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Novel 5- and 6-subtituted benzothiazoles with improved physicochemical properties: Potent $S1P_1$ agonists with in vivo lymphocyte-depleting activity

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ABSTRACT

An SAR campaign designed to increase polarity in the 'tail' region of benzothiazole **1** resulted in two series of structurally novel 5-and 6-substituted $S1P_1$ agonists. Structural optimization for potency ultimately delivered carboxamide (+)-**11f**, which in addition to possessing improved physicochemical properties relative to starting benzothiazole **1,** also displayed good $S1P_3$ selectivity and acceptable in vivo lymphocyte-depleting activity.

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Sphingosine-1-phosphate (S1P, Fig. 1) is an endogenous lysophospholipid that modulates various cellular processes, including migration, adhesion, proliferation, and differentiation.[1] The molecule produces many of its effects via high affinity interaction(s) with the sphingosine-1-phosphate ($S1P_{1-5}$) G-protein coupled receptors.[2] The $S1P_1$ receptor subtype, which is highly expressed on lymphocytes, regulates lymphocyte egress from secondary lymphoid tissue. Therefore, the receptor has received considerable attention as a potentially important target for therapeutic intervention. Seminal studies with the aminodiol prodrug fingolimod (FTY720, Fig. 1) have provided much of the validation for $S1P_1$ as a therapeutic target in autoimmunity and inflammation.[3] Sphingosine kinase 2 phosphorylates fingolimod in vivo,[4] which generates the corresponding (*S*)-phosphate (FTY720P); this active metabolite has high affinity for $S1P_{1,3-5}$.[5] Upon binding $S1P_1$, FTY720P causes immunosuppression via $S1P_1$ receptor internalization, which ultimately results in lymphocyte sequestration in secondary lymphoid tissue. Fingolimod has proven to be an effective treatment for relapsing-remitting multiple sclerosis in clinical studies, and its efficacy and safety profile has prompted the US Food & Drug Administration to approve the agent for treatment of this disease.[6] One of several adverse events observed with fingolimod therapy is dose-dependent reduction of heart rate.[7] Whereas this side effect has been linked to $S1P_3$ agonism in rodents,[8] additional research has indicated it is more likely $S1P_1$- rather than $S1P_3$-mediated in humans.[9] Nevertheless, we and others have aimed for the development of selective $S1P_1$ agonists to address the cardiovascular findings.[10]

Benzothiazole **1** was first prepared during a structure–activity relationship (SAR) study of benzannulated compounds that produced selective $S1P_1$ receptor agonists.[10e,f] Although **1** was a potent $S1P_1$ agonist (EC_{50} = 0.041 μM) and was selective against $S1P_3$ (EC_{50} = 1.21 μM), the overall physicochemical properties—low total polar surface area (tPSA = 52.9 $Å^2$) and relatively high lipophilicity (*c*log*P* = 3.92)—predicted a higher-than-average likelihood for attrition during development because of an unfavorable safety profile.[11] We therefore initiated an SAR campaign designed to increase

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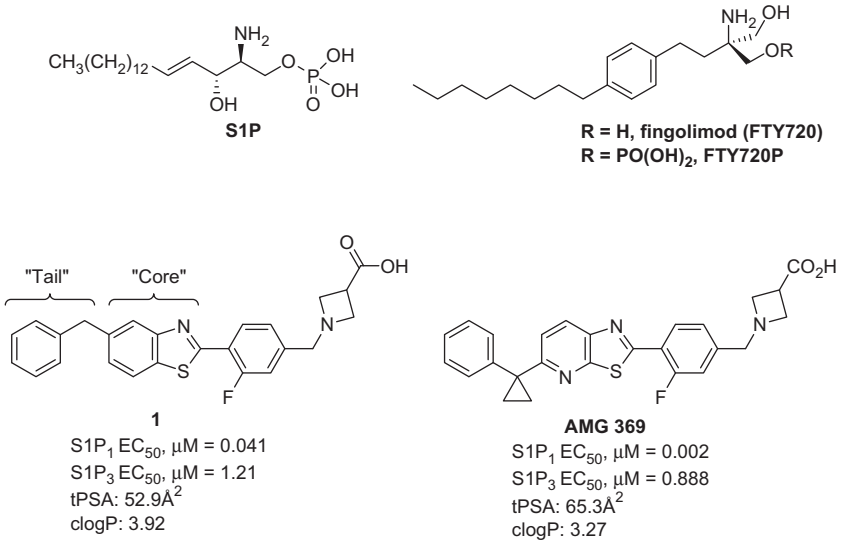
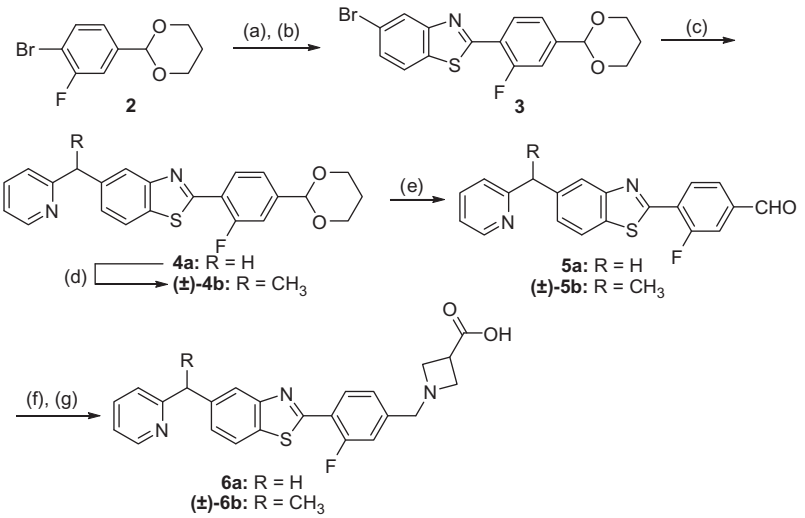


Figure 1. Sphingosine-1-phosphate (S1P), fingolimod (FTY720), FTY720P, benzothiazole **1**, **AMG 369**.

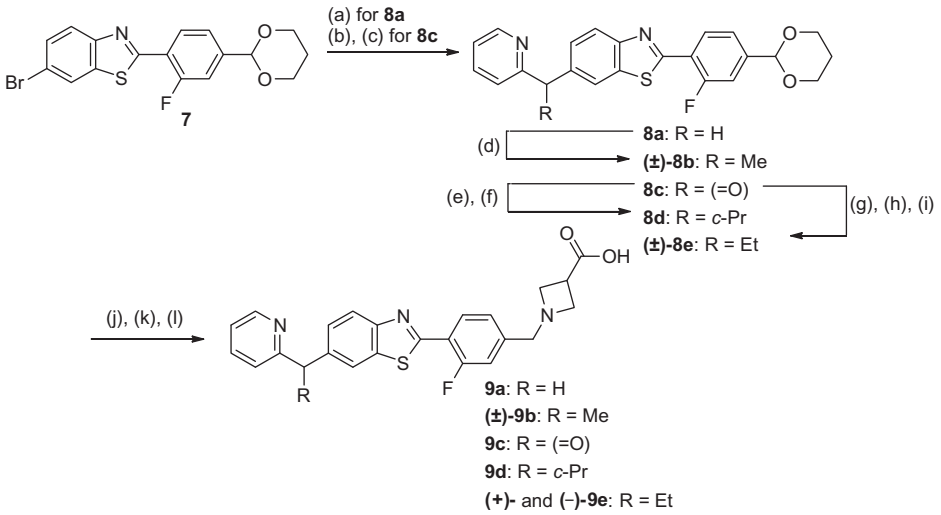
the polarity of **1** to levels associated with a higher probability for successful development. Specifically, our goal was to deliver a potent, selective $S1P_1$ agonist with acceptable in vivo lymphocyte-depleting activity, lower *c*log*P*, and higher tPSA. One facet of this strategy involved introducing polarity to the 'core' region of **1**. That work resulted in **AMG 369**, a potent and selective $S1P_1$ agonist that became a development candidate (Fig. 1).[10f] In parallel to these efforts, we also attempted to increase polarity in the 'tail' region of the molecule, and that is the focus of this manuscript.

Analogues for this study were prepared as shown in Schemes 1–3. Compounds **6a** and (±)-**6b** were constructed according to Scheme 1. Aryl bromide **2**[10e,10f] was lithiated at −78 °C and then exposed to 4-bromo-1-fluoro-2-isothiocyanatobenzene to give an intermediate thioamide. That compound was subsequently cyclized under basic conditions at elevated temperature to give benzothiazole **3** in good overall yield.[12] Subsequent palladium-catalyzed coupling with 2,4-dimethyl-3-(pyridin-2-ylmethyl)pentan-3-ol resulted in picoline-substituted compound **4a**.[13] Methyl substitution at the newly-formed methylene group was achieved by deprotonation with lithium (bis)trimethylsilylamide followed by alkylation with iodomethane, which produced compound (±)-**4b**. Removal of the 1,3-dioxane from both **4a** and (±)-**4b** with aqueous hydrochloric acid, reductive amination of the liberated aldehydes with methyl azetidine-3-carboxylate, and saponification of the resulting methyl esters with lithium hydroxide delivered analogues **6a** and (±)-**6b**.

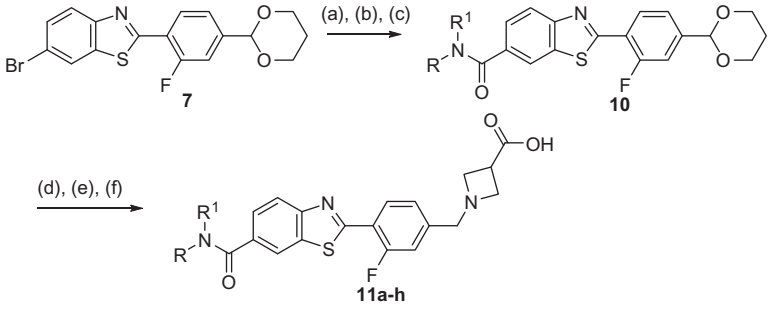
Whereas C-6 substituted analogues **9a** and (±)-**9b** (Scheme 2) were constructed analogously to **6a** and (±)-**6b**; the remaining C-6 substituted analogues **9c–e** were prepared slightly differently. Bromide **7** was first converted to the corresponding Weinreb amide via palladium-catalyzed carbonylation[14]; subsequent treatment with the organomagnesium reagent generated by addition of ^{i}PrMgCl to 2-bromopyridine resulted in a versatile intermediate ketone (**8c**). This compound could either be transformed into final product **9c** or converted to methylene-substituted intermediates **8d** and (±)-**8e** (Scheme 2). The cyclopropyl unit of **8d** was installed



Scheme 1. Reagents and conditions: (a) *n*-BuLi, 4-bromo-1-fluoro-2-isothiocyanatobenzene, THF, −78 °C, 63% (b) Na_2CO_3, DMF, 110 °C, 93% (c) 2,4-dimethyl-3-(pyridin-2-ylmethyl)pentan-3-ol, $Pd(OAc)_2$, PPh_3, Cs_2CO_3, toluene, 120 °C, 76% (d) $LiN(TMS)_2$, CH_3I, THF, 71% (e) 5 N HCl (aq), THF, 65 °C, 68–99% (f) methyl azetidine-3-carboxylate hydrochloride, AcOH, $^{i}Pr_2NEt$, $NaBH_3CN$, $MeOH/CH_2Cl_2$, 70–83% (g) NaOH, THF; HCl, then pH 6 sodium phosphate buffer, 11–59%.



Scheme 2. Reagents and conditions: (a) 2,4-dimethyl-3-(pyridin-2-ylmethyl)pentan-3-ol, $Pd(OAc)_2$, PPh_3, Cs_2CO_3, toluene, 120 °C, 78% (b) *N*,*O*-dimethyl hydroxylamine, $Pd(OAc)_2$, XANTPHOS, Et_3N, CO (1 atm), toluene, 80 °C, 86% (c) 2-bromopyridine, ^{i}PrMgCl, THF, 0 °C to RT, THF, 45% (d) $LiN(TMS)_2$, CH_3I, THF, 11% (e) *n*-BuLi, methyltriphenylphosphonium bromide, THF, 56% (f) Me_3SOI, *t*BuOK, DMSO/THF, 34% (g) EtMgCl, $ZnCl_2$, THF, 0 °C, 84% (h) CH_3SO_2Cl, Et_3N, CH_2Cl_2, 83% (i) H_2, Pd-C, MeOH, 58% (j) 5 N HCl (aq), THF, 65 °C, 88–98% (k) methyl azetidine-3-carboxylate hydrochloride, AcOH, $^{i}Pr_2NEt$, $NaBH_3CN$, MeOH/CH_2Cl_2, 34–91% (l) NaOH, THF/H_2O; HCl, then pH 6 sodium phosphate buffer, 31–97%.



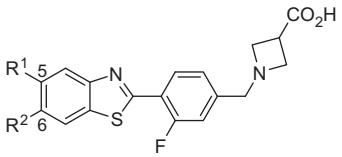
Scheme 3. Reagents and conditions: (a) $Pd(dppp)Cl_2$, Et_3N, CO (20 psi), 1:2:1 DMF/THF/EtOH, 95 °C, 79% (b) NaOH, dioxane/H_2O, 40 °C, 68% (c) $HNRR^1$, HBTU, Et_3N or $^{i}Pr_2NEt$, DMF, RT, 82–97% (d) 5 N HCl (aq), THF, 65 °C, 70–92% (e) methyl azetidine-3-carboxylate hydrochloride, AcOH, $^{i}Pr_2NEt$, $NaBH_3CN$, MeOH/CH_2Cl_2, 39–71% (f) NaOH, THF/H_2O; HCl, then pH 6 sodium phosphate buffer, 47–78%.

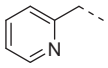
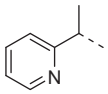
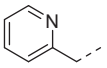
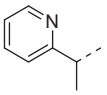
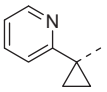
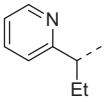
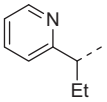
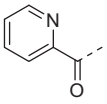
by Wittig olefination of the ketone followed by cyclopropanation with trimethylsulfoxonium ylide. The ethyl group of (±)-**8e** was produced via nucleophillic addition of ethylmagnesium bromide, dehydration of the resulting tertiary alcohol, and palladium-catalyzed hydrogenation of the olefins. Both **8d** and (±)-**8e** supplied **9d**, (+)-**9e**, and (−)-**9e** using the same end game sequence as depicted in Scheme 1.[15] Carboxamide analogues **11a**–**h** were synthesized from **7** by palladium-catalyzed carbonylation to form the corresponding ethyl ester, saponification of the ester, and amide coupling (Scheme 3). Completion of the analogues proceeded as in Schemes 1 and 2.[16,17]

Analogue effect on $S1P_1$ was evaluated by measuring receptor internalization (RI) of an $hS1P_1$–GFP fusion protein in U2 osteosarcoma (U2OS) cells. Selectivity against $S1P_3$ was measured using a Ca^{2+} flux assay in CHO cells that stably co-expressed $hS1P_3$ receptor and a chimeric $G_{q/i5}$ G-protein (Tables 1 and 2).[18] The SAR study began by evaluating single N for C–H replacements of the terminal phenyl ring. Prior SAR from a related benzofuranyl series indicated that of the three possible N for C–H substitutions, replacement of the 2-C–H was tolerated the best (data not shown). The related 2-pyridyl benzothiazole analogue **6a** ($S1P_1$ EC_{50} = 0.68 μM, $S1P_3$ EC_{50} >25 μM, >37-fold selectivity) exhibited reasonable potency for S1P1 and became the starting point for further optimization. Alkyl substitution at the methylene linker was examined next. Unfortunately, even replacement of a single hydrogen atom with a methyl group had a negative impact ([±]-**6b**, $S1P_1$ EC_{50} = 2.37 μM). Therefore, the study turned to compounds with the 'tail' region extending from C-6 of the benzothiazole core. Despite being over twofold less active than the analogous C-5 substituted analogue **6a**, derivatizing C-6 substituted picoline **9a** ultimately provided access to compounds with improved potency. Immediate gains were realized with methyl-substituted compound (±)-**9b** (EC_{50} = 0.57 μM), and additional improvements were obtained with slightly larger alkyl substituents. Cyclopropyl derivative **9d** was roughly twofold more active than (±)-**9b** (EC_{50} = 0.24 μM, >105-fold selectivity), and ethyl-substituted derivative (−)-**9e** increased potency even further (EC_{50} = 0.10 μM); however, ethyl enantiomer (+)-**9e** displayed significantly lower activity (EC_{50} = 0.515 μM) than its isomer. Whereas additional improvements were not achieved in this series, ketone **9c** ($S1P_1$ EC_{50} = 1.30 μM, $S1P_3$ EC_{50} >25 μM) was also considered a promising analogue in its own right. Since options within the 2-picoline series appeared exhausted, we adjusted our SAR strategy towards finding high-affinity agonists that incorporated the carbonyl subunit.

It was immediately clear that the linker carbonyl could be incorporated into a carboxamide function, so we began an SAR

Table 1
SAR of 2-picoline substituted benzothiazole analogues[a]



Cmpd	R^1	R^2	hS1P$_1$ RI EC_{50}, μM (% efficacy)	hS1P$_3$ Ca^{2+} EC_{50}, μM (% efficacy)	*c*log *P*[b]	tPSA (Å^2)
1	Bn	H	0.042 (102)	1.21 (24)	3.92	52.9
6a		H	0.68 (94)	>25	2.42	66.3
(±)-**6b**		H	2.37 (62)	>25	2.82	66.3
9a	H		1.96 (71)	>25	2.42	66.3
(±)-**9b**	H		0.57 (79)	>25	2.82	66.3
9d	H		0.24 (90)	>25	2.86	66.3
(−)-**9e**	H		0.10 (95)	2.06 (82)	3.35	66.3
(+)-**9e**	H		0.52 (93)	9.75 (30)	3.35	66.3
9c	H		1.30 (50)	>25	2.25	83.4

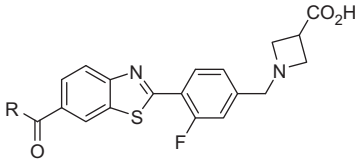
[a] Data reported as an average of at least two experimental determinations. S1P$_1$ efficacy was measured relative to 0.200 μM S1P.
[b] ACD *c*log*P*.

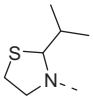
study of C-6 substituted amides (Table 2). We began with the homologous amides **11a–c**. Whereas piperidine amide **11a** (S1P$_1$ EC_{50} = 1.17 μM) and homopiperidine amide **11b** (S1P$_1$ EC_{50} = 2.72 μM) both had reasonable activity, the pyrrolidine amide **11c** did not (EC_{50} >20 μM). Thiazolidine amide **11d** (EC_{50} = 2.71 μM), a structural isostere of **11a**, restored S1P$_1$ activity; and we therefore focused on this structural unit for the remainder of the study. Additional potency increases were realized from alkyl substitution at the C-2 position of the thiazolidine ring. For example, methyl-substituted thiazolidine (±)-**11e** (EC_{50} = 0.042 μM) was fivefold more potent than **11d**; and the slightly larger ethyl-containing derivative (+)-**11f** ultimately became the most potent compound in either the picoline or amide series (EC_{50} = 0.017 μM, S1P$_3$ EC_{50} = 0.39 μM, 23-fold selectivity). Ethyl enantiomer (−)-**11f** had much lower S1P$_1$ agonistic activity (EC_{50} = 0.51 μM). A number of additional compounds with other substituents were also prepared and tested (e.g., (±)-**11g** and **11h**), but none were more active and selective than (+)-**11f**.

Since (+)-**11f** and (−)-**11f** were moderately selective against S1P$_3$, showed divergent pharmacology on S1P$_1$, and possessed improved physicochemical properties relative to **1**—*c*log*P* (2.58) and tPSA (73.7 Å^2)—the in vivo lymphocyte-depleting activity of the two compounds was evaluated.[19] Each compound was orally dosed in female Lewis rats, and total blood lymphocyte counts were taken 4 h post dose (Table 3).[20] Gratifyingly, analogue (+)-**11f** displayed statistically significant reduction of circulating lymphocytes after a single 10 mg/kg dose (55%, P <0.05). Measurement of total plasma concentration showed systemic exposure was 10-fold higher than the S1P$_1$ receptor internalization EC_{50}. Conversely, (−)-**11f** did not show significant lymphocyte sequestration, most likely because of insufficient plasma exposure. Although the lymphocyte-depleting activity of (+)-**11f** was not superior to **1** (58% lymphocyte depletion vs vehicle at 24 h post dose, 1 mg/kg dose, N = 5), this carboxamide achieved several goals of the current study: it is a potent S1P$_1$ agonist in vitro, selective against S1P$_3$, displays acceptable in vivo lymphocyte-depleting activity, and has improved physicochemical properties relative to **1**. Therefore, this molecule is attractive not only because of its in vivo activity, but also because its improved physicochemical profile positions it as a suitable template for further SAR study in the 'core' and 'head' regions of the molecule.

In conclusion, a novel series of selective S1P$_1$ benzothiazole agonists with polar functionality in the 'tail' region of the molecule was discovered. Starting from benzothiazole **1**, an SAR study led to the preparation of carboxamide (+)-**11f**, which displayed potent S1P$_1$ receptor internalization, moderate S1P$_3$ selectivity, acceptable in vivo lymphocyte-depleting activity, and improved physicochemical properties.

Table 2
SAR of ketone and carboxamide substituted benzothiazole analogues[a]



Cmpd	R	hS1P$_1$ RI EC$_{50}$, μM (% efficacy)	hS1P$_3$ Ca^{2+} EC$_{50}$, μM (% efficacy)	*c*log *P*[b]	tPSA (Å^2)
9c		1.30 (50)	>25	2.25	83.4
11a		1.17 (95)	>25	1.79	73.7
11b		2.72 (108)	>25	2.34	73.7
11c		>20	>25	1.23	73.7
11d		2.71 (110)	7.42 (21)	1.53	73.7
(±)-**11e**		0.042 (90)	2.04 (71)	2.05	73.7
(−)-**11f**		0.15 (106)	6.24 (36)	2.58	73.7
(+)-**11f**		0.017 (107)	0.39 (82)	2.58	73.7
(±)-**11g**		0.049 (96)	0.90 (87)	2.98	73.7
11h		0.43 (80)	2.68 (72)	2.57	73.7

[a] Data reported as an average of at least two experimental determinations. S1P$_1$ efficacy was measured relative to 0.200 μM S1P.
[b] ACD *clogP*.

Table 3
Effects of (+)-**11f** and (−)-**11f** on circulating lymphocytes 4 h post dose in female lewis rats

Compd	hS1P$_1$ RI EC$_{50}$, μM	Dose (mg/kg)	Lymphocyte reduction (%)	Plasma concentration (ng/mL, μM)
(−)-**11f**	0.151	10	24	65 (0.13)
(+)-**11f**	0.017	10	55*	92 (0.19)

* *P* <0.05 versus vehicle by ANOVA/Dunnett's multiple comparison test. (*N* = 5/group. Vehicle = 20% HPBCD, 1% HPMC, 1% pluronic F68 w/MSA).

Supplementary data

Supplementary data (synthesis and characterization of (+)-**11f** and (−)-**11f**, and statistical analysis of S1P$_1$ and S1P$_3$ data) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.10.069.

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15. Chiral-phase SFC of (+/−)-**9e** separately provided (+)-**9e** and (−)-**9e**. Separation conditions: Chiralpak AD-H column (21 × 250 mm), mobile phase: 40% IPA containing 0.2% diethylamine, 70 mL/min flow rate. (+)-**9e**: R_t = 3.10 min, $[\alpha]_D^{25}$ = +19.4°, (*c* 0.12, MeOH); (−)-**9e**: R_t = 3.65 min, $[\alpha]_D^{25}$ = −16.8°, (*c* 0.18, MeOH).
16. Substituted thiazolidines required for **11e–h** were synthesized using a literature procedure: Lalezari, I.; Schwartz, E. L. *J. Med. Chem.* **1988**, *31*, 1427.
17. Please refer to the Supplementary data for the synthesis and chiral separation of (+)-**11f** and (−)-**11f**. Separation conditions: chiralcel OJ-H column (250 × 21 mm), mobile phase: 40% IPA containing 0.2% diethylamine, 65 mL/min flow rate.
18. The hS1P1 receptor internalization assay was performed using a U2OS cell line expressing hS1P1–eGFP chimeric protein (Thermo Scientific, Søborg, Denmark). Upon compound treatment, the hS1P1 receptor was internalized into the cytoplasm, forming GFP-containing-endosomes. This event was detected using an ArrayScan automated microscope (Thermo Scientific Cellomics, Pittsburg, PA), and the degree of receptor internalization was quantitated by counting the number of GFP-containing endosomes per cell. hS1P1–eGFP expressing U2OS cells were starved in serum free media for two hours prior to compound treatment. Compounds were incubated with the starved cells at 37 °C for one hour. Compound-treated cells were subsequently fixed using 4% formaldehyde, and nuclei were stained using Hoechst dye (Invitrogen/Molecular Probes, Cat. #H3570). The cells were then imaged by ArrayScan, and the potency and efficacy of the compounds were determined by plotting the number of GFP-containing endosomes per cell against corresponding compound concentration.
19. The measured solubility in 0.1 N HCl, PBS buffer, and simulated intestinal fluid was >200 ng/mL Tan, H.; Semin, D.; Wacker, M.; Cheetham, J. *J. Assoc. Lab Autom.* **2005**, 364.
20. Female Lewis rats (250 g, 6–8 wks) were received from Charles River Laboratories (Wilmington, MA) and allowed to acclimatize for at least one week before being placed on study. Rats (N = 5/group) were administered vehicle (20% captisol in water), (+)-**11f** or (−)-**11f** (10 mg/kg in 20% captisol/water) by oral gavage (10 mL/kg). 4 h Post-dose, animals were sacrificed by CO_2 inhalation, and blood was collected by cardiac puncture. Approximately 1 mL of blood was transferred to a Microtainer® hematology tube containing EDTA (Becton Dickinson, #365973) for CBC analysis and 500 μL of plasma was placed in a Microtainer® tube containing heparin (Becton Dickinson, #365958) for subsequent pharmacokinetic analysis (plasma exposure). Differential cell counts were obtained using an Advia® 120 hematology system (Bayer Diagnostics).