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LETTER

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4-Methoxy-*N*-[2-(trifluoromethyl)biphenyl-4-ylcarbamoyl]nicotinamide: A Potent and Selective Agonist of $S1P_1$

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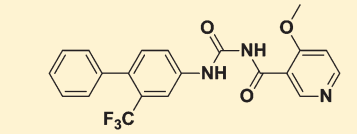
Supporting Information

ABSTRACT: The sphingosine-1-phosphate-1 receptor ($S1P_1$) and its endogenous ligand sphingosine-1-phosphate (S1P) cooperatively regulate lymphocyte trafficking from the lymphatic system. Herein, we disclose 4-methoxy-*N*-[2-(trifluoromethyl)biphenyl-4-ylcarbamoyl]nicotinamide (**8**), an uncommon example of a synthetic $S1P_1$ agonist lacking a polar headgroup, which is shown to effect dramatic reduction of circulating lymphocytes (POC = −78%) in rat 24 h after a single oral dose (1 mg/kg). The excellent potency that **8** exhibits toward $S1P_1$ (EC_{50} = 0.035 μM, 96% efficacy) and the >100-fold selectivity that it displays against receptor subtypes $S1P_{2-5}$ suggest that it may serve as a valuable tool to understand the clinical relevance of selective $S1P_1$ agonism.



KEYWORDS: Sphingosine-1-phosphate-1 receptor agonist, peripheral lymphocyte count, immunosuppression, multiple sclerosis

Sphingosine-1-phosphate (**1**, S1P) and its affiliated G-protein-coupled receptors $S1P_{1-5}$ are involved in modulating myriad physiological processes including cardiovascular function, pulmonary endothelium integrity, and cell motility (Figure 1).[1,2] S1P (**1**) is derived biosynthetically from sphingosine (**2**) via phosphorylation mediated by either sphingosine kinase 1 or sphingosine kinase 2 (SphK1 or SphK2).[3] Several years ago, S1P (**1**) and $S1P_1$ were shown to regulate lymphocyte trafficking from the lymphatic system.[4,5] At high concentrations of **1** or upon exposure to synthetic agonists of $S1P_1$, binding to $S1P_1$ located on the cell surface of lymphocytes in lymphatic tissues induces receptor internalization (RI) and prevents lymphocyte egress to the periphery and the central nervous system (CNS).[6,7] Synthetic agonists of $S1P_1$ are under intense investigation as potential therapies for medical conditions that may benefit from immunosuppression, such as multiple sclerosis (MS).[8,9]

Fingolimod (**3**, FTY720)[10,11] bears a dihydroxyamino polar headgroup akin to that of **2** and is likewise phosphorylated by SphK2 to give its bioactive congener **4** (FTY720-P),[12,13] the first non-natural agonist of $S1P_1$ to be reported (Figure 1). Notably, **4** displays agonist activity toward several S1P receptor subtypes,[14] and until recently, its activation of $S1P_3$ was believed to be exclusively responsible for its bradycardia side effects in humans and preclinical animal models.[15,16] A short time ago, our laboratory disclosed 1-(3-fluoro-4-(5-(1-phenylcyclopropyl)thiazolo-[5,4-b]-pyridin-2-yl)benzyl)azetidine-3-carboxylic acid (**5**, AMG369), a potent and selective dual agonist of $S1P_1$/$S1P_5$ bearing a polar headgroup that does not require bioactivation.[17,18] Much more rare are $S1P_1$ agonists that do not contain a polar headgroup,[8,9] such as 5-(4-phenyl-5-(trifluoromethyl)thiophen-2-yl)-3-(3-(trifluoro-

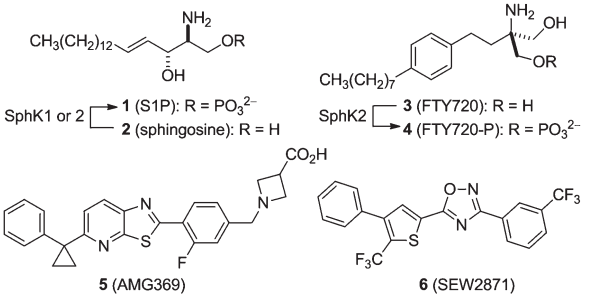


Figure 1. Endogenous and synthetic agonists of $S1P_1$.

methyl)phenyl)-1,2,4-oxadiazole (**6**, SEW2871),[19,20] a compound that is exquisitely selective for $S1P_1$ versus the other S1P receptor subtypes.[21] The agonism of $S1P_1$ by **4**, **5**, and **6** has been shown to effect reduction of circulating lymphocytes in vivo,[11,17,22] and **3** (FTY720) was recently approved by the U.S. Food and Drug Administration for the treatment of relapsing forms of MS.[23,24] As part of an effort to discover structurally novel agonists of $S1P_1$, we set out to search for atypical chemotypes like **6** that lack a polar headgroup.[25,26]

During a high-throughput screening (HTS) campaign, we identified *N*-(3-chloro-4-(piperidin-1-yl)phenylcarbamothioyl)-2-methoxybenzamide (**7**, Scheme 1).[27] The auspicious potency

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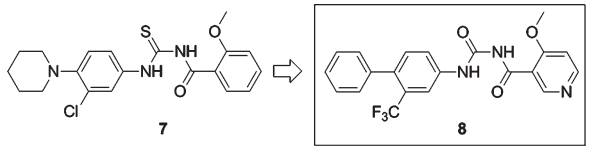
of **7** toward $S1P_1$ (EC_{50} = 0.43 μM, 87% efficacy),[28] coupled with its excellent selectivity against $S1P_3$ (EC_{50} > 25 μM)[29] and reasonable molecular and physicochemical properties (MW = 404, PSA = 54 Å^2, cLogP = 4.5, and $cLogD_{7.4}$ = 4.7),[30] prompted us to investigate analogues that might exhibit improved physicochemical and biological profiles. The highlights of this lead optimization effort and its fruition in the discovery of a potent, selective, and orally bioavailable agonist of $S1P_1$ lacking a polar headgroup, 4-methoxy-*N*-(2-(trifluoromethyl)biphenyl-4-ylcarbamoyl)nicotinamide (**8**) are the subject of this report (Scheme 1).

Carbamoylnicotinamide **8** was prepared as detailed in Scheme 2. Commercially available 2-(trifluoromethyl)biphenyl-4-amine (**9**)[31] was treated with phosgene at elevated temperature to afford isocyanate **10**,[32] which was used immediately in the next step of the synthesis without purification. Deprotonation of known 4-methoxynicotinamide (**11**)[33] with NaH followed by addition of **10** gave carbamoylnicotinamide **8** in 35% overall yield for the two-step sequence.[34] This expedient route provided a multigram quantity of **8** that was sufficiently pure (>99%, HPLC) for an in vivo toxicology study (vide infra).

Table 1 summarizes the lead optimization endeavor through which carbamothioylbenzamide **7** evolved to carbamoylnicotinamide **8**.[35] Although initially wary of the potential chemical lability of the carbamothioylbenzamide moiety of **7**,[36] we speculated that its intramolecular hydrogen-bonding network[37] might help impart hydrolytic stability and conformational rigidity to it and its carbamoylbenzamide analogues.[38−40] However, the potential toxicity of the carbamothioylbenzamide moiety or its possible metabolites[41] prompted us to exchange the sulfur atom with oxygen to give carbamoylbenzamide **12**. Whereas **12** exhibits reasonable potency toward $S1P_1$ (EC_{50} = 0.23 μM, 120% efficacy), its cell permeability is diminished (P_{app} < 1.0 × 10^{-6} cm/s). Postulating that the chloro and piperidinyl substituents of **12** may bind to the same region of $S1P_1$ as do the trifluoromethyl and phenyl groups on the thiophene ring of **6**,[25] we replaced the

Scheme 1. Evolution of HTS Hit 7 to Carbamoylnicotinamide 8



Scheme 2. Synthesis of Carbamoylnicotinamide 8

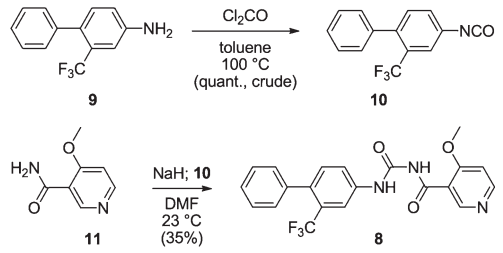
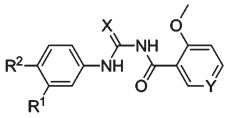


Table 2. S1P Receptor Subtype Selectivity of 8[a]

EC_{50} (μM)			
$hS1P_2$	$hS1P_3$	$hS1P_4$	$hS1P_5$ (% eff.)
>25	>25	>50	4.3 (58)

[a] See the Supporting Information for experimental details (data represent an average of at least two determinations; see ref 44).

Table 1. $S1P_1$ Structure−Activity Relationship, Physicochemical and Microsomal Stability Profiles, and PLC Activity[a]



	X	Y	R^1	R^2	$hS1P_1$, (μM)[b] EC_{50} (% eff.)	sol. (μg/mL)[c] HCl, PBS, SIF	P_{app} (ER)[d] (× 10^{-6} cm/s)	CL_{int}, RLM[e] (μL/min/mg)	PLC, POC[f] (C_{plasma}, ng/mL)
7	S	CH	Cl	*N*-piperidinyl	0.43 (87)	<1.0, < 1.0, 27	1.8 (1.0)	29	ND[g]
12	O	CH	Cl	*N*-piperidinyl	0.23 (120)	3.6, < 1.0, 8.5	<1.0 (NR)[h]	36	ND[g]
13	O	CH	CF_3	*N*-piperidinyl	0.013 (120)	3.0, < 1.0, 4.1	<1.0 (NR)[h]	26	ND[g]
14	O	CH	CF_3	phenyl	0.068 (110)	<1.0, < 1.0, 23	<1.0 (NR)[h]	15	−20 (4.9)[i,j]
15	O	CH	*i*-Pr	phenyl	0.0054 (120)	<1.0, < 1.0, 24	1.2 (1.0)	<14	+3.0 (7.0)[i]
16	O	CH	CF_3	2-pyridinyl	0.013 (110)	28, 19, 8.5	7.4 (1.1)	15	−14 (13)[i]
17	O	CH	CF_3	2-thiazolyl	0.038 (130)	1.1, < 1.0, 12	12 (1.4)	33	−18 (1.0)[i]
18	O	CH	CF_3	isopropyl	0.014 (110)	<1.0, < 1.0, 6.9	2.1 (1.0)	<14	−16 (BQL)[i,k]
19	O	CH	CF_3	CF_3CH_2O	0.0068 (83)	<1.0, < 1.0, 2.2	<1.0 (NR)[h]	25	+4.0 (1.0)[i]
8	O	N	CF_3	phenyl	0.035 (96)	17, 6.6, 16	2.3 (1.0)	<14	−67 (64)[l]

[a] See the Supporting Information for experimental details. [b] Data represent an average of at least two determinations; see ref 28. [c] Solubility in 0.010 M aqueous hydrogen chloride (HCl), phosphate-buffered saline (PBS) at pH 7.4, or simulated intestinal fluid (SIF). [d] Apparent permeability (P_{app}) through porcine proximal tubule cells (LLC-PK1 cell line) and efflux ratio (ER). [e] Estimated intrinsic clearance (CL_{int}) determined by incubation of test compound with rat liver microsomes (RLM) for 30 min and measurement of % turnover. [f] Percent-of-control (POC) reduction vs vehicle in PLC 24 h after a single oral dose (1 mg/kg; vehicle = 20% captisol, 1% HPMC, and 1% pluronic F68, pH 2.1 with MSA) administered to female Lewis rats (N = 5/group) and total compound concentration in plasma (C_{plasma}) at the 24 h time point. [g] ND, not determined. [h] NR, not reportable. [i] The measured POC reduction in PLC is not statistically significant (P > 0.05 vs vehicle by the ANOVA/Dunnett's multiple comparison test). [j] See ref 43. [k] BQL, below the quantifiable limit. [l] The measured POC reduction in PLC is statistically significant (P < 0.05 vs vehicle by the ANOVA/Dunnett's multiple comparison test).

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Table 3. Pharmacokinetic Profile of Carbamoylnicotinamide 8[a]

	1 mg/kg iv						
PPB[b] (%) rat	CL[c] (L/h/kg)	V_{dss}[c] (L/kg)	$T_{1/2}$[c] (h)	$AUC_{0\rightarrow 48\,h}$[c] (ng h/mL)	$AUC_{0\rightarrow 48\,h}$[d] (ng h/mL) 3 mg/kg po	F[e] (%)	$AUC_{0\rightarrow 48\,h}$[f] (ng h/mL) 100 mg/kg po
95	0.25	6.3	19	3400	13000	140	350000

[a] In vivo experiments were conducted using male Sprague−Dawley rats (N = 3/group). [b] Percent rat plasma protein binding (PPB) of **8** measured in vitro following separation by ultracentrifugation. [c] Clearance (CL), volume of distribution (V_{dss}), half-life ($T_{1/2}$), and area under the plasma concentration−time curve ($AUC_{0\rightarrow 48\,h}$) determined following a single intravenous dose (1 mg/kg; vehicle = DMSO). [d] $AUC_{0\rightarrow 48\,h}$ determined following a single oral dose (3 mg/kg; vehicle = 20% HPBCD, 1% HPMC, and 1% pluronic F68, pH 2.1, with MSA). [e] Percent bioavailability (F) calculated using $AUC_{0\rightarrow\infty}$ values determined from the 1 (iv) and 3 mg/kg (po) doses. [f] $AUC_{0\rightarrow 48\,h}$ determined following a single oral dose (100 mg/kg; vehicle = 20% HPBCD, 1% HPMC, and 1% pluronic F68, pH 2.1, with MSA).

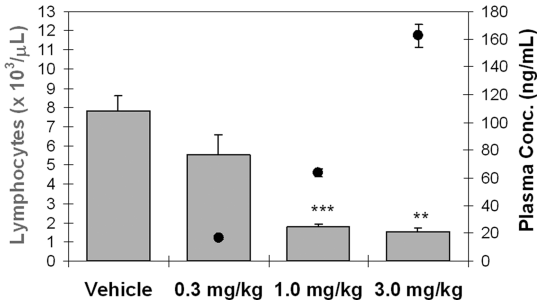

Figure 2. Female Lewis rats (vehicle, 0.30, and 1.0 mg/kg dose groups: N = 5/group; 3.0 mg/kg dose group: N = 3; vehicle = 20% HPBCD, 1% HPMC, and 1% pluronic F68, pH 2.1, with MSA) administered a single oral dose of carbamoylnicotinamide **8** (0.30, 1.0, or 3.0 mg/kg) showed dose-proportional plasma exposure (black circles represent average plasma concentration ± SE) and dose-dependent reduction in circulating lymphocytes (gray bars represent average blood lymphocyte counts ± SE) at the 24 h time point (statistical significance: ***P < 0.001 and **P < 0.01 vs vehicle by the ANOVA/Dunnett's multiple comparison test, respectively); see the Supporting Information for experimental details.

Cl atom in **12** with a CF_3 group to afford **13**. Although the improved potency of **13** was encouraging (EC_{50} = 0.013 μM, 120% efficacy), we desired to improve its stability against rat liver microsomes (CL_{int} = 26 μL/min/mg). Suspecting that the piperidine ring of **13** may be the primary site of its metabolism,[42] we replaced this ring with a phenyl group to provide **14**. Gratifyingly, carbamoylbenzamide **14** displays enhanced microsomal stability (CL_{int} = 15 μL/min/mg) and exhibits good potency toward $S1P_1$ (EC_{50} = 0.068 μM, 110% efficacy). In an attempt improve the potency of **14**, we replaced the CF_3 substituent in **14** with an isopropyl group to give **15**, which does indeed exhibit potent agonism of $S1P_1$ (EC_{50} = 0.0054 μM, 120% efficacy). We also sought to improve the solubility and cell permeability of **14** by inserting a polar N atom into the scaffold, exemplified by 2-pyridinyl derivative **16**, which shows the desired improvements (Sol. = 8.5−28 μg/mL; P_{app} = 7.4 × 10^{-6} cm/s), in addition to excellent potency on $S1P_1$ (EC_{50} = 0.013 μM, 110% efficacy). Interestingly, 2-thiazolyl congener **17** displays greater cell permeability as compared to **16** (P_{app} = 12 × 10^{-6} cm/s) but has lower solubility and microsomal stability (Sol. = < 1.0−12 μg/mL; CL_{int} = 33 μL/min/mg). To examine the effect of replacing the left-hand heterocyclic and (hetero)aromatic rings present in **7** and **12**−**17** with acyclic groups, isopropyl analogue **18** was prepared. Analogue **18** exhibits both excellent potency toward $S1P_1$ (EC_{50} = 0.014 μM, 110% efficacy) and microsomal stability (CL_{int} < 14 μL/min/mg). The 2,2,2-trifluoroethoxy compound **19** also shows potent activity toward $S1P_1$ (EC_{50} = 0.0068 μM, 83% efficacy) but has lower cell permeability and microsomal stability (P_{app} < 1.0 × 10^{-6} cm/s; CL_{int} = 25 μL/min/mg). Finally, inspired by 2-pyridinyl analogue **16**, we desired to probe the effect of placing a polar N atom into the right-hand ring of carbamoylbenzamide **14**. Capitalizing on the ready availability of 4-methoxynicotinamide (**11**),[33] carbamoylnicotinamide **8** was prepared (Scheme 2). Indeed, **8** displays good potency toward $S1P_1$ (EC_{50} = 0.035 μM, 96% efficacy), excellent stability against both human and rat liver microsomes (CL_{int} < 14 μL/min/mg), reasonable cell permeability (P_{app} = 2.3 × 10^{-6} cm/s), and moderate solubility (Sol. = 6.6−17 μg/mL).

We desired next to examine the effect of the $S1P_1$ agonists in Table 1 on circulating lymphocyte levels in vivo. Female Lewis rats were administered a single oral dose of each compound (1 mg/kg); after 24 h, lymphocyte counts in blood and compound concentrations in plasma were measured. The 24 h time point was selected for our studies, as it was found to mitigate the effects of dosing on lymphocyte counts.[17] Notably, carbamoylnicotinamide **8** reaches >5-fold higher concentration in plasma than the other compounds tested in Table 1 (64 ng/mL) and is also the only one that effects statistically significant (P < 0.05 vs vehicle by the ANOVA/Dunnett's multiple comparison test) reduction in circulating lymphocytes (POC = −67%). Given their comparable in vitro profiles, it is unclear why compounds **14**−**16** and **18** do not achieve higher plasma levels and realize statistically significant reduction of peripheral lymphocyte counts (PLCs).[43]

Because of its robust activity in our preliminary in vivo study, carbamoylnicotinamide **8** was selected for further examination. As mentioned previously, compound **8** exhibits potent activation of $S1P_1$ internalization (EC_{50} = 0.035 μM, 96% efficacy; Table 1);[28] gratifyingly, it does not display appreciable agonism of receptor subtypes $S1P_{2-4}$ (EC_{50} > 25 μM)[44] and shows only weak activation of $S1P_5$ (EC_{50} = 4.3 μM, 58% efficacy; Table 2).[44] As the agonism of both $S1P_1$ and $S1P_5$ in oligodendrocytes by **4** has been suggested to contribute to its efficacy in the treatment of MS,[45] $S1P_1$-selective agonists such as **8** may serve as valuable tool compounds to examine the clinical relevance of $S1P_5$ agonism.

A pharmacokinetic profile of carbamoylnicotinamide **8** was undertaken in preparation for more detailed studies in vivo (Table 3). In male Sprague−Dawley rats (N = 3) given a single intravenous dose (1 mg/kg), **8** displays low clearance (CL = 0.25 L/h/kg), moderate volume of distribution (V_{dss} = 6.3 L/kg), and a long half-life ($T_{1/2}$ = 19 h). Although the plasma protein binding of **8** was found to be moderately high (PPB = 95%), its excellent bioavailability (% F = 140) and dose-proportional plasma exposure over a wide range of doses ($AUC_{0\rightarrow 48\,h}$, 1 mg/kg iv =3400 ng h/mL;

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$AUC_{0\rightarrow 48\,h}$, 3 mg/kg po =13000 ng h/mL; $AUC_{0\rightarrow 48\,h}$, 100 mg/kg po = 350000 ng h/mL) enabled a subsequent in vivo toxicology study (vide infra).

We next sought to evaluate the pharmacodynamic effects of **8** in more detail (Figure 2). Following a single oral dose in female Lewis rats (0.30, 1.0, or 3.0 mg/kg), **8** exhibits dose-proportional plasma exposure (17, 63, and 160 ng/mL, respectively) and concomitant dose-dependent reduction in circulating lymphocytes at the 24 h time point. Statistically significant lymphocyte depletion is realized for the 1.0 and 3.0 mg/kg doses (78 and 81% reduction in PLCs; $P < 0.001$ and $P < 0.01$ vs vehicle by the ANOVA/Dunnett's multiple comparison test, respectively). This dose-dependent decrease in circulating lymphocytes reaching a plateau at 81% maximal reduction is consistent with the $S1P_1$ agonist activity of **8**.[19,22]

The potential safety of carbamoylnicotinamide **8** was initially examined in vitro. Encouragingly, **8** displays no appreciable activity against either hERG (IC_{50} > 10 μM) or hCYPs 3A4 and 2D6 (IC_{50} > 30 μM) and exhibits negligible induction of hPXR (POC = +3.7%, 10 μM). An in vivo toxicology study of **8** was subsequently conducted. In male Sprague—Dawley rats (N = 3/group) dosed orally at 50 or 200 mg/kg (qd) for 4 days, no mortality, adverse clinical signs, or changes in body weight were observed. However, increases in total bilirubin (both doses) and cholesterol (high dose only) suggest altered bile uptake, processing, or excretion.

In conclusion, carbamoylnicotinamide **8** is a rare example of an $S1P_1$ agonist lacking a polar headgroup. Despite its modest solubility and cell permeability, **8** is orally bioavailable and induces dose-dependent reduction in lymphocyte count in rat 24 h after a single oral dose. The excellent potency that **8** exhibits toward $S1P_1$ and the remarkable selectivity that it displays against receptor subtypes $S1P_{2-5}$ suggest that it may serve as a valuable tool to examine the proposed clinical relevance of $S1P_5$ agonism for treating MS and the cardiovascular safety issues associated with $S1P_3$ agonism.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures and characterization data for compounds **8** and **12**—**19**, as well as experimental details and statistical analysis for key biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

EC_{50}, molar concentration of compound that produces half maximal response; RI, receptor internalization; PSA, polar surface area; cLogP, calculated log P (logarithm of the octanol/water partition coefficient); $cLogD_{7.4}$, calculated log D (logarithm of the octanol/water distribution coefficient at pH 7.4); P_{app}, apparent permeability; CL_{int}, intrinsic clearance; PLC, peripheral lymphocyte count; HPMC, hydroxypropyl methylcellulose; MSA, methanesulfonic acid; C_{plasma}, total compound concentration in plasma; HPBCD, hyroxypropyl β-cyclodextrin; CL, clearance; V_{dss}, volume of distribution at steady state; $T_{1/2}$, half-life of compound in plasma; AUC, area under the plasma concentration—time curve; F, bioavailability; hERG, human ether-a-go-go related gene product (K_v11.1 potassium ion channel); hCYP 3A4, human cytochrome P450 subtype 3A4; hCYP 2D6, human cytochrome P450 subtype 2D6; IC_{50}, molar concentration of compound that produces half maximal inhibition; hPXR, human pregnane X receptor; qd, quaque die (once per day dosing)

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(21) SEW2871 (**6**) $hS1P_{1-5}$ EC_{50}, μM (% efficacy): $hS1P_1$ = 0.27 (86%) and $hS1P_3$ > 25 (see the Supporting Information for experimental details); $hS1P_{2-5}$ EC_{50} > 10 μM (see ref 22).

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(29) By measurement of Ca^{2+} mobilization in CHO-K1 cells expressing $hS1P_3$ and a chimeric $G_{q/i5}$ G-protein (% efficacy is reported relative to S1P at a concentration of 0.20 μM; > [highest concentration tested] is reported for compounds that do not achieve >10% of control activity); see the Supporting Information for experimental details.

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