4.1.18**. and **4.1.19**. The total yield from compound **2c** was 30% over 2 steps. ^{1}H NMR (400 MHz, CD_3CO_2D) δ 1.43 (s, 3H), 1.84–2.05 (m, 2H), 2.28 (s, 3H), 2.47 (s, 3H), 2.64 (br s, 2H), 2.93 (br s, 2H), 3.61 (d, 1H, J = 10.3 Hz), 3.76 (d, 1H, J = 10.3 Hz), 4.08–4.22 (m, 2H), 4.58 (d, 1H, J = 12.2 Hz), 4.64 (d, 1H, J = 12.2 Hz), 7.07 (br s, 4H), 7.27 (br s, 2H), 7.64 (br s, 1H); IR (KBr) 2924, 2608, 1685, 1611, 1549, 1448, 1179, 1065, 942, 808, 505 cm^{-1}; HRMS (ESI): calcd for $C_{23}H_{33}NO_6P$ $[M+H]^+$: 450.2045, found 450.2043.

5.1.23. (2*R*)-2-Amino-3-({2-fluoro-4-[4-(4-methylphenyl)butanoyl]benzyl}oxy)-2-methylpropyl dihydrogen phosphate (2d-P)

Compound **2d-P** was synthesized from **2d** by using the same procedure of **4.1.18**. and **4.1.19**. The total yield from compound **2d** was 31% over 2 steps. ^{1}H NMR (400 MHz, CD_3CO_2D) δ 1.50 (s, 3H), 1.98–2.06 (m, 2H), 2.27 (s, 3H), 2.66 (t, 2H, J = 7.6 Hz), 3.00 (t, 2H, J = 7.6 Hz), 3.82 (d, 2H, J = 6.6 Hz), 4.25–4.30 (m, 1H), 4.34–4.38 (m, 1H), 4.75 (s, 2H), 7.08 (s, 4H), 7.62–7.69 (m, 2H), 7.77–7.79 (m, 1H); IR (KBr) 2945, 2611, 1687, 1577, 1418, 1183, 1039, 945, 810, 513 cm^{-1}; MS (FAB) m/z: 452 $(M-H)^-$; Anal. Calcd for $C_{22}H_{29}FNO_6P$: C, 58.27; H, 6.45; N, 3.09; F, 4.19; P, 6.83. Found: C, 57.59; H, 5.68; N, 3.11; F, 4.29; P, 6.78.

5.1.24. (2*R*)-2-Amino-3-({2-chloro-4-[4-(4-methylphenyl)butanoyl]benzyl}oxy)-2-methylpropyl dihydrogen phosphate (2e-P)

Compound **2e-P** was synthesized from **2e** by using the same procedure of **4.1.18**. and **4.1.19**. The total yield from compound **2e** was 31% over 2 steps. ^{1}H NMR (400 MHz, CD_3CO_2D) δ 1.42 (s, 3H), 1.88–1.95 (m, 2H), 2.17 (s, 3H), 2.55 (t, 2H, J = 7.1 Hz), 2.90 (t, 2H, J = 7.1 Hz), 3.73 (d, 1H, J = 8.0 Hz), 3.87 (d, 1H, J = 8.0 Hz), 4.16–4.20 (m, 1H), 4.25–4.28 (m, 1H), 4.56 (s, 2H), 6.97 (br s, 4H), 7.63 (d, 1H, J = 7.8 Hz), 7.78 (d, 1H, J = 7.8 Hz), 7.82 (s, 1H); IR (KBr) 2923, 2612, 1686, 1560, 1182, 1039, 943, 811 cm^{-1}; HRMS (ESI): calcd for $C_{22}H_{30}NO_6PCl$ $[M+H]^+$: 470.1499, found 470.1499.

5.1.25. (2*R*)-2-Amino-3-({2-ethyl-4-[4-(4-methylphenyl)butanoyl]benzyl}oxy)-2-methylpropyl dihydrogen phosphate (2f-P)

Compound **2f-P** was synthesized from **2f** by using the same procedure of **4.1.18**. and **4.1.19**. The total yield from compound **2f** was 20% over 2 steps. ^{1}H NMR (400 MHz, CD_3CO_2D) δ 1.20 (t, 3H, J = 7.4 Hz), 1.39 (s, 3H), 1.95–2.06 (m, 2H), 2.24 (s, 3H), 2.60–2.69 (m, 4H), 2.97 (t, 2H, J = 7.4 Hz), 3.60 (d, 1H, J = 9.4 Hz), 3.76 (d, 1H, J = 9.4 Hz), 4.10 (d, 2H, J = 10.6 Hz), 4.65 (d, 2H, J = 19.0 Hz), 7.02 (br s, 4H), 7.48 (d, 1H, J = 7.8 Hz), 7.73 (d, 1H, J = 7.8 Hz), 7.74 (s, 1H); IR (KBr) 2958, 2609, 1689, 1455, 1205, 1104, 1065, 944, 838, 520 cm^{-1}; HRMS (ESI): calcd for $C_{24}H_{35}NO_6P$ $[M+H]^+$: 464.2202, found 464.2204.

5.1.26. (2*R*)-2-Amino-3-({2,6-dimethyl-4-[4-(4-methylphenyl)butanoyl]benzyl}oxy)-2-methylpropyl dihydrogen phosphate (2g-P)

Compound **2g-P** was synthesized from **2g** by using the same procedure of **4.1.18**. and **4.1.19**. The total yield from compound **2g** was 16% over 2 steps. ^{1}H NMR (400 MHz, CD_3CO_2D) δ 1.38 (s, 3H), 1.95–2.06 (m, 2H), 2.28 (s, 3H), 2.44 (s, 6H), 2.65 (t, 2H, J = 7.3 Hz), 2.98 (t, 2H, J = 7.3 Hz), 3.33–3.36 (m, 4H), 3.60 (d, 1H, J = 9.5 Hz), 3.79 (d, 1H, J = 9.5 Hz), 4.10 (d, 1H, J = 10.0 Hz), 4.70 (d, 1H, J = 10.0 Hz), 7.08 (s, 4H), 7.60 (s, 2H); IR (KBr) 2950, 2468, 2229, 1683, 1578, 1458, 1285, 1121, 1066, 1037, 969, 767 cm^{-1}; HRMS (ESI): calcd for $C_{24}H_{34}NO_6P$ $[M-H]^-$: 462.2045, found 462.2037.

5.1.27. (2*R*)-2-Amino-3-({2,5-dimethyl-4-[4-(4-methylphenyl)butanoyl]benzyl}oxy)-2-methylpropyl dihydrogen phosphate (2h-P)

Compound **2h-P** was synthesized from **2h** by using the same procedure of **4.1.18**. and **4.1.19**. The total yield from compound **2h** was 20% over 2 steps. ^{1}H NMR (400 MHz, CD_3CO_2D) δ 1.42 (s, 3H), 1.92–2.06 (m, 2H), 2.28 (s, 3H), 2.30 (s, 3H), 2.42 (s, 3H), 2.64 (t, 2H, J = 7.3 Hz), 2.91 (t, 2H, J = 7.3 Hz), 3.35 (br s, 2H), 3.61 (d, 1H, J = 9.8 Hz), 3.77 (d, 1H, J = 9.8 Hz), 4.09–4.19 (m, 2H), 4.59 (q, 2H, J = 12.2 Hz), 7.07 (s, 4H), 7.25 (s, 1H), 7.40 (s, 1H); IR (KBr) 2923, 2595, 1681, 1558, 1457, 1192, 1135, 1092, 1052, 971, 804 cm^{-1}; HRMS (ESI): calcd for $C_{24}H_{34}NO_6P$ $[M-H]^-$: 462.2045, found 462.2043.

5.2. Biology

5.2.1. In vitro [^{35}S] GTPγ-S binding assay

First, 58 μL of assay buffer (50 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.05 mg/mL Saponin) and 10 μL of 50 μM Guanosine 5′-diphosphate (GDP) solution (50 μMG DP, 54.1 μM 1,4-dithio-DL-threitol (DTT), 50 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.05 mg/mL Saponin) were pipetted into the wells of a 96-well polypropylene microplate (Sumitomo Bakelite Co., Ltd. SUMILON Proteosave 96 U plate, M S·3996U). Next, 2 μL of the final solution for test compounds (**2**) or their phosphate (**2-P**) was added to the individual wells, respectively. As control assays, 2 μL of either the 20 mM Pyrrolidine or MeOH solution was added to the blank well instead of the compounds. Then the assay was initiated with the addition of both 10 μL of 3.5 nM [^{35}S] GTPγ-S solution and 20 μL of 0.5 mg/mL of the membrane fraction diluted with the assay buffer and mixed by a plateshaker. After 60 min of incubation at room temperature, membrane fractions were immediately collected onto UniFilter-96, GF/C (PerkinElmer life sciences., 6005174) and repeatedly washed with 200 μL of wash buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$) 5 times by a cell harvester. The filter bound with the membrane was fully dried and BackSeal, an accessory of the filter, was attached at the bottom of the UniFilter-96, GF/C, followed by the addition of 50 μL of MICROSCINT 20 (PerkinElmer Life and Analytical Sciences) into the individual wells. After TopSeal-A: 96-Well Microplates (PerkinElmer Life Sciences.) were also attached to the front of the UniFilter-96, GF/C, bound [^{35}S] GTPγ-S radioactivity was measured as a count per minute (CPM) by TopCount (PerkinElmer Life and Analytical Sciences, TopCount.NET) and was expressed as an integral number. The individual [^{35}S] GTPγ-S binding ratios of test compounds were calculated using the following equation and were represented to one decimal place. Binding ratio (%) = 100 × (T − B)/(C − B′), where *T* represents the CPM value of a well treated with test compounds (n = 2) or Sph-l-P (n = 2 or 4) and *B* represents the mean CPM value of the blank wells (n = 4) for test compounds. *C* represents the mean CPM value of the wells treated

with 20 μM Sph-l-P ($n = 4$) and B′ represents the mean CPM value of the blank wells ($n = 4$) for Sph-l-P. The Sph-l-P stimulated-activation ratio (C/B′) was also calculated and was represented to two decimal places. When the final ratio is under 2.00, this experiment should be stopped. The calculation of both the all binding ratio and the activation ratio (C/B′) was performed using SAS System Release 8.2 (SAS Institute Inc.).

5.2.2. Lymphocyte counts following oral administration in rats

Lewis rats (male, 5 weeks of age) were used. Five rats/group were used. Vehicle (saline), 0.1 mg/kg of FTY720 or compound **2a** was orally administered to rats. Blood was collected into a tube containing EDTA before and 4 h after drug administration, and analyzed by an automatic hemacytometer to count the number of lymphocyte. The blood of untreated rats was also drawn and the lymphocyte numbers were counted.

5.2.3. Inhibitory activities against Host versus Graft Reaction in rats (HvGR)

Two strains of rats [Lewis rats (male, 6 weeks of age, Charles River Japan Inc.) and WKAH/Hkm rats (male, 7 weeks of age, Japan SLC Inc.) were used. Five rats/group were used. Splenocytes were isolated from the spleens of WKAH/Hkm and Lewis rats and suspended in RPMI1640 medium (Life Technologies Inc.) at a concentration of 1×10^8 cells/mL, 0.1 mL of the medium containing the free-floating spleen cells of WKAH/Hkm rats or Lewis rats (1×10^7 of the spleen cells) was then intracutaneously injected into the bilateral foot-pads of hindlimbs of Lewis rats. Test compounds were suspended in 0.5% tragacanth solution. The suspended compounds were orally administered to rats in the drug-treated group (Lewis rats injected with spleen cells of WKAH/Hkm rats and treated with the compound) at a volume of 5 mL/kg once daily for 4 successive days starting on the day of spleen cell injection. The tragacanth solution (0.5%) was orally administered, instead of the compound-suspended solution, to rats in the 'syngeneic group' (Lewis rats injected with spleen cells of Lewis rats) and the allogeneic group (Lewis rats injected with spleen cells of WKAH/Hkm rats and not treated with the compound). The average weight of the popliteal lymph nodes of the same strain rats was subtracted from individual weights of the popliteal lymph nodes of individual rats ('HvGR-induced changes in weight of the popliteal lymph nodes'). The inhibitory activities of compounds were calculated from the 'HvGR-induced changes in weight of the popliteal lymph nodes' of individual rats in the drug-treated group versus the average 'HvGR-induced changes in weight of the popliteal lymph nodes' in the control group. The inhibitory activities of compounds were expressed as ID_{50} values (mg/kg) as calculated by the least squares method based on the doses of compounds administered and inhibitory activities at these doses.

5.2.4. Heart rate decrease following oral administration in rats

Vehicle (1% MC solution), 10 mg/kg of FTY720 or 30 mg/kg of compound **2b** and **2e** were orally administered to Wistar Imamichi rats. Heart rate in beats per minute (bpm) of the unanesthetized and unrestrained rats were recorded at 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 4 h, 5 h, 6 h, 24 h and 48 h after oral administration. The maximum heart rate decrease (or increase) occurred during 48 h were expressed as 'Heart rate decrease (%)' as a percentage in comparison to the vehicle treated control group. Three rats were used for each group.

5.2.5. Evaluation of phosphorylation rate in in vitro

Evaluation of the phosphorylation rate of substrate in whole blood was conducted as follows. To 500 μL of rat whole blood, which was treated with aqueous 3.2% citric acid solution, was added 0.5 μL solution of test compounds in DMSO (100 μg/mL), and incubated at 37 °C for 3 h. Reactions were stopped with the addition of 1 mL of methanol, and the mixture was cleared by centrifuging twice at 15,000 rpm for 5 min at 4 °C. The supernatants were analyzed by HPLC [Column: YMC-pack ODS A-312; Eluent: 10:90 to 90:10 CH3CN: (0.1% TFA/H_2O); flow rate: 1.0 mL/min; Column temperature: 40 °C]. The phosphorylation rate was determined by the peak area ratio of the phosphorylated compound and total peak area of both the phosphorylated and remaining parent compound.

5.3. Molecular modeling

5.3.1. Induced fit docking in $S1P_1$ receptor

Compound **2f** conformations were generated using Program Ligprep[17] and used for induced fit docking[17] in the crystal structure of $S1P_1$ receptor (Protein Data Bank entry 3V2Y).[15]

5.3.2. Homology modeling of $S1P_3$ receptor

Homology model of $S1P_3$ receptor was generated with Program Prime[17] based on the $S1P_1$–compound **2f** complex model generated by induced fit docking.

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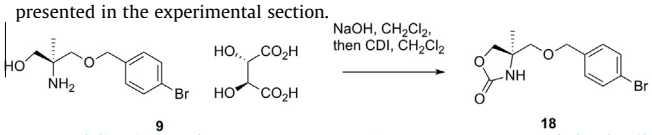

9 **18**

15. Our modeling is based on an S1P1 antagonist X-ray structure and the detail information is shown in the following report. Hanson, M. A.; Roth, C. B.; Jo, E.; Griffith, M. T.; Scott, F. L.; Reinhart, G.; Desale, H.; Clemons, B.; Cahalan, S. M.; Schuerer, S. C.; Sanna, M. G.; Han, G. W.; Kuhn, P.; Rosen, H.; Stevens, R. C. *Science* **2012**, *335*, 851.
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