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Original article

Synthesis and evaluation of CS-2100, a potent, orally active and $S1P_3$- sparing $S1P_1$ agonist

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ABSTRACT

Modulators of sphingosine phosphate receptor-1 ($S1P_1$) have recently been focused as a suppressant of autoimmunity. We have discovered a 4-ethylthiophene-based $S1P_1$ agonist 1-({4-Ethyl-5-[5-(4-phenoxyphenyl)-1,2,4-oxadiazol-3-yl]-2-thienyl}methyl)azetidine-3-carboxylic acid (CS-2100, **8**) showing potent $S1P_1$ agonist activity against $S1P_3$ and an excellent *in vivo* potency. We report herein the synthesis of CS-2100 (**8**) and pharmacological effects such as $S1P_1$ and $S1P_3$ agonist activity *in vitro*, peripheral blood lymphocyte lowering effects and the suppressive effects on adjuvant-induced arthritis and experimental autoimmune encephalomyelitis (EAE) in animal models. The pharmacokinetic data were also reported. CS-2100 (**8**) had >5000-fold greater agonist activity for human $S1P_1$ (EC_{50}; 4.0 nM) relative to $S1P_3$ (EC_{50}; >20000 nM). Following administration of single oral doses of 0.1 and 1 mg/kg of CS-2100 (**8**) in rats, lymphocyte counts decreased significantly, with a nadir at 8 and/or 12 h post-dose and recovery to vehicle control levels by 24–48 h post-dose. CS-2100 (**8**) is efficacious in the adjuvant-induced arthritis model in rats (ID_{50}; 0.44 mg/kg). In the EAE model compared to the vehicle-treated group, significant decreases in the cumulative EAE scores were observed for 0.3 and 1 mg/kg CS-2100 (**8**) groups in mice. While CS-2100 (**8**) showed potent efficacy in various animal disease models, it was also revealed that the central 1,2,4-oxadiazole ring of CS-2100 (**8**) was decomposed by enterobacteria in intestine of rats and monkeys, implicating the latent concern about an external susceptibility in its metabolic process in the upcoming clinical studies.

1. Introduction

Sphingosine 1-phosphate (S1P) receptor-1 ($S1P_1$) is one of five G-protein–coupled receptors which bind S1P, a bioactive lipid mediator involved in cell differentiation, morphogenesis, angiogenesis, motility, and multiple other processes [1,2]. Modulation of S1P receptors may have important clinical applications. In particular, targeting $S1P_1$ with synthetic modulators appears to have clinical utility in the suppression of autoimmunity by affecting lymphocyte trafficking [1–4]. Knowledge of the physiology and pharmacology of $S1P_1$ has evolved rapidly through studies involving Gilenya (fingolimod, FTY720) (**1**), which was approved for the treatment of patients with relapsing remitting multiple sclerosis (RRMS) [5,6]. According to previous reports, the active phosphorylated form of fingolimod, FTY720-P (**2**) [7–9], which is generated *in vivo* via a sphingosine kinase, acts as an $S1P_1$ agonist or functional antagonist. However, FTY720-P (**2**) lacks specificity due to its affinity for $S1P_3$, $S1P_4$, and $S1P_5$ [10,11]. The beneficial effects of fingolimod are thought to be mediated via $S1P_1$. On the other hand, $S1P_3$ is reported to be concerned with bradycardia in rodents [12–14]. Recent studies suggested that only removal of the $S1P_3$ agonism is not sufficient to exclude the cardiovascular side effect

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[15]. However, sparing the $S1P_3$ agonism has been proposed as a medicinal chemical approach to reduce potential side effects. We therefore focused our attention on synthesizing compounds with greater $S1P_1/S1P_3$ selectivity. Comprehensive evaluation of various properties of these synthesized compounds led to the identification of a promising clinical candidate, CS-0777 (**3**), conclusively [16]. CS-0777 is currently undergoing human clinical trials, and the initial clinical results for single dose administration of CS-0777 in healthy subjects and a 12-week open-label study in patients with multiple sclerosis have also been reported separately [17].

The success of **1** in RRMS clinical trials has encouraged a new and startling development of $S1P_1$ agonists [18,19]. Especially, SEW2871 (**5**), which is screening hits of non-phosphate type $S1P_1$ selective agonist from Scripps and Norvartis, has attracted attention as the second stream of $S1P_1$ agonists [20,21]. Over the past decade, a structurally-broad variety of $S1P_1$ agonists have been disclosed as exemplified by thiazolo[5,4-b]pyridine **6** (Amgen) [22] or 2-imino-thiazolidin-4-one **7** (Actelion) [23] in Fig. 1 and other compounds [14,24–27].

The reduction in peripheral blood lymphocyte counts following single doses of 1.0 and 2.5 mg CS-0777 (**3**) was sustained for more than 2 weeks. This prolonged pharmacologic effect likely reflects the relatively long pharmacokinetic half-life, although phosphorylation kinetics, selective accumulation of the drug in lymphocytes, or other factors may play a role. The half-life of CS-0777-P (**4**) in blood was ~7–8 days [17], similar to that reported for fingolimod (**1**). Achieving improved $S1P_1/S1P_3$ selectivity and decreased relative long half-life of CS-0777 (**3**) has been a consistent theme of our efforts. In this context, we focused our attention on non-phosphate type analogues to develop a potent and $S1P_3$-sparing $S1P_1$ agonist with adequate pharmacokinetic half-life. Among several non-phosphate type compounds that we explored, 4-ethylthiophene analogues appeared most promising and they were used for further scrutinization [28]. We then discovered a novel $S1P_3$-sparing $S1P_1$ agonist CS-2100 (**8**), which shows excellent *in vivo* potency.

2. Results and discussion

The synthetic route of CS-2100 (**8**) is shown in Scheme 1. The cross coupling reaction of silylether **9** [29] with ethylmagnesium bromide proceeded smoothly by using a catalytic amount of $NiCl_2$(dppp) to afford ethyl thiophene **10** in good yield. The 5-position on the thiophene ring was formylated by treatment with *n*-BuLi followed by DMF. Aldehyde **11** was treated with hydroxylamine hydrochloride in the presence of triethylamine to give oxime, which was dehydrated with DCC in toluene to afford nitrile **12**. Nitrile **12** was converted to key intermediate amidoxime **13** by treatment with 40% aqueous hydroxylamine in EtOH. Amidoxime **13** was esterified with 4-phenoxybenzoic acid in the presence of WSC–HCl and HOBt. Cyclization of the oxadiazole ring and deprotection of the TBS group proceeded smoothly by treatment with TBAF in THF to afford alcohol **15** in 89% yield. Alcohol **15** was treated with CBr_4 and PPh_3 in dichloromethane, which was then successively treated with methyl azetidine-3-carboxylate hydrochloride in the presence of diisopropylethylamine to afford methyl ester **16** in good yield. The methyl ester group was saponified with 1 M NaOH in 1,4-dioxane, and then neutralized with AcOH, which afforded CS-2100 (**8**) as a white crystalline solid.

Next, agonist-specificity of CS-2100 (**8**) was determined by measuring agonist-evoked [^{35}S]GTPγ-S binding activity to rat and human $S1P_1$ or $S1P_3$ expressed in transfected CHO-K1 cells as shown in Table 1. The active FTY720-P (**2**) and CS-0777-P (**4**) were included as a comparator. CS-0777-P (**4**) demonstrated potent agonist activity for rat and human $S1P_1$ (EC_{50} = 1.8 and 1.1 nM, respectively) and at least 100-fold greater activity for $S1P_3$ (EC_{50} = 200 and 350 nM, respectively). Relative to $S1P_3$ agonist activity, CS-0777-P (**4**) appears to have greater $S1P_1/S1P_3$ selectivity

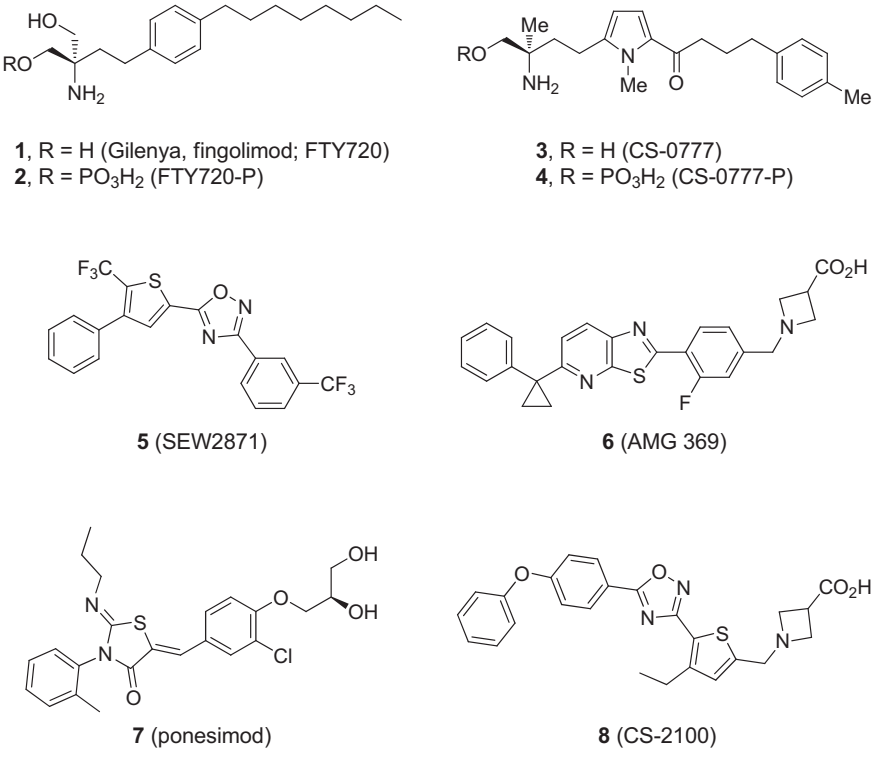
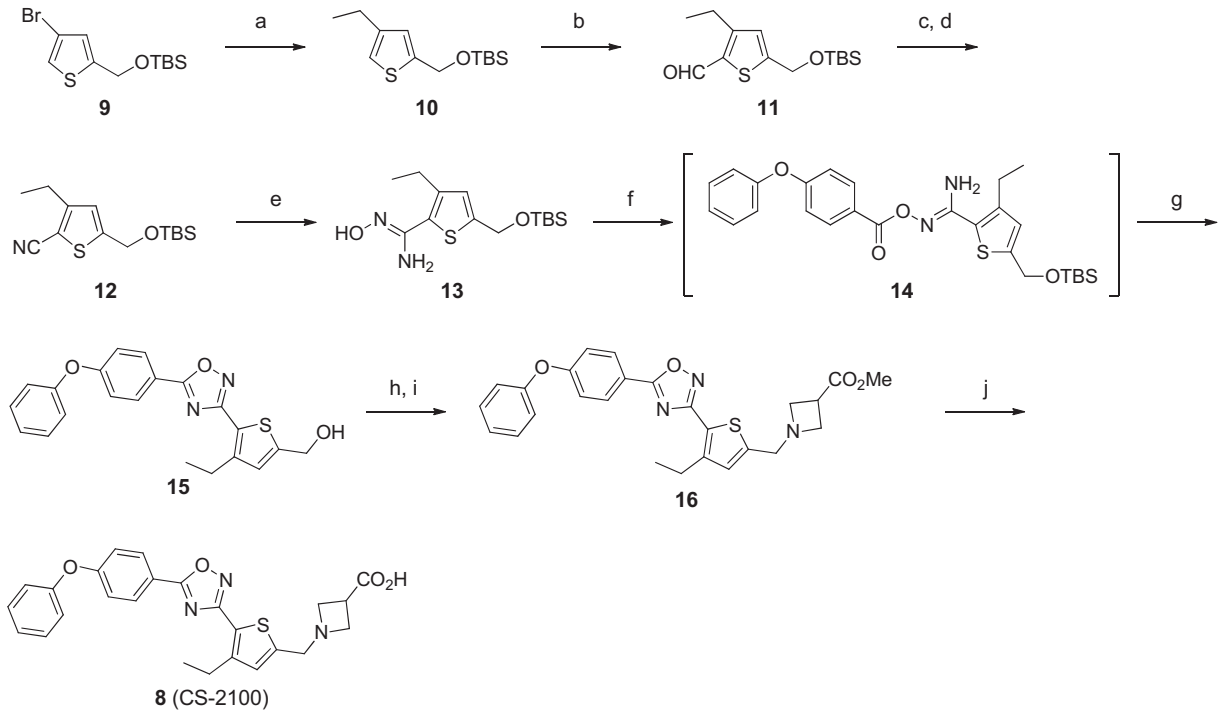


Fig. 1. Structures of FTY720 (**1**), FTY720-P (**2**), CS-0777 (**3**), CS-0777-P (**4**), SEW2871 (**5**), AMG 369 (**6**), ponesimod (**7**) and CS-2100 (**8**).



Scheme 1. Reagents and conditions: (a) EtMgBr, cat.$NiCl_2$(dppp), Et_2O, 95%; (b) *n*-BuLi then DMF, THF, 86%; (c) NH_2OH–HCl, NEt_3, MeOH, CH_2Cl_2; (d) DCC, toluene, 69% in 2 steps; (e) aq.NH_2OH, EtOH, 60%; (f) 4-Phenoxybenzoic acid, WSC–HCl, HOBt, CH_3CN, THF; (g) TBAF, THF, 89% in 2 steps; (h) CBr_4, PPh_3, CH_2Cl_2; (i) Methyl azetidine-3-carboxylate-HCl, *i*-Pr_2NEt, CH_2Cl_2, 89% in 2 steps; (j) aq.1 M NaOH, 1,4-dioxane, then neutralized with AcOH, 86%.

compared to FTY720-P (**2**). Compared with CS-0777-P (**4**), CS-2100 (**8**) has much higher $S1P_1/S1P_3$ selectivity for rat and human $S1P_1$ (EC_{50} = 1.5 and 4.0 nM, respectively) and at least 5000-fold greater activity for $S1P_3$ (EC_{50} = 7400 and >20000 nM, respectively). In addition, the agonist activity (EC_{50}) of CS-2100 (**8**) for human $S1P_4$ and $S1P_5$ were 58 and 17 nM, respectively, and it had no ability to bind human $S1P_2$ (>20000 nM).

In a second series of studies, the effects of CS-2100 (**8**) on peripheral blood lymphocyte counts were investigated in rats. In LEW/CrlCrlj (Lewis) rats administered a single oral dose of 0.1 or 1 mg/kg of CS-2100 (**8**), the peripheral blood lymphocyte number decreased to 27% and 11% of vehicle-treated control values for the 0.1 and 1 mg/kg dose levels, respectively, 8 h after CS-2100 (**8**) administration (Fig. 2). The decreased peripheral blood lymphocyte number was recovered to vehicle control levels by 24–48 h post-dose. These data suggests that CS-2100 (**8**) exhibits relatively quick recovery of the peripheral blood lymphocyte number in Lewis rats compared to CS-0777 (**3**) (30% and 72% of control for 0.1 or 1 mg/kg of CS-0777 (**3**) at 48 h oral post-dose) [16].

In addition, CS-2100 (**8**) was evaluated for its suppressive effect on adjuvant-induced arthritis in Lewis rats. The rats in the normal group were administered vehicle and the adjuvant-injected rats were administered vehicle (Control), or 0.1, 0.3, 1, or 3 mg/kg CS-2100 (**8**) from Day 0 to Day 17, orally once daily. The volumes of the right hind foot (adjuvant-injected foot) of all rats were measured on Day 0, 3, 5, 7, 10, 13, 15 and 18 with a plethysmometer (Fig. 3). A linear regression curve was obtained from the % inhibition and the logarithmic value of the dosage by least squares regression analysis, and the ID_{50} value of the compound was calculated from the curve. CS-2100 (**8**) showed a strong suppressive effect on adjuvant-induced arthritis in rats with 0.44 mg/kg of the ID_{50} value on Day 18.

Next, we evaluated the suppressive effect of CS-2100 (**8**) on experimental autoimmune encephalomyelitis (EAE) in mice. EAE has been used as a preclinical animal model for proof of concept studies for multiple sclerosis therapy. Vehicle-administered mice began to show clinical signs of EAE from Day 12, and thereafter an

Table 1
In vitro agonist-evoked GTPγ-S binding to rat and human $S1P_1$ and $S1P_3$.[a]

Compound	EC_{50} (nM)				Selectivity (human $S1P_1$ vs $S1P_3$)
	Rat		Human		
	$S1P_1$	$S1P_3$	$S1P_1$	$S1P_3$	
2 (FTY720-P)	0.29	1.3	0.37	3.3	9
4 (CS-0777-P)	1.8	200	1.1	350	320
8 (CS-2100)	1.5	7400	4.0	>20000	>5000

[a] EC_{50} is defined as the mid-point between the binding ratio of the vehicle and the maximum response of the test compound.

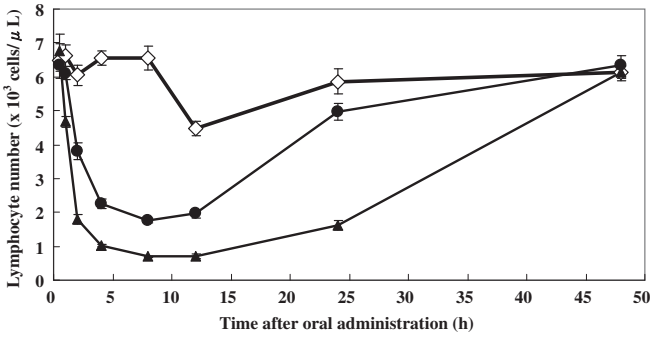


Fig. 2. Effects of a single oral dose of CS-2100 (**8**) on lymphocyte counts in rats. Vehicle (1% MC solution, ◇), 0.1 mg/kg (●), or 1 mg/kg of CS-2100 (**8**) (▲) was orally administered to rats. The rats were anesthetized and abdominally dissected 0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, and 48 h after oral administration. Lymphocyte numbers are shown as mean ± SE.

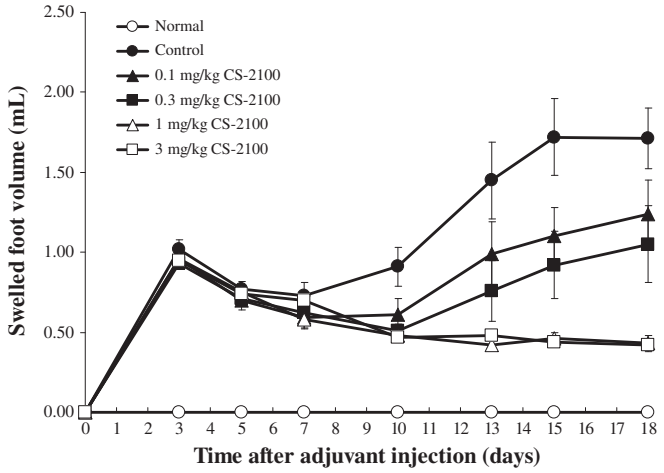


Fig. 3. Time course of the swelled foot volume of the rats with induced adjuvant arthritis. Swelled foot volumes are shown as mean ± SE.

acute increase of the EAE score was observed, reaching a maximal level on Day 18 (Fig. 4). Mean cumulative EAE scores, calculated by summing the daily scores, were 14.4, 10.0, 0, and 0 for the vehicle and 0.1, 0.3 and 1 mg/kg CS-2100 (**8**) groups, respectively (data not shown). Compared to the vehicle-treated group, statistically significant decreases in the cumulative EAE scores were observed for the 0.3 and 1 mg/kg CS-2100 (**8**) groups (nonparametric Dunnett test [joint ranking], $p < 0.01$). Thus, CS-2100 (**8**) has a potent suppressive effect on EAE in mice, and the above data represent CS-2100 (**8**) to be a potential agent for the treatment of MS.

The pharmacokinetics of CS-2100 (**8**) was evaluated in male Lewis rats and C57BL/6J mice. The plasma concentration versus time profiles of CS-2100 (**8**) in Fig. 5 and the PK parameters of CS-2100 (**8**) are presented in Table 2. Mean maximum plasma concentration (C_{max}) at a dose of 1 mg/kg in rats and mice was 186 and 135 ng/ml, respectively, and the time reaching C_{max} (T_{max}) was 2.0 and 4.0 h post-dose in rats and mice, respectively. In rats, good dose proportionality was observed for C_{max} and $AUC_{0-72\ h}$ between the doses from 0.1 to 1 mg/kg. Terminal-half life ($T_{1/2}$) in rats and mice at a dose of 1 mg/kg was 12.4 and 13.9 h, respectively.

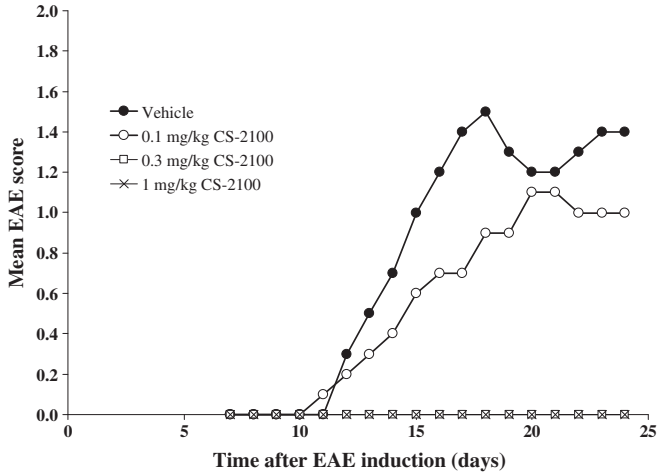


Fig. 4. Time course of mean EAE scores. The EAE score was evaluated daily from Day 7 to Day 24 in accordance with the following criteria: 0, normal; 1, flaccid tail without difficulty in picking themselves up; 2, hindlimb weakness defined as paralysis of only one hindlimb and/or difficulty in picking themselves up; 3, paralysis of both hindlimbs; 4, quadriplesia; 5, dead.

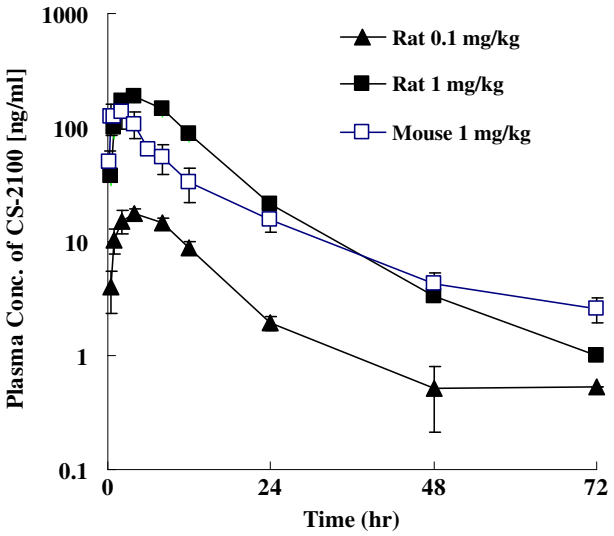


Fig. 5. Plasma concentrations of CS-2100 (**8**) after oral administration to rats (0.1 and 1 mg/kg) and mice (1 mg/kg). Each value is the mean ± standard deviation of five animals in the study.

While the promising efficacy of CS-2100 (**8**) as immunosuppressant in a variety of preclinical animal disease models was observed, it was also revealed that CS-2100 (**8**) had a latent concern about its metabolic process. From identifying studies on metabolites of CS-2100 (**8**) *in vivo*, one of the major metabolites was found to be 4-Phenoxy benzoic acid (4-PBA, **17**) in rat and monkey plasma after oral administration (Fig. 6), although the preceding *in vitro* metabolic stability studies in liver microsomes did not indicate the generation of 4-PBA (**17**) as a metabolite nor the degradation of 1,2,4-oxadiazole ring (data not shown). Further detailed analysis revealed that CS-2100 (**8**) was subjected to enterobacterial metabolism in the intestine, resulting in the decomposition of the 1,2,4-oxadiazole ring triggered by the reductive cleavage of the N–O bond to form 4-PBA (**17**) (data not shown). This undesirable ring-opening would arouse concern about potential species or individual differences in pharmacokinetics and pharmacodynamics, and accompanying unpredictable toxicological issues. It is noteworthy that whereas 1,2,4-oxadiazole has been known as an attractive and versatile heteroaromatic ring in drug design from its synthetic facility and favorable physicochemical aspect such as superior oxidative metabolic stability and characteristic moderate polarity, it has not been publicized enough that 1,2,4-oxadiazoles have a potential to be subjected to reductive metabolism in liver and intestinal bacterial flora as described in this report [30–34].

3. Conclusion

In summary, we presented CS-2100 (**8**), a structurally novel $S1P_1$ selective modulator showing potent and selective $S1P_1$ agonist activity *in vitro*; peripheral blood lymphocyte lowering and suppression of the development of adjuvant-induced arthritis in a rat model and EAE in a mice model. With an unfavorable

Table 2
PK parameters of CS-2100 (**8**) after oral administration to rats and mice.

Species	Dose (mg/kg)	C_{max} (ng/ml)	T_{max} (h)	$T_{1/2}$ (h)	$AUC_{0-72\ h}$ (ng*h/ml)
Lewis rats	0.1	17.4	4.0	12.4	268
	1.0	186	4.0	8.73	2670
C57BL mice	1.0	135	2.0	13.9	1540

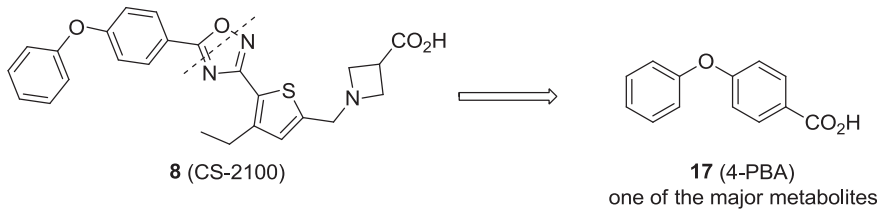


Fig. 6. 4-Phenoxy benzoic acid resulted from reductive cleavage of the 1,2,4-oxadiazole ring of CS-2100 (**8**) in rat and monkey.

metabolite 4-PBA (**17**) and the externally susceptible metabolic pathway identified, our research effort focused to the substitution of the central 1,2,4-oxadiazole ring to identify the robust back-up compound of CS-2100 (**8**). This continuous study will be reported elsewhere.

4. Experimental

4.1. Chemistry

4.1.1. General

NMR spectra were recorded on a Varian Mercury 400 or 500 spectrometer with tetramethylsilane as an internal reference. The Infrared spectra were recorded on a Jasco FT/IR-830 spectrophotometer, and the peaks were recorded in cm^{-1}. The mass spectra were recorded on a JEOL JMS-AX505H. TLC analysis was performed on $60F_{254}$ plates. Column chromatography was performed on Silica gel 60 (Merck, 230-400).

4.1.2. tert-Butyl[(4-ethyl-2-thienyl)methoxy]dimethylsilane (*10*)

To a solution of [(4-bromo-2-thienyl)methoxy]-*tert*-butyldimethylsilane **9** (0.61 g, 2.0 mmol) and [1,3-bis(diphenylphosphino) propane]dichloronickel (54 mg, 0.1 mmol) in Et_2O (5 ml) was slowly added ethylmagnesium bromide (1.0 M in THF, 3.0 ml, 3.0 mmol) at 0 °C. The resulting mixture was warmed to 25 °C and stirred for 1 h, at which time the reaction was quenched with sat. aq. NH_4Cl (5 ml). The resulting biphasic mixture was poured into water (20 ml) and extracted with Et_2O. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered and concentrated. The residue was purified by flash column chromatography (silica gel, hexane:EtOAc 0:10 to 5:95) to afford the title compound **10** (0.44 g, 1.7 mmol, 95%), as a pale yellow oil. ^1H NMR (400 MHz, $CDCl_3$) δ 0.10 (s, 6H), 0.93 (s, 9H), 1.21 (t, 3H, $J = 7.4$ Hz), 2.58 (q, 2H, $J = 7.4$ Hz), 4.82 (s, 2H), 6.77 (s, 1H), 6.81 (s, 1H). IR (liquid film): 1077, 1131, 1174, 1255, 1463, 1471 cm^{-1}. MS (FAB): *m*/*z* 255 $(M - H)^+$.

4.1.3. 5-({[tert-Butyl(dimethyl)silyl]oxy}methyl)-3-ethylthiophene-2-carboxaldehyde (*11*)

To a solution of **10** (1.3 g, 5.6 mmol) in THF (10 ml) was slowly added *n*-butyllithium (1.6 M in hexane, 4.2 ml, 6.7 mmol) at −78 °C, and warmed to 0 °C. After stirring for 30 min at 0 °C, the reaction mixture was cooled to −78 °C again and was slowly added *N*,*N*-dimethylformamide (0.86 ml, 11 mmol). After stirring for 30 min at −78 °C, the reaction was quenched with sat. aq. NH_4Cl (5 ml). The resulting biphasic mixture was poured into water (20 ml) and extracted with Et_2O. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered and concentrated. The residue was purified by flash column chromatography (silica gel, hexane:EtOAc 0:10 to 5:95) to afford the title compound **11** (1.3 g, 4.6 mmol, 86%) as a pale yellow oil. ^1H NMR (400 MHz, $CDCl_3$) δ 0.12 (s, 6H), 0.94 (s, 9H), 1.29 (t, 3H, $J = 7.4$ Hz), 2.94 (q, 2H, $J = 7.4$ Hz), 4.86 (s, 2H), 6.84 (s, 1H), 9.99 (s, 1H). IR (liquid film): 1092, 1156, 1225, 1256, 1461, 1659 cm^{-1}. MS (EI): *m*/*z* 285 M^+.

4.1.4. 5-({[tert-Butyl(dimethyl)silyl]oxy}methyl)-3-ethylthiophene-2-carbonitrile (*12*)

To a suspension of **11** (1.3 g, 4.8 mmol) and hydroxylamine hydrochloride (0.37 g, 5.3 mmol) in CH_2Cl_2 (20 ml) and MeOH (2 ml) were added triethylamine (1.3 ml, 9.6 mmol) at 25 °C. The resulting mixture was stirred at 25 °C for 2 h. After removing the solvent *in vacuo*, toluene (10 ml) was added to the resulting mixture and evaporated azeotropically *in vacuo*. The residue was suspended in toluene (20 ml) and added dicyclohexylcarbodiimide (1.1 g, 5.3 mmol) at 25 °C. The reaction mixture was stirred at 90 °C for 15 h. After cooling to room temperature, hexane (20 ml) was added to the reaction mixture, and the resulting mixture was filtered through Celite. The filtrate was evaporated *in vacuo*, and the residue was purified by flash column chromatography (silica gel, hexane:EtOAc 0:10 to 5:95) to afford the title compound **12** (0.94 g, 3.3 mmol, 69%) as a pale yellow oil. ^1H NMR (400 MHz, $CDCl_3$) δ 0.11 (s, 6H), 0.93 (s, 9H), 1.25 (t, 3H, $J = 7.8$ Hz), 2.75 (q, 2H, $J = 7.8$ Hz), 4.83 (s, 2H), 6.74 (s, 1H). IR (liquid film): 1093, 1149, 1256, 2212 cm^{-1}. MS (EI): *m*/*z* 282 M^+.

4.1.5. 5-({[tert-Butyl(dimethyl)silyl]oxy}methyl)-3-ethyl-N′-hydroxythiophene-2-carboximidamide (*13*)

To a solution of **12** (0.93 g, 3.3 mmol) in EtOH (5 ml) was added hydroxylamine (40 wt% in water, 0.5 ml, 6.1 mmol), and stirred at 60 °C for 1 h. After removal of the solvent *in vacuo*, the residue was diluted with Et_2O, poured into water (20 ml) and extracted with Et_2O. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered and concentrated. The residue was purified by recrystallization (hexane:AcOEt 6:4) to afford the title compound **13** (0.60 g, 1.9 mmol, 60%) as a white crystalline solid. ^1H NMR (400 MHz, $CDCl_3$) δ 0.11 (s, 6H), 0.93 (s, 9H), 1.20 (t, 3H, $J = 7.8$ Hz), 2.76 (q, 2H, $J = 7.8$ Hz), 4.80 (s, 4H), 6.76 (s, 1H), 7.10 (br, 1H). IR (KBr): 1059, 1590, 1643, 3284, 3357, 3491 cm^{-1}. MS (EI): *m*/*z* 315 M^+.

4.1.6. {4-Ethyl-5-[5-(4-phenoxyphenyl)-1,2,4-oxadiazol-3-yl]-2-thienyl}methanol (*15*)

To a solution of 4-phenoxybenzoic acid (0.12 g, 0.53 mmol) in acetonitrile (4 ml) and tetrahydrofuran (2 ml) was added successively 1-hydroxybenzotriazole (74 mg, 0.55 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.11 g, 0.55 mmol) and **13** (0.16 g, 0.50 mmol), and the resulting mixture was stirred at 50 °C for 30 min. After cooling to room temperature, the reaction was quenched with water (5 ml), and the resulting biphasic mixture was poured into water (20 ml) and extracted with ethyl acetate. The combined organic layers were washed successively with 0.1 M hydrochloric acid, sat. aq. $NaHCO_3$ and brine, and dried over Na_2SO_4, filtered and concentrated. The residue was diluted with THF (5 ml) and added tetrabutylammonium fluoride (1.0 M in THF, 1.0 ml, 1.0 mmol), and the resulting mixture was stirred at 50 °C for 2 h. After cooling to room temperature, the reaction mixture was poured into water (20 ml) and extracted with Et_2O. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash column chromatography (silica gel, hexane:EtOAc 1:3) to afford the title compound **15** (0.17 g, 0.45 mmol, 89%) as a pale

yellowish crystalline solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.29 (t, 3H, J = 7.4 Hz), 1.88 (t, 1H, J = 5.9 Hz), 3.05 (q, 2H, J = 7.4 Hz), 4.83 (d, 2H, J = 5.9 Hz), 6.96 (s, 1H), 7.04–7.10 (m, 4H), 7.20 (t, 1H, J = 7.4 Hz), 7.39 (t, 2H, J = 7.4 Hz), 8.12 (d, 2H, J = 9.0 Hz). IR (KBr): 1248, 1353, 1490, 1496, 1515, 1588, 1612, 3356 cm^{-1}. MS (FAB): m/z 379 $(M + H)^+$.

4.1.7. Methyl 1-({4-ethyl-5-[5-(4-phenoxyphenyl)-1,2,4-oxadiazol-3-yl]-2-thienyl}methyl)azetidine-3-carboxylate (**16**)

To a solution of **15** (0.17 g, 0.44 mmol) in CH_2Cl_2 (6.0 ml) was successively added carbon tetrabromide (0.19 g, 0.57 mmol) and triphenylphosphine (0.15 g, 0.57 mmol) at 0 °C and stirred for 1 h. The reaction mixture was added successively methyl 3-azetidinecarboxylate hydrochloride (0.10 g, 0.66 mmol) and *N*,*N*-diisopropylethylamine (0.23 ml, 1.3 mmol) at 0 °C, and warmed to 25 °C. After stirring for 2 h, the reaction was quenched with sat. aq. $NaHCO_3$ (1.0 ml). The resulting biphasic mixture was poured into water (20 ml) and extracted with ethyl acetate. The extract was washed with brine, dried over $MgSO_4$, filtered and concentrated. The residue was purified by flash column chromatography (silica gel, hexane:EtOAc 1:3 to 1:2) to afford the title compound **16** (0.19 g, 0.40 mmol, 89%) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ : 1.27 (t, 3H, J = 7.4 Hz), 3.03 (q, 2H, J = 7.4 Hz), 3.30–3.40 (m, 3H), 3.59–3.67 (m, 2H), 3.71 (s, 3H), 3.78 (s, 2H), 6.84 (s, 1H), 7.04–7.10 (m, 4H), 7.20 (t, 1H, J = 7.4 Hz), 7.39 (t, 2H, J = 7.4 Hz), 8.11 (d, 2H, J = 9.0 Hz). IR (liquid film): 1168, 1200, 1245, 1346, 1489, 1514, 1589, 1613, 1737 cm^{-1}. MS (FAB): m/z 476 $(M + H)^+$.

4.1.8. 1-({4-Ethyl-5-[5-(4-phenoxyphenyl)-1,2,4-oxadiazol-3-yl]-2-thienyl}methyl)azetidine-3-carboxylic acid (**8**)

To a solution of **16** (0.18 g, 0.39 mmol) in 1,4-dioxane (3 ml) was added NaOH (1.0 M in water, 1.2 ml, 1.2 mmol), and stirred at 25 °C for 2 h. The reaction was quenched with acetic acid (68 μl, 1.2 mmol), and the resulting mixture was evaporated *in vacuo*. To the residue thus obtained were added successively MeOH (3 ml) and water (2 ml) with stirring, and the white solid precipitated was collected by filtration using a Kiriyama funnel, washed with a mixed solvent of water and methanol (3:7) and dried *in vacuo* to afford the title compound **8** (0.15 g, 0.33 mmol, 86%) as a white crystalline solid. ^{1}H NMR (400 MHz, CD_3CO_2D) δ 1.30 (t, 3H, J = 7.4 Hz), 3.09 (q, 2H, J = 7.4 Hz), 3.76–3.87 (m, 1H), 4.33–4.45 (m, 2H), 4.45–4.57 (m, 2H), 4.68 (s, 2H), 7.15 (d, 4H, J = 9.0 Hz), 7.24 (t, 1H, J = 7.4 Hz), 7.32 (s, 1H), 7.45 (t, 2H, J = 7.4 Hz), 8.17 (d, 2H, J = 9.0 Hz). ^{13}C NMR (500 MHz, CD_3CO_2D) δ 176.3, 175.9, 165.6, 163.2, 156.5, 149.1, 135.0, 134.0, 131.3, 131.2, 126.3, 125.9, 121.2, 119.2, 119.0, 56.3, 52.9, 33.9, 24.2, 14.7. IR (KBr): 1167, 1249, 1347, 1489, 1517, 1557, 1592, 1613, 3422 cm^{-1}. MS (FAB): m/z 462 $(M + H)^+$.

4.2. Biology

4.2.1. In vitro agonist-evoked GTPγ-S binding assay

To measure the functional activation of the S1P receptors, an agonist stimulation of *in vitro* [^{35}S] GTPγ-S binding assay was performed as follows. The membrane was homogenized from CHO cells expressing rat $S1P_1$, rat $S1P_3$, human $S1P_1$ or human $S1P_3$, respectively, with assay buffer (5 mM Tris–HCl, pH7.4, 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA) and centrifuged at 100,000 × g for 60 min at 4 °C. For *in vitro* [^{35}S] GTPγ-S binding assay, serial dilutions of test compound (FTY720-P, CS-0777-P or CS-2100) were added to aliquots (1–10 μg protein/well) of the membrane and assayed as described in reference [35].

4.2.2. Counting of peripheral lymphocytes

Lewis rats (male, 5 weeks of age, Charles River Japan Inc.) were used. Five rats/group were used. The compound was suspended in 1% (w/v) methyl cellulose #400 solution (vehicle). Suspended solution of the compound was orally administered to rats at a volume of 5 ml/kg. In control rats, vehicle instead of the suspended solution of the compound was orally administered. Blood was collected from the postcaval vein of the rats under ether anesthesia at the indicated time in Fig. 2. Then, the collected blood was placed into a tube containing EDTA. The absolute number of lymphocytes in the collected blood was counted using a full blood count analyzer.

4.2.3. Evaluation of antiarthritic activity

Lewis rats aged 8 weeks were used for the study. Heat-killed dried *Mycobacterium butyricum* were ground on an agate mortar and then suspended in dry-sterilized liquid paraffin to make a 2 mg/ml suspension. The resulting suspended solution was then sonicated and used as adjuvant. Arthritis was induced by intradermal injection of the prepared adjuvant (0.05 ml) into the foot pad of the right hindlimb of rats in the compound-treated group and in the control group. Rats that were not treated with adjuvant were separately used as normal control group. The compound was suspended in 1% (w/v) methyl cellulose #400 solution and orally administered to rats in the compound-treated group at a volume of 5 ml/kg once daily from the injection day of the adjuvant (Day 0) for 18 successive days. To rats in the control groups 1% (w/v) methyl cellulose #400 solution alone was similarly administered. The right foot volume of each rat was measured by customized apparatus for determination of the volume at the indicated time in Fig. 3. The mean swelled volume of each group was thus calculated. Percent inhibition of swelling of the injected foot of treated animals as compared with that of the control animals was calculated according to the following equation:

Percentage inhibition of swollen foot volume (%) = {1 − [(swollen foot volume of animals treated with a compound) − (foot volume of normal control animals)]/[(swollen foot volume of control animals) − (foot volume of normal control animals)]} × 100.

4.2.4. Evaluation of the suppressive effect on EAE in mice

Mycobacterium tuberculosis H37 RA (Difco Laboratories) suspension (8 mg/ml) in incomplete Freund's adjuvant (Difco Laboratories) was mixed with myelin oligodendrocyte glycoprotein$_{35–55}$ ($MOG_{35–55}$, Peptide Institute, Inc.) (4 mg/ml solution) in physiological saline at equal volumes, and the mixture was emulsified with a sonicator on ice. C57BL/6J mice (female, 6 weeks of age, Japan SLC, Inc.) were immunized by subcutaneous injection of 50 μl of emulsion into each of the right and left axillas. After immunization on Day 0, 200 μl of pertussis toxin, *Bordetella pertussis* (PT, Calbiochem, 1 μg/ml solution) in physiological saline was injected via the tail vein. On Day 2, the same volume of PT solution (1 μg/ml) was injected. Vehicle (1% (w/v) methyl cellulose #400 solution) or 0.1, 0.3 or 1 mg/kg compound was administered orally once daily from Day 0 to Day 23. The EAE score was evaluated daily from Day 7–24 using the following criteria: 0, normal; 1, flaccid tail; 2, hindlimb weakness; 3, paralysis of both hindlimbs; 4, quadriplegia; 5, dead (Mendel et al., 1995). The cumulative EAE score was calculated by summing up daily scores (Days 7–24).

4.3. Pharmacokinetics

4.3.1. Pharmacokinetics in rats

Pharmacokinetics of CS-2100 (**8**) in male Lewis rats was evaluated using an aliquot of blood collected at each time point in the blood lymphocyte count evaluation study (section 4.2.2). The blood was centrifuged to prepare plasma and the plasma concentration was measured using liquid chromatography-tandem mass spectrometry (LC–MS/MS) system after solid phase extraction. The LC–MS/MS system consisted of a Prominence LC-20A system

(Shimadzu Corp., Japan) and an API 5000 (Applied Biosystems/MDS SCIEX). The column used was a Capcell pak C_{18} ACR (column size: 3.0 mm I.D. × 150 mm, particle size: 5 μm, Shiseido Co., Japan). Analysis was performed using WinNonlin™ (Pharsight, Palo Alto, CA) and parameters were estimated by noncompartmental analysis using mean concentration data from five animals for each time point.

4.3.2. Pharmacokinetics in mice

Male C57BL16J mice were orally dosed with CS-2100 (**8**) as a solution in an independent experiment. CS-2100 (**8**) was solubilized in dimethyl acetamide/25% (w/v) Purebright™ (NOF Corp., Japan) solution/distilled water (1/8/1, v/v/v). Suspended solution of the compound was orally administered to mice at a volume of 5 ml/kg. Blood was collected from the inferior vena cava of the mice under ether anesthesia at the indicated time in Fig. 2. All the other procedures were the same as aforementioned study in rats (section 4.3.1).

Author contributions

T. Nakamura, M. Asano, Y. Sekiguchi, Y. Mizuno, K. Tamaki, and T. Nishi contributed to the Design, Synthesis of CS-2100; F. Nara, Y. Kawase, and M. Nagasaki contributed to the *in vitro* study of CS-2100, CS-0777-P and FTY720-P; Y. Yabe, D. Nakai, E. Kamiyama, and Y. Urasaki-Kaneno contributed to the PK study of CS-2100; and T. Shimozato, H. Doi-Komuro, T. Kagari, W. Tomisato, R. Inoue, H. Yuita, K. Oguchi-Oshima, and R. Kaneko contributed to the *in vivo* study of CS-2100.

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