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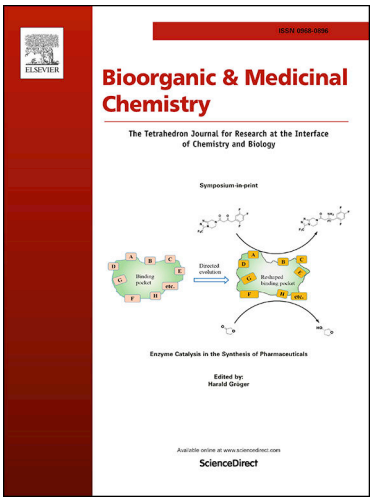

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PII: S0968-0896(20)30357-6
DOI: https://doi.org/10.1016/j.bmc.2020.115531
Reference: BMC 115531

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 5 March 2020
Revised Date: 22 April 2020
Accepted Date: 23 April 2020

Please cite this article as: H. Tanaka, K. Negoro, T. Koike, I. Tsukamoto, K. Yokoyama, J. Maeda, Y. Inagaki, Y. Shimoshige, K. Ino, K. Ishizu, T. Takahashi, Discovery and Structure-Activity Relationships Study of Positive Allosteric Modulators of the M_3 Muscarinic Acetylcholine Receptor, *Bioorganic & Medicinal Chemistry* (2020), doi: https://doi.org/10.1016/j.bmc.2020.115531

Discovery and Structure-Activity Relationships Study of Positive Allosteric Modulators of the M_3 Muscarinic Acetylcholine Receptor

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Abstract

The M_3 muscarinic acetylcholine receptor (mAChR) is a member of the family of mAChRs, which are associated with a variety of physiological functions including the contraction of various smooth muscle tissues, stimulation of glandular secretion, and regulation of a range of cholinergic processes in the central nerve system. We report here the discovery and a comprehensive structure-activity relationships (SARs) study of novel positive allosteric modulators (PAMs) of the M_3 mAChR through a high throughput screening (HTS) campaign. Compound **9** exhibited potent *in vitro* PAM activity towards the M_3 mAChR and significant enhancement of muscle contraction in a concentration-dependent manner when applied to isolated smooth muscle strips of rat bladder. Compound **9** also showed excellent subtype selectivity over other subtypes of mAChRs including M_1, M_2, and M_4 mAChRs, and moderate selectivity over the M_5 mAChR, indicating that compound **9** is an M_3-preferring M_3/M_5 dual PAM. Moreover, compound **9** displayed acceptable pharmacokinetics profiles after oral dosing to rats. These results suggest that compound **9** may be a promising chemical probe for the M_3 mAChR for further investigation of its pharmacological function both *in vitro* and *in vivo*.

Keywords

M_3 muscarinic acetylcholine receptor; positive allosteric modulators; allosteric enhancers.

1. Introduction

Muscarinic acetylcholine receptors (mAChRs) are class A G-protein-coupled receptors (GPCRs), with five receptor subtypes M_1 to M_5 having been identified to date. M_1, M_3 and M_5 mAChR subtypes preferentially couple to G_q protein and activate the phospholipase C/inositol 1,4,5-riphosphate (IP_3)/Ca^{2+} signaling pathway, whereas M_2 and M_4 mAChR subtypes couple to $G_{i/o}$ protein and lead to inhibition of adenylyl cyclase activity.[1] mAChRs mediate a broad range of actions throughout the peripheral organs and central nervous system (CNS).

The M_3 mAChR is expressed in smooth muscle, glands, and the brain. It plays an essential role in the contraction of many smooth muscle tissues such as the bladder and gastrointestinal tract, the stimulation of glandular secretion, and the regulation of a variety of cholinergic processes in the CNS.[2] For example, solifenacin (VESIcare®),[3] a competitive M_3 mAChR antagonist, blocks the binding of ACh to its receptors and helps relax the smooth muscle of the bladder, enabling the bladder to accumulate larger volumes of urine. As a result, it may be effective for controlling and reducing overactive bladder (OAB) symptoms such as urinary frequency, urinary urgency and incontinence episodes.[4] However, the pharmacological effects of direct agonistic stimulation of M_3 mAChRs are hampered by dose limiting side effects and remain unclear due to the poor subtype selectivity of available ligands.[5] mAChRs are known to have highly homologous sequences, thereby hampering the identification of subtype selective ligands. Moreover, recent studies have revealed that prolonged exposure of GPCRs to orthosteric agonists cause a reduction in receptor-mediated signaling via phosphorylation, G protein uncoupling, internalization and down-regulation.[6]

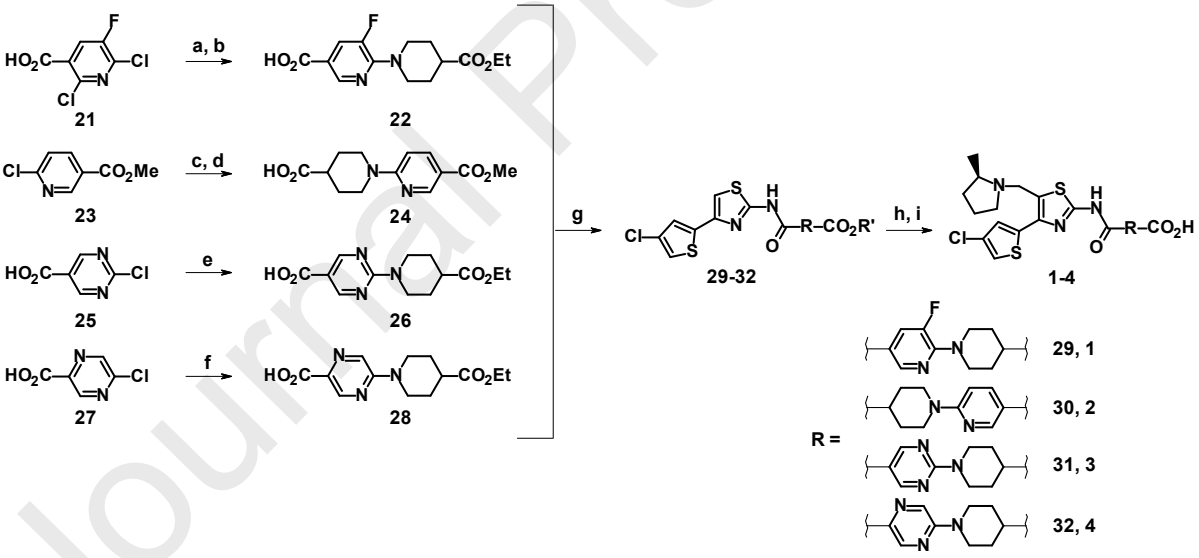
Based on these facts, we focused on an alternative approach to further study the pharmacological functions of the M_3 mAChR by targeting the receptor using positive allosteric modulators (PAMs). A PAM is a compound that binds to an allosteric site other than the orthosteric binding site, primarily to cause a change in the structure of the receptor, with the effect of increasing the affinity of an agonist to the receptor and changing the agonist's activity. In the living body, PAMs themselves do not exhibit an agonistic effect, but increase the effect of an endogenous agonist. The general advantages of using a PAM over an agonist include: (1) reduced side effects because PAMs stimulation-dependently do not primarily induce receptor activation but only enhance the activity of endogenous agonists; (2) high subtype selectivity because PAMs bind to a site other than the ligand-binding site; and (3) low chance of desensitization that can be observed with an agonist.[7] This strategy has been applied to M_1 and M_4 mAChRs, which are considered attractive therapeutic targets for the treatment of neurodegenerative and neuropsychiatric diseases.[8] For example, Beshore *et al.* recently reported the discovery of MK-7622, a highly selective PAM of the M_1 muscarinic receptor, which is

currently under phase 2 clinical trial for the treatment of Alzheimer's disease.[9] In contrast to emerging studies on selective PAMs for M_1 and M_4 mAChRs, to our knowledge, only a few studies have reported selective PAMs for the M_3 mAChR, most of which have relatively low affinity and limited selectivity.[5] Therefore, the identification of potent and selective PAMs of the M_3 mAChR are needed for further investigations into the receptor's pharmacological functions.

Here, we report the discovery of novel PAMs of the M_3 mAChR through a high throughput screening (HTS) campaign and the subsequent structure-activity relationships (SARs) study.

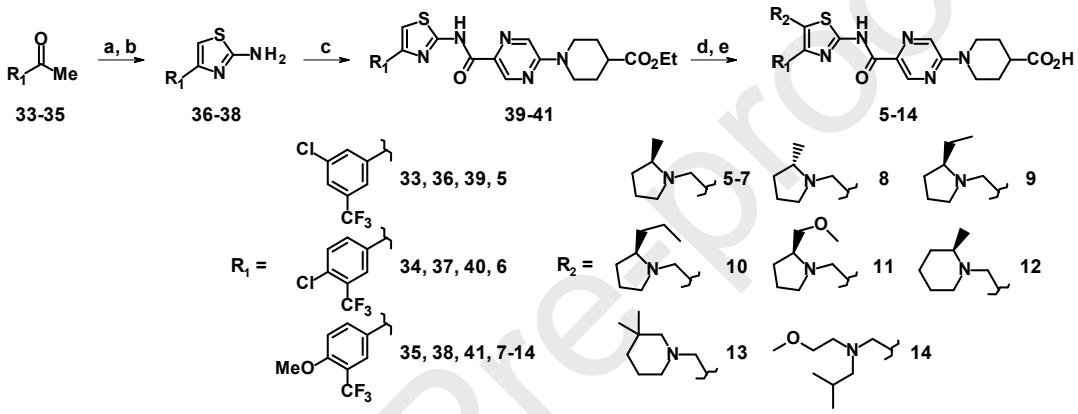
2. Chemistry

The synthesis of compounds **1**–**4** were performed as outlined in Scheme 1.[10] Commercially available aryl chlorides were converted to the corresponding aryl piperidines **22**, **24**, **26**, and **28**, which were subjected to condensation with 4-(4-chloro-2-thienyl)-1,3-thiazol-2-amine to yield **29**–**32**. The benzylamine unit was then attached through a Mannich reaction of (2*R*)-2-methylpyrrolidine, and subsequent hydrolysis produced compounds **1**–**4**.



Scheme 1. General procedure for preparation of compounds **1**–**4**. Reagents and conditions: (a) ethyl piperidine-4-carboxylate, DMAc, 80ºC; (b) 10% Pd/C, Et_3N, THF-EtOH, rt; (c) *tert*-butyl piperidine-4-carboxylate, K_2CO_3, DMF, 100ºC; (d) 4M HCl/AcOEt, AcOEt, rt, then TFA, CH_2Cl_2, rt; (e) ethyl piperidine-4-carboxylate, DIPEA, DMF, 80ºC; (f) ethyl piperidine-4-carboxylate, DIPEA, DMF, 90ºC; (g) $SOCl_2$, DCE, reflux, then 4-(4-chloro-2-thienyl)-1,3-thiazol-2-amine, Et_3N, 1,4-dioxane, reflux; (h) (2*R*)-2-methylpyrrolidine L-tartrate, 35% HCHO aq. AcOH, 80ºC; (i) 1 M NaOH aq. EtOH, 50ºC.

Synthesis of compounds **5**–**14** started with the corresponding acetophenones **33**–**35** (Scheme 2). Compounds **33**–**35** were treated with phenyltrimethylammonium tribromide, followed by cyclization with thiourea to yield 2-aminothiazoles **36**–**38**. Amide formation using the carboxylic acid chloride in pyridine gave compounds **39**–**41**. Subsequently, the amine moiety was directly attached using a Mannich reaction or via a 5-acetoxymethyl thiazole intermediate, and subsequent hydrolysis gave the desired analogues **5**–**14**.

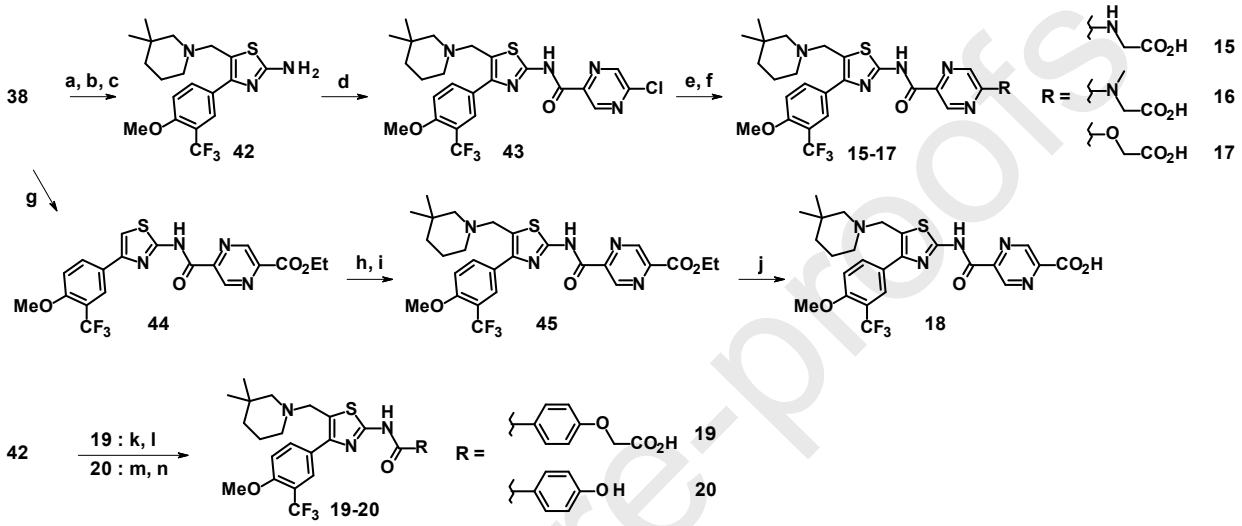


Scheme 2. General procedure for preparation of compounds **5**–**14**.[a] Reagents and conditions: (a) phenyltrimethylammonium tribromide, THF, rt; (b) thiourea, EtOH, 80ºC; (c) **28**, $POCl_3$, pyridine, rt or TBTU, DIPEA, THF, microwave 150ºC; (d) amine, 36% HCHO aq., DIPEA, AcOH, 90ºC or 36% HCHO aq., Ac_2O, AcOH, microwave 170ºC then amine, DIPEA, DMF, 100ºC; (e) 1 M NaOH aq. EtOH, THF, 50ºC.

[a] For the synthesis of **6**, amine was attached in the presence of DIPEA in THF at 150ºC under irradiation with microwaves.

Functionalization at the 5-position of the 2-aminothiazole in compound **38** was accomplished through trifluoroacetyl protection (Scheme 3). Compound **38** was first treated with trifluoroacetic anhydride (TFAA) to yield the trifluoroacetylated intermediate, which underwent a Mannich reaction to attach the 3,3-dimethyl piperidine, followed by removal of the trifluoroacetyl group to give compound **42**. Subsequently, amide formation using *N*-[({[(1*Z*)-1-cyano-2-ethoxy-2-oxoethylidene]amino}oxy)(morpholin-4-yl)methylene]-*N*-methylmethanaminium hexafluorophosphate (COMU)[11] as a condensation reagent gave compound **43**. The carboxylic acid

fragments were connected by nucleophilic aromatic substitution, and subsequent hydrolysis produced the desired analogues **15**–**17**. Alternatively, pyrazinoic acid analogue **18** was attained via thiazole intermediate **38**, and compounds **19**–**20** were successfully transformed from **42** using the procedure described above.



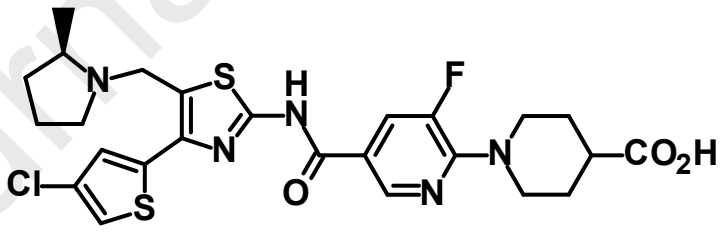
Scheme 3. General procedure for preparation of compounds **15**–**20**. Reagents and conditions: (a) TFAA, Et_3N, CH_2Cl_2, rt; (b) 3,3-dimethyl piperidine hydrochloride, 36% HCHO aq., DIPEA, AcOH, 60ºC; (c) 6 M NaOH aq., EtOH, THF, 80ºC; (d) 5-chloropyrazine-2-carboxylic acid, COMU, DIPEA, 1,4-dioxane, rt; (e) amine, DIPEA, DMF, 80ºC or alcohol, NaH, NMP, 80ºC; (f) 1 M NaOH aq., EtOH, 60ºC; (g) **29**: 5-(ethoxycarbonyl)pyrazine-2-carboxylic acid, $POCl_3$, pyridine, rt; **30**: HATU, DIPEA, DMF, 100ºC; **31**–**32**: TBTU, DIPEA, 1,4-dioxane, microwave 130ºC; (h) 36% HCHO aq., Ac_2O, AcOH, 90ºC; (i) 3,3-dimethyl piperidine, DIPEA, DMF, 80ºC; (j) 1 M NaOH aq., EtOH, 60ºC; (k) 4-(2-*tert*-butoxy-2-oxoethoxy)benzoic acid, COMU, DIPEA, 1,4-dioxane, 80ºC; (l) 1 M NaOH aq. EtOH, THF, 50ºC; (m) 4-acetoxybenzoic acid, COMU, DIPEA, 1,4-dioxane, 80ºC; (n) 1 M NaOH aq., MeOH, rt.

3. Results and Discussion

All analogues described in this study were screened with a Fluorometric Imaging Plate Reader (FLIPR^{TM}) assay using CHO-K1 cells stably expressing the human M_3 mAChR with carbachol (CCh) as a ligand. The PAM activity of the M_3 mAChR was determined from the shift in the CCh concentration-response curve (CCh-CRC) toward lower concentrations following exposure to the test substance. That is, PAM activity was determined by dividing the EC_{50} value of CCh in the absence

of the test substance by the EC_{50} value of CCh in the presence of the test substance. For example, when the EC_{50} value of CCh in the absence of the test substance was 200 nM and the EC_{50} value of CCh in the presence of the test substance was 10 nM, the PAM activity of the test substance showed a 20-fold shift. In the tables below, values in the presence of the test substances at 10 μM are shown for human M_3 PAMs (-fold shift).

To identify PAMs of M_3 mAChR, we performed a HTS campaign of the Astellas internal chemical library, which culminated in the identification of compound **1** (Figure 1).[12] In functional assays, **1** produced a 132-fold shift in CCh-CRC at 10 μM, and the CCh-CRC shifted toward lower concentrations at increasing concentrations of the compound (Figure 2). Because analogues of **1** reportedly have agonistic activity for c-myeloproliferative leukemia virus type P (c-Mpl),[13] also known as human thrombopoietin receptor (TPOR), we assessed the c-Mpl agonistic activity of **1** using a human c-Mpl-introduced Ba/F3 cell proliferative assay and found that **1** showed c-Mpl agonistic activity with an EC_{30} value of 3.2 nM. Thrombopoietin (Tpo) is the cytokine responsible for the primary regulation of megakaryocytes and platelet production via activation of the intracellular JAK-STAT signaling pathway, which is triggered when Tpo binds to c-Mpl.[14] Therefore, the agonistic activity for c-Mpl may potentially hamper clinical studies because it has been shown to increase platelet production in humans[15] and is associated with the risk of adverse side effects such as thromboembolism.[16] To elucidate whether SARs of M_3 PAM activity and c-Mpl agonistic activity are distinct, we next evaluated c-Mpl agonistic activity and the M_3 PAM activity of the test compounds.



1

Figure 1. Structure of HTS hit **1**[a].

[a] hydrochloride salt

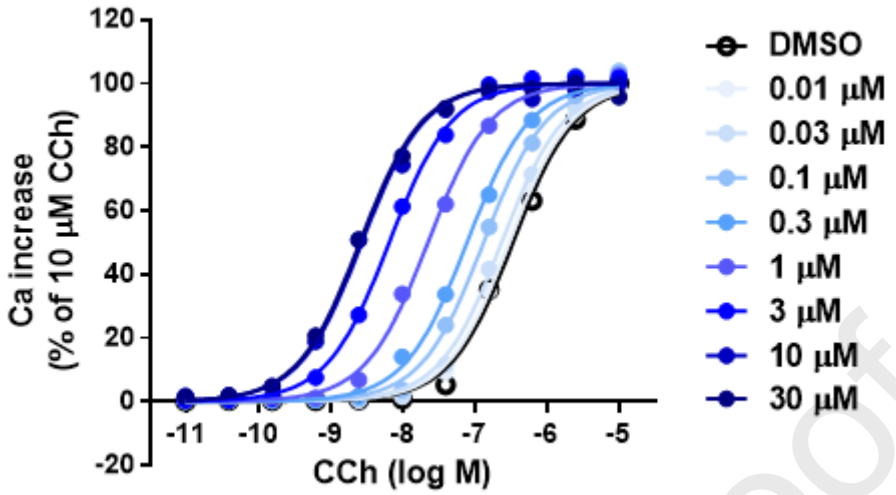
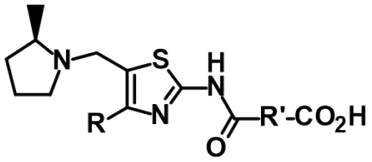


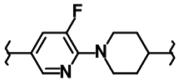
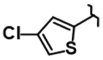
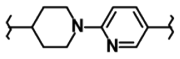
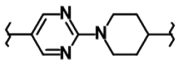
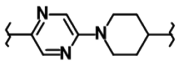
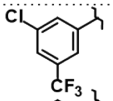
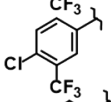
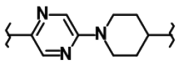
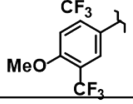
Figure 2. *In vitro* human M_3 PAM activity of **1**.

CCh-CRCs in human M_3-expressing cells in the absence (DMSO) or presence of the indicated concentrations of **1**.

Initial efforts focused on attempts to replace the pyridyl-piperidine moiety of **1** (Table 1). Compound **2**, in which the positions of pyridine and piperidine were switched, decreased M_3 PAM activity to a 2.8-fold shift. Although exposure to pyrimidine analogue **3** reduced the shift in CCh-CRC by half compared to **1**, pyrazine analogue **4** produced a substantial increase in activity, resulting in a 186-fold shift. Interestingly, replacement of 4-chlorothiophene at the 4-position of 2-aminothiazole with substituted benzenes resulted in a significant reduction in c-Mpl agonistic activity and a slight reduction in M_3 PAM activity (compounds **5**–**7)**, suggesting that SARs for c-Mpl agonistic activity and M_3 PAM activity were distinct. We noted that **7** caused a drastic reduction in c-Mpl agonistic activity (EC_{30} value >10 μM). The result also suggested that c-Mpl differently recognized this region in accordance with the position (compound **5** and **6**), the lipophilicity and the electrical property (compound **6** and **7**) of substituents. Therefore, analogue **7** was adopted as a template and used to investigate the amine moiety at the 5-position of 2-aminothiazole.

Table 1. *In vitro* SARs for human M_3 PAM activity and human c-Mpl-introduced Ba/F3 cell proliferative activity for analogues following replacement of the fluoro pyridine core and 4-chlorothiophene.

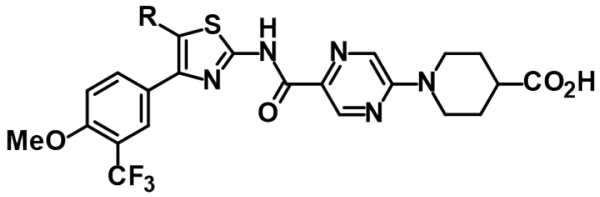


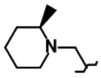
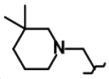
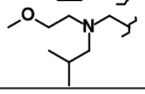
Compound	R	R'	human M_3 PAM (-fold shift)[a]	h-cmpl-Ba/F3 cell proliferation EC_{30} (nM)
1[b]			132	3.2
2[b, c]			2.8	NT[d]
3			65	NT[d]
4[b]			186	350
5[b]			168	150
6[b]			118	1720
7[b]			73	>10000

[a] 10 μM of test substance was used, [b] hydrochloride salt, [c] racemate, [d] NT = Not Tested

The next set of investigations involved modifications of the amine moiety (Table 2). Replacing (2*R*)-2-methylpyrrolidine (**7**) with (2*S*)-2-methylpyrrolidine (**8**) decreased M_3 PAM activity to a 25-fold shift, demonstrating that the (2*R*)-substituent was more favorable for activity. (2*R*)-Alkylated pyrrolidine analogues such as ethyl (**9**) and *n*-propyl (**10**) led to increased M_3 PAM activity compared with the methyl analogue (**7**), whereas hydrophilic methoxymethyl (**11**) retained the same level of activity as **7**. While (2*R*)-2-methylpiperidin (**12**) resulted in a slight increase in M_3 PAM activity, 3,3-dimethylpiperidin (**13**) caused a 238-fold shift in activity. In contrast, replacement with a branched alkyl chain (**14**), in which the (2*S*)-2-methoxymetylpyrrolidin of **11** was cracked into an alkyl chain, had little impact on M_3 PAM activity, suggesting that this benzyl amine region was only capable of accepting some flexible conformations. We also noted that none of the analogues prepared in Table 2 showed c-Mpl agonistic activity.

Table 2. *In vitro* SARs for analogues following replacement of amines at the 5-position of the 2-aminothiazole.

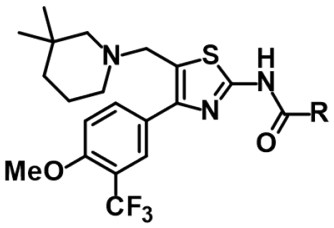


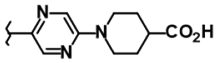
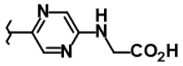
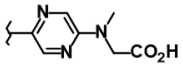
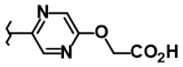
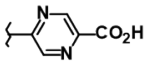
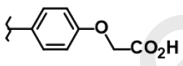
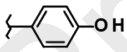
Compound	R	human M_3 PAM (-fold shift)[a]	h-cmpl-Ba/F3 cell proliferation EC_{30} (nM)
7[b]		73	>10000
8[b]		25	>10000
9[b]		269	>10000
10[b]		128	>10000
11[b]		72	>10000
12[b]		94	>10000
13[b]		238	>10000
14		84	>10000

[a] 10 μM of test substance was used, [b] hydrochloride salt

With potent amine moieties at hand, we conducted a SARs study around the pyrazyl-piperidine moiety using **13** as a template scaffold (Table 3). Transformation of the piperidine (**13**) to *N*- or *O*-linked analogues (**15**–**17**) attenuated M_3 PAM activity, and the pyrazinoic acid analogue (**18**) drastically reduced activity. Despite the fact that an aromatic amine was not necessarily required at the terminal region (**17**), a measurable distance between pyrazine and carboxylic acid was thought to be essential for retaining M_3 PAM activity because the directly attached carboxylic acid in **18** negatively affected activity. Interestingly, benzene analogue **19** exhibited almost the same level of M_3 PAM activity as its direct analogue **17**. Of note, our attempt to replace the carboxylic acid with phenol (**20**) caused a marked decrease in M_3 PAM activity, suggesting that the carboxylic acid might play a crucial role in activity.

Table 3. *In vitro* SARs for analogues following replacement of the pyrazyl-piperidine moiety.



Compound	R	human M_3 PAM (-fold shift)[a]
13[b]		238
15[c]		162
16[c]		137
17[c]		175
18		39
19[b]		204
20[b]		8.9

[a] 10 μM of test substance was used, [b] hydrochloride salt, [c] potassium salt

4. Further evaluation of a selected compound

Among the compounds synthesized in this study, compound **9**, which showed the most potent PAM activity towards the M_3 mAChR, was selected for further evaluation.

As shown in Figure 3, compound **9** concentration-dependently shifted CCh-CRC for human M_3 mAChR to the left. The selectivity profiles of compound **9** for other subtypes of human and rat mAChRs were also evaluated. We found that **9** displayed specific modulation of the M_3 mAChR over the other human and rat mAChR subtypes with the exception of the M_5 mAChR, for which it possessed moderately lower PAM activity than for M_3 mAChR (Table 4).

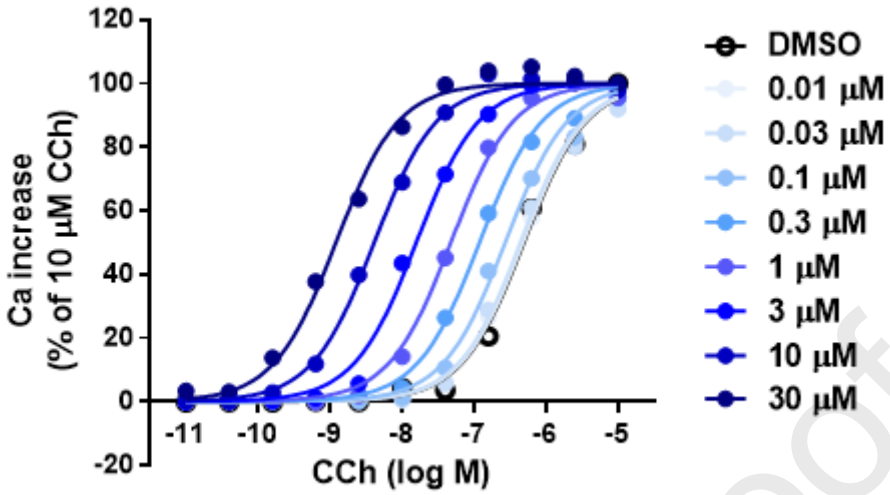


Figure 3. *In vitro* human M_3 PAM activity of **9**.

CCh-CRCs in human M_3-expressing cells in the absence (DMSO) or presence of the indicated concentrations of **9**.

Table 4. Selectivity profile of **9** for all subtypes of human and rat mAChRs.

	mAChR PAM (-fold shift at 10 µM)				
	M_1	M_2	M_3	M_4	M_5
Human	2.3	1.3	269	1.0	94
Rat	2.1	1.6	158	0.7	110

To assess whether compound **9** enhances the physiological functions mediated by the M_3 AChR, we examined the effect of compound **9** on the electrical field stimulation (EFS)-induced contraction of isolated rat bladder strips (Table 5). Compound **9** at 3 and 10 µmol/L significantly enhanced EFS-induced bladder contractions in a concentration-dependent manner. These results suggest that through M_3 PAM activity, compound **9** enhanced the action of ACh, which was released from nerves through EFS, resulting in increasing contraction of isolated rat bladder strips.

Table 5. Effect of **9** on electrical field stimulation-induced contraction of isolated rat bladder strips

Concentration of **9** (µM)	Tension of isolated bladder contraction (g)
pre	2.21 ± 0.26
1	2.34 ± 0.32
3	2.79 ± 0.41**

10	3.49 ± 0.50**

Data are expressed as the mean ± SEM (n=7).

** P<0.005: significantly different from the pre-value (Williams' test using within subject error).

The pharmacokinetics profiles of compound **9** in rats are shown in Table 6. After intravenous administration (0.3 mg/kg), compound **9** showed moderate total clearance (917.7 mL/h/kg), a steady-state volume of distribution (1.59 L/kg), and a half-life of 2.43 h. Compound **9** was rapidly absorbed after oral administration at 1 mg/kg and showed acceptable bioavailability (19.0%). Therefore, **9** has potential as a chemical probe for investigation *in vivo*.

Table 6. Pharmacokinetics data for compound **9** in rats.[a]

Route	Dose (mg/kg)	C_{max} (ng/mL)	t_{max} (h)	AUC_{inf} (ng·h/mL)	$t_{1/2}$ (h)	CL_{tot} (mL/h/kg)	V_{ss} (L/kg)	*F* (%)[b]
iv	0.3	−	−	330.2	2.43	917.7	1.59	−
po	1	85.29	0.31	208.8	2.16	−	−	19.0

− : not applicable

[a] Mean values of individual parameters calculated from the plasma concentrations in each animal (n=4).

[b] *F* = Bioavailability.

5. Conclusion

We reported the discovery of the first potent PAM of the M_3 mAChR and a comprehensive SARs study. Compound **1**, initially identified by a HTS campaign, caused a 132-fold shift in CCh-CRC for the human M_3 mAChR at 10 μM. Compound **1** was successfully converted to analogue **7**, which diminished human c-Mpl agonistic activity. In the SARs study around the terminal amine and pyrazyl-piperidine moiety, **9** was identified as a more promising chemical tool, causing a 269-fold shift in CCh-CRC at 10 μM and contraction of isolated smooth muscle strips of rat bladder. Moreover, **9** exhibited acceptable PK profiles in rats, excellent subtype selectivity over both human and rat M_1, M_2, and M_4, and moderate selectivity over M_5 mAChRs, indicating that compound **9** is an M_3-preferring M_3/M_5 dual PAM. Therefore, we anticipate that **9**, a newly identified PAM of the M_3 mAChR, will be useful as a new class of chemical probe for investigating an array of pharmacological

functions for future drug discovery campaigns. To evaluate the utilities of compound **9** as a potential pharmacological tool, further experiments are needed (manuscripts are under preparation).

Experimental

Biology

Evaluation of mAChR Positive Allosteric Modulator Activity

Human and rat M_1, M_2, M_3, M_4, and M_5 mAChR genes were inserted into pcDNA3.1™ expression vectors (Life Technologies) and the mAChR-expressing vectors were transfected into CHO-K1 cells (ATCC No. CCL-61) using Lipofectoamine 2000 reagent (Life Technologies). The cells were cultured in alpha Modified Eagle Minimum Essential Medium (α-MEM) containing 2 mM glutamic acid, 10% fetal bovine serum, and 2.0 mg/mL Geneticin (Life Technologies) for 4 weeks to acquire drug-resistant clones. To convert the signaling pathway of M_2 and M_4 mAChR subtypes, which are coupled to $G_{i/o}$ proteins, into a G_q signaling pathway, we also transfected a human G16 G protein α subunit-expressing vector into human and rat M_2- and M_4-expressing cells as described above and drug-resistant clones were selected in the presence of 0.5 mg/mL Hygromycin B.

The day before the experiment, M_1, M_3, and M_5 receptor-expressing CHO-K1 cells were suspended in α-MEM containing 2 mM glutamic acid, 10% fetal bovine serum, and 0.2 mg/mL Geneticin, dispensed into a 384-well plate (Lot number 355962, BD Biosciences) at 1.2 to 1.5 × 10^4 cells/well, and cultured at 37°C and 5% CO_2 overnight. M_2 and M_4 receptor-expressing CHO-K1 cells were suspended in α-MEM containing 2 mM glutamic acid, 10% fetal bovine serum, 0.2 mg/mL Geneticin, and 50 μg/mL Hygromycin B, seeded and cultured as described above.

The culture medium was replaced with loading buffer (Assay Buffer [Hanks Balanced Salt Solution (HBSS)], 1 g/L BSA, 20 mM HEPES (pH 7.5), and 2.5 mM Probenecid) containing 3.1 μM Fluo 4-AM (Dojindo Laboratories), and left at room temperature for about 2 hours. Thereafter, the cells were washed with assay buffer using plate washing equipment (ELx405™; BIO-TEK Instruments), and placed in a FLIPR Tetra™, a system that measures the intracellular Ca^{2+} concentration (Molecular Devices). A test substance (final concentration 10 μM) and CCh (Sigma, final concentration 0.0024 nM to 100 μM), each of which were dissolved in assay buffer in advance, were placed in the FLIPR Tetra™. Test substances were added to the cells inside the device, and CCh was added to the cells about 5 minutes later. The Ca^{2+} flux induced by CCh was measured at an excitation wavelength of 470 nm to 495 nm and fluorescence wavelength of 515 nm to 575 nm.

The activity of mAChR PAMs was determined from the shift in the CCh concentration-response curve toward lower concentrations following exposure to the test substance. That is, the

minimum and maximum value of the CCh response from the concentration-response curve of CCh were set to 0% and 100%, respectively. Using logistic regression, the concentration of CCh showing a 50% response was calculated as the EC_{50} value, and the activity was determined by dividing the EC_{50} value of CCh in the absence of the test substance by the EC_{50} value in the presence of the test substance.

Human c-Mpl-Introduced Ba/F3 Cell Proliferative Assay

A human c-Mpl receptor gene (GenBank Accession No. M90102.1) was inserted into the pEF-BOS expression vector (Nucleic Acids Res. 18, 5322, 1990), and the human c-Mpl receptor-expressing vector was transfected into Ba/F3 cells (RIKEN BRC: RCB0805) using electroporation. pEF-BOS- c-Mpl (10 μg), pSV2bsr (1 μg; Kaken Pharmaceutical Co., Ltd.) and 1×10^7 Ba/F3 cells were added to a cuvette with a gap width of 0.4 cm, and electroporated at 1.5 kV (25 μF) using a Gene Pulser (BioRad). The cells were cultured in RPMI-1640 culture medium containing 0.5% WEHI conditioned media (BD Biosciences) and 10% fetal bovine serum for 3 days, and subsequently cultured in RPMI-1640 culture medium containing 10 μg/mL blasticidin for 30 days to acquire a drug-resistant clone.

The obtained cells were cultured in RPMI-1640 culture medium containing 0.5% WEHI conditioned media and 10% fetal bovine serum. The day before the experiment, test substances (final concentration 100 nM to 10 μM) that had been dissolved in culture medium for the assay (RPMI-1640 culture medium containing 10% fetal bovine serum) were added to a 384-well plate (Lot No.781185, Greiner Bio-One). The cell medium was replaced with culture medium for the assay. The cells were then dispensed into a 384-well plate containing the test substances at 1×10^4 cells/well, and cultured at 37°C and 5% CO_2 overnight. On the day of the experiment, a solution from the Cell Counting Kit (Dojindo Laboratories) was added to each well of the 384-well plate and cultured at 37°C and 5% CO_2 for about 5 hours. Thereafter, the absorbance (absorption wavelength 450 nm) in each well was measured using Safire2™ (TECAN) and used as an index for the number of cells. A well without the test substance was used the negative control. The absorbance for the negative control was set to 0%, and the absorbance for the positive control, where 1-(5-{[4-(4-chloro-2-thienyl)-5-{[(2*R*)-2-methylpyrrolidin-1-yl]methyl}-1,3-thiazol-2-yl]carbamoyl}-3-fluoropyridin-2-yl)piperidine-4-carboxylic acid hydrochloride was added at a final concentration of 1 μM, was set to 100%. The cell proliferation rate (%) was calculated in wells containing the test substance, and the test substance concentration showing 30% proliferation was calculated as the EC_{30} value using logistic regression.

Effects on EFS-Induced Contraction of Isolated Rat Bladder Strips

Nine- and ten-week-old female Sprague-Dawley rats (SLC Japan Inc., Shizuoka, Japan) were euthanized by exsanguination under isoflurane anesthesia. The urinary bladder was isolated, and a longitudinal bladder strip (approximately 10 mm in length and 2 mm in width) was placed between a pair of electrodes in 10 mL organ baths containing Krebs solution, maintained at 37°C and constantly aerated with 95% O_2 and 5% CO_2. Celphines tied with silk ligatures were applied to each end of the bladder strip. One end was attached to a holder and the other to an isometric tension transducer (TB-611T, Nihon Kohden, Tokyo, Japan) coupled to a PowerLab system (Power Lab8/30, ADInstruments, Aichi, Japan) to record contractile responses. The resting tension of the strips was adjusted to approximately 1 g, and the strips were left to equilibrate for approximately 1 hour. The strips were pre-contracted with KCl (final concentration 60 mmol/L in the organ bath) and washed out with Krebs solution. This step was repeated 2 times.

Neurogenic contraction of bladder strips was induced by applying EFS. Approximately 20 V of EFS (trains of electrical pulses at 8 Hz, 10 s duration, 0.3 ms pulse width) was applied 2 times at 4-minute intervals to observe the maximum response, and the voltage required to elicit approximately half the maximum response was determined and fixed.

After observing the pre-contractions, potentiating effects of compound **9** (final concentration 1, 3, 10 μM in the organ bath) on EFS (same conditions as above)-induced contraction were tested. Compound **9** was added cumulatively and EFS was applied 10 times at each concentration.

Bladder contraction induced by EFS was calculated as the difference between the peak contraction and the basal tone (the average tension for 2 minutes before contraction). The average of the contractions and the basal tone measured from the last 3 contractions before the application of compound **9** was defined as the pre-value. The effect of compound **9** (1, 3 μM) was evaluated from the average of the contractions and the basal tone measured from the last 3 contractions before the application of the next concentration of compound **9**. The effect of compound **9** (10 μM) was evaluated using the average of the contractions and the basal tone measured from the last 3 contractions before the end of the experiment.

The contractions and basal tone were compared between pre-value and compound **9**-treated values using Williams' test with within subject error, and a probability value of less than 0.025 was considered significant. Data are expressed as the mean ± SEM (n=7).

Pharmacokinetics study

Pharmacokinetic characterization of compound 9 was conducted in male SD rats (Sprague-Dawley strain SPF rats Crl:CD(SD), 8 weeks of age). Compound 9 was administered intravenously at 0.3 mg/kg and orally at 1 mg/kg in a mixture of DMF/propylene glycol (PG)/water for injection containing 1 M sodium hydroxide solution (2 equivalents of 9) (1/1/8). The animals were food deprived. After each administration, blood samples were drawn at assigned time points up to 24 hours, and the concentrations of compound 9 in plasma were determined using a validated LC-MS/MS method. Pharmacokinetic parameters were calculated from the plasma concentrations in each animal by noncompartmental analysis using the pharmacokinetic software Phoenix WinNonlin.

Chemistry

^{1}H-NMR spectra were recorded on JEOL JNM-EX400, Brucker DPX200, Avance400, AV400M, Avance III HD500, or Varian VNS-400 spectrometer and were referenced against the internal standard, tetramethylsilane. The abbreviations used for the signal patterns are as follows: s, singlet; br, broad; brs, broad singlet; d, doublet; t, triplet; q, quartet; dd, double doublet; tt, triple triplet; m, multiplet. Mass spectra (MS) were recorded on JEOL LX-2000, Agilent 1100 LC/MSD, Thermo Fisher Scientific LCQ Advantage, or Waters UPLC/SQD mass spectrometer. Elemental analyses were performed using a Yanako MT-5 microanalyzer (C, H, N), Elementar Vario EL III (C, H, N), Dionex ICS-3000 (S, halogen), and Yokogawa IC-7000S ion chromatographic analyzer (S, halogens), where the analytical results are within ±0.4% of the theoretical values. Electrospray ionization positive high-resolution mass spectrometry (HRMS) was performed using Thermo Scientific Exactive Plus. Column chromatography was performed using Wakogel C-200 or Merck silica gel 60 N (Kanto Chemical, 63–210 μm) or HI-FLASH™ Column (Yamazen). All reactions were conducted using commercially available reagents and solvents without further purification.

6-[4-(Ethoxycarbonyl)piperidin-1-yl]-5-fluoronicotinic acid (22)

To a mixture of 2,6-dichloro-5-fluoronicotinic acid (**21**, 21.0 g) and *N*,*N*-dimethylacetamide (200 mL) was added ethyl piperidine-4-carboxylate (39.1 mL), followed by stirring at 80°C overnight. The mixture was concentrated under reduced pressure, and ethyl acetate and a 1 M aqueous hydrogen chloride solution were added thereto. The organic layer was separated, washed with a 1 M aqueous hydrogen chloride solution and a saturated aqueous sodium chloride solution, and dried over anhydrous sodium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure to give a brown solid (34.3 g). The obtained solid was washed with ethyl acetate/diisopropyl ether to obtain a colorless solid (18.3 g).

To the above obtained solid (15.0 g) in ethanol (150 mL) and tetrahydrofuran (150 mL) was added triethylamine (19.0 mL) and 10% palladium-supported carbon (50% wet, 1.50 g), followed by stirring at room temperature overnight at 3 atm under a hydrogen atmosphere. The insoluble materials were separated by filtration, and 1 M aqueous hydrogen chloride solution (500 mL) was added to the filtrate, followed by extraction with ethyl acetate. The organic layer was washed with a saturated aqueous sodium chloride solution, and dried over anhydrous sodium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure to give **22** (12.2 g, 50%) as a colorless solid: ^{1}H-NMR ($CDCl_3$) δ 1.27 (3H, t, J = 6.8 Hz), 1.77–1.90 (2H, m), 1.98–2.06 (2H, m), 2.60 (1H, tt, J = 10.8, 4.0 Hz), 3.12–3.22 (2H, m), 4.16 (2H, q, J = 6.8 Hz), 4.33–4.42 (2H, m), 7.75 (1H, dd, J = 14.0, 2.0 Hz), 8.66 (1H, t, J = 2.0 Hz).

1-[5-(Methoxycarbonyl)pyridin-2-yl]piperidine-4-carboxylic acid (24)

Methyl 6-chloronicotinate (**23**, 5.56 g), *tert*-butyl piperidine-4-carboxylate (5.50 g), dipotassium carbonate (11.2 g), and *N*,*N*-dimethylformamide (80 mL) were mixed, followed by stirring at 100°C overnight. The mixture was cooled to room temperature and diluted with ethyl acetate. The insoluble materials were separated by filtration and the filtrate was washed with water and a saturated aqueous sodium chloride solution, and dried over anhydrous magnesium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure. The obtained solid was washed with diisopropyl ether and dried to obtain a white solid (7.97 g).

The obtained solid (7.97 g), ethyl acetate (60 mL), and a 4 M hydrogen chloride/ethyl acetate solution (63 mL) were mixed, followed by stirring at room temperature for 1 hour. The mixture was concentrated under reduced pressure, and water and a 1M aqueous sodium hydroxide solution were added thereto, followed by extraction with ethyl acetate. The organic layer was washed with water and a saturated aqueous sodium chloride solution, and dried over anhydrous magnesium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure.

The obtained residue was mixed with dichloromethane (60 mL) and trifluoroacetic acid (20 mL), followed by stirring at room temperature overnight. The mixture was concentrated under reduced pressure, and water and a 1 M aqueous sodium hydroxide solution were added thereto, followed by extraction with ethyl acetate. The organic layer was dried over anhydrous magnesium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure. The obtained residue was washed with diisopropyl ether and dried to obtain **24**

(5.68 g, 72%) as a white solid: ^{1}H-NMR (DMSO-d_6) δ 1.44–1.57 (2H, m), 1.84–1.94 (2H, m), 2.57 (1H, tt, J = 10.9, 4.0 Hz), 3.04–3.14 (2H, m), 3.78 (3H, s), 4.26–4.36 (2H, m), 6.89 (1H, d, J = 9.2 Hz) 7.93 (1H, dd, J = 9.2, 2.5 Hz), 8.63 (1H, d, J = 2.5 Hz), 12.24 (1H, brs).

2-[4-(Ethoxycarbonyl)piperidin-1-yl]pyrimidine-5-carboxylic acid (26)

2-Chloropyrimidine-5-carboxylic acid (**25**, 4.06 g), ethyl piperidine-4-carboxylate (4.74 mL), *N,N*-diisopropylethylamine (9.65 mL), and *N*,*N*-dimethylformamide (40 mL) were mixed, followed by stirring at 80°C overnight. The mixture was cooled to room temperature and diluted with ethyl acetate. The organic layer was washed with an aqueous citric acid solution, water, and a saturated aqueous sodium chloride solution, and dried over anhydrous magnesium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure. The obtained solid was washed with diisopropyl ether and dried to obtain **26** (5.71 g, 80%) as a beige solid: ^{1}H-NMR (DMSO-d_6) δ 1.19 (3H, t, J = 7.1 Hz), 1.44–1.58 (2H, m), 1.86–1.98 (2H, m), 2.69 (1H, tt, J = 10.9, 3.9 Hz), 3.12–3.24 (2H, m), 4.08 (2H, q, J = 7.1 Hz), 4.54–4.66 (2H, m), 8.75 (2H, s), 12.79 (1H, brs).

5-[4-(Ethoxycarbonyl)piperidin-1-yl]pyrazine-2-carboxylic acid (28)

5-Chloropyrazine-2-carboxylic acid (**27**, 7.84 g), *N*,*N*-dimethylformamide (120 mL), ethyl piperidine-4-carboxylate (16.0 mL), and *N,N*-diisopropylethylamine (25.0 mL) were mixed, followed by stirring at 90°C for 8 hours. The reaction mixture was cooled to room temperature and ethyl acetate was added. The mixture was washed with a dilute hydrochloric acid solution and dried over anhydrous sodium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure. The obtained solid was washed with diisopropyl ether and dried to obtain **28** (6.80 g, 49%) as a milky white solid: ^{1}H-NMR ($CDCl_3$) δ 1.27 (3H, t, J = 7.0 Hz), 1.74–1.86 (2H, m), 2.02–2.11 (2H, m), 2.66 (1H, tt, J = 10.5, 4.1 Hz), 3.20–3.30 (2H, m), 4.17 (2H, q, J = 7.1 Hz), 4.33–4.42 (2H, m), 8.02 (1H, d, J = 1.3 Hz), 8.86 (1H, d, J = 1.3 Hz); ESI-MS m/z 280 [$(M+H)^+$].

Ethyl 1-(5-{[4-(4-chloro-2-thienyl)-1,3-thiazol-2-yl]carbamoyl}-3-fluoropyridin-2-yl) piperidine-4-carboxylate (29)

To a mixture of 6-[4-(ethoxycarbonyl)piperidin-1-yl]-5-fluoronicotinic acid (**22**, 14.0 g) and 1,2-dichloroethane (140 mL) was added thionyl dichloride (6.89 mL), followed by heating to reflux

for 110 minutes. The mixture was cooled to room temperature and concentrated under reduced pressure, and the residual solvents were chased with toluene.

The resulting residue was mixed with 1,4-dioxane (180 mL), and 4-(4-chloro-2-thienyl)-1,3-thiazol-2-amine (6.83 g) and triethylamine (17.5 mL) were added thereto, followed by heating to reflux overnight. The mixture was cooled to room temperature and concentrated under reduced pressure. The residue was diluted with ethyl acetate, washed with a saturated aqueous sodium hydrogen carbonate solution twice and a saturated aqueous sodium chloride solution, and then dried over anhydrous sodium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure to give a brown amorphous solid (21.2 g). The amorphous solid was recrystallized from ethanol to obtain **29** (14.1 g, 90%) as a brown powder: ^{1}H-NMR ($CDCl_3$) δ 1.27 (3H, t, J = 6.8 Hz), 1.77–1.90 (2H, m), 1.98–2.08 (2H, m), 2.61 (1H, tt, J = 11.2, 4.0 Hz), 3.12–3.23 (2H, m), 3.73 (2H, q, J = 6.8 Hz), 4.33–4.42 (2H, m), 7.04 (1H, d, J = 1.6 Hz), 7.07 (1H, s), 7.22 (1H, d, J = 1.6 Hz), 7.73 (1H, dd, J = 14.4, 2.0 Hz), 8.51 (1H, s), 9.79 (1H, brs); FAB-MS m/z 495 [$(M+H)^+$].

Methyl 6-(4-{[4-(4-chloro-2-thienyl)-1,3-thiazol-2-yl]carbamoyl}piperidin-1-yl)nicotinate (30)

4-(4-Chloro-2-thienyl)-1,3-thiazol-2-amine (500 mg), 1-[5-(methoxycarbonyl)pyridin-2-yl]piperidine-4-carboxylic acid (**24**, 611 mg), *N*-[(dimethylamino)(3*H*-[1,2,3]triazolo[4,5-b]pyridin-3-yloxy)methylene]-*N*-methylmethanaminium hexafluorophosphate (878 mg), *N,N*-diisopropylethylamine (395 μL), and *N,N*-dimethylformamide (5.0 mL) were mixed, followed by stirring at 100°C for 2 hours. *N,N*-diisopropylethylamine (395 μL) was added thereto, followed by stirring at the same temperature for 15 minutes. *N*-[(dimethylamino)(3*H*-[1,2,3]triazolo[4,5-b]pyridin-3-yloxy)methylene]-*N*-methylmethanaminium hexafluorophosphate (878 mg) was added thereto, followed by stirring at the same temperature for 15 minutes. 1-[5-(methoxycarbonyl)pyridin-2-yl]piperidine-4-carboxylic acid (**24**, 182 mg) was added thereto, followed by stirring at the same temperature for 1 hour. The mixture was cooled to room temperature, and diluted with ethyl acetate. The organic layer was washed with water, a 1 M aqueous sodium hydroxide solution, and a saturated aqueous sodium chloride solution, and then dried over anhydrous magnesium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexanes-ethyl acetate) and then washed with hexanes to obtain **30** (291 mg, 27%) as a beige solid: ^{1}H-NMR (DMSO-d_6) δ 1.61 (2H, ddd, J = 24.7, 12.7, 3.9 Hz), 1.87–1.97 (2H, m), 2.86 (1H, tt, J = 11.3, 3.9 Hz), 2.97–3.10 (2H, m), 3.79 (3H,

s), 4.42–4.56 (2H, m), 6.91 (1H, d, *J* = 9.0 Hz), 7.53 (1H, d, *J* = 1.5 Hz), 7.55 (1H, d, *J* = 1.5 Hz), 7.60 (1H, s), 7.94 (1H, dd, *J* = 9.0, 2.3 Hz), 8.65 (1H, d, *J* = 2.3 Hz), 12.44 (1H, s).

Ethyl 1-(5-{[4-(4-chloro-2-thienyl)-1,3-thiazol-2-yl]carbamoyl}pyrimidin-2-yl)piperidine-4-carboxylate (31)

4-(4-Chloro-2-thienyl)-1,3-thiazol-2-amine (300 mg), 2-[4-(ethoxycarbonyl)piperidin-1-yl]pyrimidine-5-carboxylic acid (**26**, 464 mg), *N*-[(1*H*-benzotriazol-1-yloxy)(dimethylamino)methylene]-*N*-methylmethanaminium tetrafluoroborate (533 mg), *N,N*-diisopropylethylamine (592 μL), and 1,4-dioxane (3.0 mL) were mixed, followed by stirring at 130°C for 30 minutes under irradiation with microwaves. The mixture was cooled to room temperature and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexanes-ethyl acetate) and then washed with hexanes/ethyl acetate to obtain **31** (313 mg, 47%) as a white powder: ^{1}H-NMR (DMSO-d_6) δ 1.19 (3H, t, *J* = 7.1 Hz), 1.46–1.61 (2H, m), 1.88–1.99 (2H, m), 2.71 (1H, tt, *J* = 10.9, 3.9 Hz), 3.15–3.26 (2H, m), 4.09 (2H, q, *J* = 7.1 Hz), 4.57–4.66 (2H, m), 7.53 (1H, d, *J* = 1.5 Hz), 7.58 (1H, d, *J* = 1.5 Hz), 7.66 (1H, s), 9.00 (2H, s), 12.69 (1H, brs); ESI-MS m/z 478, 480 [$(M+H)^+$].

The following compound (**32**) was prepared using a procedure similar to that described for **31**.

Ethyl 1-(5-{[4-(4-chloro-2-thienyl)-1,3-thiazol-2-yl]carbamoyl}pyrazin-2-yl)piperidine-4-carboxylate (32)

Pale brown solid (yield 77%): ^{1}H-NMR (DMSO-d_6) δ 1.20 (3H, t, *J* = 7.1 Hz), 1.53–1.66 (2H, m), 1.88–2.02 (2H, m), 2.73 (1H, tt, *J* = 10.9, 3.9 Hz), 3.16–3.27 (2H, m), 4.09 (2H, q, *J* = 7.1 Hz), 4.37–4.49 (2H, m), 7.54 (1H, d, *J* = 1.5 Hz), 7.57 (1H, d, *J* = 1.5 Hz), 7.67 (1H, s), 8.39 (1H, d, *J* = 1.2 Hz), 8.76 (1H, d, *J* = 1.2 Hz), 11.82 (1H, brs).

1-(5-{[4-(4-Chloro-2-thienyl)-5-{[(2*R*)-2-methylpyrrolidin-1-yl]methyl}-1,3-thiazol-2-yl] carbamoyl}-3-fluoropyridin-2-yl)piperidine-4-carboxylic acid dihydrochloride (1)

To a mixture of ethyl 1-(5-{[4-(4-chloro-2-thienyl)-1,3-thiazol-2-yl]carbamoyl}-3-fluoropyridin-2-yl)piperidine-4-carboxylate (**29**, 10.0 g) and acetic acid (300 mL) was added (2*R*)-2-methylpyrrolidine L-tartrate (6.52 g) and 35% formaldehyde aqueous solution (4.39 mL), followed by stirring at 80°C overnight. The reaction mixture was cooled to room temperature and concentrated under reduced pressure. The residue was mixed with a saturated aqueous sodium hydrogen carbonate

solution and ethyl acetate. The organic layer was separated, washed with a saturated aqueous sodium chloride solution, and dried over anhydrous sodium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure to give a brown amorphous solid (11.5 g).

Ethyl acetate (550 mL) and a 4 M hydrogen chloride/ethyl acetate solution (4.86 mL) were added to the resulting amorphous solid, and the mixture was filtered to give a pale brown solid (10.2 g). The obtained solid was mixed with a saturated aqueous sodium hydrogen carbonate solution, followed by extraction with ethyl acetate twice. The organic layers were combined, washed with a saturated aqueous sodium chloride solution, and dried over anhydrous sodium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure to give a brown amorphous solid (10.0 g). The amorphous solid was recrystallized from ethanol to obtain a slightly brown powder (7.70 g).

The obtained powder (7.66 g) and ethanol (115 mL) were mixed, and a 1 M aqueous sodium hydroxide solution (38.8 mL) was added thereto, followed by stirring at 50°C for 1.5 hours. The reaction mixture was cooled to room temperature and a 1 M aqueous hydrochloric acid solution (38.8 mL) was added. The precipitated solid was collected by filtration to give a pale brown solid (7.58 g).

The obtained solid, acetonitrile (258 mL), and a 1 M aqueous hydrochloric acid solution (28.7 mL) were mixed, followed by stirring at room temperature for 30 minutes. Acetonitrile (10% aqueous, 130 mL) was further added, and the solid was dissolved under heating. After cooling to room temperature, the precipitated solid was collected by filtration and dried to obtain **1** (3.54 g, 28%) as a grey solid: ^{1}H-NMR (DMSO-d_6) δ ^{1}H-NMR (DMSO-d_6) δ 1.47 (3H, d, J = 6.4 Hz), 1.55–1.80 (3H, m), 1.85–2.03 (4H, m), 2.15–2.27 (1H, m), 2.60 (1H, tt, J = 10.7, 3.9 Hz), 3.10–3.25 (3H, m), 3.40–3.63 (2H, m), 4.16–4.30 (2H, m), 4.63 (1H, dd, J = 14.9, 7.1 Hz), 4.87 (1H, d, J = 14.9 Hz), 5.50–7.50 (2H, m), 7.70–7.75 (2H, m), 8.14 (1H, dd, J = 15.4, 1.7 Hz), 8.72–8.77 (1H, m), 11.13 (1H, brs), 12.89 (1H, brs); FAB-MS m/z 564 [$(M+H)^+$]; HRMS $(M+H)^+$ calcd for $C_{25}H_{28}O_3N_5ClFS_2$ 564.1301; found 564.1303. Anal. calcd for $C_{25}H_{27}ClFN_5O_3S_2 \cdot 2HCl \cdot 2H_2O$: C, 44.61; H, 4.94; N, 10.41; S, 9.53; Cl, 15.80; F, 2.82. Found: C, 44.82; H, 5.07; N, 10.66; S, 9.57; Cl, 15.65; F, 2.82.

The following compounds (**2**–**4**) were prepared using a procedure similar to that described for **1**.

6-[4-({4-(4-Chloro-2-thienyl)-5-[(2-methylpyrrolidin-1-yl)methyl]-1,3-thiazol-2-yl} carbamoyl)piperidin-1-yl]nicotinic acid dihydrochloride (2)

White solid (yield 34%): ^{1}H-NMR (DMSO-d_6) δ ^{1}H-NMR (DMSO-d_6) δ 1.42 (3H, d, J = 6.5 Hz), 1.59–1.72 (3H, m), 1.82–2.02 (4H, m), 2.12–2.26 (1H, m), 2.92 (1H, tt, J = 11.2, 3.8 Hz), 3.05–3.20 (3H, m), 3.38–3.60 (2H, m), 3.60—5.00 (2H, m), 4.41–4.53 (2H, m), 4.59 (1H, dd, J = 15.0, 7.4 Hz), 4.88 (1H, dd, J = 15.0, 3.1 Hz), 7.06 (1H, d, J = 9.2 Hz), 7.66 (1H, d, J = 1.3 Hz), 7.72 (1H, d, J = 1.3 Hz), 8.01 (1H, dd, J = 9.2, 2.3 Hz), 8.56 (1H, d, J = 2.3 Hz), 10.51 (1H, brs), 12.65 (1H, s); ESI-MS m/z 546, 548 [(M+H)$^+$]; HRMS (M+H)$^+$ calcd for $C_{25}H_{29}O_3N_5ClS_2$ 546.1395; found 546.1395.

1-(5-{[4-(4-Chloro-2-thienyl)-5-{[(2*R*)-2-methylpyrrolidin-1-yl]methyl}-1,3-thiazol-2-yl] carbamoyl}pyrimidin-2-yl)piperidine-4-carboxylic acid (3)

White powder (yield 63%): ^{1}H-NMR (DMSO-d_6) δ ^{1}H-NMR (DMSO-d_6) δ 1.16 (3H, d, J = 5.7 Hz), 1.32–1.45 (1H, m), 1.45–1.59 (2H, m), 1.59–1.76 (2H, m), 1.84–2.02 (3H, m), 2.12–2.27 (1H, m), 2.50–2.66 (2H, m), 2.95–3.09 (1H, m), 3.14–3.26 (2H, m), 3.56 (1H, d, J = 14.7 Hz), 4.19 (1H, d, J = 14.7 Hz), 4.54–4.66 (2H, m), 7.46 (1H, s), 7.57 (1H, s), 8.98 (2H, s), 12.29 (1H, brs), 12.51 (1H, brs); ESI-MS m/z 547, 549 [(M+H)$^+$]; HRMS (M+H)$^+$ calcd for $C_{24}H_{28}O_3N_6ClS_2$ 547.1347; found 547.1351.

1-(5-{[4-(4-Chloro-2-thienyl)-5-{[(2*R*)-2-methylpyrrolidin-1-yl]methyl}-1,3-thiazol-2-yl] carbamoyl}pyrazin-2-yl)piperidine-4-carboxylic acid dihydrochloride (4)

Pale yellow solid (yield 68%): ^{1}H-NMR (DMSO-d_6) δ ^{1}H-NMR (DMSO-d_6) δ 1.47 (3H, d, J = 6.4 Hz), 1.51–1.65 (2H, m), 1.65–1.80 (1H, m), 1.86–2.03 (4H, m), 2.14–2.28 (1H, m), 2.65 (1H, tt, J = 10.8, 4.0 Hz), 3.10–3.30 (3H, m), 3.40–3.63 (2H, m), 4.35–4.50 (2H, m), 4.63 (1H, dd, J = 15.0, 7.2 Hz), 4.87 (1H, dd, J = 15.0, 3.1 Hz), 7.72 (1H, d, J = 1.3 Hz), 7.73 (1H, d, J = 1.3 Hz), 8.40 (1H, d, J = 1.2 Hz), 8.76 (1H, d, J = 1.2 Hz), 9.89 (3H, brs), 11.22 (1H, brs), 12.09 (1H, brs); ESI-MS m/z 547, 549 [(M+H)$^+$]; HRMS (M+H)$^+$ calcd for $C_{24}H_{28}O_3N_6ClS_2$ 547.1347; found 547.1346.

4-[4-Methoxy-3-(trifluoromethyl)phenyl]-1,3-thiazol-2-amine (38)

1-[4-Methoxy-3-(trifluoromethyl)phenyl]ethanone (**35**, 15 g) and tetrahydrofuran (270 mL) were mixed, and phenyltrimethylammonium tribromide (28.42 g) was added thereto, followed by stirring at room temperature for 30 minutes. The precipitated insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure.

The obtained residue and ethanol (260 mL) were mixed, and thiourea (6.81 g) was added thereto, followed by stirring at 80°C for 3 hours. The reaction mixture was cooled to room temperature, and

water, a 1 M aqueous sodium hydroxide solution, and ethyl acetate were added. The organic layer was washed with a 1 M aqueous sodium hydroxide solution, water, and a saturated aqueous sodium chloride solution, and dried over anhydrous magnesium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane-ethyl acetate) to obtain **38** (16.18 g, 86%) as a white solid: ^{1}H-NMR (DMSO-d_6) δ 3.91 (3H, s), 7.04 (1H, s), 7.10 (2H, s), 7.27 (1H, d, J = 8.6 Hz), 8.01–8.05 (1H, m), 8.02 (1H, s); ESI-MS m/z 275 [(M+H)$^+$].

The following compounds (**36**–**37**) were prepared using a procedure similar to that described for **38**.

4-[3-Chloro-5-(trifluoromethyl)phenyl]-1,3-thiazol-2-amine (36)

Yellow solid (yield 107%): ^{1}H-NMR (DMSO-d_6) δ ^{1}H-NMR (DMSO-d_6) δ 4.36 (2H, brs), 7.48 (1H, s), 7.75 (1H, brs), 8.10 (1H, brs), 8.17 (1H, brs); ESI-MS m/z 279, 281 [(M+H)$^+$].

4-[4-Chloro-3-(trifluoromethyl)phenyl]-1,3-thiazol-2-amine (37)

Off-white solid (yield 93%): ^{1}H-NMR (DMSO-d_6) δ ^{1}H-NMR ($CDCl_3$) δ 5.03 (2H, brs), 6.81 (1H, s), 7.50 (1H, d, J = 8.4 Hz), 7.86 (1H, d, J = 8.4, 2.1 Hz), 8.12 (1H, d, J = 2.1 Hz); ESI-MS m/z 279, 281 [(M+H)$^+$].

Ethyl 1-[5-({4-[3-chloro-5-(trifluoromethyl)phenyl]-1,3-thiazol-2-yl}carbamoyl)pyrazin-2-yl] piperidine-4-carboxylate (39)

A mixture of 4-[3-chloro-5-(trifluoromethyl)phenyl]-1,3-thiazol-2-amine (**36**, 1.03 g), 5-[4-(ethoxycarbonyl)piperidin-1-yl]pyrazine-2-carboxylic acid (**28**, 1.13 g), [(1*H*-benzotriazol-1-yl)oxy](dimethylamino)-*N*,*N*-dimethylmethaniminium tetrafluoridoborate (1.30 g), *N*,*N*-diisopropylethylamine (1.6 mL), and THF (10 mL) was stirred at 150°C for 30 minutes under irradiation with microwaves. The mixture was diluted with water and then extracted with chloroform. The organic layer was washed with a saturated aqueous sodium chloride solution and dried over anhydrous sodium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure. The resulting residue was purified by silica gel column chromatography (hexanes-ethyl acetate) and then washed with hexanes/ethyl acetate to obtain **39** (1.07 g, 54%) as a pale yellow solid: ^{1}H-NMR (DMSO-d_6) δ ^{1}H-NMR (DMSO-d_6) δ 1.20 (3H, t, J = 7.1 Hz), 1.54–1.67 (2H, m), 1.92–2.02 (2H, m), 2.68–2.78 (1H, m), 3.17–3.28 (2H, m), 4.09 (2H, q,

J = 7.1 Hz), 4.39–4.49 (2H, m), 7.81 (1H, brs), 8.11 (1H, s), 8.28 (1H, brs), 8.35 (1H, brs), 8.40 (1H, d, *J* = 1.3 Hz), 8.77 (1H, d, *J* = 1.3 Hz), 11.89 (1H, brs); ESI-MS m/z 540, 542 [$(M+H)^+$].

The following compound (**40**) was prepared using a procedure similar to that described for **39**.

Ethyl 1-[5-({4-[4-chloro-3-(trifluoromethyl)phenyl]-1,3-thiazol-2-yl}carbamoyl)pyrazin-2-yl] piperidine-4-carboxylate (40)

Pale yellow powder (yield 76%): ^{1}H-NMR (DMSO-d_6) δ 1.20 (3H, t, *J* = 7.0 Hz), 1.53–1.67 (2H, m), 1.92–2.02 (2H, m), 2.73 (1H, tt, *J* = 10.9, 3.9 Hz), 3.17–3.27 (2H, m), 4.09 (2H, q, *J* = 7.0 Hz), 4.38–4.50 (2H, m), 7.80 (1H, d, *J* = 8.5 Hz), 7.99 (1H, s), 8.24 (1H, dd, 8.5, 2.0 Hz), 8.36–8.44 (2H, m), 8.77 (1H, d, *J* = 1.2 Hz), 11.87 (1H, brs).

Ethyl 1-[5-({4-[4-methoxy-3-(trifluoromethyl)phenyl]-1,3-thiazol-2-yl}carbamoyl)pyrazin -2-yl]piperidine-4-carboxylate (**41**)

Phosphorous oxychloride (2.25 mL) was added dropwise to a mixture of 4-[4-methoxy-3-(trifluoromethyl)phenyl]-1,3-thiazol-2-amine (**38**, 6.00 g), 5-[4-(ethoxycarbonyl)piperidin-1-yl]pyrazine-2-carboxylic acid (**28**, 6.72 g), and pyridine (106 mL) at an internal temperature of -5°C to 5°C over 10 minutes, and the mixture was warmed to room temperature and stirred for 1 hour. The reaction mixture was concentrated under reduced pressure. An aqueous citric acid solution was added to the residue and the mixture was extracted with ethyl acetate. The organic layer was dried over anhydrous magnesium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure to about half the volume. Silica gel was added to the residue and the mixture was stirred. The insoluble materials were separated by filtration, and the filtrate was concentrated under reduced pressure. Diisopropyl ether was added to the residue, and the mixture was stirred. The solid was collected by filtration and dried to obtain **41** (7.59 g, 65%) as a yellow brown solid: ^{1}H-NMR ($CDCl_3$) δ 1.28 (3H, t, *J* = 7.0 Hz), 1.76–1.89 (2H, m), 2.03–2.13 (2H, m), 2.66 (1H, tt, *J* = 4.1, 10.5 Hz), 3.20–3.30 (2H, m), 3.95 (3H, s), 4.18 (2H, q, *J* = 7.0 Hz), 4.40 (2H, dt, *J* = 3.9, 13.7 Hz), 7.05 (1H, d, *J* = 8.7 Hz), 7.11 (1H, s), 7.99 (1H, dd, *J* = 8.7, 2.2 Hz), 8.07 (1H, d, *J* = 1.3 Hz), 8.10 (1H, d, *J* = 2.2 Hz), 8.94 (1H, d, *J* = 1.3 Hz), 10.64 (1H, s); ESI-MS m/z 536 [$(M+H)^+$].

1-[5-({5-[(3,3-Dimethylpiperidin-1-yl)methyl]-4-[4-methoxy-3-(trifluoromethyl)phenyl] -1,3-thiazol-2-yl}carbamoyl)pyrazin-2-yl]piperidine-4-carboxylic acid dihydrochloride (**13**)

Ethyl 1-[5-({4-[4-methoxy-3-(trifluoromethyl)phenyl]-1,3-thiazol-2-yl}carbamoyl)pyrazin-2-yl]piperidine-4-carboxylate (**41**, 200 mg), acetic acid (4.0 mL), a 36% aqueous formaldehyde solution (145 μL), and 3,3-dimethylpiperidine (215 mg) were mixed and the mixture was stirred at 90°C for 1 hour. The reaction mixture was cooled to room temperature and concentrated under reduced pressure, and the residue was diluted with ethyl acetate. The mixture was washed with an aqueous sodium hydrogen carbonate solution and a saturated aqueous sodium chloride solution, and dried over anhydrous sodium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by basic silica gel column chromatography (hexanes-ethyl acetate).

The obtained residue was mixed with ethanol (4.0 mL) and tetrahydrofuran (2.0 mL), and a 1 M aqueous sodium hydroxide solution (1.90 mL) was added thereto, followed by stirring at 50°C for 20 minutes. The reaction mixture was cooled to room temperature, and water and a 1 M aqueous hydrochloric acid solution (1.90 mL) were added, followed by extraction with ethyl acetate. The organic layer was washed with a saturated aqueous sodium chloride solution and dried over anhydrous magnesium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure.

The obtained solid was mixed with ethyl acetate, and a 4 M hydrogen chloride/ethyl acetate solution (500 μL) was added, and the mixture was concentrated under reduced pressure and washed with ethyl acetate to obtain **13** (130 mg, 49%) as a yellow solid: ^{1}H-NMR (DMSO-d_6) δ 0.84 (3H, s), 1.06 (3H, s), 1.25 (1H, dt, J = 3.7, 13.4 Hz), 1.33–1.41 (1H, m), 1.51–1.71 (3H, m), 1.77–1.88 (1H, m), 1.90–2.00 (2H, m), 2.44–2.55 (1H, m), 2.64 (1H, tt, J = 4.0, 10.8 Hz), 2.69–2.80 (1H, m), 2.84 (1H, d, J = 11.7 Hz), 3.16–3.31 (3H, m), 3.97 (3H, s), 4.37–4.48 (3H, m), 4.57 (1H, dd, J = 14.8, 3.1 Hz), 4.60–6.20 (2H, m), 7.39 (1H, d, J = 8.7 Hz), 7.83 (1H, d, J = 2.0 Hz), 7.87 (1H, dd, J = 8.7, 2.0 Hz), 8.40 (1H, d, J = 1.1 Hz), 8.77 (1H, d, J = 1.1 Hz), 9.86 (1H, brs), 12.06 (1H, s); ESI-MS m/z 633 [(M+H)$^+$]; HRMS (M+H)$^+$ calcd for $C_{30}H_{36}O_4N_6F_3S$ 633.2465; found 633.2463.

The following compounds (**7**–**12**) were prepared using a procedure similar to that described for **13**.

1-{5-[(4-[4-Methoxy-3-(trifluoromethyl)phenyl]-5-{[(2*R*)-2-methylpyrrolidin-1-yl]methyl}-1,3-thiazol-2-yl)carbamoyl]pyrazin-2-yl}piperidine-4-carboxylic acid dihydrochloride (**7**)

Yellow solid (yield 71%): ^{1}H-NMR (DMSO-d_6) δ 1.35 (3H, d, J = 6.4 Hz), 1.51–1.70 (3H, m), 1.84–2.00 (4H, m), 2.11–2.22 (1H, m), 2.64 (1H, tt, J = 3.9, 10.7 Hz), 3.06–3.18 (1H, m), 3.17–3.28 (2H, m), 3.36–3.56 (2H, m), 3.60–4.10 (2H, m), 3.97 (3H, s), 4.38–4.52 (3H, m), 4.74 (1H, dd, J =

14.9, 2.4 Hz), 7.40 (1H, d, *J* = 8.8 Hz), 7.93 (1H, d, *J* = 2.0 Hz), 7.99 (1H, dd, *J* = 8.8, 2.0 Hz), 8.40 (1H, d, *J* = 1.2 Hz), 8.77 (1H, d, *J* = 1.2 Hz), 10.52 (1H, brs), 12.10 (1H, s); ESI-MS m/z 605 [(M+H)$^+$]; HRMS (M+H)$^+$ calcd for $C_{28}H_{32}O_4N_6F_3S$ 605.2152; found 605.2153.

1-{5-[(4-[4-Methoxy-3-(trifluoromethyl)phenyl]-5-{[(2*S*)-2-methylpyrrolidin-1-yl]methyl} -1,3-thiazol-2-yl)carbamoyl]pyrazin-2-yl}piperidine-4-carboxylic acid dihydrochloride (**8**)

Pale yellow solid (yield 55%): ^{1}H-NMR (DMSO-d_6) δ 1.34 (3H, d, *J* = 6.5 Hz), 1.51–1.68 (3H, m), 1.83–1.99 (4H, m), 2.12–2.22 (1H, m), 2.59–2.68 (1H, m), 3.07–3.27 (3H, m), 3.37–3.56 (2H, m), 3.62–3.92 (2H, m), 3.97 (3H, s), 4.38–4.52 (3H, m), 4.72–4.79 (1H, m), 7.40 (1H, d, J = 8.8 Hz), 7.92 (1H, d, *J* = 2.0 Hz), 7.95–7.99 (1H, m), 8.37–8.42 (1H, m), 8.77 (1H, d, *J* = 1.2 Hz), 10.25 (1H, brs), 12.10 (1H, s); ESI-MS m/z 605 [(M+H)$^+$]; HRMS (M+H)$^+$ calcd for $C_{28}H_{32}O_4N_6F_3S$ 605.2152; found 605.2162.

1-{5-[(5-{[(2*R*)-2-Ethylpyrrolidin-1-yl]methyl}-4-[4-methoxy-3-(trifluoromethyl)phenyl] -1,3-thiazol-2-yl)carbamoyl]pyrazin-2-yl}piperidine-4-carboxylic acid dihydrochloride (9)

Yellow solid (yield 55%): ^{1}H-NMR (DMSO-d_6) δ 0.80 (3H, t, *J* = 7.3 Hz), 1.45–2.01 (9H, m), 2.04–2.20 (1H, m), 2.56–2.70 (1H, m), 3.05–3.28 (4H, m), 3.44–3.56 (1H, m), 3.97 (3H, s), 4.07–4.77 (6H, m), 7.40 (1H, d, *J* = 8.8 Hz), 7.93 (1H, d, *J* = 2.0 Hz), 7.99 (1H, dd, *J* = 8.7, 2.0 Hz), 8.40 (1H, d, *J* = 1.2 Hz), 8.77 (1H, d, *J* = 1.2 Hz), 10.62 (1H, brs), 12.10 (1H, s); ESI-MS m/z 619 [(M+H)$^+$]; HRMS (M+H)$^+$ calcd for $C_{29}H_{34}O_4N_6F_3S$ 619.2309; found 619.2306.

1-{5-[(4-[4-Methoxy-3-(trifluoromethyl)phenyl]-5-{[(2*R*)-2-propylpyrrolidin-1-yl]methyl} -1,3-thiazol-2-yl)carbamoyl]pyrazin-2-yl}piperidine-4-carboxylic acid dihydrochloride (**10**)

Yellow solid (yield 57%): ^{1}H-NMR (DMSO-d_6) δ 0.81 (3H, t, *J* = 7.2 Hz), 0.97–1.11 (1H, m), 1.19–1.32 (1H, m), 1.50–1.66 (5H, m), 1.82–2.00 (4H, m), 2.06–2.20 (1H, m), 2.58–2.69 (1H, m), 3.08–3.28 (4H, m), 3.46–3.57 (1H, m), 3.97 (3H, s), 4.03–4.86 (6H, m), 7.40 (1H, d, *J* = 8.7 Hz), 7.93 (1H, d, *J* = 2.0 Hz), 7.98 (1H, dd, *J* = 8.6, 2.0 Hz), 8.40 (1H, d, *J* = 1.1 Hz), 8.77 (1H, d, *J* = 1.2 Hz), 10.55 (1H, brs), 12.11 (1H, s); ESI-MS m/z 633 [(M+H)$^+$]; HRMS (M+H)$^+$ calcd for $C_{30}H_{36}O_4N_6F_3S$ 633.2465; found 633.2467.

1-{5-[(5-{[(2*S*)-2-(Methoxymethyl)pyrrolidin-1-yl]methyl}-4-[4-methoxy-3-(trifluoromethyl) phenyl]-1,3-thiazol-2-yl)carbamoyl]pyrazin-2-yl}piperidine-4-carboxylic acid dihydrochloride (**11**)

Pale yellow solid (yield 30%): ^{1}H-NMR (DMSO-d_6) δ 1.52–1.69 (3H, m), 1.80–2.01 (4H, m), 2.06–2.16 (1H, m), 2.58–2.68 (1H, m), 3.16–3.28 (5H, m), 3.48–3.78 (4H, m), 3.94–4.01 (4H, m), 4.31–4.99 (6H, m), 7.39 (1H, d, J = 8.8 Hz), 7.92 (1H, d, J = 2.0 Hz), 8.03 (1H, dd, J = 8.6, 2.0 Hz), 8.39—8.40 (1H, m), 8.77 (1H, d, J = 1.2 Hz), 10.62 (1H, brs), 12.09 (1H, s); ESI-MS m/z 635 [(M+H)$^+$]; HRMS (M+H)$^+$ calcd for $C_{29}H_{34}O_5N_6F_3S$ 635.2258; found 635.2259.

1-{5-[(4-[4-Methoxy-3-(trifluoromethyl)phenyl]-5-{[(2*R*)-2-methylpiperidin-1-yl]methyl}-1,3-thiazol-2-yl)carbamoyl]pyrazin-2-yl}piperidine-4-carboxylic acid dihydrochloride (12)

Yellow solid (yield 19%): ^{1}H-NMR (DMSO-d_6) δ 1.34 (3H, d, J = 6.3 Hz), 1.31–1.74 (7H, m), 1.76–1.85 (1H, m), 1.90–2.00 (2H, m), 2.58–2.74 (2H, m), 3.09–3.30 (4H, m), 3.60–4.40 (2H, m), 3.97 (3H, s), 4.37–4.48 (3H, m), 4.79 (1H, dd, J = 14.9, 1.7 Hz), 7.39 (1H, d, J = 8.7 Hz), 7.88 (1H, d, J = 2.0 Hz), 7.93 (1H, dd, J = 8.7, 2.0 Hz), 8.40 (1H, d, J = 1.0 Hz), 8.77 (1H, d, J = 1.0 Hz), 10.34 (1H, brs), 12.12 (1H, s); ESI-MS m/z 619 [(M+H)$^+$]; HRMS (M+H)$^+$ calcd for $C_{29}H_{34}O_4N_6F_3S$ 619.2309; found 619.2318.

1-{5-[(5-{[Isobutyl(2-methoxyethyl)amino]methyl}-4-[4-methoxy-3-(trifluoromethyl)phenyl]-1,3-thiazol-2-yl)carbamoyl]pyrazin-2-yl}piperidine-4-carboxylic acid (14)

Ethyl 1-[5-({4-[4-methoxy-3-(trifluoromethyl)phenyl]-1,3-thiazol-2-yl}carbamoyl)pyrazin-2-yl]piperidine-4-carboxylate (**41**, 1.45 g), acetic acid (10 mL), a 36% aqueous formaldehyde solution (1.50 mL), and acetic anhydride (1.8 mL) were mixed, followed by stirring at 170°C for 30 minutes under irradiation with microwaves. The reaction mixture was concentrated under reduced pressure, and water and a saturated aqueous sodium hydrogen carbonate solution were added to the residue, followed by extraction with ethyl acetate. The organic layer was washed with water and a saturated aqueous sodium chloride solution, and dried over anhydrous magnesium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (chloroform-ethyl acetate).

The obtained residue was mixed with *N*,*N*-dimethylformamide (15 mL), *N*-(2-methoxyethyl)-2-methylpropane-1-amine hydrochloride (685 mg), and *N*,*N*-diisopropylethylamine (1.4 mL), and the mixture was stirred at 100°C for 1 hour. The reaction mixture was cooled to room temperature and ethyl acetate was added. The mixture was washed with water and a saturated aqueous sodium chloride solution, and dried over anhydrous magnesium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by silica

gel column chromatography (hexane-ethyl acetate) and the obtained solid was washed with diisopropyl ether.

The obtained solid was mixed with ethanol (5 mL), and a 1 M aqueous sodium hydroxide solution (2.8 mL) was added, and the mixture was stirred at 60°C for 15 minutes. The reaction mixture was cooled to room temperature, and water and a 1 M aqueous hydrochloric acid solution (2.8 mL) were added thereto, followed by extraction with ethyl acetate. The organic layer was washed with water and a saturated aqueous sodium chloride solution, and dried over anhydrous magnesium sulfate. The insoluble materials were then separated by filtration and the filtrate was concentrated under reduced pressure. The obtained solid was washed with diisopropyl ether and dried to obtain **14** (224 mg, 13%) as a white solid: ^{1}H-NMR (DMSO-d_6) δ 0.85 (6H, d, J = 6.6 Hz), 1.52–1.65 (2H, m), 1.68–1.80 (1H, m), 1.90–2.00 (2H, m), 2.26 (2H, d, J = 7.2 Hz), 2.58–2.69 (1H, m), 2.61 (2H, t, J = 6.1 Hz), 3.16–3.26 (2H, m), 3.19 (3H, s), 3.42 (2H, t, J = 6.1 Hz), 3.87 (2H, s), 3.94 (3H, s), 4.38–4.47 (2H, m), 7.34 (1H, d, J = 9.1 Hz), 7.90–7.96 (2H, m), 8.36–8.41 (1H, m), 8.75 (1H, d, J = 1.1 Hz), 11.53 (1H, brs), 12.31 (1H, brs); ESI-MS m/z 651 [(M+H)$^+$]; HRMS (M+H)$^+$ calcd for $C_{30}H_{38}O_5N_6F_3S$ 651.2571; found 651.2571.

The following compounds (**5**–**6**) were prepared using a procedure similar to that described for **14**.

1-{5-[(4-[3-Chloro-5-(trifluoromethyl)phenyl]-5-{[(2*R*)-2-methylpyrrolidin-1-yl]methyl}-1,3-thiazol-2-yl)carbamoyl]pyrazin-2-yl}piperidine-4-carboxylic acid dihydrochloride (**5**)

Yellow powder (yield 45%): ^{1}H-NMR (DMSO-d_6) δ 1.39 (3H, d, J = 6.4 Hz), 1.50–1.72 (3H, m), 1.85–2.01 (4H, m), 2.14–2.24 (1H, m), 2.58–2.69 (1H, m), 3.13–3.28 (3H, m), 3.29–3.65 (4H, m), 4.38–4.52 (3H, m), 4.81 (1H, dd, J = 14.6, 1.5 Hz), 7.99 (1H, brs), 8.05 (1H, brs), 8.13 (1H, brs), 8.40 (1H, d, J = 1.1 Hz), 8.78 (1H, d, J = 1.1 Hz), 10.42 (1H, brs), 12.21 (1H, s); ESI-MS m/z 609, 611 [(M+H)$^+$]; HRMS (M+H)$^+$ calcd for $C_{27}H_{29}O_3N_6ClF_3S$ 609.1657; found 609.1661.

1-{5-[(4-[4-Chloro-3-(trifluoromethyl)phenyl]-5-{[(2*R*)-2-methylpyrrolidin-1-yl]methyl}-1,3-thiazol-2-yl)carbamoyl]pyrazin-2-yl}piperidine-4-carboxylic acid dihydrochloride (**6**)

Pale yellow powder (yield 20%): ^{1}H-NMR (DMSO-d_6) δ 1.35 (3H, d, J = 6.5 Hz), 1.51–1.69 (3H, m), 1.83–2.01 (4H, m), 2.12–2.23 (1H, m), 2.64 (1H, tt, J = 10.8, 3.9 Hz), 3.00–3.60 (4H, m), 3.04–3.17 (1H, m), 3.17–3.28 (2H, m), 4.38–4.54 (3H, m), 4.81 (1H, dd, J = 14.8, 2.4 Hz), 7.88 (1H, d, J = 8.4 Hz), 8.02 (1H, dd, J = 8.4, 1.9 Hz), 8.15 (1H, d, J = 1..9 Hz), 8.40 (1H, d, J = 1.1 Hz), 8.78

(1H, d, J = 1.1 Hz), 10.32 (1H, brs), 12.20 (1H, s); ESI-MS m/z 609, 611 [$(M+H)^+$]; HRMS $(M+H)^+$ calcd for $C_{27}H_{29}O_3N_6ClF_3S$ 609.1657; found 609.1653.

5-[(3,3-Dimethylpiperidin-1-yl)methyl]-4-[4-methoxy-3-(trifluoromethyl)phenyl] -1,3-thiazol-2-amine (**42**)

To a solution of 4-[4-methoxy-3-(trifluoromethyl)phenyl]-1,3-thiazol-2-amine (**38**, 5.00 g) and triethylamine (4.06 mL) in dichloromethane (100 mL) was added trifluoroacetic anhydride (5.74 g) under ice-cooling, followed by stirring at room temperature for 3 hours. Water was added to the reaction mixture, followed by extraction with chloroform. The organic layer was washed with a saturated aqueous sodium chloride solution and dried over anhydrous magnesium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane-ethyl acetate) and then washed with hexanes/diisopropyl ether to obtain 2,2,2-trifluoro-*N*-{4-[4-methoxy-3-(trifluoromethyl)phenyl]-1,3-thiazol-2-yl}acetamide (4.80 g, 71%) as a white solid.

To a mixture of 2,2,2-trifluoro-*N*-{4-[4-methoxy-3-(trifluoromethyl)phenyl]-1,3-thiazol-2-yl}acetamide (10.0 g) and acetic acid (167 mL) was added 36% formaldehyde aqueous solution (4.80 mL), 3,3-dimethylpiperidine hydrochloride (10.0 g), and *N,N*-diisopropylethylamine (23.3 mL), followed by stirring at 60°C for 2 hours. The reaction mixture was cooled on ice and a 1 M aqueous sodium hydroxide solution was added thereto, and the mixture was extracted with ethyl acetate. The aqueous layer was extracted with ethyl acetate, and the combined organic layers were washed with water and a saturated aqueous sodium chloride solution, and dried over anhydrous magnesium sulfate. The insoluble materials were then separated by filtration and the filtrate was concentrated under reduced pressure.

The obtained residue was dissolved in tetrahydrofuran (50 mL) and ethanol (50 mL), and a 6 M aqueous sodium hydroxide solution (45 mL) was added, and the mixture was stirred at 80°C overnight. The reaction mixture was cooled to room temperature and water was added thereto, followed by extraction with ethyl acetate. The organic layer was washed with water and a saturated aqueous sodium chloride solution, and dried over anhydrous magnesium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure. The residue was washed with hexanes/diisopropyl ether to obtain **42** (7.70 g, 71%) as a white solid: ^{1}H-NMR (DMSO-d_6) δ 0.90 (6H, s), 1.15–1.23 (2H, m), 1.48–1.58 (2H, m), 1.99–2.11 (2H, m), 2.24–2.44 (2H, m), 3.40 (2H, s), 3.91 (3H, s), 6.90 (2H, s), 7.27 (1H, d, J = 8.8 Hz), 7.88 (1H, d, J = 8.8 Hz), 7.96 (1H, s); ESI-MS m/z 400 [$(M+H)^+$].

5-Chloro-*N*-{5-[(3,3-dimethylpiperidin-1-yl)methyl]-4-[4-methoxy-3-(trifluoromethyl) phenyl]-1,3-thiazol-2-yl}pyrazine-2-carboxamide (43)

To a mixture of 5-[(3,3-dimethylpiperidin-1-yl)methyl]-4-[4-methoxy-3-(trifluoromethyl)phenyl]-1,3-thiazol-2-amine (**42**, 2.00 g) and 1,4-dioxane (40 mL) was added 5-chloropyrazine-2-carboxylic acid (910 mg), *N*-[({[(1*Z*)-1-cyano-2-ethoxy-2-oxoethylidene]amino}oxy)(morpholin-4-yl)methylene]-*N*-methylmethanaminium hexafluorophosphate (2.55 g), and *N,N*-diisopropylethylamine (1.82 mL), followed by stirring at room temperature for 3 hours. The reaction mixture was concentrated under reduced pressure and the residue was purified by silica gel column chromatography (hexanes-ethyl acetate). The obtained residue was washed with diisopropyl ether to obtain **43** (1.40 g, 52%) as a yellow solid: ^{1}H-NMR (DMSO-d_6) δ 0.91 (6H, s), 1.18–1.25 (2H, m), 1.53–1.61 (2H, m), 2.04–2.16 (2H, m), 2.35–2.47 (1H, m), 3.65 (2H, s), 3.95 (3H, s), 7.36 (1H, d, J = 8.8 Hz), 8.00 (2H, dd, J = 8.8, 2.1 Hz), 8.08 (1H, d, J = 2.1 Hz), 8.98 (1H, d, J = 1.5 Hz), 9.15 (1H, d, J = 1.5 Hz), 12.56 (1H, s); ESI-MS m/z 540 [(M+H)$^+$].

Potassium {[5-({5-[(3,3-dimethylpiperidin-1-yl)methyl]-4-[4-methoxy-3-(trifluoromethyl) phenyl]-1,3-thiazol-2-yl}carbamoyl)pyrazin-2-yl]amino}acetate (15)

To a mixture of 5-chloro-*N*-{5-[(3,3-dimethylpiperidin-1-yl)methyl]-4-[4-methoxy-3-(trifluoromethyl)phenyl]-1,3-thiazol-2-yl}pyrazine-2-carboxamide (**43**, 100 mg) and *N,N*-dimethylformamide (1.5 mL) was added ethyl glycinate hydrochloride (52 mg), and *N,N*-diisopropylethylamine (159 μL), followed by stirring at room temperature for 1 hour. The reaction mixture was then heated to 80°C and further stirred for 2 hours. The reaction mixture was cooled to room temperature and water was added thereto, followed by extraction with ethyl acetate. The organic layer was washed with a saturated aqueous sodium chloride solution and dried over anhydrous magnesium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexanes-ethyl acetate) to obtain the intermediate (80 mg, 71%) as a pale yellow solid.

A 1 M aqueous sodium hydroxide solution (418 μL) was added to a solution of the obtained intermediate (70.0 mg) in ethanol (875 μL), and the mixture was stirred at 60°C for 1 hour. The reaction mixture was cooled to room temperature, and a 1 M aqueous hydrochloric acid solution (418 μL), water, and chloroform were added. The organic layer was separated, washed with a saturated aqueous sodium chloride solution, and dried over anhydrous magnesium sulfate. The insoluble

materials were then separated by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (chloroform-methanol).

The obtained residue was mixed with ethanol, and a 0.5 M potassium hydroxide in ethanol solution (194 μL) was added, and the mixture was concentrated under reduced pressure, and washed with hexanes/diisopropyl ether to obtain **15** (60.0 mg, 84%) as a yellow powder: ^{1}H-NMR (DMSO-d_6) δ 0.90 (6H, s), 1.16–1.27 (2H, m), 1.51–1.61 (2H, m), 2.00–2.17 (2H, m), 2.30–2.46 (2H, m), 3.42–3.62 (4H, m), 3.93 (3H, s), 7.32 (1H, d, J = 8.5 Hz), 7.40–8.40 (4H, m), 8.67 (1H, s), 11.32 (1H, s); ESI-MS m/z 579 [(M+H)$^+$]; HRMS (M+H)$^+$ calcd for $C_{26}H_{30}O_4N_6F_3S$ 579.1996; found 579.2001.

The following compound (**16**) was prepared using a procedure similar to that described for **15**.

Potassium {[5-({5-[(3,3-dimethylpiperidin-1-yl)methyl]-4-[4-methoxy-3-(trifluoromethyl) phenyl]-1,3-thiazol-2-yl}carbamoyl)pyrazin-2-yl](methyl)amino}acetate (16)

Pale yellow powder (yield 87%): ^{1}H-NMR (DMSO-d_6) δ 0.90 (6H, s), 1.17–1.28 (2H, m), 1.52–1.62 (2H, m), 2.04–2.17 (2H, m), 2.34–2.45 (2H, m), 3.12 (3H, s), 3.53–3.63 (2H, m), 3.74–3.86 (2H, m), 3.94 (3H, s), 7.34 (1H, d, J = 8.8 Hz), 7.84–8.20 (3H, m), 8.70 (1H, s), 11.42 (1H, s); ESI-MS m/z 593 [(M+H)$^+$]; HRMS (M+H)$^+$ calcd for $C_{27}H_{32}O_4N_6F_3S$ 593.2152; found 593.2155.

Potassium {[5-({5-[(3,3-dimethylpiperidin-1-yl)methyl]-4-[4-methoxy-3-(trifluoromethyl) phenyl]-1,3-thiazol-2-yl}carbamoyl)pyrazin-2-yl]oxy}acetate (17)

Under a nitrogen atmosphere, a 55% oil dispersion of sodium hydride (40 mg) was added to a solution of ethyl glycolate (96 mg) in *N*-methylpyrrolidone (1.5 mL) under ice-cooling, followed by stirring at the same temperature for 15 minutes. A solution of 5-chloro-*N*-{5-[(3,3-dimethylpiperidin-1-yl)methyl]-4-[4-methoxy-3-(trifluoromethyl)phenyl]-1,3-thiazol-2-yl}pyrazine-2-carboxamide (**43**, 100 mg) in *N*-methylpyrrolidone was added dropwise to the reaction mixture, and the mixture was stirred at room temperature for 1 hour. The reaction mixture was then heated to 80°C and further stirred for 5 hours. The reaction mixture was cooled to room temperature and water was added thereto, followed by extraction with ethyl acetate. The organic layer was washed with a saturated aqueous sodium chloride solution and dried over anhydrous magnesium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexanes-ethyl acetate) to obtain the intermediate (20 mg, 18%) as a white amorphous solid.

To a solution of the obtained intermediate (16.0 mg) in ethanol (1.0 mL) was added a 1 M aqueous sodium hydroxide solution (100 μL), followed by stirring at 60°C for 1 hour. The reaction mixture was cooled to room temperature, and a 1 M aqueous hydrochloric acid solution (100 μL), water, and chloroform were added. The organic layer was separated, washed with a saturated aqueous sodium chloride solution, and dried over anhydrous magnesium sulfate. The insoluble materials were then separated by filtration and the filtrate was concentrated under reduced pressure.

The obtained residue was mixed with ethanol, and a 0.5 M potassium hydroxide solution in ethanol (41 μL) was added, and the mixture was concentrated under reduced pressure, and washed with hexanes/diisopropyl ether to obtain **17** (9.0 mg, 55%) as a yellow powder: ^{1}H-NMR (DMSO-d_6) δ 0.91 (6H, s), 1.18–1.27 (2H, m), 1.52–1.63 (2H, m), 2.02–2.21 (2H, m), 2.33–2.48 (2H, m), 3.60 (2H, s), 3.94 (3H, s), 4.50 (2H, s), 7.35 (1H, d, J = 8.8 Hz), 8.03 (1H, d, J = 8.8 Hz), 8.13 (1H, s), 8.29 (1H, s), 8.83 (1H, s), 12.00 (1H, brs); ESI-MS m/z 580 [(M+H)$^+$].

Ethyl 5-({4-[4-methoxy-3-(trifluoromethyl)phenyl]-1,3-thiazol-2-yl}carbamoyl)pyrazine -2-carboxylate (**44**)

To a solution of 4-[4-methoxy-3-(trifluoromethyl)phenyl]-1,3-thiazol-2-amine (**38**, 1.05 g) and 5-(ethoxycarbonyl)pyrazine-2-carboxylic acid (800 mg) in pyridine (21 mL) was added phosphoric trichloride (420 μL) at -10°C under a N_2 atmosphere, followed by stirring at room temperature for 1 hour. Water was added to the mixture and the mixture was extracted with ethyl acetate. The aqueous layer was extracted twice with ethyl acetate, and the combined organic layers were washed with a saturated aqueous sodium chloride solution and dried over anhydrous magnesium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexanes-ethyl acetate) to obtain **44** (1.60 g, 92%) as a white powder: ^{1}H-NMR (DMSO-d_6) δ 1.39 (3H, t, J = 7.1 Hz), 3.94 (3H, s), 4.45 (2H, q, J = 7.1 Hz), 7.37 (1H, d, J = 9.3 Hz), 7.86 (1H, s), 8.19–8.25 (2H, m), 9.31 (1H, s), 9.42 (1H, s), 12.85 (1H, s); ESI-MS m/z 453 [(M+H)$^+$].

Ethyl 5-({5-[(3,3-dimethylpiperidin-1-yl)methyl]-4-[4-methoxy-3-(trifluoromethyl)phenyl] -1,3-thiazol-2-yl}carbamoyl)pyrazine-2-carboxylate (**45**)

To a mixture of ethyl 5-({4-[4-methoxy-3-(trifluoromethyl)phenyl]-1,3-thiazol-2-yl}carbamoyl)pyrazine-2-carboxylate (**44**, 1.60 g) and acetic acid (22.4 mL) was added 36% formaldehyde aqueous solution (2.82 mL) and acetic anhydride (3.40 mL), followed by stirring at 90°C for 2 hours. The reaction mixture was cooled to room temperature, and a saturated aqueous

sodium hydrogen carbonate solution and ethyl acetate were added. The organic layer was separated, washed with water and a saturated aqueous sodium chloride solution, and dried over anhydrous magnesium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure. Pyridine (19.0 mL) and acetic anhydride (441 μL) were added to the resulting residue, and the mixture was stirred at room temperature for 1 hour. Water was added to the mixture and the mixture was extracted with ethyl acetate. The organic layer was washed with water and a saturated aqueous sodium chloride solution, and dried over anhydrous magnesium sulfate. The insoluble materials were then separated by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (chloroform-ethyl acetate) and washed with diisopropyl ether to obtain the intermediate (500 mg, 27%) as a white solid.

To a mixture of the obtained intermediate (250 mg) and *N,N*-dimethylformamide (2.5 mL) was added *N,N*-diisopropylethylamine (410 μL) and 3,3-dimethylpiperidine (108 mg), followed by stirring at 80°C for 1 hour. The reaction mixture was cooled to room temperature, and water and ethyl acetate were added. The organic layer was washed with a saturated aqueous sodium chloride solution and dried over anhydrous magnesium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexanes-ethyl acetate) and then washed with diisopropyl ether to obtain **45** (100 mg, 36%) as a yellowish-white solid: ^{1}H-NMR (DMSO-d_6) δ 0.91 (6H, s), 1.18–1.26 (2H, m), 1.38 (3H, t, J = 7.1 Hz), 1.53–1.62 (2H, m), 2.06–2.17 (2H, m), 2.36–2.48 (2H, m), 3.65 (2H, s), 3.95 (3H, s), 4.45 (2H, q, J = 7.1 Hz), 7.36 (1H, d, J = 8.8Hz), 8.01 (1H, dd, J = 8.8, 2.1 Hz), 8.10 (1H, d, J = 2.1 Hz), 9.29 (1H, d, J = 1.3 Hz), 9.42 (1H, d, J = 1.3 Hz), 12.37 (1H, brs); ESI-MS m/z 578 [(M+H)$^+$].

5-({5-[(3,3-Dimethylpiperidin-1-yl)methyl]-4-[4-methoxy-3-(trifluoromethyl)phenyl] -1,3-thiazol-2-yl}carbamoyl)pyrazine-2-carboxylic acid (18)

To a solution of ethyl 5-({5-[(3,3-dimethylpiperidin-1-yl)methyl]-4-[4-methoxy-3-(trifluoromethyl)phenyl]-1,3-thiazol-2-yl}carbamoyl)pyrazine-2-carboxylate (**45**, 100 mg) in ethanol (1.2 mL) was added a 1 M aqueous sodium hydroxide solution (661 μL), followed by stirring at 60°C for 1 hour. The reaction mixture was cooled to room temperature, and a 1 M aqueous hydrochloric acid solution (661 μL), water, chloroform, and methanol were added. The organic layer was separated and dried over anhydrous magnesium sulfate. The insoluble materials were separated by filtration and the filtrate was concentrated under reduced pressure to obtain **18** (90 mg, 95%) as a yellow solid: ^{1}H-NMR (DMSO-d_6) δ 0.91 (6H, s), 1.18–1.28 (2H, m), 1.53–1.63 (2H, m), 2.05–2.20 (2H, m), 2.36–

2.48 (2H, m), 3.67 (2H, s), 3.95 (3H, s), 7.37 (1H, d, J = 8.8Hz), 8.00 (1H, dd, J = 8.8, 2.1 Hz), 8.09 (1H, d, J = 2.1 Hz), 9.27 (1H, d, J = 1.2 Hz), 9.40 (1H, d, J = 1.2 Hz), 12.64 (1H, s); ESI-MS m/z 550 [$(M+H)^+$]; HRMS $(M+H)^+$ calcd for $C_{25}H_{27}O_4N_5F_3S$ 550.1730; found 550.1735.

_N_-{5-[(3,3-Dimethylpiperidin-1-yl)methyl]-4-[4-methoxy-3-(trifluoromethyl)phenyl] -1,3-thiazol-2-yl}-4-hydroxybenzamide hydrochloride (20)

To a mixture of 5-[(3,3-dimethylpiperidin-1-yl)methyl]-4-[4-methoxy-3-(trifluoromethyl)phenyl]-1,3-thiazol-2-amine (**42**, 162 mg) and 1,4-dioxane (3.5 mL) was added 4-acetoxybenzoic acid (110 mg), *N*-[({[(1*Z*)-1-cyano-2-ethoxy-2-oxoethylidene]amino}oxy)(morpholin-4-yl)methylene]-*N*-methylmethanaminium hexafluorophosphate (222 mg), and *N,N*-diisopropylethylamine (150 μL), followed by stirring at 80°C for 4 hours. The reaction mixture was cooled to room temperature and a saturated aqueous sodium hydrogen carbonate solution was added thereto, followed by extraction with ethyl acetate. The organic layer was washed with a saturated aqueous sodium chloride solution and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexanes-ethyl acetate).

The obtained residue was mixed with methanol (4.0 mL), and a 1 M aqueous sodium hydroxide solution (300 μL) was added, and the mixture was stirred at room temperature for 30 minutes. A 1 M aqueous hydrochloric acid solution (300 μL) and water were added to the reaction mixture, and the mixture was extracted with chloroform. The organic layer was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexanes-ethyl acetate) and purified by silica gel column chromatography (chloroform-ethyl acetate). The obtained solid was mixed with ethyl acetate, and a 4 M hydrogen chloride/ethyl acetate solution (40 μL) was added, and the mixture was concentrated under reduced pressure to obtain **20** (37 mg, 16%) as a white solid: ^{1}H-NMR (DMSO-d_6) δ 0.84 (3H, s), 1.05 (3H, s), 1.20–1.31 (1H, m), 1.32–1.42 (1H, m), 1.62–1.72 (1H, m), 1.74–1.90 (1H, m), 2.46–2.56 (1H, m), 2.68–2.80 (1H, m), 2.82–2.90 (1H, m), 3.22–3.32 (1H, m), 3.97 (3H, s), 4.43 (1H, dd, J = 14.7, 5.6 Hz), 4.57 (1H, dd, J = 14.7, 3.4 Hz), 6.90 (2H, d, J = 8.8 Hz), 7.39 (1H, d, J = 8.6 Hz), 7.85 (1H, s), 7.87 (1H, d, J = 8.6 Hz), 8.03 (2H, d, J = 8.8 Hz), 9.63 (1H, s), 10.41 (1H, brs), 12.65 (1H, s); ESI-MS m/z 520 [$(M+H)^+$]; HRMS $(M+H)^+$ calcd for $C_{26}H_{29}O_3N_3F_3S$ 520.1876; found 520.1880.

The following compound (**19**) was prepared using a procedure similar to that described for **20**.

[4-({5-[(3,3-Dimethylpiperidin-1-yl)methyl]-4-[4-methoxy-3-(trifluoromethyl)phenyl] -1,3-thiazol-2-yl}carbamoyl)phenoxy]acetic acid hydrochloride (19)

White solid (yield 18%): ^{1}H-NMR (DMSO-d_6) δ 0.84 (3H, s), 1.04 (3H, s), 1.20–1.31 (1H, m), 1.33–1.41 (1H, m), 1.62–1.72 (1H, m), 1.74–1.88 (1H, m), 2.47–2.55 (1H, m), 2.69–2.81 (1H, m), 2.83–2.91 (1H, m), 3.23–3.31 (1H, m), 3.40–3.80 (1H, m), 3.97 (3H, s), 4.44 (1H, dd, J = 14.7, 5.3 Hz), 4.57 (1H, dd, J = 14.7, 3.4 Hz), 4.82 (2H, s), 7.07 (2H, d, J = 8.9 Hz), 7.40 (1H, d, J = 8.6 Hz), 7.85 (1H, s), 7.87 (1H, d, J = 8.6 Hz), 8.12 (2H, d, J = 8.9 Hz), 9.57 (1H, brs), 12.80 (1H, s); ESI-MS m/z 578 [$(M+H)^+$]; HRMS $(M+H)^+$ calcd for $C_{28}H_{31}O_5N_3F_3S$ 578.1931; found 578.1937.

Acknowledgments

The authors thank Ms. Risa Okimoto for evaluating the compounds. The authors also thank Dr. Chikashi Saitoh and Dr. Hajime Takamatsu for their support in the research. In addition, the authors are also grateful to the staff of the Division of Analytical Science Laboratories for conducting the elemental analysis and spectral measurements.

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10. The following abbreviations are used: DMAc, *N,N*-dimethylacetamide; Pd/C, palladium on carbon; Et_3N, triethylamine; THF, tetrahydrofuran; EtOH, ethanol; K_2CO_3, potassium carbonate; DMF, *N,N*-dimethylformamide; HCl, hydrochloric acid; AcOEt, ethyl acetate; DIPEA, *N,N*-diisopropylethylamine; $SOCl_2$, thionyl chloride; DCE, 1,2-dichloroethane; HATU, *N*-[(dimethylamino)(3*H*-[1,2,3]triazolo[4,5-b]pyridin-3-yloxy)methylene]-*N*-methylmethanaminium hexafluorophosphate; TBTU, [(1*H*-benzotriazol-1-yl)oxy](dimethylamino)-*N*,*N*-dimethylmethaniminium tetrafluoridoborate; HCHO, formaldehyde; AcOH, acetic acid; NaOH, sodium hydroxide; $POCl_3$, phosphoryl chloride; Ac_2O, acetic anhydride; TFAA, trifluoroacetic anhydride; CH_2Cl_2, dichloromethane; MeOH, methanol; COMU, *N*-[({[(1*Z*)-1-cyano-2-ethoxy-2-oxoethylidene]amino}oxy)(morpholin-4-yl)methylene]-*N*-methylmethanaminium hexafluorophosphate; NaH, sodium hydride; NMP, *N*-methyl-2-pyrrolidone.
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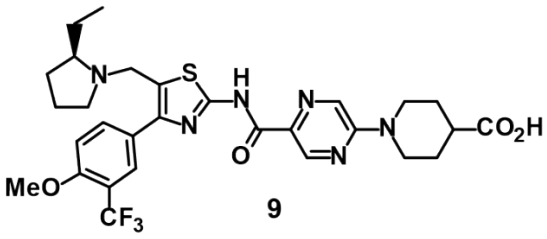
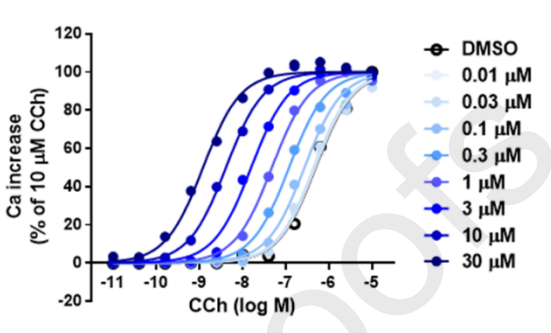
Graphical abstract



9

human mAChR PAM activity
-fold shift at 10 μM

M_1	2.3
M_2	1.3
M_3	269
M_4	1.0
M_5	94



In vitro human M_3 PAM activity