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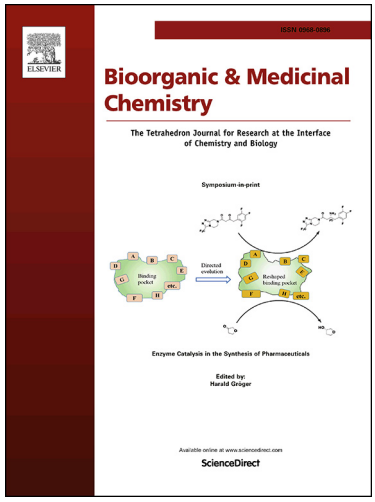

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Discovery of novel serine palmitoyltransferase inhibitors as cancer therapeutic agents

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Abstract

We pursued serine palmitoyltransferase (SPT) inhibitors as novel cancer therapeutic agents based on a correlation between SPT inhibition and growth suppression of cancer cells. High-throughput screening and medicinal chemistry efforts led to the identification of structurally diverse SPT inhibitors **4** and **5**. Both compounds potently inhibited SPT enzyme and decreased intracellular ceramide content. In addition, they suppressed cell growth of human lung adenocarcinoma HCC4006 and acute promyelocytic leukemia PL-21, and displayed good pharmacokinetic profiles. Reduction of 3-ketodihydrosphingosine, the direct downstream product of SPT, was confirmed under in vivo settings after oral administration of compounds **4** and **5**. Their anti-tumor efficacy was observed in a PL-21 xenograft mouse model. These results suggested that SPT inhibitors might have potential to be effective cancer therapeutics.

Keywords: SPT; 3-KDS; antitumor efficacy

Introduction

Serine palmitoyltransferase (SPT) is a heterodimeric membrane protein localized in endoplasmic reticulum. It catalyzes condensation of L-serine and palmitoyl-coenzyme A to generate 3-ketodihydrosphingosine (3-KDS) as shown in Figure 1. SPT is an initial and rate-limiting enzyme in the de novo biosynthesis of sphingolipids such as ceramide and sphingomyelin.[1,2] Sphingolipids are known to play important biological roles (i.e. cell membrane formation, signal transduction, plasma lipoprotein metabolism) and to be associated with various diseases including cancer.[3-8] Intradermal and intraperitoneal administrations of Myriocin (**1**, Fig. 2), an SPT inhibitorisolated from fungal extracts, suppressed tumor formation in a mouse B16F10 melanoma xenograft model with decrease in sphingolipids.[9] In addition, our studies revealed an apparent correlation between SPT enzyme inhibition and growth suppression of a human lung adenocarcinoma cell line (HCC4006)[10]. Therefore, SPT could be an attractive target for cancer therapy.

Recently, SPT inhibitors **2** and **3** were reported as potential treatments for diabetes and dyslipidemia.[11] In our laboratories, to develop orally bioavailable SPT inhibitors, we conducted high-throughput screening (HTS) of in-house compound libraries and subsequent optimization via medicinal chemistry. These efforts successfully identified structurally diverse SPT inhibitors such as tetrahydropyrazolopyridine **4** and 3-phenylpiperidine **5** (Fig. 2), which exhibited potent SPT inhibition with favorable pharmacokinetic profiles. In this report, we describe the design and synthesis of these compounds as wells as their biological profiles as anti-cancer agents.

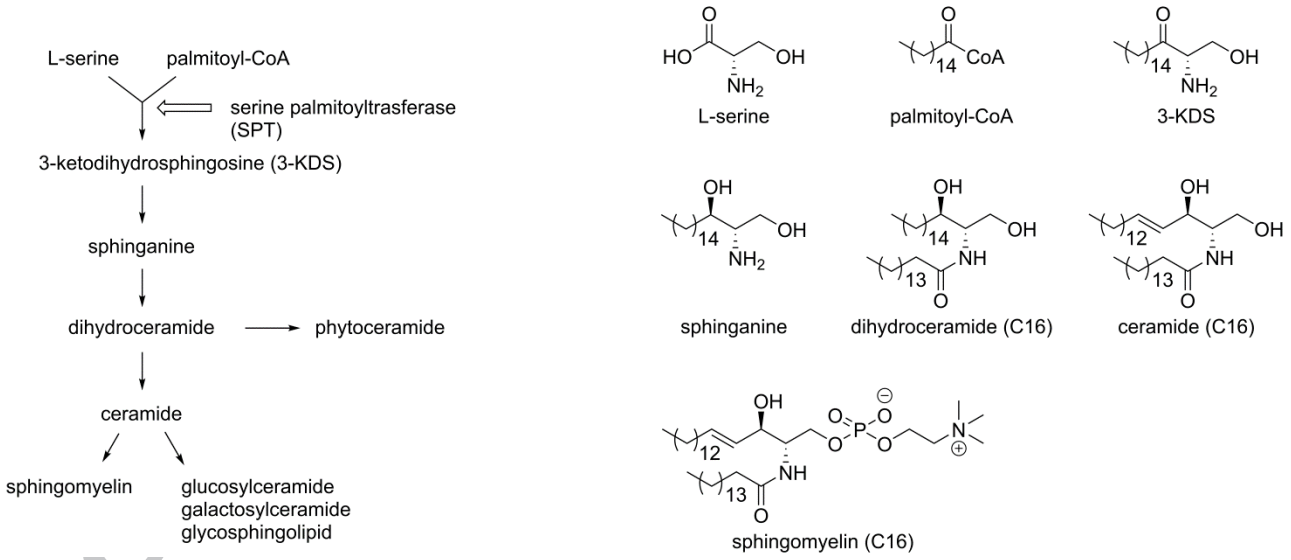


Figure 1. Biosynthesis pathway for sphingolipids and their chemical structures.

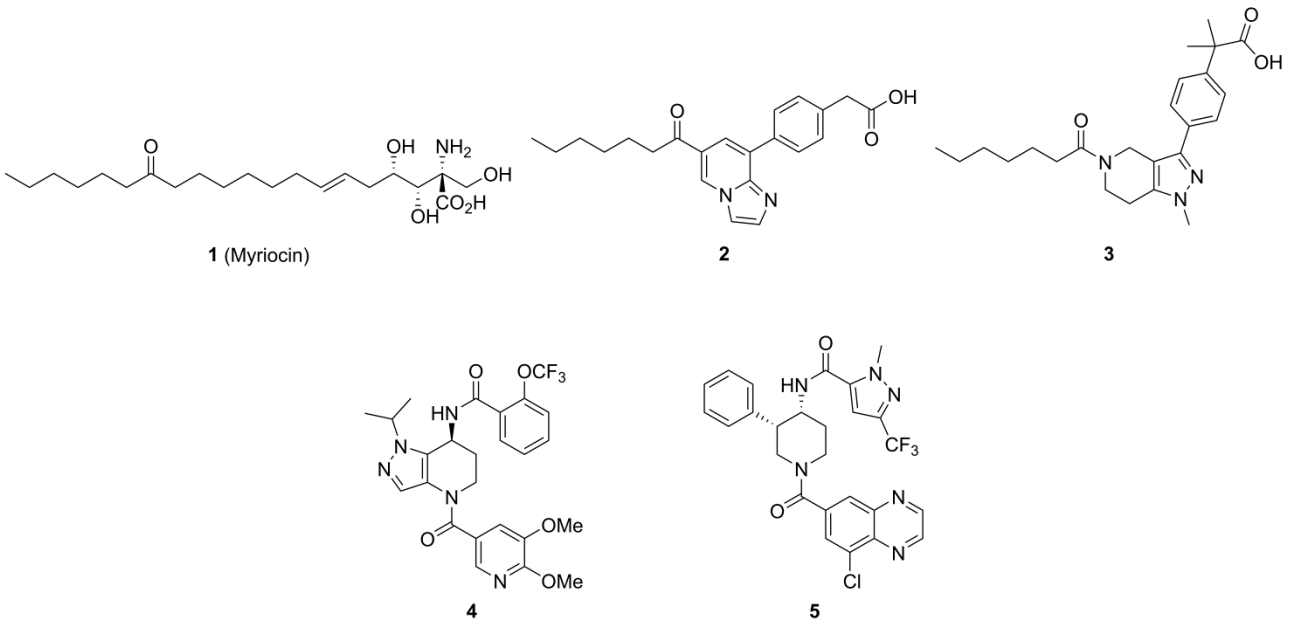
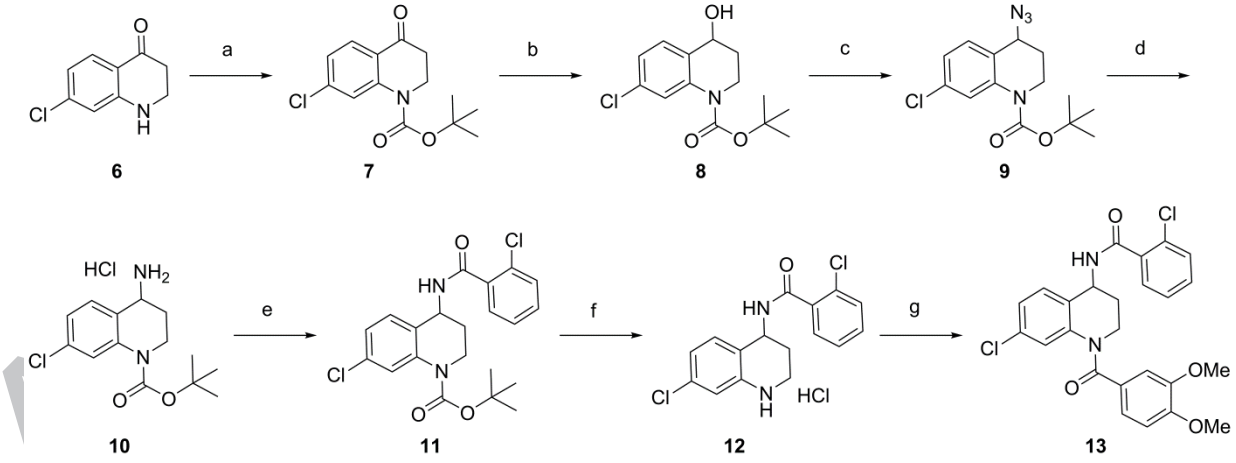


Figure 2. Reported SPT inhibitors **1**-**3** and our compounds **4** and **5**.

Chemistry

The synthesis of 1,2,3,4-tetrahydroquinoline derivative **13** is shown in Scheme 1. Starting from compound **6**[12], protection of the amine **6** with a Boc group and following reduction of the ketone **7** gave alcohol **8**. After conversion of **8** to azide **9** by reaction with DPPA, reduction of **9** with PPh_3 and subsequent amidation yielded compound **11**. Deprotection of the Boc group and an acylation of the secondary amine **12** afforded target compound **13**.

Scheme 1. Synthesis of 1,2,3,4-tetrahydroquinoline derivative **13**[a]

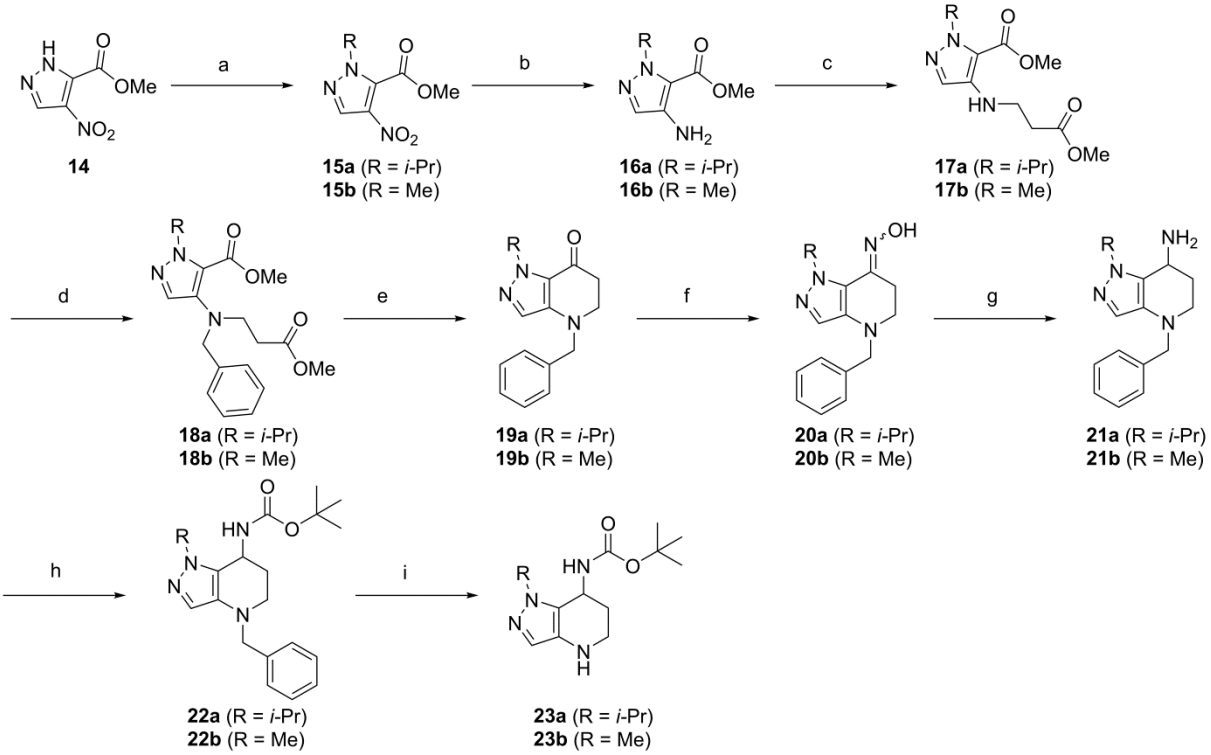


[a]Reagents and conditions: (a) $(Boc)_2O$, DMAP, THF, rt; (b) $NaBH_4$, THF, MeOH, 0 °C to rt, 87% in 2 steps; (c) DPPA, DBU, THF, rt, quant; (d) PPh_3, THF, H_2O, 100 °C, then 4 M HCl in EtOAc, rt, 44%; (e) 2-chlorobenzoyl chloride, Et_3N, THF, rt, 94%; (f) 4 M HCl in EtOAc, rt, 91%; (g) 3,4-dimethoxybenzoyl chloride, Et_3N, THF, 50 °C, 83%.

The synthesis of 4,5,6,7-tetrahydro-1*H*-pyrazolo[4,3-*b*]pyridine derivative **4** started from commercially available compound **14** (Scheme 2). Mitsunobu reaction of compound **14** proceeded in a regioselective manner to give N1-isopropyl (**15a**) and N1-methyl (**15b**) derivatives, and hydrogenation of their nitro groups afforded amines **16a** and **16b**. Michael reaction of **16a** and **16b** with methyl acrylate gave compounds **17a** and **17b**, which were converted to compounds **18a** and **18b** by benzylation. Bicyclic ring was constructed by intramolecular condensation of compounds

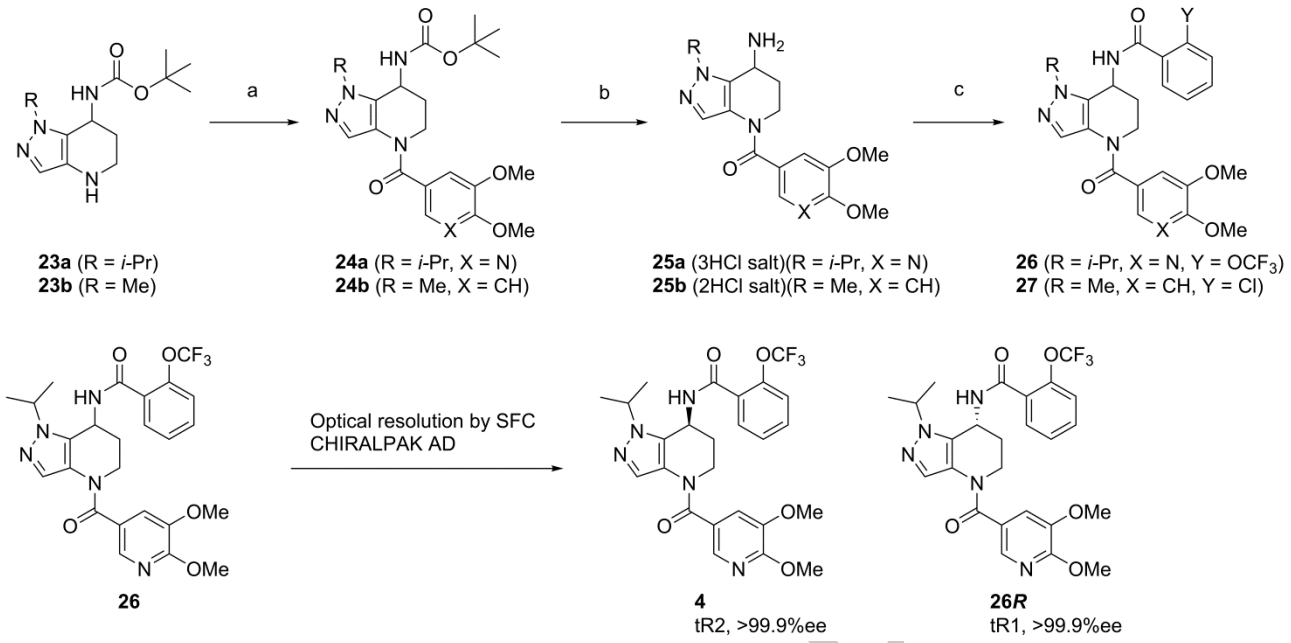
18a and **18b** in the presence of LiHMDS, and following decarboxylation under basic conditions afforded compounds **19a** and **19b**. Compounds **22a** and **22b** were obtained through oxime formation and its hydrogenation, followed by protection with a Boc group. Deprotection of the benzyl group gave key intermediates **23a** and **23b**. After acylation of the secondary amines, deprotection of the Boc group and amidation provided target compounds **26** and **27** as a racemates (Scheme 3). Optical resolution of **26** was conducted by Chiralpak AD to provide **4** and **26R** with ≥99.9%ee, respectively. Absolute configuration of compound **4** was determined to be *S* by its X-ray crystallographic analysis.

Scheme 2. Synthesis of 4,5,6,7-tetrahydro-1*H*-pyrazolo[4,3-*b*]pyridine intermediates **23a** and **23b**[a]



[a]Reagents and conditions: (a) for **15a**, DTBAD, PBu_3, *i*-PrOH, THF, rt, 73%; for **15b**, ADDP, PBu_3, MeOH, toluene, rt, 61%; (b) Pd/C, H_2, MeOH, rt, 97-98%; (c) methyl acrylate, DMAP, DMF, 100 °C; (d) BnBr, K_2CO_3, DMF, rt, 68-76% in 2 steps; (e) NaHMDS, THF, 0 °C to rt, then NaOH aq., 70 °C, 91%-quant; (f) NH_2OH-HCl, pyridine, EtOH, 80-90 °C; (g) Raney Ni, H_2, MeOH, rt, 58-71% in 2 steps; (h) Boc_2O, Et_3N, THF, rt; (i) Pd/C, H_2, AcOH, MeOH, rt, 59-93% in 2 steps.

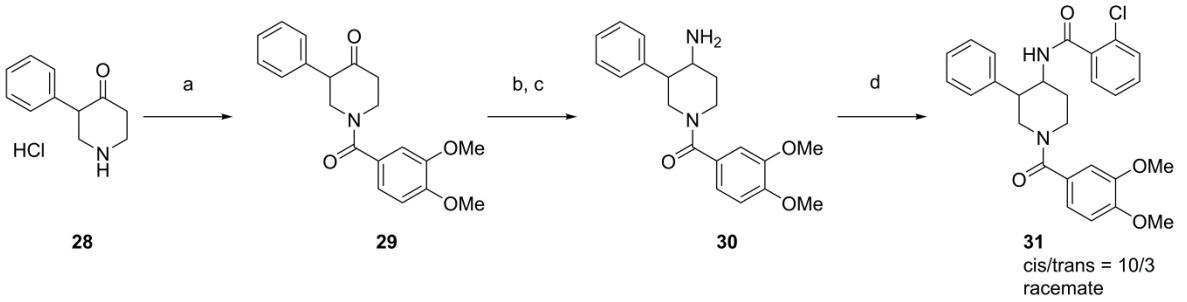
Scheme 3. Synthesis of 4,5,6,7-tetrahydro-1*H*-pyrazolo[4,3-*b*]pyridine derivatives **26** and **27**, and chiral resolution of racemic **26**[a]



[a]Reagents and conditions: (a) 5,6-dimethoxynicotinic acid, HATU, Et_3N, DMF, rt, 95%; (b) 4 M HCl in EtOAc, MeOH, rt, 98%; (c) 2-(trifluoromethoxy)benzoic acid, HATU, Et_3N, DMF, rt, 70%; (d) 3,4-dimethoxybenzoyl chloride, pyridine, rt, 98%; (e) 4 M HCl in CPME, MeOH, rt, 97%; (f) 2-chlorobenzoic acid, HATU, Et_3N, DMF, rt, 83%.

The synthesis of 3-phenylpiperidine derivative **31** is shown in Scheme 4. The amidation of 3-phenylpiperidine-4-one hydrochloride **28**[13] with 3,4-dimethoxybenzoyl chloride, subsequent condensation with hydroxylamine, and reduction of the resulting oxime by Raney nickel gave cis/trans mixture of 4-amino-3-phenylpiperidine **30**. Finally, amidation with 2-chlorobenzoyl chloride afforded compound **31** (cis/trans = 10/3, racemate).

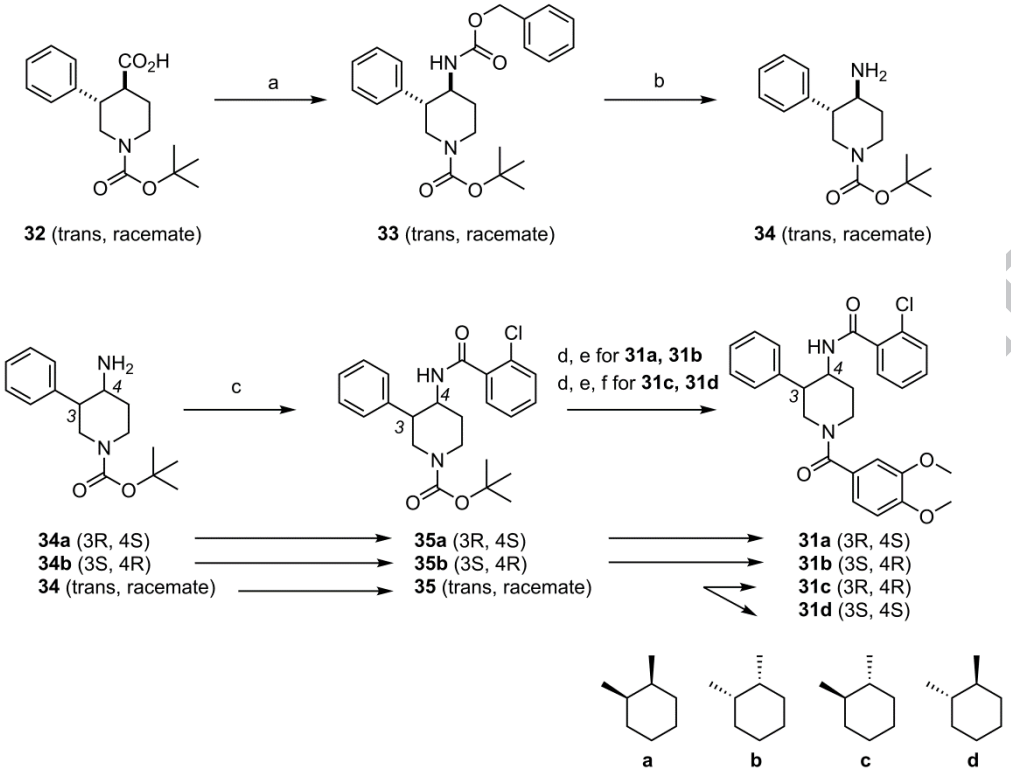
Scheme 4. Synthesis of 3-phenylpiperidine derivative **31**[a]



[a]Reagents and conditions: (a) 3,4-$(MeO)_2C_6H_3COCl$, pyridine, 0 °C to rt, 71%; (b) NH_2OH-HCl, Et_3N, EtOH, reflux, 99%; (c) Raney Ni, H_2, MeOH, rt, 89%; (d) 2-Cl-C_6H_4COCl, Et_3N, THF, 0 °C to rt, 83%.

Scheme 5 shows the synthesis of four stereoisomers of **31** (**31a**−**d**). Curtius rearrangement of commercially available racemic trans carboxylic acid **32** followed by reaction with benzyl alcohol yielded carbamate **33**, which was converted to intermediate amine **34** by removal of the benzyloxycarbonyl group. The target compounds **31a**−**31d** were obtained through acylation of primary amine followed by deprotection of the Boc group and subsequent acylation of the secondary amine. Cis isomers **31a** and **31b** were prepared from the corresponding chiral materials **34a**[13] and **34b**[13], respectively, while trans isomers **31c** and **31d** were prepared from the racemic material **35** by chiral resolution at the final step.

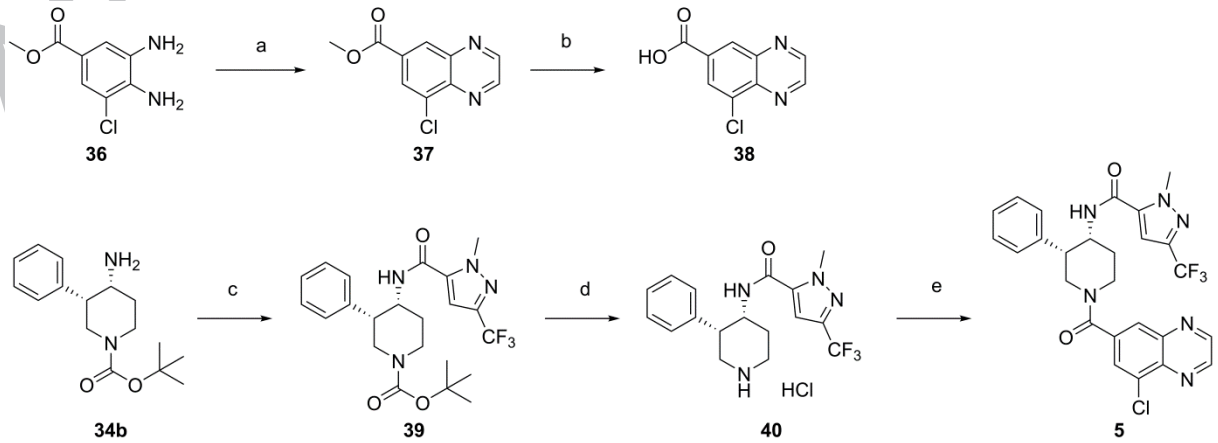
Scheme 5. Synthesis of 3-phenylpiperidine derivatives **31a**–**31d**[a]



[a]Reagents and conditions: (a) DPPA, Et_3N, toluene, then BnOH, reflux, quant; (b) Pd/C, H_2, MeOH, rt, 99%; (c) 2-Cl-C_6H_4COCl, Et_3N, THF, 0 °C to rt, 38-94%; (d) 4 M HCl in EtOAc, 0 °C to rt; (e) 3,4-dimethoxybenzoyl chloride, pyridine, 0 °C to rt, 89-98% in 2 steps; (f) Chiral HPLC resolution, 67-70% in 3 steps.

The synthetic route to 3-phenylpiperidine derivative **5** is illustrated in Scheme 6. Condensation of diamine **36** with glyoxal followed by hydrolysis of the ester group provided carboxylic acid **38**. Amidation of the chiral 4-aminopiperidine **34b**[13] afforded compound **39**. Finally, target compound **5** was obtained through the removal of the Boc group followed by acylation of the piperidine nitrogen. Absolute configuration of **5** was confirmed to be 3*S*,4*R* by its X-ray crystallographic analysis.

Scheme 6. Synthesis of 3-phenylpiperidine derivative **5**[a]



[a]Reagents and conditions: (a) glyoxal, MeOH, THF, rt, 98%; (b) 4 M NaOH aq., THF, MeOH, rt, 98%; (c) 1-methyl-3-(trifluoromethyl)-1H-pyrazole-5-carboxylic acid, WSC, HOBt, Et_3N, DMF, rt, 95%; (d) 4 M HCl in EtOAc,

rt., 98%; (e) **38**, WSC, HOBt, Et_3N, DMF, rt, 59%.

Results and discussion

The synthesized compounds were evaluated for their SPT inhibitory activity (IC_{50}) and growth inhibitory activity against HCC4006 cells. The growth inhibition was assessed based on inflection point (IP), since the maximum inhibition rate was around 50% in the HCC4006 growth assay.

Figure 3 highlights our medicinal chemistry approach to discover tetrahydropyrazolopyridine compound **4**. Our initial efforts for exploring SAR of HTS hit tetrahydroquinoline derivative **41** to avoid basic amine moiety led to the identification of amide compound **13**, which exhibited significant improvement in SPT inhibitory activity (IC_{50} = 650 nM vs 5.1 nM). Next, we focused on scaffold hopping of the central core ring system, in order to acquire drug-like properties such as metabolic stability and oral bioavailability. In the course of screening of the core systems to reduce lipophilicity, 4,5,6,7-tetrahydro-1*H*-pyrazolo[4,3-*b*]pyridine **27** was found to have favorable metabolic and pharmacokinetic profiles (F = 28%) while keeping the activity (IC_{50} = 7.7 nM). It was selected as a lead compound for the next optimization step. We optimized the lead **27** to improve inhibitory activity against HCC4006 cell growth based on the SAR information - modification of both substituent on pyrazole ring and upper amide were effective to boost the cellular activity. As a result of optimization, we identified compound **26** as the most promising compound, showing enhanced inhibitory activity against SPT enzyme (IC_{50} = 1.2 nM) and HCC4006 cell growth (IP = 23 nM). Chiral resolution of **26** afforded compound **4** as a eutomer and compound **26*R*** as a distomer. The compound **4** potently inhibited both SPT enzyme (IC_{50} = 1.2 nM) and HCC4006 cell growth (IP = 3.9 nM), and showed good pharmacokinetic properties in mice (F = 53%).

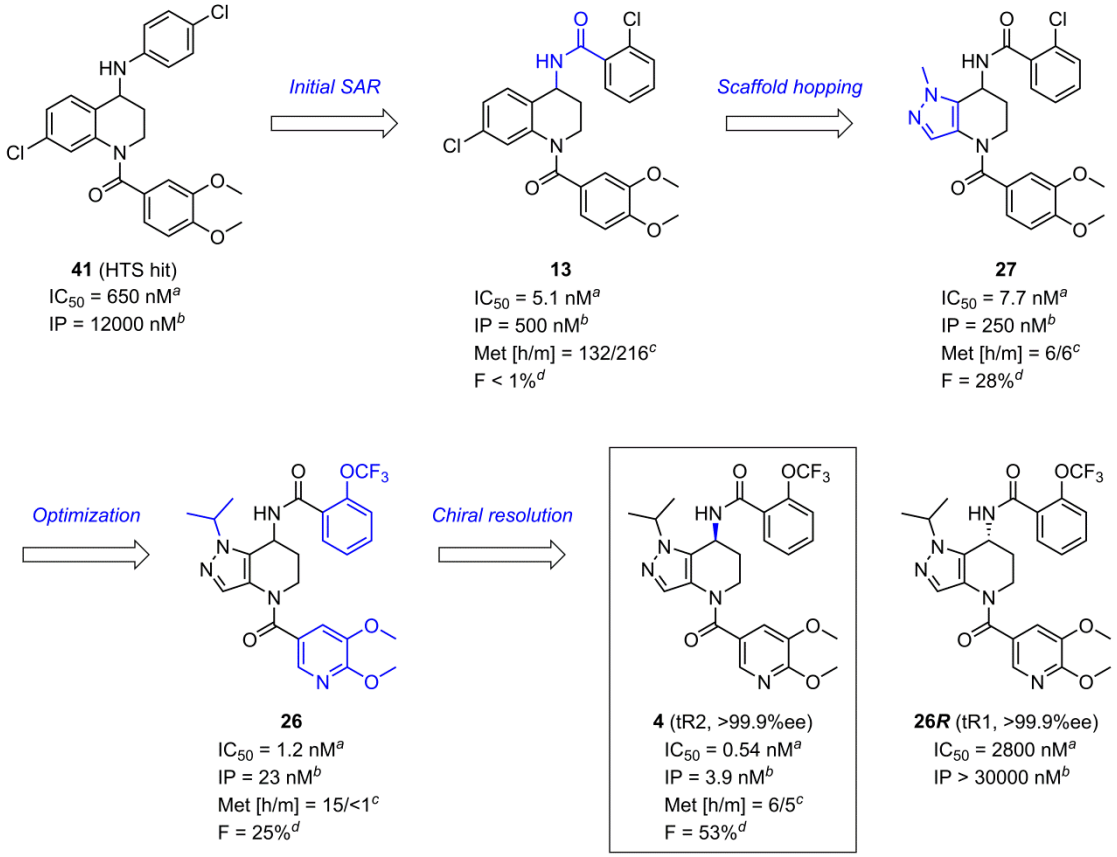


Figure 3. Identification of compound **4** from HTS hit **41**.

[a] SPT inhibitory activity (n = 2). [b] Growth inhibition of HCC4006 cells (n = 2). [c] Metabolic clearance in [human/mouse]

hepatic microsomes (n = 2, μL/min/mg). [d] Oral bioavailability in mice following cassette dosing (n = 3). [e] NC: not calculated because the measured plasma concentrations after oral administration were basically below the quantitation limit and less than two available data points.

To expand the diversity of structure, we continued the generation of another chemotype from compound **13**. SAR study of the tetrahydropyrazolopyridine series revealed that introducing sterically bulky groups on the pyrazole nitrogen was well tolerated and was rather preferred. This suggested that there would be a space to accommodate large substituents. Therefore, we aimed to replace the tetrahydroquinoline (**13**) core by 3-phenlypiperidine (**31**) to occupy the space. As expected, the designed compound **31** was found to show excellent inhibitory activity against SPT enzyme (IC_{50} = 1.2 nM) as well as HCC4006 cell growth (IP = 41 nM).

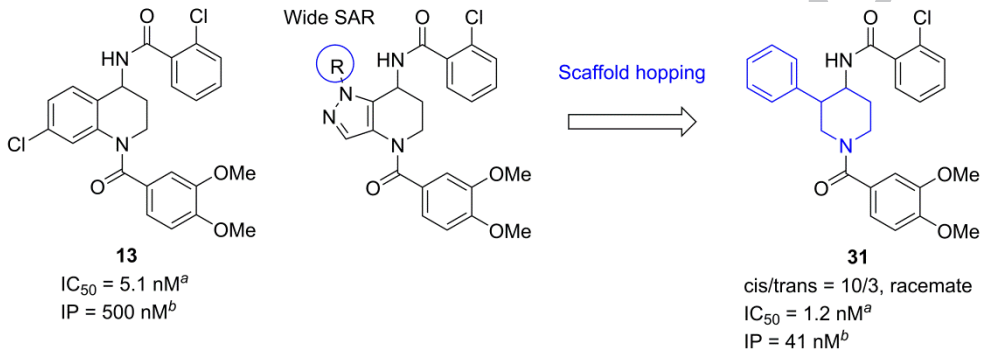


Figure 4. Design concept of 3-phenylpiperidine derivative **31**.
[a] SPT inhibitory activity (n = 2). [b] Growth inhibition of HCC4006 cells (n = 2).

Since the prototype compound **31** was a mixture of four stereoisomers, preparation and evaluation of the individual isomers were carried out. Consequently, compound **31b** with 3*S*,4*R* absolute configuration was found as the most potent isomer (Figure 5). Judging from the comparison of four isomers, stereo configuration of the 4-position (IC_{50} = 0.36 nM vs 3000 nM) on the piperidine ring seemed to have a greater impact on potency than that of the 3-position (IC_{50} = 0.36 nM vs 5.0 nM). Our strategy for optimizing compound **31b** to improve bioavailability was adjusting lipophilicity and replacing dimethoxyphenyl ring of **31b** with quinoxaline ring, which led to the identification of compound **5** as the most promising compound among this series. It was surprising that the absolute configuration of the nitrogen substituent of compound **4** and compound **5** are opposite, but superposition of both compounds is still inconclusive.

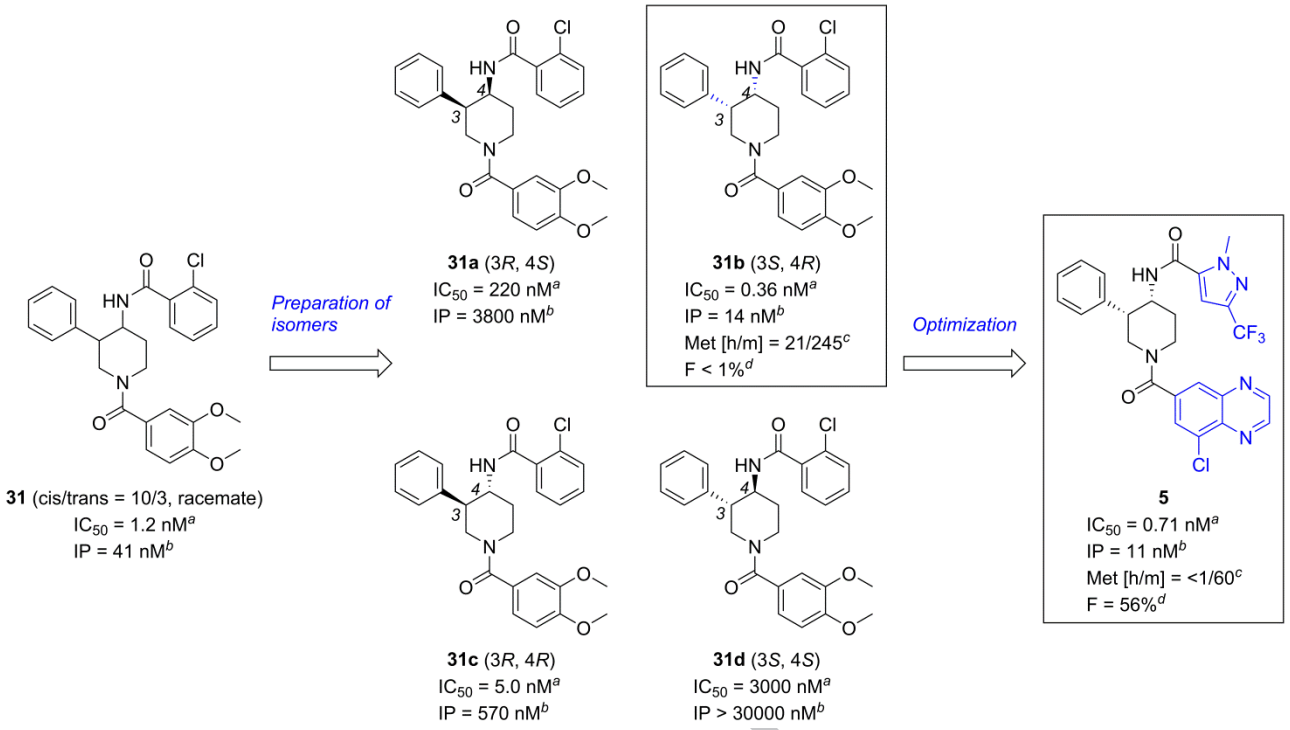


Figure 5. Identification of compound **5** from the prototype **31**.
[a] SPT inhibitory activity (n = 2). [b] Growth inhibition of HCC4006 cells (n = 2). [c] Metabolic clearance in [human/mouse] hepatic microsomes (n = 2, μL/min/mg). [d] Oral bioavailability in mice following cassette dosing (n = 3). [e] NC: not calculated because the measured plasma concentrations after oral administration were basically below the quantitation limit and less than two available data points.

In vitro profiles of compounds **4** and **5** are summarized in Table 1. Both compounds **4** and **5** were as potent as Myriocin in a human SPT enzyme inhibition assay. Compounds **4** and **5** were also tested in a ceramide content assay using HCC4006 cells, measuring the ceramide content[14]. In this assay, both compounds **4** and **5** exhibited inhibitory activity almost equal to that of Myriocin, indicating that the compounds definitely suppressed de novo synthesis of sphingolipids by SPT inhibition. Next, cancer cell growth assay was conducted. While we evaluated potency in growth inhibition using HCC4006 cells during the compound optimization stage, our efforts to search for cell lines more sensitive to SPT inhibitors led to the identification of PL-21 cells derived from an acute promyelocytic leukemia (maximum inhibition rate > 90%). Compounds **4** and **5** displayed potent growth inhibitory activity against both HCC4006 and PL-21 cells on the same level with Myriocin. Once the potency of compounds **4** and **5** as SPT inhibitors was validated in vitro, we then performed their in vivo evaluation.

Table 1. In vitro profiles of compounds **1**, **4**, and **5**[a]

Assays	**1** (Myriocin)	**4**	**5**
SPT enzyme inhibition IC_{50} (nM)[a,b,d]	0.13 (0.11–0.16)	0.54 (0.44–0.66)	0.71 (0.53–0.95)
Ceramide content, HCC4006 cells IC_{50} (nM)[a,b,e]	1.5 (0.95–2.2)	2.4 (1.4–4.0)	2.3 (1.3–3.8)
Growth inhibition, HCC4006 cells	2.1 (1.0–4.5)	3.9 (3.0–5.0)	11 (6.2–18)

IP (nM)[c,d]			
Growth inhibition, PL-21 cells IC_{50} (nM)[a,b,f]	7.8 (6.3–9.3)	4.1 (3.4–4.7)	11 (9.8–13)
Metabolic clearance, human (μL/min/mg)[g]	-	6	<1
Metabolic clearance, mouse (μL/min/mg)[g]	-	5	60
Solubility pH 6.8 (μg/mL)[h]	-	24	8
PAMPA pH7.8 A to B (nm/sec)	-	167	203

[a] Numbers in parentheses represent 95% confidence intervals. [b] Concentration where dose-inhibition curve passes through 50% inhibition. [c] Concentration at the inflection point of dose-inhibition curve. [d] n = 2. [e] n = 4. [f] n = 3. [g] Metabolic clearance was measured using liver microsomes and NADPH. [h] Thermodynamic solubility in Japanese Pharmacopoeia disintegration test solution 2 (pH 6.8).

Pharmacokinetic profiles of compounds **4** and **5** following cassette dosing to mice are summarized in Table 2. Both compounds possessed appropriate volume of distribution and clearance rate. Moreover, compound **4** and **5** displayed different pattern of the plasma concentration-time curve – compound **5** had 2-fold longer mean residence time and time to peak concentration compared to compound **4**. The oral bioavailability of compounds **4** and **5** were 53% and 56%, respectively. The good pharmacokinetic properties in mice are presumably due to their appropriate drug-like properties, including metabolic stability, aqueous solubility, and permeability as shown in Table 1.

Table 2. Pharmacokinetic parameters of compounds **4** and **5** in mice[a]

Compd	iv (0.1 mg/kg)		po (1 mg/kg)				F (%)
	Vdss (mL/kg)	CLtot (mL/h/kg)	Cmax (ng/mL)	Tmax (h)	AUC (ng·h/mL)	MRT (h)	

4	1128	954	309	0.5	572	1.4	53
5	750	507	282	1.2	1113	2.7	56

[a] Pharmacokinetic parameters were obtained by cassette dosing. Data represent mean values (n = 3).

The in vivo PD profiles of compounds **4** and **5** are shown in Figure 6. Quantity of 3-KDS, the direct product of SPT, in tumors was measured 8 h after oral administration of compounds **4** or **5** (10 mg/kg) in mouse HCC1806 (breast cancer) xenograft model. As with HCC4006, HCC1806 was found to be a sensitive cell line to SPT inhibitor, and its xenograft model was more easily prepared than that of HCC4006. As a result, both compounds **4** and **5** significantly suppressed the level of 3-KDS in tumors 8 h after oral administration (about 40% suppression relative to vehicle).

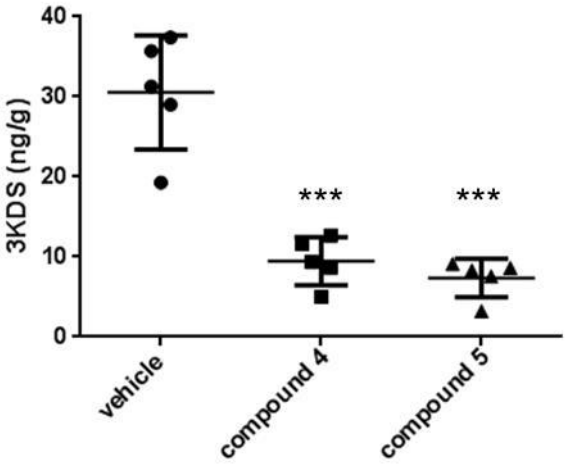


Figure 6. 3-KDS levels in the tumors of HCC1806 xenografted mice were detected by LC/MS 8 h after oral administration of compound **4** or **5** at 10 mg/kg. Data are expressed as mean ± SD (n = 5). ***p < 0.001 vs vehicle (Dunnett's test).

Antitumor efficacy study of compound **4** was carried out in a PL-21 xenograft mouse model. Oral administration of compound **4** (10 and 30 mg/kg, Q2D, 2 weeks) exhibited antitumor efficacy with T/C values, which meaned the median tumor weight in the treated group (T) divided by the median tumor weight in the control group (C), of 52% at 10 mg/kg and 38% at 30 mg/kg without severe body weight loss (Figure 7). These data indicated that compound **4** is an orally bioavailable SPT inhibitor with in vivo antitumor efficacy, which provides a useful tool for assessing the pharmaceutical potential of SPT inhibitors especially against cancer. Significant antitumor effect of compound **5** was also observed in the same in vivo model.[15]

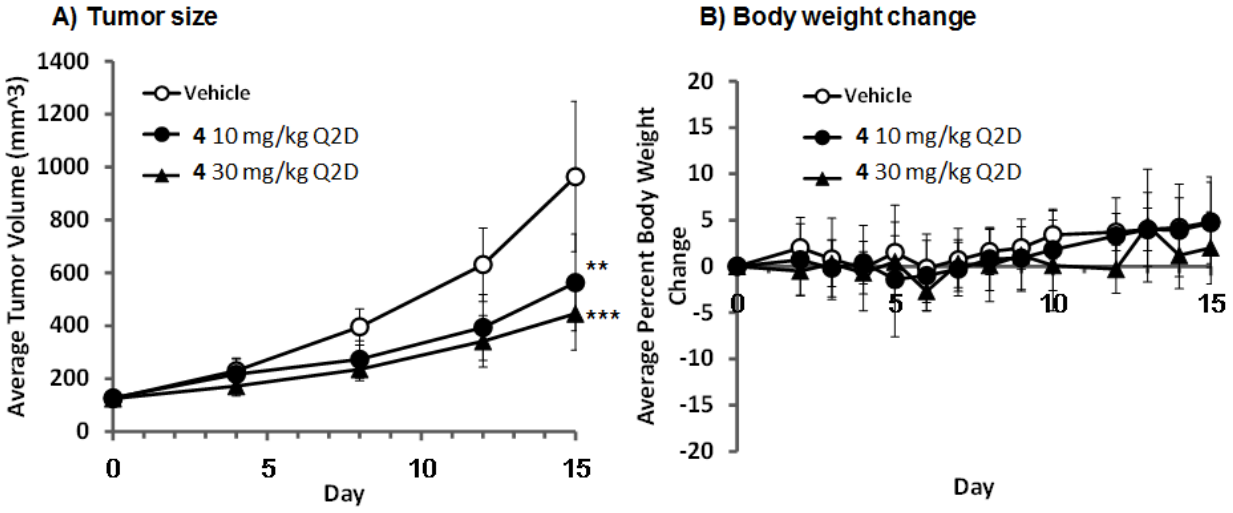


Figure 7. Mean tumor volume in PL-21 xenograft mice dosed with **4** or vehicle. Data are expressed as mean ± SD (n = 6/group). Compound **4** was suspended in 0.5% aqueous methylcellulose solution and orally administered every other day for two weeks. *** $p < 0.001$, **$p < 0.01$ vs vehicle (Dunnett's test). T/C values were as follows: 52% (10 mg/kg, Q2D), 38% (30 mg/kg, Q2D).

3. Conclusion

We established a good correlation between SPT inhibition and growth suppression of HCC4006 cell line and discovered structurally diverse SPT inhibitors **4** and **5** by an HTS campaign and iterative compound design including scaffold hopping. Both compounds **4** and **5** potently inhibited SPT enzyme activity and de novo synthesis of ceramide. They suppressed growth of HCC4006 and PL-21 cancer cell lines in vitro at the same level as Myriocin. In addition, compounds **4** and **5** displayed favorable pharmacokinetic profiles. Significant reduction of 3-KDS levels in HCC1806 xenograft mice was observed by oral administration of compounds **4** and **5** (10 mg/kg). Furthermore, antitumor efficacy of an SPT inhibitor was demonstrated in a PL-21 xenograft mouse model. Oral administration of compound **4** (30 mg/kg, Q2D, 14 days) reduced tumor growth with a T/C value of 38%. These results along with recent reported efficacy study of compound **5**[15] show that SPT inhibitors might have the potential to be effective cancer therapeutics.

Acknowlwdgement

We thank Mitsuyoshi Nishitani for X-ray structure analysis.

Experimental Section

Chemistry

General Chemistry Information. All commercially available solvents and reagents for reactions were reagent-grade and were used as purchased. Analytical thin layer chromatography (TLC) was performed on silica gel 60 F254 plates (Merck) or NH TLC plates (Fuji Silysia Chemical Ltd.). Flash column chromatography separations were performed on Purif-Pack (SI or NH, Fuji Silysia Chemical Ltd.). Melting points (mp.), determined on a SRS OptiMelt automated melting point apparatus, are uncorrected. The proton nuclear magnetic resonance (1H NMR) spectra were recorded using Bruker AVANCE II (300 MHz), Bruker AVANCE III (300 MHz, 400 MHz), or Varian mercury plus (300 MHz) spectrometers with tetramethylsilane (TMS) as an internal standard. The NMR data are given as follows: chemical shift (δ) in ppm, multiplicity (where applicable), coupling constants (*J*) in Hz (where applicable), and integration (where applicable). Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), dd (double doublets), dt,

(double triplet), dq (double quartet), br s (broad singlet), or m (multiplet). Mass spectra (MS) were acquired using an Agilent LC/MS system (Agilent1200SL/Agilent6130MS), Shimadzu LC/MS system (LC-10ADvp high pressure gradient system/LCMS-2010A) or Shimadzu UFLC/MS (Prominence UFLC high pressure gradient system/LCMS-2020) operating in electron spray ionization mode (ESI+). The column used was an L-column 2 ODS (3.0 x 50 mm I.D., 3 μm, CERI, Japan) with a temperature of 40 °C and a flow rate of 1.2 or 1.5 mL/min. Mobile phase A was 0.05% TFA in ultrapure water. Mobile phase B was 0.05% TFA in acetonitrile which was increased linearly from 5% to 90% over 2 minutes, 90% over the next 1.5 minutes, after which the column was equilibrated to 5% for 0.5 minutes. Elemental analyses were carried out by Takeda Pharmaceutical Company Ltd. Medicinal Chemistry Research Laboratories. Each compound was confirmed to be ≥95% purity by either LC/MS or elemental analysis. Yields were not optimized.

***tert*-Butyl 7-chloro-4-oxo-3,4-dihydroquinoline-1(2*H*)-carboxylate (7).** Boc_2O (10.1 mL, 44.1 mmol) and DMAP (2.1 g, 17.2 mmol) were added to a stirred solution of **6** (7.6 g, 41.8 mmol) in THF (100 mL) at room temperature, and the mixture was stirred at room temperature for 14 h. The mixture was concentrated in vacuo, diluted with water, and extracted with EtOAc. The organic layer was washed with water and brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography (silica gel, eluted with 50% EtOAc in hexane) to give **7** (13.1 g, quant) as a yellow oil. This product was used in the next step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.57 (9H, s), 2.70-2.81 (2H, m), 4.11-4.20 (2H, m), 7.12 (1H, dd, J = 8.5, 2.1 Hz), 7.87 (1H, d, J = 1.9 Hz), 7.93 (1H, d, J = 8.5 Hz).

***tert*-Butyl 7-chloro-4-hydroxy-3,4-dihydroquinoline-1(2*H*)-carboxylate (8).** Sodium borohydride (1.6 g, 42.3 mmol) was added to a solution of **7** (13.1 g) in THF (150 mL) and MeOH (75 mL) at 0 °C. The mixture was stirred at 0 °C for 1 h and at room temperature for 14 h. The mixture was quenched with water and concentrated in vacuo. The residue was diluted with water and extracted with EtOAc. The organic layer was washed with water and brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to give **8** (10.3 g, 87% yield in 2 steps) as a yellow jelly. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.48-1.61 (9H, m), 1.71-1.85 (1H, m), 1.86-2.12 (2H, m), 3.47-3.75 (1H, m), 3.89-4.08 (1H, m),4.65-4.78 (1H, m), 7.04 (1H, dd, J = 8.3, 2.1 Hz), 7.30 (1H, d, J = 8.3 Hz), 7.91 (1H, d, J = 1.9 Hz).

***tert*-Butyl 4-azido-7-chloro-3,4-dihydroquinoline-1(2*H*)-carboxylate (9).** DPPA (4.5 mL, 21.1 mmol) was added to a solution of **8** (5.0 g, 17.6 mmol) and DBU (3.1 mL, 21.1 mmol) in toluene (100 mL) at room temperature, and the mixture was stirred at room temperature for 18 h. The mixture was quenched with water and extracted with EtOAc. The organic layer was washed with water and brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with 50% EtOAc in hexane) to give **9** (6.37 g, quant) as a yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.55 (9H, s), 2.00-2.09 (2H, m), 3.49-3.65 (1H, m), 3.98-4.10 (1H, m), 4.56 (1H, t, J = 4.5 Hz), 7.02-7.08 (1H, m), 7.16-7.23 (1H, m), 7.99 (1H, d, J = 2.1 Hz).

***tert*-Butyl 4-amino-7-chloro-3,4-dihydroquinoline-1(2*H*)-carboxylate hydrochloride (10).** A mixture of **9** (5.4 g, 17.6 mmol) and PPh_3 (8.3 g, 31.7 mmol) in water (40 mL) and THF (400 mL) was stirred at 100 °C for 2 h. The mixture was concentrated in vacuo, diluted with water, and extracted with EtOAc. The organic layer was washed with water and brine, dried over $MgSO_4$, and filtered. To the filtrate was added 4 M HCl (EtOAc solution), and the resulting precipitate was collected by filtration to give **10** (2.5 g, 44% yield) as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.49 (9H, s), 1.94-2.10 (1H, m), 2.10-2.24 (1H, m), 3.59-3.77 (1H, m), 3.79-3.92 (1H, m), 4.49 (1H, brs), 7.22 (1H, dd, J = 8.3, 2.3 Hz), 7.55 (1H, d, J = 8.3 Hz), 7.88 (1H, d, J = 2.3 Hz), 8.60 (3H, brs).

***tert*-Butyl 7-chloro-4-((2-chlorobenzoyl)amino)-3,4-dihydroquinoline-1(2*H*)-carboxylate (11).** To a mixture of **10** (2.5 g, 7.83 mmol) and Et_3N (2.5 mL, 23.4 mmol) in THF (50 mL) was added 2-chlorobenzoyl chloride (1.1 mL, 8.67 mmol) at room temperature, and the mixture was stirred at room temperature for 2 h. The mixture was concentrated in

vacuo, diluted with water, and extracted with EtOAc. The organic layer was washed with water and brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography (silica gel, eluted with 10-50% EtOAc in hexane) to give **11** (3.1 g, 94% yield) as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.55 (9H, s), 2.09-2.32 (2H, m), 3.65-3.81 (1H, m), 3.87-4.03 (1H, m), 5.29-5.39 (1H, m), 6.39 (1H, d, J = 7.5 Hz), 7.04 (1H, dd, J = 8.3, 2.3 Hz), 7.27-7.43 (4H, m), 7.67-7.75 (1H, m), 7.88 (1H, d, J = 2.1 Hz).

2-Chloro-*N*-(7-chloro-1,2,3,4-tetrahydroquinolin-4-yl)benzamide hydrochloride (12). A mixture of **11** (3.1 g, 7.36 mmol) and 4 M HCl (EtOAc solution, 20 mL) was stirred at room temperature for 1 h. The mixture was treated with Et_2O and stirred at room temperature for 30 min. The precipitate was collected by filtration to give **12** (2.4 g, 91% yield) as a colorless powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.87-1.98 (2H, m), 3.27 (2H, t, J = 5.7 Hz), 4.98-5.18 (1H, m), 6.46 (2H, brs), 6.54-6.66 (2H, m), 7.13 (1H, d, J = 7.9 Hz), 7.32-7.53 (4H, m), 8.82 (1H, d, J = 8.3 Hz).

2-Chloro-*N*-(7-chloro-1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydroquinolin-4-yl)benzamide (13). Oxalyl chloride (280 uL, 3.26 mmol) was added to a mixture of 3,4-dimethoxybenzoic acid (480 mg, 2.63 mmol) and DMF (3 drops) in THF (15 mL) at room temperature, and the mixture was stirred at room temperature for 1 h. The mixture was concentrated in vacuo, and the residue was suspended in THF (15 mL). To the suspension, **12** (790 mg, 2.21 mmol) and Et_3N (690 uL, 4.94 mmol) were added at room temperature, and the mixture was stirred at 50 °C for 3 h. The mixture was concentrated in vacuo, diluted with water, and extracted with EtOAc. The organic layer was washed with aqueous 1 M HCl, aqueous saturated $NaHCO_3$, and brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography (silica gel, eluted with 10-40% EtOAc in hexane) to give **13** (891 mg, 83% yield) as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.08-2.25 (1H, m), 2.28-2.49 (1H, m), 3.76-3.89 (1H, m), 3.84 (3H, s), 3.91 (3H, s), 4.00-4.17 (1H, m), 5.33-5.49 (1H, m), 6.56 (1H, d, J = 8.0 Hz), 6.80 (1H, d, J = 8.3 Hz), 6.98-7.15 (4H, m), 7.29-7.44 (4H, m), 7.68-7.78 (1H,m). LC-MS (ESI): m/z [M + H^+] 484.9

Methyl 1-isopropyl-4-nitro-1*H*-pyrazole-5-carboxylate (15a). To a solution of **14** (10 g, 58.4 mmol), propan-2-ol (22.0 mL, 292 mmol), and tributylphosphine (17.5 mL, 70.1 mmol) in dry THF (200 mL) was added dropwise a solution of di-*tert*-butyl azodicarboxylate (17.5 g, 76.0 mmol) in dry THF (100 mL) at 20 °C. After being stirred at room temperature overnight, the mixture was partitioned between EtOAc and water. The organic layer was separated, washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with 5-50% EtOAc in hexane) to give **15a** (9.05 g, 73% yield) as a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.53 (6H, d, J = 6.6 Hz), 4.03 (3H, s), 4.68 (1H, spt, J = 6.6 Hz), 8.05 (1H, s).

Methyl 1-methyl-4-nitro-1*H*-pyrazole-5-carboxylate (15b). To a solution of **14** (5 g, 29.2 mmol), methanol (5.93 ml, 146 mmol) and tributylphosphine (14.6 mL, 58.4 mmol) in toluene (500 mL) was added 1,1'-(azodicarbonyl)dipiperidine (14.8 g, 58.4 mmol) at room temperature. The mixture was stirred at room temperature overnight. The insoluble material was removed by filtration, and the filtrate was partitioned between EtOAc and water. The organic layer was separated, washed with aqueous saturated $NaHCO_3$ and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, 30 g, eluted with 0-70% EtOAc in hexane) to give **15b** (3.28 g, 61% yield) as a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.03 (3H, s), 4.03 (3H, s), 8.02 (1H, s).

Methyl 4-amino-1-isopropyl-1*H*-pyrazole-5-carboxylate (16a). To a solution of **15a** (3.57 g, 16.8 mmol) in MeOH (60 mL) was added Pd/C (50% wet, 0.178 g), and the mixture was stirred at room temperature for 2 h under hydrogen atmosphere. The insoluble materials were filtered off by using a pad of Celite, and the Celite was washed with MeOH. The filtrate was concentrated in vacuo. The residue was diluted with EtOAc, washed with brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to afford **16a** (3.02 g, 98% yield) as a brown oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.30 (6H, d, J = 6.6 Hz), 3.79 (3H, s), 5.00 (2H, s), 5.21 (1H, dt, J = 13.1, 6.5 Hz), 7.05 (1H, s). LC-MS

(ESI): m/z [M + H^+] 183.9

Methyl 4-amino-1-methyl-1*H*-pyrazole-5-carboxylate (16b). The compound **16b** was prepared from **15b** by the same method as that described for **16a**. Colorless amorphous powder (97% yield). LC-MS (ESI): m/z [M + H^+] 156.2

Methyl 4-(benzyl(3-methoxy-3-oxopropyl)amino)-1-isopropyl-1*H*-pyrazole-5-carboxylate (18a). A mixture of **16a** (2.92 g, 15.9 mmol), methyl acrylate (13.7 g, 159 mmol), and DMAP (0.389 g, 3.19 mmol) in DMF (20 mL) was stirred at 100 ^oC for 3 days. The resulting mixture was cooled to room temperature, and the excess methyl acrylate was removed in vacuo. To the residue were added benzyl bromide (2.84 mL, 23.9 mmol) and K_2CO_3 (3.30 g, 23.9 mmol). The mixture was stirred at room temperature for 8 h. The mixture was diluted with water (200 mL) and extracted with EtOAc (200 mL). The organic layer was washed with brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography (silica gel, eluted with 0-20% EtOAc in hexane) to give **18a** as a pale yellow oil (4.34 g, 76% yield in 2 steps). ^1H NMR (300 MHz, DMSO-d_6) δ 1.34 (6H, d, J = 6.6 Hz), 2.40-2.47 (2H, m), 3.22 (2H, t, J = 7.3 Hz), 3.50 (3H, s), 3.80 (3H, s), 4.14 (2H, s), 5.12-5.25 (1H, m), 7.17-7.26 (1H, m), 7.27-7.31 (4H, m), 7.41 (1H, s). LC-MS (ESI): m/z [M + H^+] 360.0

Methyl 4-(benzyl(3-methoxy-3-oxopropyl)amino)-1-methyl-1*H*-pyrazole-5-carboxylate (18b). The compound **18b** was prepared from **16b** by the same method as that described for **18a**. Yellow oil (68% yield in 2 steps). ^1H NMR (500 MHz, $CDCl_3$): δ 2.51 (2H, t, *J* = 7.5 Hz), 3.34 (2H, t, *J* = 7.5 Hz), 3.62 (3H, s), 3.89 (3H, s), 4.10 (3H, s), 4.21 (2H, s), 7.25-7.33 (6H, m). LC-MS (ESI): m/z [M + H^+] 332.0

4-Benzyl-1-isopropyl-5,6-dihydro-1*H*-pyrazolo[4,3-*b*]pyridin-7(4*H*)-one (19a). To a solution of **18a** (4.24 g, 11.8 mmol) in dry THF (80 mL) was added dropwise NaHMDS in THF (6.83 mL, 13.0 mmol) at room temperature. After 1 h, NaHMDS in THF (3.10 mL, 5.90 mmol) was added dropwise. After 30 min, additional NaHMDS in THF (1.86 mL, 3.54 mmol) was added dropwise. After 1 h, 2 M NaOH (59.0 mL, 118 mmol) was added, and the mixture was stirred at 70 °C. After 3 h, the mixture was poured into water (150 mL), and extracted with EtOAc (150 mL + 50 mL). The organic layer was washed with brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to afford **19a** as a pale yellow oil (2.89 g, 91% yield). ^1H NMR (300 MHz, DMSO-d_6) δ 1.33 (6H, d, J = 6.6 Hz), 2.45-2.53 (2H, m), 3.12-3.19 (2H, m), 4.30 (2H, s), 5.16 (1H, quin, J = 6.6 Hz), 7.25-7.42 (6H, m). LC-MS (ESI): m/z [M + H^+] 270.3

4-Benzyl-1-methyl-5,6-dihydro-1*H*-pyrazolo[4,3-*b*]pyridin-7(4*H*)-one (19b). The compound **19b** was prepared from **18b** by the same method as that described for **19a**. Yellow solid (quant in 2 steps). ^1H NMR (500MHz, $CDCl_3$): δ 2.51 (2H, t, *J* = 7.0 Hz), 3.15 (2H, t, *J* = 7.0 Hz), 4.01 (3H, s), 4.18 (2H, s), 6.95 (1H, s), 7.20-7.31 (5H, m). LC-MS (ESI): m/z [M + H^+] 242.2

4-Benzyl-1-isopropyl-4,5,6,7-tetrahydro-1*H*-pyrazolo[4,3-*b*]pyridin-7-amine (21a). A mixture of **19a** (900 mg, 3.34 mmol), pyridine (1.32 g, 16.7 mmol), and hydroxylamine hydrochloride (1.16 g, 16.7 mmol) in EtOH (20 mL) was stirred at 80-90 °C for 6 h. The mixture was concentrated in vacuo. The residue was diluted with aqueous saturated $NaHCO_3$, and extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to afford **20a** as a pale yellow solid. A mixture of **20a** and Raney nickel (slurry in water, 8 g) in MeOH (20 mL) was stirred under hydrogen atmosphere at room temperature for 2 h. The insoluble materials were filtered off and washed with MeOH-water (3:1). The filtrate was concentrated in vacuo. The residue was diluted with aqueous saturated $NaHCO_3$, and extracted with EtOAc, washed with brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography (silica gel, eluted with 0%- 100% EtOAc in hexane) to give **21a** as colorless oil (643 mg, 71% yield in 2 steps). LC-MS (ESI): m/z [M + H^+] 271.0

4-Benzyl-1-methyl-4,5,6,7-tetrahydro-1*H*-pyrazolo[4,3-*b*]pyridin-7-amine (21b). The compound **21b** was prepared from **19b** by the same method as that described for **21a**. Colorless solid (58% yield in 2 steps). ^1H NMR (500MHz, DMSO-d_6): δ 1.65-1.69 (1H, m), 1.74 (2H, d, *J* = 6.5 Hz), 1.82-1.85 (1H, m), 2.68-2.80 (2H, m), 3.72 (3H, s), 3.89-3.91

(1H, m), 3.94 (1H, d, *J* = 14.0 Hz), 4.14 (1H, d, *J* = 14.0 Hz), 6.87 (1H, s), 7.23-7.26 (1H, m), 7.31-7.34 (4H, m). LC-MS (ESI): m/z [M + H^+] 243.0

***tert*-Butyl (1-isopropyl-4,5,6,7-tetrahydro-1*H*-pyrazolo[4,3-*b*]pyridin-7-yl)carbamate (23a).** To a solution of **21a** (811 mg, 3 mmol) and Et_3N (0.836 mL, 6.00 mmol) in dry THF (10 mL) was added dropwise Boc_2O (0.766 mL, 3.30 mmol) at room temperature, and the mixture was stirred at room temperature for 1 h. The mixture was diluted with water at room temperature and extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with 20-50% EtOAc in hexane) to give **22a** as a beige solid. A mixture of **22a** (1.06 g, 2.86 mmol) and 10% Pd/C (319 mg) in MeOH (20 mL) and AcOH (2 mL) was stirred under hydrogen atmosphere at room temperature overnight. The catalyst was removed by filtration, and the filtrate was concentrated in vacuo. The residue was diluted with aqueous saturated $NaHCO_3$ and extracted with EtOAc-THF (3/1). The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to afford **23a** as a white solid (780 mg, 93% yield in 2 steps). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.24-1.45 (17H, m), 1.63-1.81 (2H, m), 2.89-3.03 (2H, m), 4.23-4.43 (2H, m), 4.72-4.83 (1H, m), 6.87 (1H, s), 7.39 (1H, d, J = 9.2 Hz). LC-MS (ESI): m/z [M + H^+] 281.0

***tert*-Butyl (1-methyl-4,5,6,7-tetrahydro-1*H*-pyrazolo[4,3-*b*]pyridin-7-yl)carbamate (23b).** The compound **23b** was prepared from **21b** by the same method as that described for **23a**. Colorless solid (59% yield in 2 steps). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.47 (9H, s), 1.86-2.09 (2H, m), 2.89-3.05 (1H, m), 3.11 (1H, brs), 3.24 (1H, dt, J = 12.2, 3.7 Hz), 4.71-5.06 (2H, m), 7.03 (1H, s). LC-MS (ESI): m/z [M + H^+] 253.0

***tert*-Butyl (4-(5,6-dimethoxynicotinoyl)-1-isopropyl-4,5,6,7-tetrahydro-1*H*-pyrazolo[4,3-*b*]pyridin-7-yl)carbamate (24a).** A mixture of **23a** (1.40 g, 4.99 mmol), 5,6-dimethoxynicotinic acid (1.19 g, 6.49 mmol), HATU (2.47 g, 6.49 mmol), and Et_3N (2.09 mL, 15.0 mmol) in dry DMF (15 mL) was stirred at room temperature overnight. The mixture was diluted with water at room temperature and extracted with EtOAc. The organic layer was separated, washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography (NH silica gel, eluted with 10-50% EtOAc in hexane) to give **24a** (2.11 g, 95% yield) as a colorless solid. LC-MS (ESI): m/z [M + H^+] 446.1

***N*-(4-(5,6-Dimethoxynicotinoyl)-1-isopropyl-4,5,6,7-tetrahydro-1*H*-pyrazolo[4,3-*b*]pyridin-7-yl)-2-(trifluoromethoxy)benzamide (26).** To a solution of **24a** (2.10 g, 4.71 mmol) in MeOH (20 mL) was added 4 M HCl (EtOAc solution, 11.8 mL, 47.1 mmol) at room temperature, and the mixture was stirred at room temperature overnight. The mixture was concentrated in vacuo, and the residue was washed with EtOAc-IPE to give (7-amino-1-isopropyl-6,7-dihydro-1H-pyrazolo[4,3-b]pyridin-4(5H)-yl)(5,6-dimethoxypyridin-3-yl)methanone tri-hydrochloride **25a** (2.11 g, 98% yield) as a colorless powder. A mixture of **25a** (2.11 g, 4.64 mmol), 2-(trifluoromethoxy)benzoic acid (1.24 g, 6.03 mmol), HATU (2.29 g, 6.03 mmol), and Et_3N (2.59 mL, 18.6 mmol) in dry DMF (15 mL) was stirred at room temperature under N_2 for 3 h. The mixture was diluted with aqueous saturated $NaHCO_3$ at room temperature and extracted with EtOAc. The organic layer was separated, washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography (NH silica gel, eluted with 50-100% EtOAc in hexane) and triturated with EtOAc-IPE to give **26** (1.74 g, 70% yield) as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.42 (3H, d, J = 6.6 Hz), 1.48 (3H, d, J = 6.6 Hz), 2.25 (2H, d, J = 3.0 Hz), 3.56 (1H, brs), 3.91 (3H, s), 4.07 (3H, s), 4.22 (1H, d, J = 10.4 Hz), 4.47 (1H, dt, J = 13.0, 6.6 Hz), 5.53-5.68 (1H, m), 6.79 (1H, d, J = 8.5 Hz), 7.25-7.27 (1H, m), 7.28-7.36 (1H, m), 7.39-7.50 (1H, m), 7.51-7.62 (1H, m), 7.89 (1H, d, J = 1.9 Hz), 7.99 (1H, dd, J = 7.7, 1.9 Hz), 8.22 (1H, brs). LC-MS (ESI): m/z [M + H^+] 534.2, Anal. Calcd for $C_{25}H_{26}F_3N_5O_5$: C, 56.28; H, 4.91; N, 13.13. Found: C, 56.30; H, 4.99; N, 13.00.

***tert*-Butyl (4-(3,4-dimethoxybenzoyl)-1-methyl-4,5,6,7-tetrahydro-1*H*-pyrazolo[4,3-*b*]pyridin-7-yl) -carbamate**

(24b). To a solution of **23b** (800 mg, 3.17 mmol) in pyridine (10 mL) was added 3,4-dimethoxybenzoyl chloride (700 mg, 3.49 mmol) at room temperature, and the mixture was stirred at room temperature overnight. The mixture was diluted with water at room temperature and concentrated in vacuo. The residue was diluted with water at room temperature and extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography (NH silica gel, eluted with 50-100% EtOAc in hexane) to give **24b** (1.29 g, 98% yield) as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.47 (9H, s), 2.09 (2H, d, J = 3.6 Hz), 3.36-3.56 (1H, m), 3.81 (3H, s), 3.90 (3H, s), 3.92 (3H, s), 4.11 (1H, brs), 4.92 (1H, brs), 4.96-5.09 (1H, m), 6.87 (1H, d, J = 8.1 Hz), 7.00-7.11 (2H, m). LC-MS (ESI): m/z [M + H^+] 417.0

(7-Amino-1-methyl-6,7-dihydro-1*H*-pyrazolo[4,3-*b*]pyridin-4(5*H*)-yl)(3,4-dimethoxyphenyl)methanone di-hydrochloride (25b). To a solution of **24b** (1.29 g, 3.10 mmol) in MeOH (10 mL) was added 4 M HCl (CPME solution, 7.74 mL, 31.0 mmol) at room temperature, and the mixture was stirred at room temperature overnight. The mixture was concentrated in vacuo, and the residue was collected by filtration, and triturated with EtOAc to give **25b** (1.17 g, 97% yield) as a colorless powder. LC-MS (ESI): m/z [M + H^+] 317.0.

2-Chloro-*N*-(4-(3,4-dimethoxybenzoyl)-1-methyl-4,5,6,7-tetrahydro-1*H*-pyrazolo[4,3-*b*]pyridin-7-yl)benzamide (27). A mixture of **25b** (645 mg, 1.66 mmol), 2-chlorobenzoic acid (285 mg, 1.82 mmol), HATU (819 mg, 2.15 mmol), and Et_3N (0.69 mL, 4.97 mmol) in DMF (5.0 mL) was stirred at room temperature for 5h. The mixture was diluted with water at room temperature and extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography (NH silica gel, eluted with 30-100% EtOAc in hexane) and triturated with IPE to give **27** (623 mg, 83% yield) as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.13-2.40 (2H, m), 3.50 (1H, t, J = 11.0 Hz), 3.85 (3H, s), 3.90 (3H, s), 3.92 (3H, s), 4.03-4.30 (1H, m), 5.54 (1H, dt, J = 7.9, 4.0 Hz), 6.61 (1H, brs), 6.88 (1H, d, J = 8.1 Hz), 6.99-7.12 (2H, m), 7.31-7.49 (3H, m), 7.62-7.74 (1H, m). LC-MS (ESI): m/z [M + H^+] 455.0

(*S*)-*N*-(4-(5,6-Dimethoxynicotinoyl)-1-isopropyl-4,5,6,7-tetrahydro-1*H*-pyrazolo[4,3-*b*]pyridin-7-yl)-2-(trifluoromethoxy)benzamide (4). Optical resolution of **26** (300 mg, 0.56 mmol) was conducted by SFC (CHIRALPAK AD, 50 mm ID × 500 mm L, CO_2/MeOH = 700/300). The obtained solid (tR2) was recrystallized from EtOAc-IPE to give **4** (123 mg) as colorless crystals. 99.9%ee. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.42 (3H, d, J = 6.4 Hz), 1.48 (3H, d, J = 6.6 Hz), 2.16-2.30 (2H, m), 3.45-3.66 (1H, m), 3.91 (3H, s), 4.07 (3H, s), 4.12-4.35 (1H, m), 4.47 (1H, quin, J = 6.6 Hz), 5.56-5.67 (1H, m), 6.78 (1H, d, J = 8.5 Hz), 7.24-7.28 (1H, m), 7.32 (1H, d, J = 8.3 Hz), 7.41-7.50 (1H, m), 7.52-7.61 (1H, m), 7.89 (1H, d, J = 1.7 Hz), 8.00 (1H, dd, J = 7.7, 1.9 Hz), 8.23 (1H, brs). LC-MS (ESI): m/z [M + H^+] 534.3, Anal. Calcd for $C_{25}H_{26}F_3N_5O_5$: C, 56.28; H, 4.91; N, 13.13. Found: C, 56.15; H, 4.97; N, 13.08. mp: 211-212 °C.

1-(3,4-Dimethoxybenzoyl)-3-phenylpiperidin-4-one (29). To a solution of **28** (1.0 g, 5.71 mmol) in pyridine (10 mL) was added 3,4-dimethoxybenzoyl chloride (1.37 g, 6.85 mmol) at 0 °C, and the mixture was stirred at room temperature overnight. The volatile components were evaporated in vacuo, and the residue was partitioned between EtOAc and aqueous 1 M HCl. The organic layer was separated, washed with aqueous saturated $NaHCO_3$ and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with 30-100% EtOAc in hexane) to give **29** (1.38 g, 71% yield) as a pale yellow solid. ^{1}H NMR ($CDCl_3$) δ: 2.65 (2H, brs), 3.55-3.83 (3H, m), 3.90 (3H, s), 3.91 (3H, s), 4.26-4.65 (2H, m), 6.87 (1H, d, *J* = 8.7 Hz), 7.01-7.10 (2H, m), 7.15 (2H, d, *J* = 6.2 Hz), 7.28-7.41 (3H, m). LC-MS (ESI): m/z [M + H^+] 340.3

(4-Amino-3-phenylpiperidin-1-yl)(3,4-dimethoxyphenyl)methanone (30). To a solution of **29** (1.35 g, 3.98 mmol) and Et_3N (0.83 mL, 5.97 mmol) in EtOH (50 mL) was added hydroxylamine hydrochloride (415 mg, 5.97 mmol) at room temperature. After being refluxed for 2 h, the mixture was concentrated in vacuo. The residue was partitioned between EtOAc and water, and the organic layer was separated, washed with brine, dried over Na_2SO_4, filtered, and

concentrated in vacuo to give oxime derivative (1.40 g, 3.95 mmol, 99%) as a colorless solid. A mixture of the oxime (1.35 g, 3.81 mmol) and Raney Ni (slurry in water, 10 g, Kawaken NDHT-90) in MeOH (40 mL) was stirred under hydrogen atmosphere at room temperature overnight. The catalyst was removed by filtration, and the filtrate was concentrated in vacuo. The residue was purified by column chromatography (NH silica gel, eluted with 50-100% EtOAc in hexane) to give **30** (1.15 g, 89% yield) as a colorless amorphous solid. LC-MS (ESI): m/z [M + H^+] 341.2

2-Chloro-*N*-(1-(3,4-dimethoxybenzoyl)-3-phenylpiperidin-4-yl)benzamide (31). To a solution of **30** (500 mg, 1.47 mmol) and Et_3N (0.307 mL, 2.20 mmol) in THF (10 mL) was added 2-chlorobenzoyl chloride (0.223 mL, 1.76 mmol) at 0 °C. After being stirred at room temperature overnight, the mixture was partitioned between EtOAc and water. The organic layer was separated, washed with aqueous 1 M HCl, aqueous saturated $NaHCO_3$, and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with 0-100% EtOAc in hexane) to give **31** (582 mg, 83% yield) as a colorless amorphous solid. LC-MS (ESI): m/z [M + H^+] 479.0, 481.1

***tert*-Butyl *trans*-4-(((benzyloxy)carbonyl)amino)-3-phenylpiperidine-1-carboxylate (33).** To a solution of **32** (1.0 g, 3.27 mmol) in toluene (25 mL) was added DPPA (0.774 mL, 3.60 mmol) followed by Et_3N (0.50 mL, 3.60 mmol) at room temperature, and the mixture was stirred at reflux for 1 h. Benzyl alcohol (0.409 mL, 3.93 mmol) was added to the mixture at room temperature, and the mixture was stirred at reflux for 5 h. The mixture was quenched with water at room temperature and extracted with EtOAc. The organic layer was washed with water and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with 10-100% EtOAc in hexane) to give **33** (1.35 g, quant) as a colorless oil. ^{1}H NMR (DMSO-d_6) δ: 1.46 (9H, s), 2.10-2.27 (1H, m), 2.46-2.64 (1H, m), 2.67-2.98 (2H, m), 3.83-4.03 (1H, m), 4.11-4.30 (2H, m), 4.47 (1H, brs), 4.70 (1H, d, *J* = 5.8 Hz), 4.95 (2H, s), 7.09-7.49 (10H, m).

***tert*-Butyl *trans*-4-amino-3-phenylpiperidine-1-carboxylate (34).** A mixture of **33** (1.3 g, 3.17 mmol) and 10% Pd-C (200 mg) in MeOH (30 mL) was stirred under hydrogen atmosphere at room temperature overnight. The catalyst was removed by filtration, and the filtrate was concentrated in vacuo to give **34** (864 mg, 99% yield) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ: 1.46 (9H, s), 1.74 (3H, brs), 1.83-2.04 (1H, m), 2.39 (1H, td, *J* = 11.0, 4.1 Hz), 2.66-2.93 (2H, m), 3.01 (1H, td, *J* = 10.7, 3.9 Hz), 3.99-4.35 (2H, m), 7.03-7.42 (5H, m).

***tert*-Butyl (3*R*,4*S*)-4-(2-chlorobenzamido)-3-phenylpiperidine-1-carboxylate (35a).** To a solution of **34a** (200 mg, 0.72 mmol) and Et_3N (0.151 mL, 1.09 mmol) in THF (10 mL) was added 2-chlorobenzoyl chloride (0.110 mL, 0.87 mmol) at 0 °C. After being stirred at room temperature overnight, the mixture was partitioned between EtOAc and water. The organic layer was separated, washed with aqueous 1 M HCl, aqueous saturated $NaHCO_3$, and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with 0-50% EtOAc in hexane) to give **35a** (282 mg, 94% yield) as a colorless amorphous solid. ^{1}H NMR ($CDCl_3$) δ: 1.45 (9H, brs), 1.80-2.04 (2H, m), 3.24 (1H, q, *J* = 4.8 Hz), 3.37-4.07 (4H, m), 4.63 (1H, tt, *J* = 8.2, 4.2 Hz), 5.99 (1H, brs), 7.18-7.37 (8H, m), 7.46-7.53 (1H, m).

2-Chloro-*N*-((3*R*,4*S*)-1-(3,4-dimethoxybenzoyl)-3-phenylpiperidin-4-yl)benzamide (31a). A mixture of **35a** (279 mg, 0.67 mmol) and 4 M HCl (EtOAc solution, 10 mL) was stirred at room temperature for 2 h. The volatile components were evaporated in vacuo to give amine derivative as a colorless solid. To a solution of the amine derivative (230 mg) in pyridine (10mL) was added 3,4-dimethoxybenzoyl chloride (158 mg, 0.79 mmol) at 0 °C, and the mixture was stirred at room temperature overnight. The volatile components were evaporated in vacuo, and the residue was partitioned between EtOAc and aqueous 1 M HCl. The organic layer was separated, washed with aqueous saturated $NaHCO_3$ and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with 30-100% EtOAc in hexane) to give **31a** (279 mg, 89% yield in 2 steps) as pale yellow crystals.

^{1}H NMR (DMSO-d_6) δ: 1.51-1.90 (1H, m), 1.90-2.13 (1H, m), 3.16-3.30 (1H, m), 3.35-3.50 (1H, m), 3.76 (7H, brs), 4.12 (2H, brs), 4.47-4.76 (1H, m), 6.81-7.12 (4H, m), 7.12-7.51 (8H, m), 8.57 (1H, d, *J* = 8.9 Hz). LC-MS (ESI): m/z [M + H$^+$] 479.2, 481.1

***tert*-Butyl (3*S*,4*R*)-4-(2-chlorobenzamido)-3-phenylpiperidine-1-carboxylate (35b).** The compound **35b** was prepared from **34b** by the same method as that described for **35a**. A colorless amorphous powder (38% yield): ^{1}H NMR ($CDCl_3$) δ: same as **35a**. LC-MS (ESI) same as **35a**.

2-Chloro-*N*-((3*S*,4*R*)-1-(3,4-dimethoxybenzoyl)-3-phenylpiperidin-4-yl)benzamide (31b). The compound **31b** was prepared from **35b** by the same method as that described for **31a**. A colorless amorphous powder (98% in 2 steps): ^{1}H NMR ($CDCl_3$) δ: same as **31a**. LC-MS (ESI) same as **31a**.

***tert*-Butyl *trans*-4-(2-chlorobenzamido)-3-phenylpiperidine-1-carboxylate (35).** The compound **35** was prepared from **34** by the same method as that described for **35a**. A colorless amorphous powder (82% yield): ^{1}H NMR ($CDCl_3$) δ: 1.47 (9H, s), 2.26-2.45 (1H, m), 2.61-2.76 (1H, m), 2.76-2.90 (1H, m), 2.90-3.06 (1H, m), 4.17-4.38 (2H, m), 4.38-4.59 (1H, m), 5.84 (1H, d, *J* = 8.3 Hz), 7.11-7.40 (10H, m).

2-Chloro-*N*-((3*R*,4*R*)-1-(3,4-dimethoxybenzoyl)-3-phenylpiperidin-4-yl)benzamide (31c). Trans racemate, 2-chloro-*N*-(trans-1-(3,4-dimethoxybenzoyl)-3-phenylpiperidin-4-yl)benzamide, was prepared from **35** by the same method as that described for **31a**. Chiral resolution of the trans racemate was conducted by chiral preparative HPLC (CHIRALPAK AS-H, 20 x 250 mm, CO_2 (770) / MeOH (230), tR1). A colorless crystals (70% yield): ^{1}H NMR (DMSO-d_6) δ 1.37-1.68 (1H, m), 1.82-2.17 (1H, m), 2.70-2.90 (1H, m), 2.90-3.30 (2H, m), 3.78 (6H, s), 3.61-4.06 (1H, m), 4.42 (2H, d, *J* = 10.9 Hz), 6.88 (1H, d, *J* = 6.8 Hz), 6.92-7.05 (3H, m), 7.10-7.49 (8H, m), 8.27 (1H, d, *J* = 8.9 Hz). LC-MS (ESI): m/z [M + H$^+$] 479.2, 481.1.

2-Chloro-*N*-((3*S*,4*S*)-1-(3,4-dimethoxybenzoyl)-3-phenylpiperidin-4-yl)benzamide (31d). Trans racemate, 2-chloro-*N*-(trans-1-(3,4-dimethoxybenzoyl)-3-phenylpiperidin-4-yl)benzamide, was prepared from **35** by the same method as that described for **31a**. Chiral resolution of the trans racemate was conducted by chiral preparative HPLC (CHIRALPAK AS-H, 20 x 250 mm, CO_2 (770) / MeOH (230), tR2). A colorless crystals (67% yield): ^{1}H NMR (DMSO-d_6) δ: same as **31c**, LC-MS (ESI) same as **31c**.

Methyl 8-chloroquinoxaline-6-carboxylate (37). Glyoxal (40% aqueous solution, 20.0 mL, 175 mmol) was added to a solution of **36** (23.4 g, 117 mmol) in MeOH (160 mL) and THF (80 mL) at room temperature. The mixture was stirred at room temperature overnight. The organic solvent was evaporated in vacuo, and the resulting solid was collected, washed with water, and dried in vacuo to give **37** (25.5 g, 98% yield) as a light brown powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.97 (3H, s), 8.39 (1H, d, J = 1.7 Hz), 8.59 (1H, d, J = 1.7 Hz), 8.94-9.48 (2H, m).

8-Chloroquinoxaline-6-carboxylic acid (38). To a solution of **37** (25.5 g, 115 mmol) in THF (200 mL) and MeOH (200 mL) was added aqueous 4 M NaOH (100 mL, 400 mmol) at room temperature. After being stirred at room temperature overnight, the mixture was concentrated in vacuo. The residue was dissolved in water and neutralized with 6 M HCl (pH = 5). The precipitate was collected by filtration, washed with water and dried in vacuo to give **38** (23.3 g, 98% yield) as light brown powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.75 (1H, brs), 8.42 (1H, d, J = 1.7 Hz), 8.50 (1H, d, J = 1.5 Hz), 9.09 (2H, s).

***tert*-Butyl (3*S*,4*R*)-4-(1-methyl-3-(trifluoromethyl)-1*H*-pyrazole-5-carboxamido)-3-phenylpiperidine-1-carboxylate (39).** To a solution of **34b** (5 g, 18.1 mmol), 1-methyl-3-(trifluoromethyl)-1*H*-pyrazole-5-carboxylic acid (4.21 g, 21.7 mmol), and HOBt (3.67 g, 27.1 mmol) in DMF (60 mL) was added EDC (4.76 mL, 27.1 mmol) at 0 °C. After being stirred at room temperature overnight, the mixture was concentrated in vacuo. The residue was partitioned between EtOAc and water, and the organic layer was separated, washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with 10-100% EtOAc in

hexane) to give **39** (7.76 g, 95% yield) as a colorless amorphous solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.41 (9H, brs), 1.60-1.91 (2H, m), 3.14 (1H, d, J = 3.6 Hz), 3.37-3.51 (1H, m), 3.58-3.87 (3H, m), 3.90 (3H, s), 4.49 (1H, brs), 6.80-7.47 (6H, m), 8.31 (1H, d, J = 8.9 Hz).

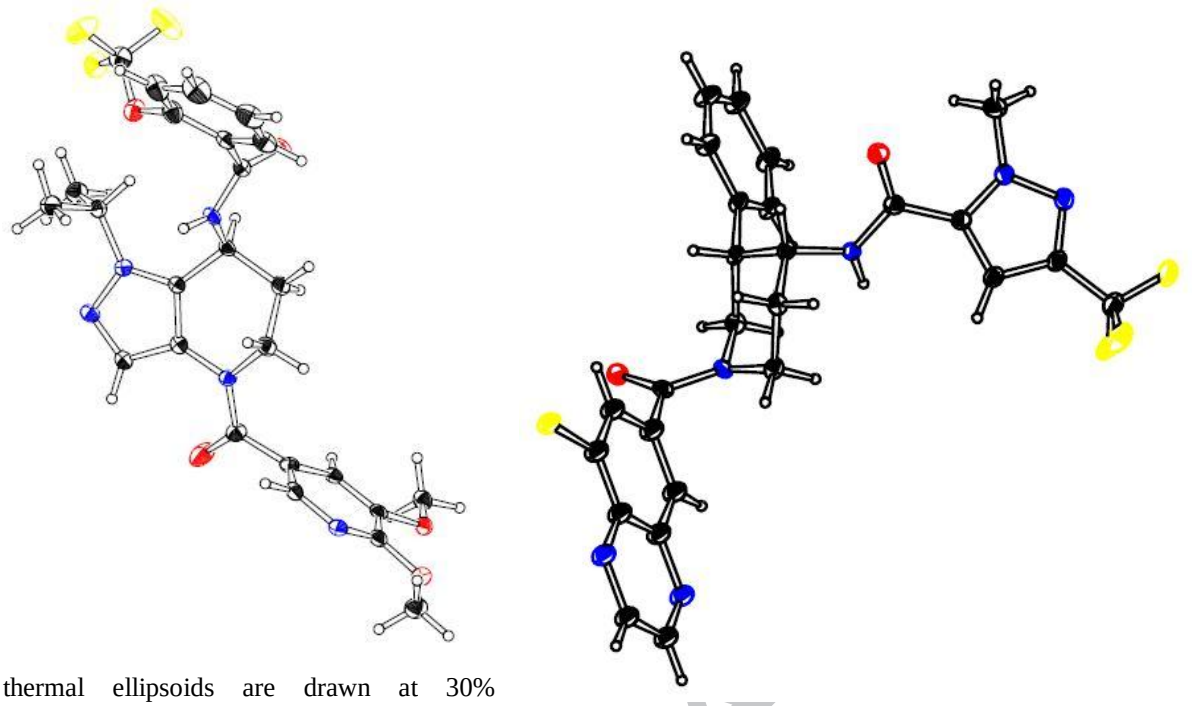
1-Methyl-*N*-((3*S*,4*R*)-3-phenylpiperidin-4-yl)-3-(trifluoromethyl)-1*H*-pyrazole-5-carboxamide hydrochloride (40). A mixture of **39** (7.75 g, 17.1 mmol) and 4 M HCl (EtOAc solution, 50 mL, 200 mmol) was stirred at room temperature for 3 h, and the mixture was concentrated in vacuo to give **40** (6.50 g, 98% yield) as a colorless solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.87 (1H, d, J = 12.6 Hz), 2.23 (1H, ddd, J = 14.1, 10.1, 4.1 Hz), 3.13-3.34 (3H, m), 3.45 (1H, dt, J = 13.4, 3.9 Hz), 3.83 (3H, s), 4.05 (1H, t, J = 13.0 Hz), 4.66 (1H, dd, J = 9.7, 3.1 Hz), 7.08-7.34 (5H, m), 7.40 (1H, s), 8.75 (1H, d, J = 9.8 Hz), 9.08 (1H, brs), 9.65 (1H, brs). LC-MS (ESI): m/z [M + H^+] 353.2

***N*-((3*S*,4*R*)-1-(8-Chloroquinoxaline-6-carbonyl)-3-phenylpiperidin-4-yl)-1-methyl-3-(trifluoromethyl)-1*H*-pyrazole-5-carboxamide (5).** To a solution of **40** (6.50 g, 16.72 mmol), **38** (3.49 g, 16.7 mmol), and HOBt (3.39 g, 25.1 mmol) in DMF (150 mL) was added EDC (4.40 mL, 25.1 mmol) at 0 °C. After being stirred at room temperature for 3 h, the mixture was concentrated in vacuo. The residue was partitioned between EtOAc and water, and the organic layer was separated, washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, eluted with 30-100% EtOAc in hexane) to give **5** (5.39 g, 59% yield) as a colorless amorphous solid. The obtained amorphous (1.50 g) was recrystallized from EtOAc-heptane to give **5** (1.18 g) as colorless crystals. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.10-1.40 (2H, m), 1.51-2.19 (2H, m), 3.42 (2H, brs), 3.62 (2H, d, J = 13.6 Hz), 3.89-4.07 (1H, m), 4.09-4.44 (1H, m), 4.62 (1H, brs), 6.84-7.57 (6H, m), 7.87-8.23 (2H, m), 8.46 (1H, d, J = 9.0 Hz), 8.87-9.26 (2H, m). LC-MS (ESI): m/z [M + H^+] 543.1. Anal. Calcd for $C_{26}H_{22}ClF_3N_6O_2$: C,57.52; H,4.08; N,15.48. Found: C,57.39; H,3.98; N,15.37. mp: 129-131 °C.

X-ray structure analysis

Crystal data for compound 4: $C_{25}H_{26}F_3N_5O_5$, *MW* = 533.51; crystal size, 0.12 x 0.10 x 0.02 mm; colourless, plate; triclinic, space group *P*1, *a* = 5.00246(8) Å, *b* = 22.5164(3) Å, *c* = 24.0321(5) Å, *α* = 109.6190(15)°, *β* = 92.2852(15)°, *γ* = 90.7764(12)°, *V* = 2546.69(8) Å^3, *Z* = 4, *Dx* = 1.391 g/cm^3, *T* = 100 K, *μ* = 0.967 mm^{-1}, *λ* = 1.54187 Å, R_1 = 0.064, wR_2 = 0.178, Flack ParameterDD = -0.08(10). All measurements were made on a Rigaku XtaLAB P200 diffractometer using graphite monochromated Cu-K*α*radiation. The structure was solved by direct methods with SIR2008[16] and was refined using full-matrix least-squares on F^2 with SHELXL-2014/7.[17] All non-H atoms were refined with anisotropic displacement parameters. CCDC 1511207 for compound **4** contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via http://www.ccdc.cam.ac.uk/Community/Requestastructure/Pages/DataRequest.aspx?.

Crystal data for compound 5: $C_{26}H_{22}ClF_3N_6O_2$, *MW* = 542.95; crystal size, 0.06 x 0.05 x 0.04 mm; colourless, block; triclinic, space group *P*1, *a* = 10.2798(3) Å, *b* = 10.6917(3) Å, *c* = 11.8027(3) Å, *α* = 103.716(2)°, *β* = 91.308(2)°, *γ* = 96.677(2)°, *V* = 1249.97(6) Å^3, *Z* = 2, *Dx* = 1.442 g/cm^3, *T* = 100 K, *μ* = 1.878 mm^{-1}, *λ* = 1.54187 Å, R_1 = 0.088, wR_2 = 0.233, Flack Parameter[18] = 0.043(13). All measurements were made on a Rigaku XtaLAB P200 diffractometer using graphite monochromated Cu-K*α*radiation. The structure was solved by direct methods with SIR2008[16] and was refined using full-matrix least-squares on F^2 with SHELXL-2014/7.[17] All non-H atoms were refined with anisotropic displacement parameters. CCDC 1511402 for compound **5** contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via http://www.ccdc.cam.ac.uk/Community/Requestastructure/Pages/DataRequest.aspx?.



(A) (B) **Figure 8.** (A) ORTEP of compound **4**, thermal ellipsoids are drawn at 30% probability. (B) ORTEP of compound **5**, thermal ellipsoids are drawn at 20% probability.

Biology

Preparation of human SPT2 enzyme

cDNA encoding human SPT1, human SPT2, and human ssSPTa were isolated by polymerase chain reaction (PCR) with primers and subcloned to make expression vectors. For preparation of SPT2 enzyme, FreeStyle293 cells (Life Technologies, Carlsbad, CA) were transfected with expression plasmids for human SPT1, human SPT2, and human ssSPTa and cultured for 3 days. Cells were homogenized in 50 mM Hepes buffer (pH 7.5) containing 250 mM sucrose, 5 mM EDTA, 5 mM DTT, and Complete, EDTA-free (Roche Applied Science, Penzberg, Upper Bavaria, Germany). Cell homogenates were centrifuged and the supernatant was recovered. Total membrane fractions were isolated by ultracentrifugation. Pellets were re-suspended in 50 mM Hepes buffer (pH 7.5) containing 5 mM EDTA, 5 mM DTT, and Complete, EDTA-free and stored at -80°C. The protein concentration was determined with the CBB Protein Assay.

Enzyme assay

The enzyme reaction was run in assay buffer, which consisted of 100 mM Hepes (pH 8.0), 2.5 mM EDTA, 5 mM DTT, and 0.01% bovine serum albumin (fatty acid free) in a 384-well assay plate at a volume of 20 µL format. Briefly, 5 µL of a tested compound and 10 µL of 100 µg/mL SPT2-expressed-membrane dissolved in the assay buffer, were mixed and incubated for 60 minutes. Then, the enzyme reaction was started with the addition of 5 µL of substrate solution containing 2 mM L-serine and 20 µM palmitoyl-CoA in the assay buffer. After 15-minute incubation at room temperature, the reaction was terminated by adding 20 µL of 2% formic acid. Then, the reaction was added with 40 µL of acetonitrile containing 600 nM C17-sphinganine as internal standard. High-throughput online solid phase extraction was performed using a RapidFire® 300 (Agilent Technologies, Santa Clara, CA). Mass spectrometric analysis was performed using an API-4000^{TM} triple quadrupole mass spectrometer (AB SCIEX, Framingham, MA) in positive SRM mode. The SRM transitions for 3-ketodihydrosphingosine as a reaction product and C17-sphinganine were set as 300.5/270.3 and 288.4/60.2, respectively. Analytical data was acquired using Analyst software version 1.5.0 (AB SCIEX, Framingham, MA) and 300.5/270.3 was divided by 288.4/60.2 for calibration. IC_{50} values for the test

compounds were calculated using XLfit software (IDBS, London, UK).

Growth inhibition assay

HCC4006 cells were cultured in RPMI medium containing 10% fetal bovine serum and penicillin/streptomycin. Cells were dispensed into a 384 well culture plate at a density of 250 cells/well in 40 μL of the culture medium and cultured overnight. The cells were treated with 10 μL of test compounds and cultured for 5 days. The medium was removed and replaced with 30 μL of CellTiter-Glo Luminescent Cell Viability Assay reagent (Promega, Fitchburg, WI). Luminescence was measured with EnVision (PerkinElmer, Waltham, MA). IP values (concentration at the inflection point of dose-response curve) for the test compounds were calculated using XLfit software.

Cassette dosing test to mice

Test compounds were administered intravenously (0.1 mg/kg, 10 compounds in one) or orally (1 mg/kg, 5 compounds in one) by cassette dosing to non-fasted mice. After administration, blood samples were collected and centrifuged to obtain the plasma fraction. The plasma samples were deproteinized by mixing with acetonitrile followed by centrifugation. The compound concentrations in the supernatant were measured by LC/MS/MS.

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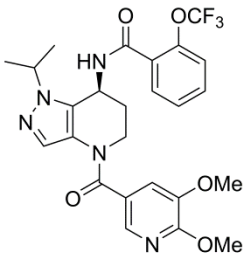
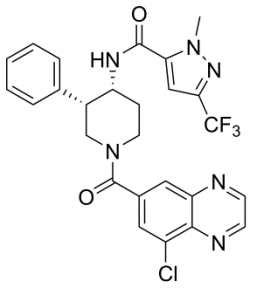


4

SPT enzyme inhibition
IC_{50} = 0.54 nM



5

SPT enzyme inhibition
IC_{50} = 0.71 nM