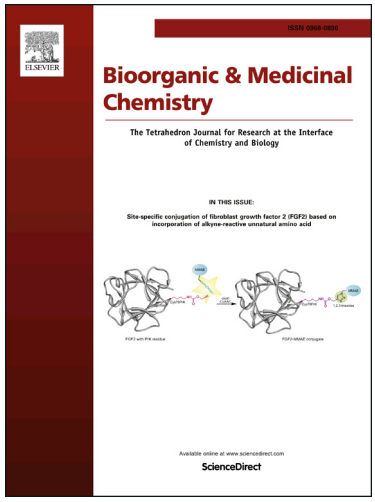
Accepted Manuscript



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Discovery of novel 5-oxa-2,6-diazaspiro[3.4]oct-6-ene derivatives as potent, selective, and orally available somatostatin receptor subtype 5 (SSTR5) antagonists for treatment of type 2 diabetes mellitus

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Abstract

Somatostatin receptor subtype 5 (SSTR5) has emerged as a novel attractive drug target for type 2 diabetes mellitus. Starting from *N*-benzyl azetidine derivatives **1** and **2** as in-house hit compounds, we explored the introduction of a carboxyl group into the terminal benzene of **1** to enhance SSTR5 antagonistic activity by the combination of the substituents at the 3-position of the isoxazoline. Incorporation of a carboxyl group at the 4-position of the benzene ring resulted in a significant enhancement in potency, however, the 4-benzoic acid derivative **10c** exhibited moderate human ether-a-go-go related gene (hERG) inhibitory activity. A subsequent optimization study revealed that replacement of the 4-benzoic acid with an isonipecotic acid dramatically reduced hERG inhibition (5.6% inhibition at 30 μM) by eliminating π–related interaction with hERG K^+ channel, which resulted in the identification of 1-(2-((2,6-diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)piperidin-4-carboxylic acid **25a** (hSSTR5 / mSSTR5 IC_{50} = 9.6 / 57 nM). Oral administration of **25a** in high-fat diet fed C57BL/6J mice augmented insulin secretion in a glucose-dependent manner and lowered blood glucose concentration.

Keywords: somatostatin, SSTR5, SSTR5 antagonist, anti-diabetic drug, hERG inhibition, OGTT

Abbreviations: SSTR5, somatostatin receptor subtype 5; OGTT, oral glucose tolerance test; hERG, human ether-a-go-go related gene; DM, diabetes mellitus; HFD, high-fat diet; NCS,

N-chlorosuccinimide; WSC, 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; Et_3N, triethylamine; MS4A, molecular sieve 4A; $LiAlH_4$, lithium aluminium hydride; ADDP, 1,1'-(azodicarbonyl)dipiperidine; $P(^nBu)_3$, tributylphosphine; SAR, structure-activity relationship; ; HBA, hydrogen bond acceptor; HBD, hydrogen bond donor; Fsp^3, fraction of sp^3 carbons; CHO, Chinese hamster ovary; HLM, human liver microsome; MLM, mouse liver microsome; KO, knock out.

1. Introduction

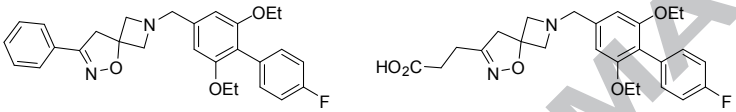
Diabetes mellitus (DM) is a disease characterized by pathologically elevated blood sugar levels due to insulin secretion failure or insulin resistance, and is known to be a risk factor for various severe complications, such as diabetic neuropathy, retinopathy and nephropathy.[1,2] The development of DM is affected by various environmental factors, such as lack of exercise, overeating, and obesity, and genetic factors.[1] The number of diabetic patients will increase as the number of obese people increases in the future. DM is classified into an insulin-dependent type 1 diabetes mellitus (T1DM) and non-insulin-dependent type 2 diabetes mellitus (T2DM), and a majority of patients with DM have T2DM. T2DM is a disease caused by decrease in the amount of β cell insulin secretion in response to glucose as well as decrease in insulin sensitivity in the peripheral tissues. T2DM is treated with drug therapy in addition to diets and exercise therapy. Examples of typical drugs presently used for therapy include insulin, insulin analogues, glucagon-like peptide-1 (GLP-1) analogues, sulfonylurea agents, biguanide agents, α-glucosidase inhibitors, PPARγ agonists, DPP-4 inhibitors, and SGLT-2 inhibitors.[3] However

some patients with T2DM have insufficient glycemic control and fail to reach target HbA1c levels.[3] To improve glycemic control for these patients, it is of considerable importance to develop novel anti-diabetic drugs that act via novel mechanisms.[1,3]

Somatostatin (SST) is a peptide hormone comprising 14 or 28 amino acids. It is widely distributed throughout the body, such as the hypothalamus, pancreatic islets of Langerhans, and intestinal mucosa, and it plays an important role in gastrointestinal motility, secretion of digestive juice, and regulation of glucose or lipid metabolism.[4,5] In particular, somatostatin inhibits production or secretion of various hormones, proliferation factors, and physiologically active substances, such as growth hormone, thyroid stimulating hormone, prolactin, insulin, glucagon, gastrin, secretin, peptide YY (PYY), gastric inhibition polypeptide (GIP), GLP-1, cholecystokinin (CCK), vasoactive intestinal peptide (VIP), and oxyntomodulin.[6–11] Furthermore, somatostatin also acts as a paracrine hormone in the islets of Langerhans or the digestive tract mucosa.[12,13] Thus, somatostatin has various endocrine system, exocrine system, and nervous system impacts.

SST acts via five distinct somatostatin receptors (SSTR1–SSTR5), which are characterized as seven-transmembrane G-protein coupled receptors.[14–16] Among them, SSTR5 is involved in the regulation of insulin and incretin secretion. It is also reported that SSTR5 knockout mice fed high-fat diets display anti-diabetic and anti-obese phenotypes.[11,17] In contrast, SSTR2 is involved in the regulation of glucagon secretion, and its inhibition results in elevation of blood-glucose level.[18] Therefore, SSTR5 selective antagonists are expected to improve hyperglycemia, hyperinsulinemia, and obesity.[19–23] On the basis of this background, we initiated a program to develop novel SSTR5 antagonists for the treatment of T2DM and obesity, and identified

N-benzyl azetidine derivatives **1** and **2** with moderate SSTR5 antagonistic activity from high-throughput screening (HTS) of our in-house compound library (Figure 1). In this article, we will report the design and synthesis of the 5-oxa-2,6-diazaspiro[3.4]oct-6-ene derivatives as new SSTR5 antagonists as well as the pharmacological effect of representative compound **25a**. In the course of this report, we will also discuss the elimination of hERG inhibition of 4-benzoic acid derivative **10c** by modification at the benzyl position of *N*-benzyl azetidine or modification of the benzene ring in the 4-benzoic acid moiety.



1
hSSTR5 / mSSTR5 IC_{50} = 2800 / 980 nM

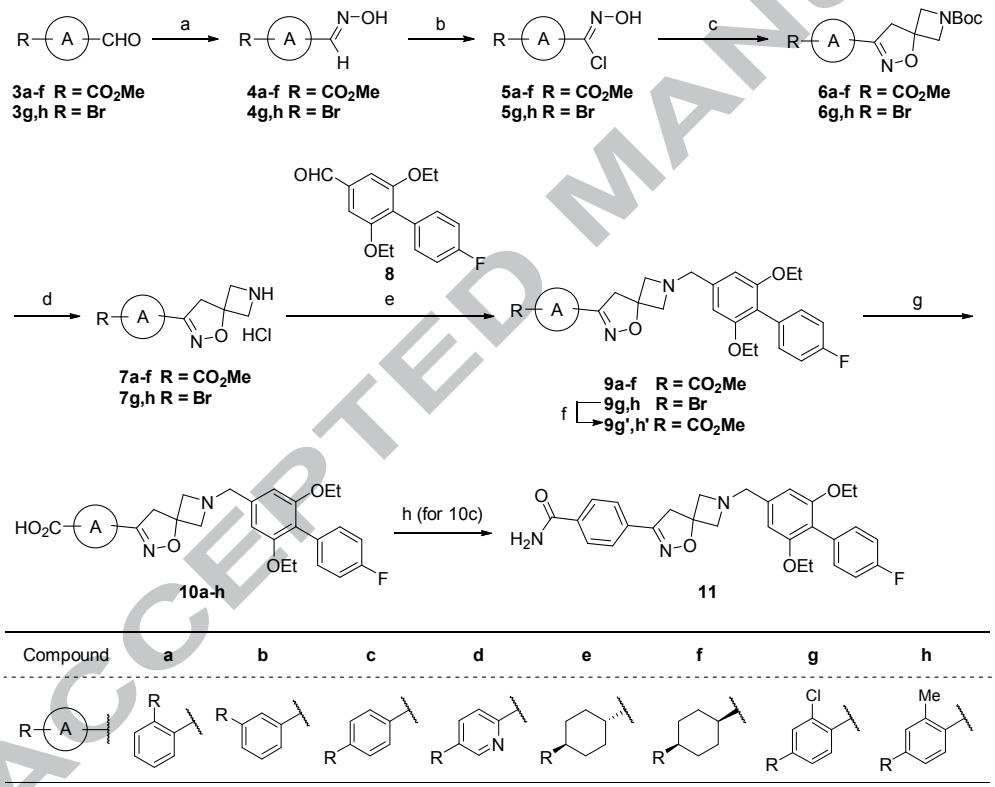
2
hSSTR5 / mSSTR5 IC_{50} = 3200 / 220 nM

Figure 1. HTS hit compounds **1** and **2**.

2. Chemistry

Scheme 1 describes the synthesis of spiroisoxazoline derivatives **10a–h**, and **11** with various substituents at the 3-position of the isoxazoline ring. The synthesis was started from the reaction of aldehydes **3a–h** with hydroxylamine, followed by chlorination by using *N*-chlorosuccinimide (NCS) to furnish intermediates **5a–h**. 1,3-Dipolar cycloaddition of **5a–h** and *tert*-butyl 3-methyleneazetidine-1-carboxylate gave spiroisoxazoline derivatives **6a–h**, which were subjected to

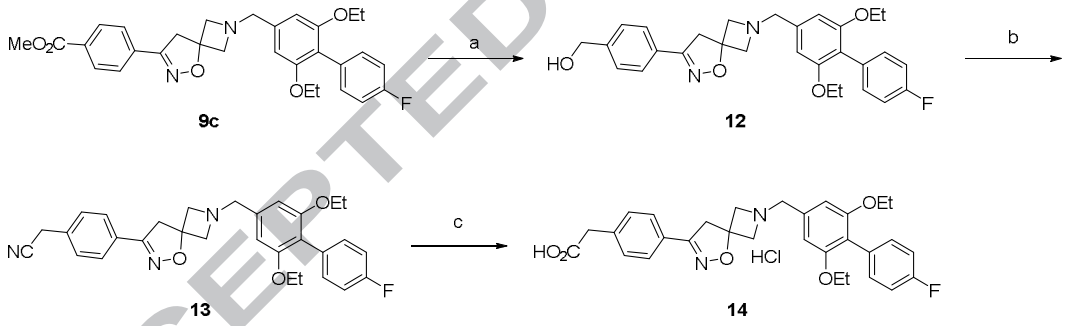
Boc deprotection under acidic conditions to yield azetidine hydrochlorides **7a–h**. Subsequent reductive amination of **7a–h** with 2,6-diethoxy-4'-fluorobiphenyl-4-carbaldehyde (**8**) provided *N*-benzyl azetidine derivatives **9a–h**. Hydrolysis reaction of **9a–f** gave carboxylic acid derivatives **10a–f**. Palladium-catalyzed carbonylative cross coupling reaction of **9g** and **9h** provided methyl ester derivatives **9g'** and **9h'**, which were subsequently hydrolyzed to yield carboxylic acid derivatives **10g** and **10h**. Carboxylic acid derivative **10c** was converted to the corresponding carboxamide **11**.



Scheme 1. Synthesis of spiroisoxazoline derivatives **10a–h**, and **11**. Reagents and conditions: (a) hydroxylamine hydrochoride, $NaHCO_3$, MeOH, rt, 35–88%; (b) NCS, DMF, rt; (c) *tert*-butyl

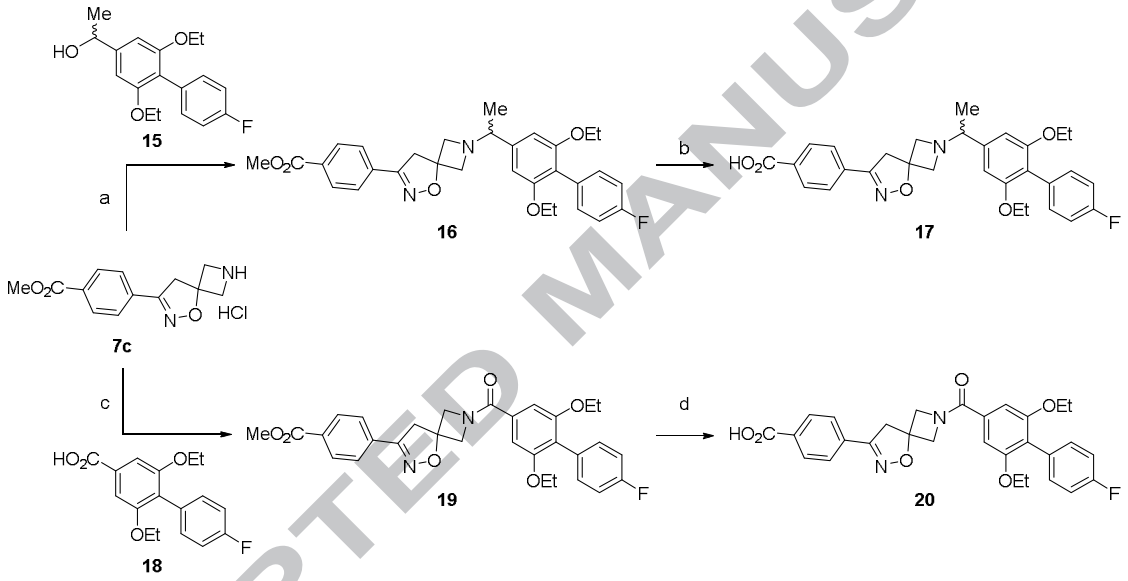
3-methyleneazetidine-1-carboxylate, Et_3N, THF, reflux; (d) 4 M HCl/AcOEt, rt, 25–84% over 3 steps; (e) **8**, $NaBH(OAc)_3$, Et_3N, THF, rt, 42–86%; (f) CO (0.5 MPa), $PdCl_2(dppf)\cdot CH_2Cl_2$, Et_3N, MeOH, DMF, 90 °C, 66–80%; (g) 1 M NaOH aq, EtOH, THF, 50 °C, 66–89%; (h) WSC, HOBt ammonia salt, DMF, rt, 66%.

The synthesis of benzyl alcohol derivative **12** and phenylacetic acid derivative **14** is described in Scheme 2. Reduction of the methyl ester of **9c** gave benzyl alcohol **12**. Mitsunobu reaction of **12** with acetone cyanohydrin furnished phenylacetonitrile **13**, which was subsequently hydrolyzed to yield the desired compound **14**.



Scheme 2. Synthesis of spiroisoxazoline derivatives **12** and **14**. Reagents and conditions: (a) $LiAlH_4$, THF, 0 °C to rt, 64%; (b) acetone cyanohydrin, ADDP, $P(^nBu)_3$, THF, rt, 75%; (c) 1 M NaOH aq, EtOH, 180 °C, microwave irradiation, then 6 M HCl, 75%.

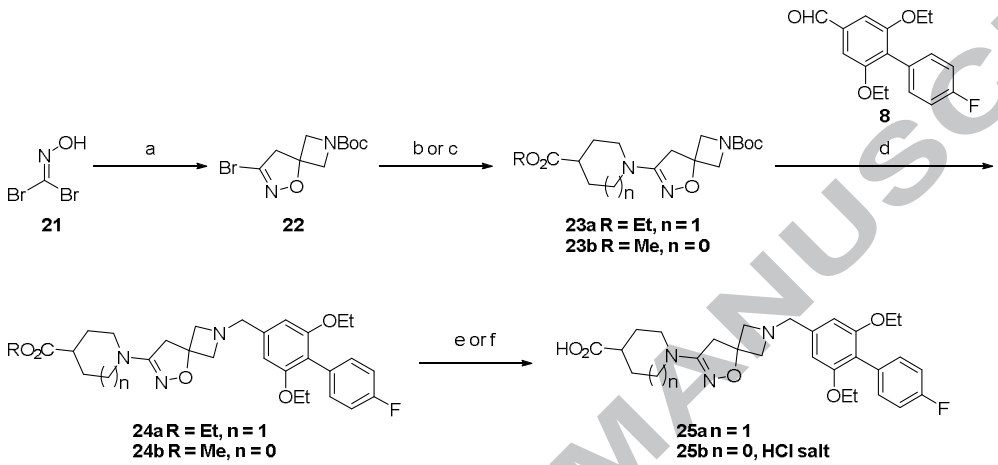
Synthesis of compounds **17** and **20** is depicted in Scheme 3. Alkylation of **7c** with benzyl chloride derived from 1-(2,6-diethoxy-4'-fluorobiphenyl-4-yl)ethanol (**15**) and subsequent hydrolysis reaction afforded the desired compound **17**. Compound **7c** was subjected to a condensation reaction with 2,6-diethoxy-4'-fluorobiphenyl-4-carboxylic acid (**18**) to give amide **19**, which was hydrolyzed to yield the desired compound **20**.



Scheme 3. Synthesis of spiroisoxazoline derivatives **17** and **20**. Reagents and conditions: (a) (i) **15**, $SOCl_2$, toluene, rt, (ii) Et_3N, DMF, 70 ^oC, 35% over 2 steps; (b) 1 M NaOH aq, EtOH, THF, 50 ^oC, 59%; (c) **18**, WSC, HOBt, Et_3N, DMF, rt, 75%; (d) 1 M NaOH aq, EtOH, THF, rt, 76%.

Synthesis of isonipecotic acid derivative **25a** and pyrrolidine-3-carboxylic acid derivative **25b** is illustrated in Scheme 4. Intermediate **22** was synthesized by 1,3-dipolar cycloaddition of *tert*-butyl

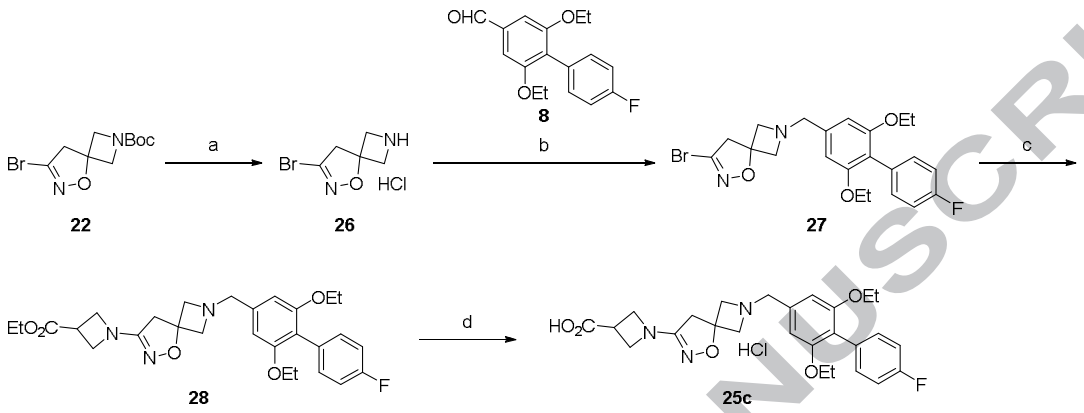
3-methyleneazetidine-1-carboxylate and 1,1-dibromoformaldoxime (**21**) and then an S_NAr reaction with cyclic amines afforded **23a** and **23b**. The desired compounds **25a** and **25b** were prepared by Boc deprotection of **23a** and **23b** and hydrolysis after reductive amination with **8**.



Scheme 4. Synthesis of spiroisoxazoline derivatives **25a** and **25b**. Reagents and conditions: (a) *tert*-butyl 3-methyleneazetidine-1-carboxylate, $NaHCO_3$, AcOEt, rt to 50 °C, 79%; (b) ethyl piperidine-4-carboxylate, Na_2CO_3, DMF, 130 °C, 80% (for **23a**); (c) methyl pyrrolidine-3-carboxylate hydrochloride, Na_2CO_3, DMF, 130 °C (for **23b**); (d) formic acid, then **8**, $NaBH(OAc)_3$, Et_3N, THF, rt, 79% (for **24a**), 11% over 2 steps (for **24b**); (e) 2 M NaOH aq, EtOH, 70 °C, 78% (for **25a**); (f) 2 M NaOH aq, EtOH, 70 °C, then 1 M HCl, 97% (for **25b**).

Scheme 5 describes the synthetic steps to prepare azetidine-3-carboxylic acid derivative **25c**. After the removal of the Boc group of intermediate **22**, reductive amination of azetidine hydrochloride **26**

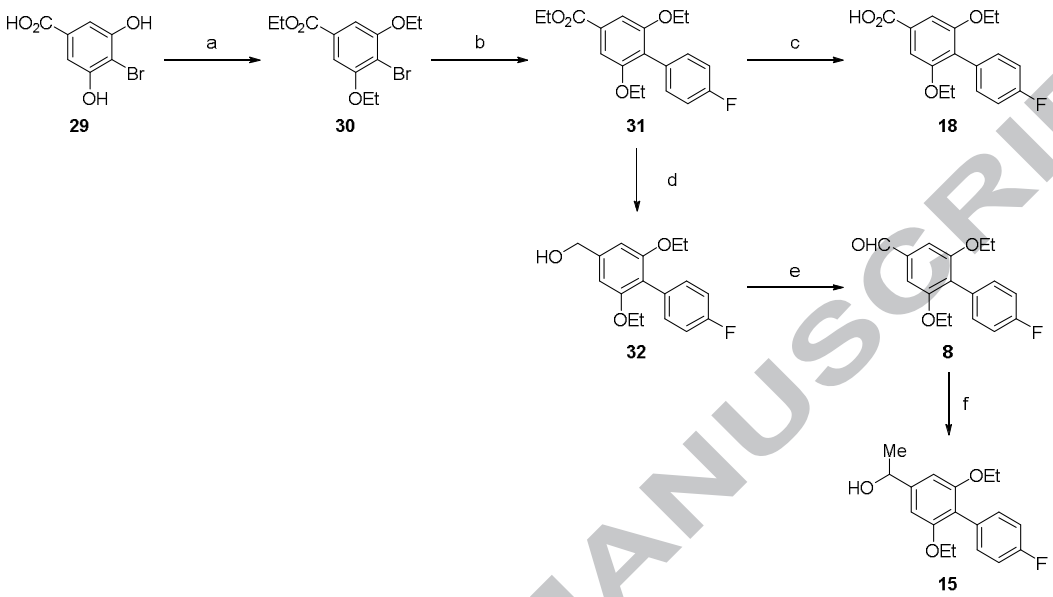
with **8** provided *N*-benzyl azetidine **27**. An S_NAr reaction of ethyl azetidine-3-carboxylate with **27** gave intermediate **28**, which was subsequently hydrolyzed to yield the desired compound **25c**.



Scheme 5. Synthesis of spiroisoxazoline derivative **25c**. Reagents and conditions: (a) 4 M HCl/AcOEt, AcOEt, rt, 60%; (b) **8**, $NaBH(OAc)_3$, Et_3N, THF, rt, 84%; (c) ethyl azetidine-3-carboxylate hydrochloride, Na_2CO_3, ^{n}BuOH, 120 ^{o}C; (d) 2 M NaOH aq, EtOH, 70 ^{o}C, then 2 M HCl, 13% over 2 steps.

Scheme 6 describes the synthesis of benzaldehyde **8**, benzyl alcohol **15**, and benzoic acid **18**. Alkylation reaction of 4-bromo-3,5-dihydroxybenzoic acid (**29**) with iodoethane, followed by palladium-catalyzed coupling reaction with (4-fluorophenyl)boronic acid gave biphenyl intermediate **31**. The hydrolysis reaction of **31** provided benzoic acid **18**. Reduction of the ethyl ester of **31** furnished alcohol **32**, which was then oxidized to give benzaldehyde **8**. Reaction of **8** with

methylmagnesium bromide furnished benzyl alcohol **15**.



Scheme 6. Synthesis of benzaldehyde **8**, benzyl alcohol **15**, and benzoic acid **18**. Reagents and conditions: (a) iodoethane, K_2CO_3, DMF, 60 ^oC, 82%; (b) (4-fluorophenyl)boronic acid, Palladium acetate, tricyclohexylphosphine, K_3PO_4, toluene, water, 90 ^oC, 87%; (c) 2M NaOH aq, MeOH, THF, 60 ^oC, 89%; (d) $LiAlH_4$, THF, 0 ^oC; (e) Sulfer trioxide-pyridine complex, Et_3N, DMSO, rt, 84% over 2 steps; (f) MeMgBr (1M in THF solution), THF, rt, 77%.

3. Results and discussion

The antagonistic activities of the compounds synthesized in this study against human SSTR5

(hSSTR5) and mouse SSTR5 (mSSTR5) were evaluated in an assay of their functional ability to alter cAMP levels in Chinese hamster ovary (CHO) cells stably expressing hSSTR5 and mSSTR5, with somatostatin 14 (SST14) serving as a ligand.

N-Benzyl azetidine derivatives **1** and **2** were identified as hit compounds with moderate SSTR5 antagonistic activity via in-house high-throughput screening (HTS). The core structure of the hit compounds was 5-oxa-2,6-diazaspiro[3.4]oct-6-ene (a unique spiro ring system), and the substituent at the 1-position of the azetidine ring was a (2,6-diethoxy-4'-fluorobiphenyl-4-yl)methyl group. Since the substituent at the 3-position of the isoxazoline ring of the hit compounds was a phenyl group or a propionic acid moiety, we hypothesized that a polar group could be introduced through a hydrophobic linker to the 3-position of the isoxazoline ring, and effective interactions of the substituent at the 3-position with the binding site of SSTR5 would enhance SSTR5 antagonistic activity. Hence, we attempted to introduce a carboxyl group into the benzene ring at the 3-position of the isoxazoline ring and replace the carboxyl group with other polar groups to discover a lead compound, such as model compound **I** (Figure 2).

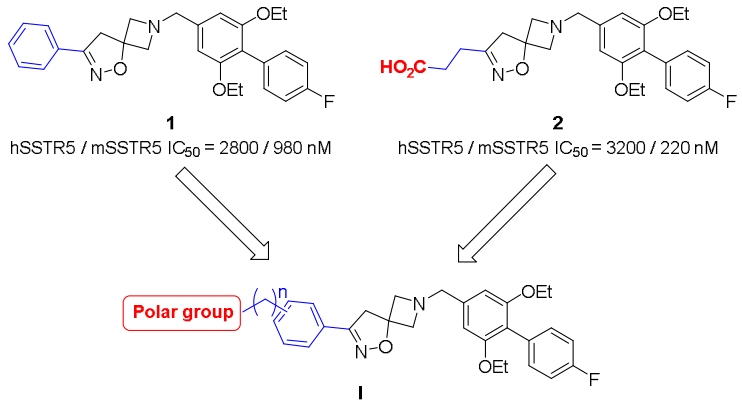


Figure 2. Design of model compound **I** from HTS hit compounds **1** and **2**.

Table 1 summarizes the SSTR5 antagonistic activity of compounds with a variety of substituents at the 3-position of the isoxazoline ring. Initially, we examined the effect of placing a carboxyl group on the terminal benzene ring. The benzoic acid derivatives from most to least potent were **10a** (*ortho*) < **10b** (*meta*) < **10c** (*para*). Notably, the 4-benzoic acid derivative **10c** exhibited dramatically enhanced SSTR5 antagonistic activity, with IC_{50} values of 9.0 and 33 nM for hSSTR5 and mSSTR5, respectively. In contrast, replacement of the 4-benzoic acid with a 4-phenyl acetic acid (**14**) caused a 25-fold decline in potency. These results suggested that the 4-position of the terminal benzene ring was the best substitution position for the carboxyl group. In addition, we assumed that the significant enhancement in potency resulted from the optimal positioning of the carboxyl group on the terminal benzene ring as well as from π–related and/or hydrophobic interactions of the benzene ring with the binding site of SSTR5.

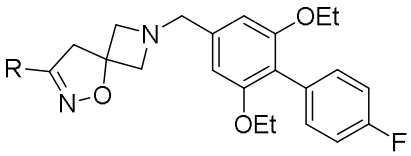
Subsequently, we examined the effect of replacing the carboxyl group with other polar groups to confirm the interaction mode of the carboxyl group of **10c** with SSTR5. On the assumption that the

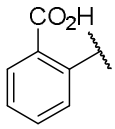
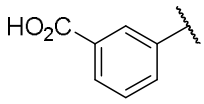
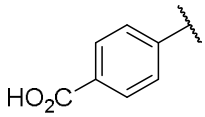
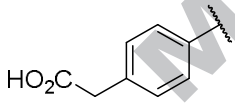
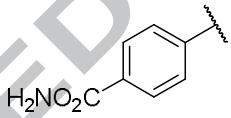
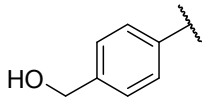
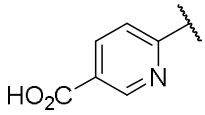
carboxyl group would form hydrogen bonds with putative amino acid residue(s) of SSTR5, we synthesized compound **11** with a carboxamide moiety as HBD (hydrogen bond donor) or HBA (hydrogen bond acceptor) and compound **12** with a hydroxymethyl group as HBD. As a result, these replacements led to profound loss of potencies. Notably, replacement with a neutral hydroxymethyl group (**12**) caused a significant decline in potency in comparison with a weakly acidic carboxamide (**11**). These results suggested that putative basic amino acid residue(s) in the binding site of SSTR5 interact with the carboxyl group of **10c** by electrostatic interaction.

Replacement of the benzene ring at the 3-position of the isoxazoline ring with a pyridine ring (**10d**) caused a 7-fold decline in hSSTR5 antagonistic activity in comparison with **10c**. Since this modification displayed limited reduction in potency in comparison with carboxamide derivative **11** and hydroxymethyl derivative **12**, we suggested that some hydrophilic structures would be tolerable for the neighborhood of the 3-position of the isoxazoline ring. Through our initial structure-activity relationship (SAR) study on a substituent at the 3-position of the isoxazoline ring, we successfully discovered 4-benzoic acid derivative **10c** as a lead compound with excellent SSTR5 antagonistic activity.

Table 1

SSTR5 antagonistic activity: SAR of the 3-position of isoxazoline ring



Compound	R	IC_{50}[a] (nM)	
		hSSTR5[b]	mSSTR5[c]
10a		>10000	>10000
10b		550 (340–910)	260 (170–390)
10c		9.0 (4.5–18)	33 (13–81)
14[d]		220 (120–390)	74 (37–150)
11		150 (110–220)	150 (110–210)
12		1000 (530–2100)	430 (250–720)
10d		61 (36–110)	20 (6.5–62)

[a]IC_{50} values were presented as the means of duplicate experiments with 95% confidence intervals in parentheses.

[b]Antagonistic activity against human SSTR5.

[c]Antagonistic activity against mouse SSTR5.

[d]Hydrochloride salt.

Because 4-benzoic acid derivative **10c** was found to exhibit moderate hERG inhibitory activity (65% inhibition at 30 μM) in our ADME/Tox study, elimination of the hERG inhibition associated with potential lethal arrhythmia was extremely important for the development of SSTR5 antagonists as anti-diabetic drugs.[24,25] Accordingly, our lead optimization study was directed toward addressing the issue of undesirable hERG inhibition.

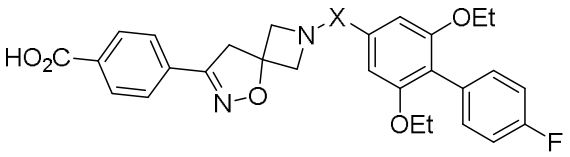
It was reported that a number of existing hERG inhibitors possessed a basic amine moiety as a positively charged group, and cation–π interaction of the basic amine with hERG K^+ channel affected hERG inhibitory activity.[26–28] On the basis of this report, we initially assumed that the basic amine of the azetidine ring caused the hERG inhibition of **10c**. Therefore, we attempted to reduce hERG inhibitory activity of **10c** by following two synthetic strategies: introduction of a methyl group at the alpha-position of the basic amine to prevent cation–π interaction by steric hindrance, or removal of basicity by amidation of the azetidine ring (Table 2).

Addition of a methyl group (**17**) on the benzyl position or amidation (**20**) of the azetidine resulted in significant reduction of hERG inhibitory activity regardless of change in lipophilicity, suggesting that these modifications would be effective for the removal of cation–π interaction of the basic amine in **10c** with the hERG K^+ channel. In contrast, given the significantly decreased potency of these compounds, we concluded that the basic amine of the azetidine ring played an important role in SSTR5 antagonistic activity as well as hERG inhibitory activity. Consequently, further modification

of the benzyl position for reduction of hERG inhibition was thought to be difficult because of the narrow SAR.

Table 2

SSTR5 antagonistic activity and hERG inhibitory activity: SAR of benzyl position of *N*-benzyl azetidine



Compound	R	IC_{50}[a] (nM)		hERG inhibition[d]	$LogD_{7.4}$[e]
		hSSTR5[b]	mSSTR5[c]	(% inhibition)	
10c		9.0 (4.5–18)	33 (13–81)	65	3.3
17		95 (46–200)	2000 (1200–3500)	14	3.6
20		>10000	>10000	7.9	2.8

[a]IC_{50} values were presented as the means of duplicate experiments with 95% confidence intervals in parentheses.

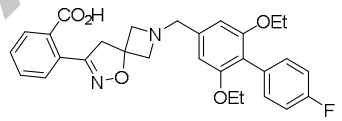
[b]Antagonistic activity against human SSTR5.

[c]Antagonistic activity against mouse SSTR5.

[d]Percent inhibition of hERG inhibitory activity at 30 μM compound concentration.

[e]The log *D* value at pH 7.4.[29]

Subsequently, we focused on the interaction of the benzoic acid with hERG K^+ channel. Although introduction of a carboxyl group into a hERG inhibitor was generally reported to attenuate hERG inhibition by electron repulsion,[30,31] the 4-benzoic acid derivative **10c** still showed moderate hERG inhibitory activity, unfortunately. In contrast, after an in-depth analysis of hERG inhibition by analogous compounds, we recognized that the 2-benzoic acid derivative **10a** exhibited a significant reduction of hERG inhibitory activity (Figure 3). Because the introduction of a carboxyl group at the 2-position of the terminal benzene ring creates torsion between the benzene and the isoxazoline ring, the 2-carboxyl group was thought to alter the conformation of the benzene ring as well as change the position of the carboxyl group from that of the 4-benzoic acid. In addition, given that the aromatic rings in existing hERG inhibitors interact with hERG K^+ channel in π–related manner, [26–28] we surmised that the reduced hERG inhibition of **10a** was due not only to the electron repulsion of the 2-carboxyl group but also to the change in benzene ring conformation. Accordingly, we attempted to reduce the hERG inhibition by introducing substituents at the 2-position of the 4-benzoic acid of **10c** to change its conformation.



10a
hERG inhibition (at 30 μM) = 4.5 % inhibition
$LogD_{7.4}$ = 3.1

Figure 3. Profiles of 2-benzoic acid derivative **10a**.

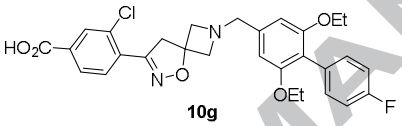
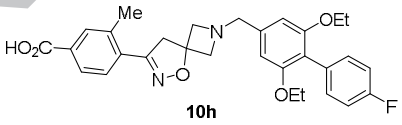
To validate the effects of the 2-substituents against hERG inhibition of **10c**, a chlorine atom (**10g**) and a methyl group (**10h**) were introduced at the 2-position of the 4-benzoic acid (Table 3). These modifications attenuated hERG inhibition relative to **10c** (**10g**: 40% inhibition, **10h**: 18% inhibition at 30 μM), and decreased SSTR5 antagonistic activity (5-fold for 10g and 15-fold for 10h). Because these reductions in hERG inhibition were not attributable to the electron-withdrawing or electron-donating effects of the 2-substituent, we hypothesized that the torsion between the benzene and isoxazoline had disrupted the interactions within hERG K^+ channel.

According to the calculation of the dihedral angle of simplified models **10c'**, **10g'**, and **10h'** by using MOE software,[32] the 4-benzoic acid and the isoxazoline ring of **10c'** positioned on the same plane, however, the introduction of a chlorine atom (**10g'**) or a methyl group (**10h'**) gave a torsion with the dihedral angle of 12 or 30 degrees, respectively (Figure 4). Relating torsion to hERG inhibition, hERG inhibitory activity was well correlated with dihedral angle, and **10h** had the largest dihedral angle and the lowest hERG inhibitory activity. From these results, we suspected that a π–related interaction occurred between the terminal benzene ring and hERG K^+ channel and that torsion between the benzene and isoxazoline could effectively prevent the interaction. Consequently, we found that the interaction with the terminal benzene ring was one of the key contributors to the hERG inhibition of **10c**. Regarding the balance between SSTR5 antagonistic activity and hERG inhibition, we found that the modification of the terminal benzene ring resulted in more limited

reduction in potency in comparison with that of the benzyl position. Accordingly, we focused on further structural modification of the terminal benzene ring to reduce hERG inhibitory activity without a loss of potency.

Table 3

SSTR5 antagonistic activity and hERG inhibitory activity: SAR of substituents at the 2-position of the 4-benzoic acid

Compound	 **10g**	 **10h**
IC_{50}[a] (nM) hSSTR5[b] / mSSTR5[c]	46 (24–90) / 31 (19–52)	140 (71–270) / 21 (14–32)
hERG inhibition[d] (% of inhibition)	40	18
$LogD_{7.4}$[e]	3.5	3.5

[a]IC_{50} values were presented as the means of duplicate experiments with 95% confidence intervals in parentheses.

[b]Antagonistic activity against human SSTR5.

[c]Antagonistic activity against mouse SSTR5.

[d]Percent inhibition of hERG inhibitory activity at 30 μM compound concentration.

[e]The log *D* value at pH 7.4.[29]

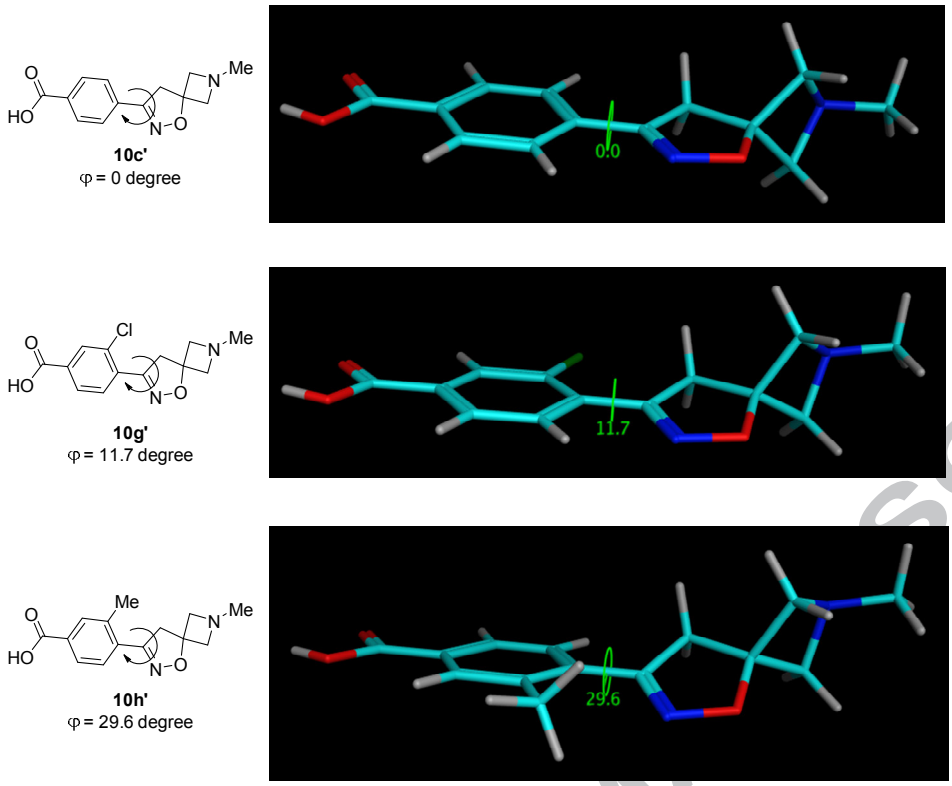


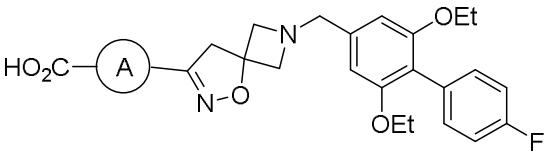
Figure 4. Dihedral angles between the benzene and the isoxazoline.

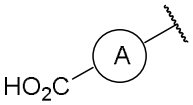
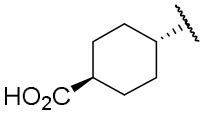
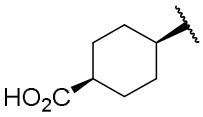
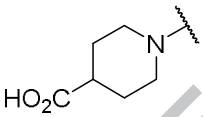
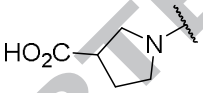
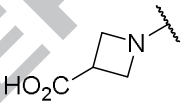
To eliminate the π–related interaction of the terminal benzene with hERG K^+ channel, we replaced a benzene ring in the 4-benzoic acid with a saturated ring (Table 4). As a result, the hERG inhibitory activity of cyclohexane derivatives **10e** and **10f**, and cyclic amine derivatives **25a–c** decreased dramatically, while maintaining a lipophilicity comparable to that of **10c**. These results were consistent with our reasoning that the terminal benzene ring of **10c** interacted with hERG K^+ channel by π–related interaction. Based on the data in Tables 3 and 4, we concluded that structural modifications, such as torsion of a benzene ring or replacement with a saturated ring, could reduce the hERG inhibitory activity of **10c** without decreasing lipophilicity.

In terms of the SSTR5 antagonistic activity, replacement of the benzene ring with a *trans*-cyclohexane (**10e**) resulted in potency comparable to that of **10c**; however, replacement with a *cis*-cyclohexane (**10f**) significantly reduced potency. These results suggested that the carboxyl group at the equatorial-position of **10e** (as observed in **10c**) is preferable to that at the axial-position of **10f**. In addition, the isonipecotic acid derivative **25a** displayed potent SSTR5 antagonistic activity as mentioned above, with IC_{50} values of 9.6 and 57 nM for hSSTR5 and mSSTR5, respectively. Conversion into a pyrrolidine (**25b**) or an azetidine (**25c**) exhibited significant decline in hSSTR5 antagonistic activities, probably because their carboxyl groups were unable to effectively interact with the basic amino acid residue(s). Consequently, we showed that replacement of the 4-benzoic acid with the isonipecotic acid dramatically reduces hERG inhibitory activity while maintaining potent SSTR5 antagonistic activity. The isonipecotic acid derivative **25a** was selected as a promising compound for further in vivo evaluation.

Table 4

SSTR5 antagonistic activity and hERG inhibitory activity: SAR of saturated rings at the 3-position of the isoxazoline ring



Compound		IC_{50}[a] (nM)		hERG inhibition[d] (% inhibition)	$LogD_{7.4}$[e]
		hSSTR5[b]	mSSTR5[c]		
10e		30 (13–67)	38 (18–81)	15	3.2
10f		130 (80–210)	210 (120–350)	33	3.3
25a		9.6 (4.6–20)	57 (33–99)	5.6	3.0
25b[f]		190 (140–270)	48 (33–69)	17	2.9
25c[f]		200 (150–260)	120 (61–240)	8.1	2.9

[a]IC_{50} values were presented as the means of duplicate experiments with 95% confidence intervals in parentheses.

[b]Antagonistic activity against human SSTR5.

[c]Antagonistic activity against mouse SSTR5.

[d]Percent inhibition of hERG inhibitory activity at 30 μM compound concentration.

[e]The log *D* value at pH 7.4.[29]

[f]Hydrochloride salt.

Because inhibition of SSTR2 was known to induce the elevation of blood-glucose level by glucagon secretion, we examined the selectivity of **25a** for SSTRs1–4 (Table 5). The binding affinities of **25a** to SSTRs1–4 were low (i.e. less than 15% inhibition at 10 μM), indicating that **25a** is a highly selective SSTR5 antagonist.

Table 5

Selectivity of **25a** over SSTRs1–4[a]

Compound	hSSTR1 (% inhibition)	hSSTR2 (% inhibition)	hSSTR3 (% inhibition)	hSSTR4 (% inhibition)
25a	11	8	14	10

[a]Inhibitory activities on SSTRs1–4 were evaluated at a drug concentration of 10 μM by radioligand binding assay.[33]

Prior to the in vivo evaluation, the selected compound **25a** was evaluated for metabolic stability, pharmacokinetic properties in mice, and solubility (Tables 6 and 7). Compound **25a** exhibited good metabolic stability toward both human and mouse microsomes. Pharmacokinetic screening in mice indicated that **25a** was orally available with acceptable plasma exposure. In addition, **25a** exhibited excellent solubility at pH 6.8 (260 μg/ml). Indeed, **25a** displayed a 200-fold higher solubility than

10c (1.3 μg/ml), suggesting that the replacement with an isonipecotic acid also contributed to the improvement of solubility. This result was connected with increase of molecular flexibility explained by the fraction of sp^3 carbons (Fsp^3 = the number of sp^3 hybridized carbons/total carbon count),[34] and **25a** showed larger Fsp^3 value than **10c** (Fsp^3 values: **10c**; 0.31, **25a**; 0.51). On the basis of these results, the isonipecotic acid derivative **25a** was found to be suitable for in vivo evaluation.

Table 6

Microsomal clearance and solubility of **25a**

Compound	in vitro CLint[a] (μL/min/mg)		Solubility at pH6.8[b] (μg/ml)
	HLM	MLM	
25a	<10	19	260

[a]Human/mouse liver microsomal clearance.

[b]The second fluid for the disintegration test is described in the Japanese Pharmacopoeia 15th edition (pH6.8).

Table 7

Pharmacokinetic profiles of **25a**[a]

Compound	iv (0.1 mg/kg)			po (1 mg/kg)			F^h (%)
	CLtotal[b] (mL/h/kg)	Vss[c] (mL/kg)	MRT_{iv}[d] (h)	Cmax[e] (ng/mL)	Tmax[f] (h)	$AUC_{0\text{-}8\ h}$[g] (ng·h/mL)	
25a	1761	3052	1.7	74.8	2.0	332	58

[a]ICR mouse (male, 8 weeks, n = 3).

[b]Total clearance.

[c]Volume of distribution at steady state.

[d]Mean residence time.

[e]Maximal plasma concentration.

[f]Time of maximal concentration.

[g]Area under the plasma concentration–time curve (0–8 h).

[h]Bioavailability.

Compound **25a** was examined for its effect on postprandial hyperglycemia in an oral glucose tolerance test (OGTT) performed in high-fat diet (HFD) fed C57BL/6J mice. Compound **25a** (100 mg/kg), Alogliptin (30 mg/kg), and Glibenclamide (10 mg/kg) were administered orally 1 h before the oral glucose load (5 g/kg), and blood glucose and insulin concentrations were monitored over 2 h. As shown in Figure 3, the maximum efficacy of **25a** was superior to that of Glibenclamide and comparable to that of Alogliptin (Figure 5A and 5B). In addition, **25a** augmented insulin secretion in a glucose-dependent manner and displayed a blood glucose-lowering effect, indicating its anti-diabetic efficacy in vivo (Figure 5C and 5D). Furthermore, **25a** did not evoke hypoglycemia induced by the administration of Glibenclamide before the oral glucose load.

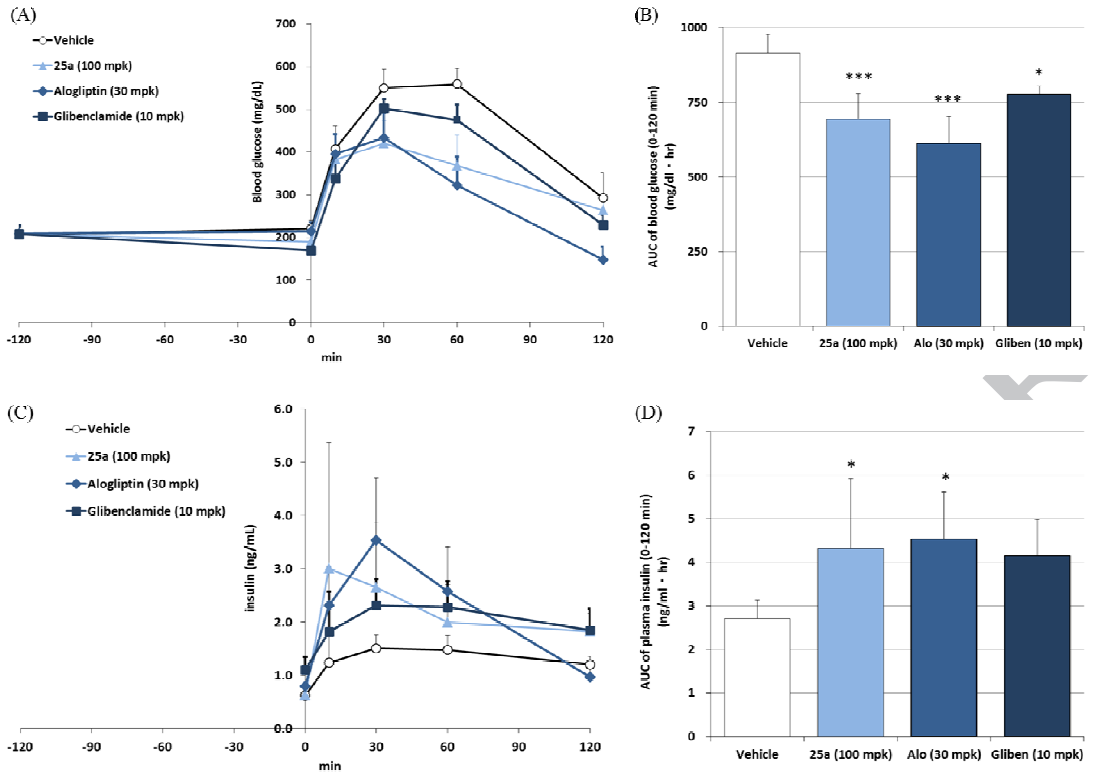


Figure 5. Effects of **25a** on glucose excursion and insulin secretion measured during oral glucose tolerance tests in HFD fed C57BL/6J mice. Compound **25a** (100 mg/kg), Alogliptin (30 mg/kg), and Glibenclamide (10 mg/kg) were orally administered 1 h before the oral glucose load (5 g/kg). (A) and (C) show time–dependent changes in blood glucose and insulin levels after a 5 g/kg glucose challenge, respectively. (B) represents the area under the curve (AUC) of blood glucose levels shown in (A). (D) represents the AUC of plasma insulin levels shown in (C). Data are presented as mean ± SD (n = 6). (*) $p < 0.05$ versus vehicle by Dunnett's test. (**) $p < 0.01$ versus vehicle by Dunnett's test. (***) $p < 0.001$ versus vehicle by Dunnett's test.

To evaluate its dose-dependent effect on the OGTT, **25a** (1, 3, 10, 30 mg/kg) was orally

administered to HFD fed C57BL/6J mice. As shown in Figure 6, the blood glucose-lowering effect of **25a** was dose-dependent detectable from 1 mg/kg and reached a maximum at 10 mg/kg. In addition, **25a** did not display blood glucose-lowering effect in OGTT in SSTR5 KO mice, indicating that the efficacy of **25a** depended on its SSTR5 antagonistic activity (data not shown). From these in vivo evaluations, we concluded that this SSTR5 antagonist is potentially an attractive anti-diabetic drug without hypoglycemic risk.

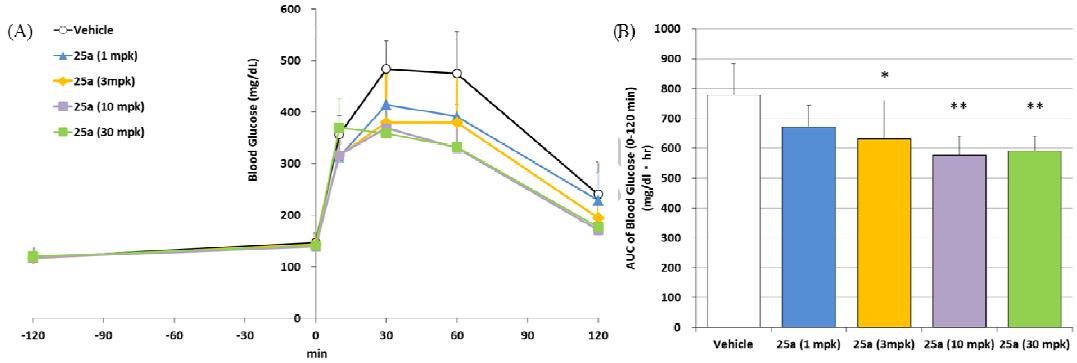


Figure 6. Dose-dependent effect of **25a** on glucose excursion measured during the oral glucose tolerance test in HFD fed C57BL/6J mice. Compound **25a** (1, 3, 10, 30 mg/kg) was orally administered. (A) shows time–dependent changes in blood glucose levels after a 5 g/kg glucose challenge. (B) represents the area under the curve (AUC) of blood glucose levels shown in (A). Data are presented as mean ± SD (n = 5). (*) $p < 0.05$ versus vehicle by Williams's test. (**) $p < 0.01$ versus vehicle by Williams's test.

4. Conclusion

Aiming to develop novel anti-diabetic drugs, we conducted synthetic studies in an effort to obtain novel and potent SSTR5 antagonists. Starting from *N*-benzyl azetidine derivatives **1** and **2** as in-house hit compounds, we introduced a carboxyl group into the terminal benzene ring of **1** to enhance SSTR5 antagonistic activity through the combination of a phenyl group and a propionic acid moiety as the substituents at the 3-position of the isoxazoline ring. As a result, incorporation of a carboxyl group at the 4-position of the benzene ring resulted in significant enhancement in potency, and the 4-carboxy group efficiently interacted with basic amino acid residue(s) in the binding site of SSTR5. Since the 4-benzoic acid derivative **10c** exhibited moderate hERG inhibitory activity in the ADME/Tox study, our optimization study addressed the issue of this undesirable hERG inhibition. Structural modifications were focused on the benzyl position of the *N*-benzyl azetidine or the benzene ring of the 4-benzoic acid. Among these modifications, increasing the torsion between the benzene and the isoxazoline ring, or replacement of the benzene ring with a saturated ring were effective strategies for eliminating π–related interaction of the terminal benzene ring with hERG K^+ channel. Notably, replacement of the 4-benzoic acid with an isonipecotic acid dramatically reduced hERG inhibition while maintaining SSTR5 antagonistic potency, which led to the discovery of a novel and potent SSTR5 antagonist **25a**. The introduction of an isonipecotic acid also significantly improved solubility at pH 6.8 by increasing molecular flexibility. In addition, **25a** displayed oral bioavailability in mice with excellent plasma exposure. Oral administration of **25a** in HFD fed C57BL/6J mice augmented insulin secretion in a glucose-dependent manner to lower blood glucose without

hypoglycemic risk. These results indicated that SSTR5 antagonist is a promising drug target for the treatment of T2DM. Further optimization efforts and pharmacological effects of this series of compounds will be described in due course.

5. Experimental section

5.1. Chemistry

Melting points were determined in open capillary tubes on a Büchi melting point apparatus B545 and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE III (300 MHz), or a Bruker Advance III plus (400 MHz) spectrometer. Chemical shifts are given in parts per million (ppm) downfield from tetramethysilane (δ) as the internal standard in deuterated solvent, and coupling constants (*J*) are in Hertz (Hz). Data are reported as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, td = triplet of doublets, qd = quartet of doublets, and brs = broad singlet), and coupling constants. Reagents and solvents were obtained from commercial sources and used without further purification. Reaction progress was determined by thin layer chromatography (TLC) analysis on Merck Kieselgel 60 F254 plates or Fuji Silysia NH plates. Chromatographic purification was performed on silica gel columns [(Merck Kieselgel 60, 70−230 mesh size or 230−400 mesh size, Merck) or (Chromatorex NH-DM 1020, 100−200 mesh size)] or on Purif-Pack (SI or NH, particle size: 60 μm, Fuji Silysia

Chemical, Ltd.). LC−MS analysis was performed on a Shimadzu Liquid Chromatography−Mass Spectrometer System, operating in APCI (+ or −) or ESI (+ or −) ionization mode. Analytes were eluted using a linear gradient of 0.05% TFA containing water/acetonitrile or 5 mM ammonium acetate containing water/acetonitrile mobile phase and detected at 220 nm. The purities of compounds submitted for biological evaluation were >95% as determined by elemental analyses within ±0.4% of the calculated values. Yields are not optimized.

5.1.1. Methyl 2-((*E*)-(hydroxyimino)methyl)benzoate (4a)

Hydroxylamine hydrochloride (1.50 g, 21.9 mmol) was added to a mixture of methyl 2-formylbenzoate (**3a**) (3.22 g, 19.6 mmol) and $NaHCO_3$ (1.81 g, 21.9 mmol) in MeOH (60 mL) at room temperature. The mixture was stirred at the same temperature for 4 h. The mixture was quenched with water at room temperature and extracted with AcOEt. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/AcOEt = 93/7 to 60/40) to give the title compound as a colorless solid (1.41 g, 7.87 mmol, 40%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.93 (3H, s), 7.42−7.49 (1H, m), 7.50−7.57 (1H, m), 7.76 (1H, s), 7.84−7.89 (1H, m), 7.94−8.01 (1H, m), 8.94 (1H, s).

5.1.2. Methyl 3-((*E*)-(hydroxyimino)methyl)benzoate (4b)

Compound **4b** was prepared from methyl 3-formylbenzoate (**3b**) in a manner similar to that

described for compound **4a** (purification: crystallization from AcOEt−hexane). Colorless crystals. Yield 53%. MS (ESI/APCI) m/z 180.1 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.94 (3H, s), 7.47 (1H, t, *J* = 7.7 Hz), 7.56 (1H, s), 7.80 (1H, d, *J* = 7.8 Hz), 8.06 (1H, d, *J* = 7.8 Hz), 8.18 (1H, s), 8.22 (1H, s).

5.1.3. Methyl 4-((*E*)-(hydroxyimino)methyl)benzoate (4c)

Compound **4c** was prepared from methyl 4-formylbenzoate (**3c**) in a manner similar to that described for compound **4a** (purification: crystallization from AcOEt−hexane). Colorless solid. Yield 60%. MS (ESI/APCI) m/z 180.1 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.93 (3H, s), 7.65 (2H, d, *J* = 8.3 Hz), 7.84 (1H, s), 8.06 (2H, d, *J* = 8.3 Hz), 8.17 (1H, s).

5.1.4. Methyl 6-((*E*)-(hydroxyimino)methyl)nicotinate (4d)

Compound **4d** was prepared from methyl 6-formylnicotinate (**3d**) in a manner similar to that described for compound **4a**. White solid. Yield 71%. MS (ESI/APCI) m/z 181.2 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.90 (3H, s), 7.93 (1H, d, *J* = 1.6 Hz), 8.16 (1H, s), 8.29 (1H, d, *J* = 1.6 Hz), 9.07 (1H, s), 12.07 (1H, s).

5.1.5. Methyl *trans*-4-((*E*)-(hydroxyimino)methyl)cyclohexanecarboxylate (4e)

Compound **4e** was prepared from methyl *trans*-4-formylcyclohexanecarboxylate (**3e**) in a manner similar to that described for compound **4a**. Colorless solid. Yield 77%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.17−1.35 (2H, m), 1.40−1.57 (2H, m), 1.88−1.99 (2H, m), 2.00−2.11 (2H, m), 2.15−2.35 (2H, m), 3.67 (3H, s), 7.33 (1H, d, *J* = 5.9 Hz), 7.60 (1H, s).

5.1.6. Methyl *cis*-4-((*E*)-(hydroxyimino)methyl)cyclohexanecarboxylate (4f)

Compound **4f** was prepared from methyl *cis*-4-formylcyclohexanecarboxylate (**3f**) in a manner similar to that described for compound **4a**. Colorless solid. Yield 79%. MS (ESI/APCI) m/z 186.1 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.57−1.75 (6H, m), 1.92−2.07 (2H, m), 2.32−2.43 (1H, m), 2.54 (1H, brs), 3.68 (3H, s), 7.37 (1H, d, *J* = 5.6 Hz), 7.67 (1H, s).

5.1.7. (*E*)-1-(4-Bromo-2-chlorophenyl)-*N*-hydroxymethanimine (4g)

Compound **4g** was prepared from 4-bromo-2-chlorobenzaldehyde (**3g**) in a manner similar to that described for compound **4a** (purification: crystallization from AcOEt−hexane). Colorless crystals. Yield 80%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.56−7.62 (1H, m), 7.75 (1H, d, *J* = 8.5 Hz), 7.81 (1H, d, *J* = 2.0 Hz), 8.31 (1H, s), 11.82 (1H, s). ^{13}C NMR (101 MHz, DMSO-d_6) δ 122.3, 127.6, 129.2, 130.1, 131.4, 132.5, 143.3.

5.1.8. (*E*)-1-(4-Bromo-2-methylphenyl)-*N*-hydroxymethanimine (4h)

Compound **4h** was prepared from 4-bromo-2-methylbenzaldehyde (**3h**) in a manner similar to that described for compound **4a** (purification: crystallization from AcOEt−hexane). Colorless crystals. Yield 88%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.37 (3H, s), 7.37−7.44 (1H, m), 7.47 (1H, d, *J* = 1.6 Hz), 7.56 (1H, d, *J* = 8.4 Hz), 8.28 (1H, s), 11.40 (1H, s). ^{13}C NMR (101 MHz, DMSO-d_6) δ 18.5, 121.5, 127.4, 128.4, 129.9, 132.6, 138.0, 145.7.

5.1.9. Methyl 2-(5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)benzoate hydrochloride (7a)

N-Chloro succinimide (1.10 g, 8.26 mmol) was added to a mixture of compound **4a** (1.41 g, 7.87

mmol) in DMF (35 mL) at room temperature. The mixture was stirred at room temperature for 1 h. The mixture was quenched with water at room temperature and extracted with Et_2O. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated under reduced pressure. Triethylamine (0.96 g, 9.44 mmol) was added to a mixture of the residue and *tert*-butyl 3-methyleneazetidine-1-carboxylate (1.59 g, 9.39 mmol) in THF (30 mL) at room temperature. The mixture was refluxed for 16 h. The mixture was quenched with saturated aqueous $NaHCO_3$ solution at room temperature and extracted with AcOEt. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/AcOEt = 75/25 to 20/80) to give a colorless oil. 4 M HCl/AcOEt (15 mL) was added to a solution of the oil in AcOEt (10 mL) at room temperature. The mixture was stirred at the same temperature for 1 h. The precipitate was collected by filtration, washed with AcOEt to give the title compound as colorless crystals (1.87 g, 6.63 mmol, 84% over 3 steps). MS (ESI/APCI) m/z 247.1 $[M + H]^+$. ^1H NMR (300 MHz, DMSO-d_6) δ 3.74 (2H, s), 3.82 (3H, s), 4.22−4.35 (4H, m), 7.52−7.57 (1H, m), 7.59−7.74 (2H, m), 7.82−7.87 (1H, m), 9.60 (2H, brs).

5.1.10. Methyl 3-(5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)benzoate hydrochloride (7b)

Compound **7b** was prepared from compounds **4b** and *tert*-butyl 3-methyleneazetidine-1-carboxylate in a manner similar to that described for compound **7a**. Colorless crystals. Yield 48% over 3 steps. MS (ESI/APCI) m/z 247.1 $[M + H]^+$. ^1H NMR (300 MHz, DMSO-d_6) δ 3.89 (3H, s), 3.92 (2H, s),

4.19–4.39 (4H, m), 7.62–7.69 (1H, m), 7.90–7.95 (1H, m), 8.03–8.09 (1H, m), 8.15–8.19 (1H, m), 9.37–9.69 (2H, m). ^{13}C NMR (101 MHz, DMSO-d_6) δ 43.7, 52.9, 58.4, 82.9, 127.5, 129.7, 130.1, 130.7, 131.4, 131.7, 157.6, 166.1. Mp 166–168 °C.

5.1.11. Methyl 4-(5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)benzoate hydrochloride (7c)

Compound **7c** was prepared from compounds **4c** and *tert*-butyl 3-methyleneazetidine-1-carboxylate in a manner similar to that described for compound **7a**. Colorless crystals. Yield 55% over 3 steps. MS (ESI/APCI) m/z 247.1 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.88 (5H, s), 4.20–4.28 (2H, m), 4.29–4.37 (2H, m), 7.79 (2H, d, J = 8.3 Hz), 8.04 (2H, d, J = 8.3 Hz), 9.43 (2H, brs). ^{13}C NMR (101 MHz, DMSO-d_6) δ 43.6, 52.8, 58.5, 83.1, 127.5, 130.1, 131.5, 133.4, 157.6, 166.1. Mp 198–200 °C.

5.1.12. Methyl 6-(5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)nicotinate dihydrochloride (7d)

Compound **7d** was prepared from compounds **4d** and *tert*-butyl 3-methyleneazetidine-1-carboxylate in a manner similar to that described for compound **7a**. White solid. Yield 25% over 3 steps. MS (ESI/APCI) m/z 248.2 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.90 (5H, s), 4.20–4.34 (4H, m), 8.03 (1H, d, J = 8.4 Hz), 8.35 (1H, dd, J = 2.0 Hz, 8.4 Hz), 9.13 (1H, d, J = 2.0 Hz), 9.57 (2H, s), 9.73 (1H, s).

5.1.13. Methyl *trans*-4-(5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)cyclohexanecarboxylate hydrochloride (7e)

Compound **7e** was prepared from compounds **4e** and *tert*-butyl 3-methyleneazetidine-1-carboxylate

in a manner similar to that described for compound **7a**. Colorless crystals. Yield 26% over 3 steps. MS (ESI/APCI) m/z 253.2 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.24−1.47 (4H, m), 1.82−1.90 (2H, m), 1.91−2.00 (2H, m), 2.25−2.40 (2H, m), 3.37 (2H, s), 3.59 (3H, s), 4.07−4.14 (2H, m), 4.15−4.22 (2H, m), 9.22 (2H, brs). ^{13}C NMR (101 MHz, DMSO-d_6) δ 28.4, 29.1, 36.1, 42.1, 44.8, 51.8, 58.4, 80.8, 163.6, 175.6. Mp 197−199 °C.

5.1.14. Methyl *cis*-4-(5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)cyclohexanecarboxylate hydrochloride (7f)

Compound **7f** was prepared from compounds **4f** and *tert*-butyl 3-methyleneazetidine-1-carboxylate in a manner similar to that described for compound **7a**. Colorless solid. Yield 49% over 3 steps. MS (ESI/APCI) m/z 253.1 $[M + H]^+$. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.52−1.72 (6H, m), 1.75−1.93 (2H, m), 2.48−2.53 (1H, m), 2.57 (1H, brs), 3.37 (2H, s), 3.60 (3H, s), 4.08−4.14 (2H, m), 4.16−4.22 (2H, m), 9.26 (2H, brs). ^{13}C NMR (101 MHz, DMSO-d_6) δ 25.9, 26.7, 34.8, 39.9, 45.2, 51.8, 58.4, 80.8, 163.1, 175.2. Mp 151−153 °C.

5.1.15. 7-(4-Bromo-2-chlorophenyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-ene hydrochloride (7g)

Compound **7g** was prepared from compounds **4g** and *tert*-butyl 3-methyleneazetidine-1-carboxylate in a manner similar to that described for compound **7a**. Colorless solid. Yield 62% over 3 steps. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.92 (2H, s), 4.17−4.40 (4H, m), 7.56−7.62 (1H, m), 7.66−7.72 (1H, m), 7.89 (1H, d, J = 1.9 Hz), 9.47 (2H, brs). ^{13}C NMR (101 MHz, DMSO-d_6) δ 46.0, 58.2, 82.8, 124.3, 127.5, 131.2, 132.7, 133.2, 133.3, 156.7. Anal. Calcd for $C_{11}H_{10}BrClN_2O \cdot HCl \cdot H_2O$: C, 37.11; H,

3.68; N, 7.87. Found: C, 37.18; H, 3.78; N, 7.90.

5.1.16. 7-(4-Bromo-2-methylphenyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-ene hydrochloride (7h)

Compound **7h** was prepared from compounds **4h** and *tert*-butyl 3-methyleneazetidine-1-carboxylate in a manner similar to that described for compound **7a**. Colorless solid. Yield 59% over 3 steps. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.45 (3H, s), 3.88 (2H, s), 4.18−4.27 (2H, m), 4.27−4.37 (2H, m), 7.38 (1H, d, J = 8.4 Hz), 7.51−7.56 (1H, m), 7.59 (1H, d, J = 1.3 Hz), 9.33 (2H, brs). ^{13}C NMR (101 MHz, DMSO-d_6) δ 21.4, 45.0, 57.3, 80.6, 122.4, 126.4, 128.4, 130.6, 133.2, 139.1, 157.1. Anal. Calcd for $C_{12}H_{13}BrN_2O{\cdot}HCl{\cdot}0.2H_2O$: C, 44.87; H, 4.52; N, 8.72. Found: C, 44.90; H, 4.79; N, 8.51.

5.1.17. Methyl

2-(2-((2,6-diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)benzoate (9a)

Triethylamine (245 mg, 2.42 mmol) was added to a mixture of compound **7a** (343 mg, 1.21 mmol) and 2,6-diethoxy-4'-fluorobiphenyl-4-carbaldehyde (**8**) (419 mg, 1.45 mmol) in THF (10 mL) at room temperature. After being stirred at room temperature for 10 min, sodium triacetoxyhydroborate (514 mg, 2.42 mmol) was added to the reaction mixture. The mixture was stirred at room temperature for 16 h. The mixture was quenched with saturated aqueous $NaHCO_3$ solution at room temperature and extracted with AcOEt. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/AcOEt = 25/75 to 0/100) to give the title compound as a colorless

amorphous solid (438 mg, 0.84 mmol, 70%). MS (ESI/APCI) m/z 519.2 $[M + H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.24 (6H, t, *J* = 7.0 Hz), 3.52–3.59 (4H, m), 3.65–3.72 (4H, m), 3.88 (3H, s), 3.97 (4H, q, *J* = 7.0 Hz), 6.58 (2H, s), 6.99-7.09 (2H, m), 7.29–7.37 (2H, m), 7.41–7.46 (1H, m), 7.46–7.60 (2H, m), 7.94 (1H, dd, *J* = 7.7, 1.4 Hz).

5.1.18. Methyl

3-(2-((2,6-diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)benzoate (9b)

Compound **9b** was prepared from compounds **7b** and **8** in a manner similar to that described for compound **9a**. Colorless solid. Yield 85%. MS (ESI/APCI) m/z 519.2 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25 (6H, t, *J* = 7.0 Hz), 3.55 (2H, d, *J* = 9.0 Hz), 3.63–3.69 (4H, m), 3.73 (2H, s), 3.92–4.02 (7H, m), 6.58 (2H, s), 7.04 (2H, t, *J* = 8.8 Hz), 7.33 (2H, dd, *J* = 8.6, 5.7 Hz), 7.49 (1H, t, *J* = 7.8 Hz), 7.94 (1H, d, *J* = 7.9 Hz), 8.09 (1H, d, *J* = 7.8 Hz), 8.23 (1H, s). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 43.9, 52.9, 63.3, 64.2, 66.9, 81.9, 106.0, 114.5 (d, *J* = 21.3 Hz), 117.3, 127.4, 129.9, 130.3, 130.7, 130.7, 131.0, 131.6, 133.2 (d, *J* = 8.1 Hz), 139.9, 156.7, 157.1, 161.3 (d, *J* = 242.8 Hz), 166.2. Mp 147–148 °C.

5.1.19. Methyl

4-(2-((2,6-diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)benzoate (9c)

Compound **9c** was prepared from compounds **7c** and **8** in a manner similar to that described for

compound **9a**. Colorless solid. Yield 78%. MS (ESI/APCI) m/z 519.2 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25 (6H, t, *J* = 7.0 Hz), 3.50–3.56 (2H, m), 3.60–3.67 (4H, m), 3.70 (2H, s), 3.91–4.01 (7H, m), 6.57 (2H, s), 7.04 (2H, t, *J* = 8.8 Hz), 7.33 (2H, dd, *J* = 8.7, 5.7 Hz), 7.72 (2H, d, *J* = 8.5 Hz), 8.07 (2H, d, *J* = 8.5 Hz). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 43.7, 52.8, 63.3, 64.2, 66.9, 82.1, 106.0, 114.5 (d, *J* = 21.3 Hz), 117.3, 127.3, 130.0, 130.7 (d, *J* = 2.9 Hz), 131.2, 133.2 (d, *J* = 8.1 Hz), 134.1, 139.9, 156.7, 157.2, 161.3 (d, *J* = 242.5 Hz), 166.2. Mp 160–161 °C. Anal. Calcd for $C_{30}H_{31}FN_2O_5$: C, 69.48; H, 6.03; N, 5.40. Found: C, 69.49; H, 5.96; N, 5.34.

5.1.20. Methyl

6-(2-((2,6-diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)nicotinate (9d)

Compound **9d** was prepared from compounds **7d** and **8** in a manner similar to that described for compound **9a**. White solid. Yield 55%. MS (ESI/APCI) m/z 520.5 $[M + H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.25 (6H, t, *J* = 7.0 Hz), 3.48–3.65 (4H, m), 3.71 (2H, s), 3.78 (2H, s), 3.89–4.08 (7H, m), 6.58 (2H, s), 7.04 (2H, t, *J* = 8.9 Hz), 7.28–7.39 (2H, m), 8.07 (1H, dd, *J* = 8.3, 0.8 Hz), 8.31 (1H, dd, *J* = 8.3, 2.1 Hz), 9.20 (1H, dd, *J* = 2.1, 0.9 Hz).

5.1.21. Methyl

***trans*-4-(2-((2,6-diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct -6-en-7-yl)cyclohexanecarboxylate (9e)**

Compound **9e** was prepared from compounds **7e** and **8** in a manner similar to that described for

compound **9a** (purification: silica gel column chromatography (AcOEt/MeOH = 100/0 to 70/30)). Colorless solid. Yield 79%. MS (ESI/APCI) m/z 525.3 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.24 (6H, t, *J* = 6.9 Hz), 1.31−1.43 (2H, m), 1.44−1.57 (2H, m), 1.95−2.03 (2H, m), 2.04−2.13 (2H, m), 2.24−2.43 (2H, m), 3.20 (2H, s), 3.41 (2H, d, *J* = 8.8 Hz), 3.51 (2H, d, *J* = 8.7 Hz), 3.63−3.70 (5H, m), 3.96 (4H, q, *J* = 6.9 Hz), 6.54 (2H, s), 7.04 (2H, t, *J* = 8.8 Hz), 7.32 (2H, dd, *J* = 8.5, 5.7 Hz).

5.1.22. Methyl *cis*-4-(2-((2,6-diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)cyclohexanecarboxylate (9f)

Compound **9f** was prepared from compounds **7f** and **8** in a manner similar to that described for compound **9a**. Colorless oil. Yield 73%. MS (ESI/APCI) m/z 525.6 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.24 (6H, t, *J* = 7.0 Hz), 1.60−1.75 (5H, m), 2.04 (3H, brs), 2.45−2.53 (1H, m), 2.57 (1H, brs), 3.19 (2H, s), 3.42 (2H, d, *J* = 8.2 Hz), 3.54 (2H, d, *J* = 8.3 Hz), 3.68 (5H, s), 3.96 (4H, q, *J* = 6.9 Hz), 6.54 (2H, s), 7.00−7.07 (2H, m), 7.29−7.36 (2H, m).

5.1.23. 7-(4-Bromo-2-chlorophenyl)-2-((2,6-diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-ene (9g)

Compound **9g** was prepared from compounds **7g** and **8** in a manner similar to that described for compound **9a**. Colorless solid. Yield 86%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.15 (6H, t, *J* = 6.9 Hz), 3.37 (2H, d, *J* = 8.9 Hz), 3.57 (2H, d, *J* = 8.9 Hz), 3.63 (2H, s), 3.78 (2H, s), 3.96 (4H, q, *J* = 6.9 Hz),

6.64 (2H, s), 7.10–7.19 (2H, m), 7.24–7.33 (2H, m), 7.56–7.61 (1H, m), 7.63–7.70 (1H, m), 7.87 (1H, d, J = 1.9 Hz). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 46.2, 63.3, 64.2, 66.7, 81.9, 106.0, 114.5 (d, J = 21.3 Hz), 117.3, 124.0, 128.3, 130.7 (d, J = 2.9 Hz), 131.1, 132.5, 133.2, 133.2 (d, J = 8.1 Hz), 133.3, 139.8, 156.3, 156.7, 161.3 (d, J = 242.1 Hz). Mp 125–126 °C. Anal. Calcd for $C_{28}H_{27}BrClFN_2O_3$: C, 58.60; H, 4.74; N,4.88. Found: C, 58.51; H, 5.00; N, 4.88.

5.1.24.

7-(4-Bromo-2-methylphenyl)-2-((2,6-diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-ene (**9h**)

Compound **9h** was prepared from compounds **7h** and **8** in a manner similar to that described for compound **9a**. Colorless solid. Yield 83%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.16 (6H, t, J = 6.9 Hz), 2.45 (3H, s), 3.36 (2H, d, J = 8.7 Hz), 3.56 (2H, d, J = 8.7 Hz), 3.63 (2H, s), 3.76 (2H, s), 3.96 (4H, q, J = 7.0 Hz), 6.65 (2H, s), 7.10–7.20 (2H, m), 7.23–7.33 (2H, m), 7.38–7.45 (1H, m), 7.47–7.54 (1H, m), 7.57 (1H, d, J = 1.5 Hz). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 22.5, 46.3, 63.3, 64.2, 66.9, 80.6, 106.0, 114.5 (d, J = 21.3 Hz), 117.3, 123.1, 128.2, 129.4, 130.7 (d, J = 3.7 Hz), 131.6, 133.2 (d, J = 7.3 Hz), 134.2, 139.9, 140.1, 156.7, 157.9, 161.3 (d, J = 242.1 Hz). Mp 153–154 °C. Anal. Calcd for $C_{29}H_{30}BrFN_2O_3$: C, 62.93; H, 5.46; N, 5.06. Found: C, 62.96; H, 5.58; N, 5.06.

5.1.25. Methyl

3-chloro-4-(2-((2,6-diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)benzoate (**9g'**)

[1,1'-Bis(diphenylphosphino)ferrocene]palladium(II) dichloride dichloromethane adduct (64.0 mg, 0.08 mmol) was added to a mixture of compound **9g** (300 mg, 0.52 mmol), triethylamine (0.146 mL, 1.05 mmol) and methanol (0.635 mL, 15.68 mmol) in DMF (10 mL) at room temperature. The mixture was stirred at 90 °C under CO atmosphere of 0.5 MPa for 6 h. The mixture was quenched with water at room temperature and extracted with AcOEt. The organic layer was separated, washed with water and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/AcOEt = 55/45 to 10/90) to give the title compound as a colorless solid (230 mg, 0.416 mmol, 80 %). MS (ESI/APCI) m/z 553.2 $[M + H]^+$. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.16 (6H, t, J = 6.9 Hz), 3.39 (2H, d, J = 8.8 Hz), 3.59 (2H, d, J = 8.9 Hz), 3.64 (2H, s), 3.83 (2H, s), 3.89 (3H, s), 3.96 (4H, q, J = 6.9 Hz), 6.65 (2H, s), 7.10–7.19 (2H, m), 7.24–7.32 (2H, m), 7.81 (1H, d, J = 8.2 Hz), 7.93–7.99 (1H, m), 8.03 (1H, d, J = 1.4 Hz). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 46.1, 53.2, 63.3, 64.2, 66.7, 82.2, 106.0, 114.5 (d, J = 20.5 Hz), 117.3, 128.3, 130.7 (d, J = 2.9 Hz), 131.3, 131.6, 132.4, 133.2, 133.2, 133.3, 139.8, 156.4, 156.7, 161.3 (d, J = 242.1 Hz), 165.1. Anal. Calcd for $C_{30}H_{30}ClFN_2O_5$: C, 65.16; H, 5.47; N, 5.07. Found: C, 65.20; H, 5.62; N, 5.08.

5.1.26. Methyl

4-(2-((2,6-diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)-3-methylbenzoate (9h')

Compound **9h'** was prepared from compound **9h** in a manner similar to that described for

compound **9g'**. colorless solid. Yield 66%. MS (ESI/APCI) m/z 533.2 $[M + H]^+$. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.16 (6H, t, J = 6.9 Hz), 2.50 (3H, s), 3.38 (2H, d, J = 8.8 Hz), 3.58 (2H, d, J = 8.8 Hz), 3.64 (2H, s), 3.81 (2H, s), 3.87 (3H, s), 3.96 (4H, q, J = 6.9 Hz), 6.65 (2H, s), 7.10−7.19 (2H, m), 7.24−7.32 (2H, m), 7.62 (1H, d, J = 8.1 Hz), 7.83−7.88 (1H, m), 7.89 (1H, s).^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 22.8, 46.2, 52.7, 63.3, 64.2, 66.8, 80.9, 106.0, 114.5 (d, J = 21.3 Hz), 117.3, 127.2, 130.1, 130.4, 130.7 (d, J = 3.7 Hz), 132.2, 133.2 (d, J = 7.3 Hz), 133.3, 138.0, 139.9, 156.7, 158.1, 161.3 (d, J = 242.1 Hz), 166.2. Anal. Calcd for $C_{31}H_{33}FN_2O_5$: C, 69.91; H, 6.25; N, 5.26. Found: C, 69.83; H, 6.28; N,5.22.

5.1.27.

2-(2-((2,6-Diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)benzoic acid (10a)

1 M NaOH aqueous solution (4 ml, 4.00 mmol) was added to a solution of compund **9a** (401 mg, 0.77 mmol) in a mixed solvent of THF (5 mL) and EtOH (5 mL) at room temperature. The mixture was stirred at 50 °C for 2 h and concentrated under reduced pressure. The residue was dissolved in water. The solution was adjusted to neutral with 1 M HCl. The precipitate was collected by filtration, and washed with water. The crystals was dissoleved in EtOH. After filtration, the filtrate was concentrated under reduced pressure. The residue was crystallized from EtOH–hexane to give the title compound as colorless crystals (300 mg, 0.59 mmol, 77%). MS (ESI/APCI) m/z 505.2 $[M + H]^+$. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.14 (6H, t, J = 6.9 Hz), 3.52 (2H, d, J = 8.7 Hz), 3.59 (2H, s),

3.67−3.77 (4H, m), 3.95 (4H, q, *J* = 7.0 Hz), 6.68 (2H, s), 7.10−7.19 (2H, m), 7.23−7.32 (2H, m), 7.45 (1H, dd, *J* = 7.3, 1.5 Hz), 7.50−7.63 (2H, m), 7.82−7.88 (1H, m). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 47.6, 61.6, 64.3, 65.9, 80.6, 106.5, 114.5 (d, *J* = 20.5 Hz), 117.8, 129.8, 130.1, 130.3, 130.4, 130.5 (d, *J* = 2.9 Hz), 131.5, 133.2 (d, *J* = 8.1 Hz), 133.8, 137.6, 156.7, 160.3, 161.3 (d, *J* = 242.8 Hz), 169.3. Mp 207−208 °C. Anal. Calcd for $C_{29}H_{29}FN_2O_5$: C, 69.03; H, 5.79; N, 5.55. Found: C, 68.76; H, 5.84; N,5.43.

5.1.28.

3-(2-((2,6-Diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)benzoic acid (10b)

Compound **10b** was prepared from compound **9b** in a manner similar to that described for compound **10a** (purification: crystallization from AcOEt−hexane). Colorless crystals. Yield 75%. MS (ESI/APCI) m/z 505.2 $[M + H]^+$. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.16 (6H, t, *J* = 7.0 Hz), 3.39 (2H, d, *J* = 8.7 Hz), 3.59 (2H, d, *J* = 8.8 Hz), 3.65 (2H, s), 3.79 (2H, s), 3.97 (4H, q, *J* = 7.0 Hz), 6.66 (2H, s), 7.10−7.19 (2H, m), 7.25−7.32 (2H, m), 7.56−7.63 (1H, m), 7.87−7.93 (1H, m), 7.99−8.04 (1H, m), 8.18−8.21 (1H, m).^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 43.9, 63.2, 64.2, 66.9, 81.8, 106.0, 114.5 (d, *J* = 20.5 Hz), 117.3, 127.6, 129.7, 130.1, 130.7 (d, *J* = 2.9 Hz), 131.0, 131.2, 132.2, 133.2 (d, *J* = 8.1 Hz), 139.7, 156.7, 157.3, 161.3 (d, *J* = 242.1 Hz), 167.3. Mp 223−224 °C. Anal. Calcd for $C_{29}H_{29}FN_2O_5 \cdot 0.1H_2O$: C, 68.79; H, 5.81; N, 5.53. Found: C, 68.78; H, 5.79; N, 5.46.

5.1.29.

4-(2-((2,6-Diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)benzoic acid (10c)

Compound **10c** was prepared from compound **9c** in a manner similar to that described for compound **10a** (purification: crystallization from EtOH–AcOEt–hexane). Colorless crystals. Yield 87%. MS (ESI/APCI) m/z 505.2 $[M + H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.22 (6H, t, *J* = 7.0 Hz), 3.84 (2H, s), 3.89–4.02 (6H, m), 4.11 (2H, s), 4.33 (2H, d, *J* = 10.6 Hz), 6.73 (2H, s), 7.00–7.08 (2H, m), 7.27–7.33 (2H, m), 7.70 (2H, d, *J* = 8.5 Hz), 8.08 (2H, d, *J* = 8.5 Hz). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 43.8, 61.9, 64.3, 66.5, 81.6, 106.5, 114.5 (d, *J* = 21.3 Hz), 117.8, 127.2, 130.2, 130.5 (d, *J* = 3.7 Hz), 132.6, 133.2 (d, *J* = 8.1 Hz), 133.4, 137.9, 156.7, 157.4, 161.3 (d, *J* = 242.1 Hz), 167.3. Mp 172–173 °C. Anal. Calcd for $C_{29}H_{29}FN_2O_5 \cdot 0.8H_2O$: C, 67.12; H, 5.94; N, 5.40. Found: C, 67.08; H, 5.97; N, 5.24.

5.1.30.

6-(2-((2,6-Diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)nicotinic acid (10d)

Compound **10d** was prepared from compound **9d** in a manner similar to that described for compound **10a**. Colorless crystals. Yield 89%. MS (ESI/APCI) m/z 506.2 $[M + H]^+$. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.16 (6H, t, *J* = 6.9 Hz), 3.47 (2H, d, *J* = 8.9 Hz), 3.65 (2H, d, *J* = 9.0 Hz), 3.69 (2H, s), 3.78 (2H, s), 3.97 (4H, q, *J* = 6.9 Hz), 6.67 (2H, s), 7.08–7.21 (2H, m), 7.22–7.34 (2H, m), 8.01 (1H, dd, *J* = 8.3, 0.8 Hz), 8.32 (1H, dd, *J* = 8.3, 2.2 Hz), 9.11 (1H, dd, *J* = 2.1, 0.8 Hz). ^{13}C NMR (101 MHz,

DMSO-d_6) δ 15.0, 43.6, 62.7, 64.2, 66.7, 82.6, 106.1, 114.5 (d, *J* = 20.5 Hz), 117.5, 121.4, 128.3, 130.7 (d, *J* = 2.9 Hz), 133.2 (d, *J* = 8.1 Hz), 138.1, 139.1, 150.7, 151.9, 156.7, 159.0, 161.3 (d, *J* = 242.8 Hz), 166.5. Mp 193−194 °C. Anal. Calcd for $C_{28}H_{28}FN_3O_5 \cdot 0.4H_2O$: C, 65.59; H, 5.66; N, 8.20. Found: C, 65.85; H, 5.64; N, 8.12.

5.1.31.

***trans*-4-(2-((2,6-Diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl) cyclohexanecarboxylic acid (10e)**

Compound **10e** was prepared from compound **9e** in a manner similar to that described for compound **10a**. Colorless crystals. Yield 81%. MS (ESI/APCI) m/z 511.3 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.15 (6H, t, *J* = 7.0 Hz), 1.23−1.42 (4H, m), 1.82−1.89 (2H, m), 1.90−1.98 (2H, m), 2.12−2.22 (1H, m), 2.25−2.36 (1H, m), 3.20−3.27 (4H, m), 3.44 (2H, d, *J* = 8.3 Hz), 3.58 (2H, s), 3.95 (4H, q, *J* = 6.9 Hz), 6.61 (2H, s), 7.11−7.18 (2H, m), 7.23−7.31 (2H, m), 12.06 (1H, brs). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 28.5, 29.3, 36.5, 42.4, 45.0, 63.3, 64.2, 66.9, 79.6, 106.0, 114.5 (d, *J* = 21.3 Hz), 117.3, 130.7 (d, *J* = 2.9 Hz), 133.2 (d, *J* = 8.1 Hz), 139.9, 156.7, 161.3 (d, *J* = 242.8 Hz), 163.2, 176.9. Mp 174−175 °C. Anal. Calcd for $C_{29}H_{35}FN_2O_5 \cdot 0.2H_2O$: C, 67.74; H, 6.94; N, 5.45. Found: C, 67.78; H, 7.01; N, 5.34.

5.1.32.

***cis*-4-(2-((2,6-Diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)cy clohexanecarboxylic acid (10f)**

Compound **10f** was prepared from compound **9f** in a manner similar to that described for compound **10a** (purification: crystallization from AcOEt–hexane). Colorless crystals. Yield 66%. MS (ESI/APCI) m/z 511.3 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.15 (6H, t, J = 6.8 Hz), 1.24–1.43 (1H, m), 1.50–1.71 (5H, m), 1.75–1.91 (2H, m), 2.42–2.50 (2H, m), 3.24 (2H, brs), 3.31 (2H, brs), 3.46 (2H, brs), 3.61 (2H, brs), 3.95 (4H, q, J = 6.7 Hz), 6.63 (2H, brs), 7.14 (2H, t, J = 8.7 Hz), 7.23–7.32 (2H, m), 12.08 (1H, brs). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 26.1, 26.9, 35.2, 40.0, 45.4, 62.9, 64.2, 66.8, 79.5, 106.1, 114.5 (d, J = 20.5 Hz), 117.4, 130.7 (d, J = 3.7 Hz), 133.2, 133.3, 156.7, 161.3 (d, J = 242.1 Hz), 162.7, 176.5. Mp 161–162 °C. Anal. Calcd for $C_{29}H_{35}FN_2O_5 \cdot 0.3H_2O$: C, 67.50; H, 6.95; N, 5.43. Found: C, 67.49; H, 6.92; N, 5.40.

5.1.33.

3-Chloro-4-(2-((2,6-diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)benzoic acid (**10g**)

Compound **10g** was prepared from compound **9g'** in a manner similar to that described for compound **10a**. Colorless crystals. Yield 74%. MS (ESI/APCI) m/z 539.2 $[M + H]^+$. ^{1}H NMR (300 MHz, DMSO-d_6) δ1.16 (6H, t, J = 6.9 Hz), 3.45 (2H, d, J = 8.9 Hz), 3.63 (2H, d, J = 8.9 Hz), 3.68 (2H, s), 3.83 (2H, s), 3.96 (4H, q, J = 6.9 Hz), 6.66 (2H, s), 7.10–7.20 (2H, m), 7.23–7.33 (2H, m), 7.77 (1H, d, J = 8.0 Hz), 7.91–7.96 (1H, m), 8.00 (1H, d, J = 1.5 Hz). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 46.2, 62.7, 64.2, 66.5, 81.9, 106.2, 114.5 (d, J = 21.3 Hz), 117.5, 128.4, 130.6 (d, J = 3.7 Hz), 131.4, 132.2, 132.4, 133.2, 133.3, 134.6, 139.0, 156.6, 156.7, 161.3 (d, J = 242.1 Hz), 166.3. Mp

181−182 °C. Anal. Calcd for $C_{29}H_{28}ClFN_2O_5 \cdot 0.6H_2O$: C, 63.35; H, 5.35; N, 5.10. Found: C, 63.31; H, 5.48; N, 4.97.

5.1.34.

4-(2-((2,6-Diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)-3-methylbenzoic acid (10h)

Compound **10h** was prepared from compound **9h'** in a manner similar to that described for compound **10a**. Colorless crystals. Yield 82%. MS (ESI/APCI) m/z 519.3 $[M + H]^+$. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.16 (6H, t, *J* = 6.9 Hz), 2.51 (3H, s), 3.41 (2H, d, *J* = 8.3 Hz), 3.61 (2H, d, *J* = 8.4 Hz), 3.66 (2H, s), 3.81 (2H, s), 3.97 (4H, q, *J* = 7.0 Hz), 6.66 (2H, s), 7.10-7.20 (2H, m), 7.24−7.33 (2H, m), 7.59 (1H, d, ***J*** = 8.0 Hz), 7.81-7.85 (1H, m), 7.86 (1H, s). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 22.9, 46.3, 63.1, 64.2, 66.8, 80.8, 106.1, 114.5 (d, *J* = 21.3 Hz), 117.4, 127.3, 129.9, 130.7 (d, *J* = 3.7 Hz), 131.8, 132.4, 132.8, 133.2 (d, *J* = 8.1 Hz), 137.7, 139.5, 156.7, 158.2, 161.3 (d, *J* = 242.8 Hz), 167.4. Mp 203−204 °C. Anal. Calcd for $C_{30}H_{31}FN_2O_5 \cdot 0.2H_2O$: C, 69.00; H, 6.06; N, 5.36. Found: C, 69.01; H, 6.08; N, 5.36.

5.1.35.

4-(2-((2,6-Diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)benzamide (11)

1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (161 mg, 0.84 mmol) was added to a mixture of compound **10c** (212.3 mg, 0.42 mmol) and *1H*-benzo[*d*][1,2,3]triazol-1-ol ammonia salt

(128 mg, 0.84 mmol) in DMF (8 mL) at room temperature. The mixture was stirred at the same temperature under N_2 atmosphere for 20 h. The mixture was quenched with saturated aqueous $NaHCO_3$ solution at room temperature and extracted with AcOEt. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by NH silica gel column chromatography (AcOEt/MeOH = 100/0 to 50/50) and crystallized from EtOH–hexane to give the title compound as colorless crystals (138.8 mg, 0.276 mmol, 66%). MS (ESI/APCI) m/z 504.3 $[M + H]^+$. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.16 (6H, t, J = 6.9 Hz), 3.37 (2H, d, J = 8.7 Hz), 3.57 (2H, d, J = 8.7 Hz), 3.64 (2H, s), 3.76 (2H, s), 3.96 (4H, q, J = 6.9 Hz), 6.65 (2H, s), 7.10–7.19 (2H, m), 7.24–7.32 (2H, m), 7.46 (1H, s), 7.74 (2H, d, J = 8.6 Hz), 7.94 (2H, d, J = 8.6 Hz), 8.06 (1H, s). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 44.0, 63.3, 64.2, 67.0, 81.8, 106.0, 114.5 (d, J = 21.3 Hz), 117.3, 126.9, 128.4, 130.7 (d, J = 2.9 Hz), 132.2, 133.2 (d, J = 8.1 Hz), 136.0, 139.9, 156.7, 157.3, 161.3 (d, J = 242.8 Hz), 167.6. Mp 219–220 °C. Anal. Calcd for $C_{29}H_{30}FN_3O_4$: C, 69.17; H, 6.00; N, 8.34. Found: C, 68.87; H, 5.96; N, 8.16.

5.1.36.

(4-(2-((2,6-Diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)phenyl)methanol (12)

A solution of compound **9c** (579 mg, 1.12 mmol) in THF (5 mL) was added to a suspention of $LiAlH_4$ (127 mg, 3.35 mmol) in THF (10 mL) at 0 °C. The mixture was stirred at the same temperature under N_2 atmosphere for 30 min. Water (0.15 mL), 1 M NaOH aqueous solution (0.15

mL), and water (0.45 mL) were added to the reaction mixture in turn. Celite was added to the mixture. The mixture was stirred at room temperature for 30 min. After filtration though celite, the filtrate was concentrated under reduced pressure. The residue was crystallized from AcOEt–hexane to give the title compound as a colorless solid (350 mg, 0.712 mmol, 64 %). MS (ESI/APCI) m/z 491.3 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.16 (6H, t, J = 6.9 Hz), 3.35 (2H, d, J = 8.6 Hz), 3.56 (2H, d, J = 8.4 Hz), 3.64 (2H, s), 3.72 (2H, s), 3.96 (4H, q, J = 6.9 Hz), 4.53 (2H, d, J = 5.6 Hz), 5.28 (1H, t, J = 5.7 Hz), 6.65 (2H, s), 7.09–7.19 (2H, m), 7.24–7.32 (2H, m), 7.39 (2H, d, J = 8.1 Hz), 7.62 (2H, d, J = 8.1 Hz). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 44.3, 63.0, 63.4, 64.2, 67.0, 81.3, 105.9, 114.5 (d, J = 20.5 Hz), 117.3, 126.8, 127.1, 128.1, 130.7 (d, J = 2.9 Hz), 133.2 (d, J = 8.1 Hz), 139.9, 145.3, 156.7, 157.5, 161.3 (d, J = 242.5 Hz). Mp 194–195 °C. Anal. Calcd for $C_{29}H_{31}FN_2O_4 \cdot 0.1H_2O$: C, 70.74; H, 6.39; N, 5.69. Found: C, 70.70; H, 6.34; N, 5.64.

5.1.37.

(4-(2-((2,6-Diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)phenyl)acetonitrile (13)

ADDP (313 mg, 1.24 mmol) was added to a mixture of compound **12** (304.5 mg, 0.62 mmol), acetone cyanohydrin (158 mg, 1.86 mmol), and P(^{n}Bu)$_3$ (0.309 ml, 1.24 mmol) in THF (10 mL) at room temperature. The mixture was stirred at the same temperature under N_2 atomsphere for 2 h. After filtration through celite, the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/AcOEt = 30/70 to 0/100) to give a colorless solid. The

solid was recrystallized from AcOEt-hexane to give the title compound as colorless crystals (231 mg, 0.463 mmol, 75 %). MS (ESI/APCI) m/z 500.6 $[M + H]^+$. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.16 (6H, t, J = 6.9 Hz), 3.36 (2H, d, J = 8.8 Hz), 3.56 (2H, d, J = 8.7 Hz), 3.64 (2H, s), 3.73 (2H, s), 3.96 (4H, q, J = 6.9 Hz), 4.10 (2H, s), 6.65 (2H, s), 7.09−7.20 (2H, m), 7.24−7.32 (2H, m), 7.44 (2H, d, J = 8.5 Hz), 7.65−7.75 (2H, m). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 22.8, 44.1, 63.3, 64.2, 67.0, 81.5, 106.0, 114.5 (d, J = 20.5 Hz), 117.3, 119.4, 127.6, 129.0, 129.1, 130.7 (d, J = 2.9 Hz), 133.2 (d, J = 8.1 Hz), 133.8, 139.9, 156.7, 157.2, 161.3 (d, J = 242.8 Hz). Mp 165−166 °C. Anal. Calcd for $C_{30}H_{30}FN_3O_3 \cdot 0.2H_2O$: C, 71.61; H, 6.09; N, 8.35. Found: C, 71.72; H, 6.21; N, 8.23.

5.1.38.

(4-(2-((2,6-Diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)phenyl)acetic acid hydrochloride (14)

To a mixture of compound **13** (82 mg, 0.16 mmol) and EtOH (2 mL) was added 1 M NaOH aqueous solution (11 mL, 11.00 mmol) at room temperature. The mixture was heated at 180 °C for 30min under microwave irradiation. The mixture was acidified with 6 M HCl at 0 °C. The precipitate was collected by filtration. The solid was recrystallized from AcOEt–hexane to give the title compound as white crystals (64.0 mg, 0.123 mmol, 75%). MS (ESI/APCI) m/z 519.5 $[M + H]^+$. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.05−1.21 (6H, m), 3.35 (2H, d, J = 8.9 Hz), 3.52−3.59 (2H, m), 3.62 (4H, d, J = 8.6 Hz), 3.71 (2H, s), 3.96 (4H, q, J = 6.9 Hz), 6.65 (2H, s), 7.08−7.20 (2H, m), 7.23−7.31 (2H, m), 7.31−7.37 (2H, m), 7.56−7.65 (2H, m). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 41.1, 44.2, 63.3,

64.2, 66.9, 81.3, 106.0, 114.5 (d, J = 21.3 Hz), 117.3, 126.9, 128.1, 130.3, 130.7 (d, J = 3.7 Hz), 133.2 (d, J = 7.3 Hz), 137.9, 139.8, 156.7, 157.5, 161.3 (d, J = 242.5 Hz), 172.9. Mp 202−203 °C. Anal. Calcd for $C_{30}H_{31}FN_2O_5 \cdot HCl \cdot 0.4H_2O$: C, 64.09; H, 5.88; N,4.98. Found: C, 64.14; H, 5.87; N, 4.89.

5.1.39. Methyl

4-(2-(1-(2,6-diethoxy-4'-fluorobiphenyl-4-yl)ethyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)benzoate (16)

Thionyl chloride (624 mg, 5.25 mmol) was added to a solution of 1-(2,6-diethoxy-4'-fluorobiphenyl-4-yl)ethanol (**15**) (799 mg, 2.62 mmol) in toluene (12 mL) at room temperature. The mixture was stirred at the same temperature under N_2 atmosphere for 1 h. Thionyl chloride (468 mg, 3.94 mmol) was added to the reaction mixture. The mixture was stirred at room temperature under N_2 atmosphere for 1 h. The mixture was quenched with saturated aqueous $NaHCO_3$ solution at room temperature and extracted with AcOEt. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was added to a mixture of compound **7c** (532 mg, 1.88 mmol) and triethylamine (381 mg, 3.77 mmol) in DMF (15 mL) at room temperature. The mixture was stirred at 70 °C under N_2 atmosphere for 18 h. The mixture was quenched with water at room temperature and extracted with AcOEt. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/AcOEt = 55/45 to

0/100) to give the title compound as a colorless solid (349 mg, 0.655 mmol, 35% over 2 steps). MS (ESI/APCI) m/z 533.3 $[M + H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.25 (6H, t, *J* = 7.0 Hz), 1.29 (3H, d, *J* = 6.4 Hz), 3.32–3.41 (3H, m), 3.50 (1H, d, *J* = 8.0 Hz), 3.61–3.72 (3H, m), 3.92–4.03 (7H, m), 6.59 (2H, s), 7.04 (2H, t, *J* = 8.9 Hz), 7.34 (2H, dd, *J* = 8.9, 5.6 Hz), 7.69–7.75 (2H, m), 8.05–8.10 (2H, m).

5.1.40.

4-(2-(1-(2,6-Diethoxy-4'-fluorobiphenyl-4-yl)ethyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)benzoic acid (17)

Compound **17** was prepared from compound **16** in a manner similar to that described for compound **10a**. Colorless crystals. Yield 59%. MS (ESI/APCI) m/z 519.3 $[M + H]^+$. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.13–1.22 (9H, m), 3.24 (1H, d, *J* = 7.9 Hz), 3.32–3.44 (3H, m), 3.60 (1H, d, *J* = 7.8 Hz), 3.76 (2H, s), 3.97 (4H, q, *J* = 6.9 Hz), 6.68 (2H, s), 7.09–7.19 (2H, m), 7.24–7.33 (2H, m), 7.78 (2H, d, *J* = 8.5 Hz), 8.00 (2H, d, *J* = 8.5 Hz). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 21.8, 43.9, 64.2, 65.9, 68.2, 81.0, 104.7, 114.5 (d, J = 21.3 Hz), 117.3, 127.1, 130.2, 130.7 (d, J = 3.7 Hz), 132.7, 133.2 (d, J = 7.3 Hz), 133.6, 145.1, 156.7, 157.2, 161.3 (d, J = 242.8 Hz), 167.3. Mp 213–214 °C. Anal. Calcd for $C_{30}H_{31}FN_2O_5$: C, 69.48; H, 6.03; N, 5.40; F, 3.66. Found: C, 69.20; H, 5.99; N, 5.35.

5.1.41. Methyl

4-(2-((2,6-diethoxy-4'-fluorobiphenyl-4-yl)carbonyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)benzoate (19)

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (427 mg, 2.23 mmol) was added to a mixture of compound **7c** (573 mg, 2.02 mmol), 2,6-diethoxy-4'-fluorobiphenyl-4-carboxylic acid (**18**) (678 mg, 2.23 mmol), HOBt·1H_2O (301 mg, 2.23 mmol) and triethylamine (512 mg, 5.06 mmol) in DMF (10 mL) at room temperature. The mixture was stirred at room temperature under N_2 atmosphere for 16 h. The mixture was quenched with 10% citric acid aqueous solution at room temperature and extracted with AcOEt. The organic layer was separated, washed with saturated aqueous $NaHCO_3$ solution and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was passed through NH silica gel with AcOEt. The filtrate was concentrated under reduced pressure. The residue was crystallized from AcOEt–hexane to give a colorless solid. The solid was recrystallized from AcOEt–hexane to give the title compound as colorless crystals (811 mg, 1.52 mmol, 75%). MS (ESI/APCI) m/z 533.4 $[M + H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.26 (6H, t, *J* = 6.9 Hz), 3.66 (2H, brs), 3.94 (3H, s), 4.00 (4H, q, *J* = 6.9 Hz), 4.47 (2H, d, *J* = 10.5 Hz), 4.55–4.81 (2H, m), 6.89 (2H, s), 7.03–7.11 (2H, m), 7.29–7.36 (2H, m), 7.69–7.75 (2H, m), 8.06–8.12 (2H, m). ^{13}C NMR (101 MHz, DMSO-d_6) δ 14.9, 43.4, 52.8, 64.5, 66.5, 82.2, 105.5, 114.7 (d, J = 21.3 Hz), 121.3, 127.4, 129.9 (d, J = 3.7 Hz), 130.1, 131.4, 133.1 (d, J = 8.1 Hz), 133.7, 133.9, 156.7, 157.4, 161.5 (d, J = 242.8 Hz), 166.1, 169.2. Mp 248–249 °C.

5.1.42.

4-(2-((2,6-Diethoxy-4'-fluorobiphenyl-4-yl)carbonyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)benzoic acid (20)

1 M NaOH aqueous solution (1 mL, 1.00 mmol) was added to a solution of compound **19** (208 mg, 0.39 mmol) in a mixed solvent of THF (5 mL) and EtOH (5 mL) at room temperature. The mixture was stirred at the same temperature under N_2 atmosphere for 3 h. 1 M HCl (1 mL, 1.00 mmol) was added to the reaction mixture at room temperature. The mixture was concentrated under reduced pressure. Water was added to the residue. The mixture was stirred at room temperature for 30 min to give colorless crystals. After filtration, the crystals was dissoleved in AcOEt and THF. The solution was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was crystallized from AcOEt–hexane to give a colorless solid. The solid was recrystallized from AcOEt–THF–hexane to give the title compound as colorless crystals (154.7 mg, 0.298 mmol, 76%). MS (ESI/APCI) m/z 519.3 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.17 (6H, t, *J* = 6.9 Hz), 3.86 (2H, s), 4.03 (4H, q, *J* = 6.9 Hz), 4.30 (1H, d, *J* = 10.4 Hz), 4.43 (1H, d, *J* = 10.0 Hz), 4.68 (2H, brs), 6.95 (2H, s), 7.14–7.22 (2H, m), 7.28–7.34 (2H, m), 7.77 (2H, d, *J* = 8.4 Hz), 8.02 (2H, d, *J* = 8.5 Hz), 13.16 (1H, brs). ^{13}C NMR (101 MHz, DMSO-d_6) δ 14.9, 43.4, 64.5, 66.6, 82.1, 105.5, 114.7 (d, J = 21.3 Hz), 121.3, 127.2, 129.9 (d, J = 2.9 Hz), 130.2, 132.7, 133.1 (d, J = 8.1 Hz), 133.3, 133.9, 156.7, 157.4, 161.5 (d, J = 242.8 Hz), 167.2, 169.2. Mp 250–251 °C. Anal. Calcd for $C_{29}H_{27}FN_2O_6$: C, 66.94; H, 5.27; N, 5.38. Found: C, 67.03; H, 5.43; N, 5.26.

5.1.43. *tert*-Butyl 7-bromo-5-oxa-2,6-diazaspiro[3.4]oct-6-ene-2-carboxylate (22)

$NaHCO_3$ (101 g, 1.20 mol) was added to a mixture of hydroxycarbonimidic dibromide (**21**) (48.8 g, 241 mmol) and *tert*-butyl 3-methyleneazetidine-1-carboxylate (20.4 g, 120 mmol) in AcOEt (400 mL)

at room temperature. The mixture was stirred at the same temperature for 15 h and at 50 °C for 4 h. The mixture was quenched with water at room temperature and filtrated through celite. The filtrate was extracted with AcOEt. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/AcOEt = 85/15 to 40/60) to give the title compound as a colorless solid (27.8 g, 95.0 mmol, 79%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.44 (9H, s), 3.44 (2H, s), 4.02–4.07 (2H, m), 4.26–4.32 (2H, m).

5.1.44. *tert*-Butyl

7-(4-(ethoxycarbonyl)piperidin-1-yl)-5-oxa-2,6-diazaspiro[3.4]oct-6-ene-2-carboxylate (23a)

Compound **22** (15.0 g, 51.5 mmol), ethyl piperidine-4-carboxylate (11.3 g, 72.1 mmol) and sodium carbonate (15.3 g, 144 mmol) were added to DMF (60 mL). The reaction mixture was stirred at 130 °C for 4 h. After the reaction mixture was cooled to room temperature, water was added to the reaction mixture. The mixture was extracted with AcOEt. The obtained organic layer was washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was crystallized from ^{i}Pr$_2$O–hexane to give the title compound as pale orange crystals (15.1 g, , 41.1 mmol, 80%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.18 (3H, t, J = 7.1 Hz), 1.38 (9H, s), 1.52 (2H, d, J = 10.2 Hz), 1.80 (2H, d, J = 11.8 Hz), 2.50 (1H, brs), 2.84 (2H, t, J = 11.6 Hz), 3.33 (2H, s), 3.43 (2H, d, J = 12.9 Hz), 3.88 (2H, d, J = 9.0 Hz), 3.98–4.12 (4H, m).

5.1.45. Ethyl

1-(2-((2,6-diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)piperidine-4-carboxylate (24a)

Compound **23a** (3.50g, 9.53 mmol) was added to formic acid (14 mL). The reaction mixture was stirred at 70 °C for 1 h, and the solvent was distilled off under reduced pressure. Sodium triacetoxyborohydride (3.03 g, 14.3 mmol) was added to a mixture of the residue and compound **8** (2.75 g, 9.53 mmol) in THF (20 mL). The reaction mixture was stirred at room temperature for 1 h. Saturated aqueous $NaHCO_3$ solution was added to the reaction mixture. The mixture was extracted with AcOEt. The obtained organic layer was washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by NH silica gel column chromatography (hexane/AcOEt = 90/10 to 50/50) and crystallized from $^{i}Pr_2O$–hexane to give the title compound as colorless crystals (4.04 g, 7.49 mmol, 79%). MS (ESI/APCI) m/z 540.5 $[M + H]^+$. ^1H NMR (400 MHz, DMSO-d_6) δ 1.07−1.22 (9H, m), 1.46−1.59 (2H, m), 1.80 (2H, d, J = 11.8 Hz), 2.50 (1H, brs), 2.83 (2H, t, J = 11.4 Hz), 3.17 (2H, d, J = 7.7 Hz), 3.30 (2H, s), 3.41−3.49 (4H, m), 3.57 (2H, s), 3.95 (4H, q, J = 6.8 Hz), 4.07 (2H, q, J = 7.0 Hz), 6.61 (2H, s), 7.09−7.18 (2H, m), 7.23−7.31 (2H, m). ^{13}C NMR (101 MHz, DMSO-d_6) δ 14.6, 15.0, 27.4, 43.0, 46.2, 60.4, 63.4, 64.2, 66.9, 79.8, 105.9, 114.5 (d, J = 20.5 Hz), 117.3, 130.7 (d, J = 2.9 Hz), 133.2 (d, J = 8.1 Hz), 140.0, 156.6, 161.2, 161.3 (d, J = 242.8 Hz), 174.4. Mp 114−115 °C.

5.1.46. Methyl

1-(2-((2,6-diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-

7-yl)pyrrolidine-3-carboxylate (24b)

Compound **24b** was prepared from methyl pyrrolidine-3-carboxylate hydrochloride, compounds **8**, and **22** in a manner similar to that described for compounds **23a** and **24a**, respectively. White crystals. Yield 11% over 2 steps. MS (ESI/APCI) m/z 512.5 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.15 (6H, t, J = 6.9 Hz), 1.97-2.18 (2H, m), 3.11−3.37 (8H, m), 3.39−3.49 (3H, m), 3.57 (2H, s), 3.64 (3H, s), 3.95 (4H, q, J = 6.9 Hz), 6.61 (2H, s), 7.09−7.18 (2H, m), 7.23−7.31 (2H, m).

5.1.47.

1-(2-((2,6-Diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)piperidine-4-carboxylic acid (25a)

2 M NaOH aqueous solution (45.6 ml, 91.2 mmol) was added to a solution of compund **24a** (8.20 g, 15.2 mmol) in EtOH (30 mL) at room temperature. The mixture was stirred at 70 °C for 1 h. The mixture was neutralized with 2 M HCl at room temperature and concentrated under reduced pressure to half amount. The solid was filtered. The solid was recrystallized from EtOH–^{i}Pr$_2$O to give the title compound as colorless crystals (6.06 g, 11.9 mmol, 78%). MS (ESI/APCI) m/z 512.4 $[M + H]^+$. ^{1}H NMR (400 MHz, CD_3OD) δ 1.24 (6H, t, J = 7.0 Hz), 1.63−1.75 (2H, m), 1.88−1.98 (2H, m), 2.41−2.51 (1H, m), 2.89−3.00 (2H, m), 3.38 (2H, s), 3.55−3.66 (4H, m), 3.77 (2H, d, J = 9.7 Hz), 3.85 (2H, s), 4.00 (4H, q, J = 7.0 Hz), 6.69 (2H, s), 7.06 (2H, t, J = 8.8 Hz), 7.28 (2H, dd, J = 8.3, 5.8 Hz). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 27.5, 43.0, 46.4, 63.4, 64.2, 66.9, 79.7, 106.0, 114.5 (d, J = 21.3 Hz), 117.3, 130.7 (d, J = 3.7 Hz), 133.2 (d, J = 7.3 Hz), 140.0, 156.6, 161.2, 161.3 (d, J = 242.8

Hz), 176.1. Mp 195−196 °C. Anal. Calcd for $C_{28}H_{34}FN_3O_5$: C, 65.74; H, 6.70; N, 8.21; F, 3.71. Found: C, 65.52; H, 6.68; N, 8.16.

5.1.48.

1-(2-((2,6-Diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)pyrrolidine-3-carboxylic acid hydrochloride (25b)

Compound **25b** was prepared from compound **24b** in a manner similar to that described for compound **25a** (purification: formation of a hydrochloride with 1 M HCl and following recrystallization from AcOEt–hexane). White crystals. Yield 97%. MS (ESI/APCI) m/z 498.2 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.15 (6H, t, J = 6.9 Hz), 1.95−2.16 (2H, m), 3.01−3.12 (1H, m), 3.12−3.52 (10H, m), 3.58 (2H, s), 3.95 (4H, q, J = 6.9 Hz), 6.61 (2H, s), 7.10−7.18 (2H, m), 7.23−7.31 (2H, m). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 28.7, 42.9, 44.0, 47.6, 50.2, 63.3, 64.2, 66.8, 79.5, 106.0, 114.5 (d, J = 20.5 Hz), 117.3, 130.7 (d, J = 2.9 Hz), 133.2 (d, J = 8.1 Hz), 139.9, 156.6, 159.0, 161.3 (d, J = 242.8 Hz), 175.0. Mp 180−182 °C. Anal. Calcd for $C_{27}H_{32}FN_3O_5 \cdot HCl \cdot H_2O$: C, 58.75; H, 6.39; N,7.61. Found: C, 58.72; H, 6.63; N, 7.50.

5.1.49. 7-Bromo-5-oxa-2,6-diazaspiro[3.4]oct-6-ene hydrochloride (26)

4 M HCl/AcOEt (6.61 mL, 26.5 mmol) was added to a solution of compound **22** (770 mg, 2.64 mmol) in AcOEt (3 mL) at room temperature. The mixture was stirred at room temperature for 30 min. The precipitate was collected by filtration, washed with AcOEt to give the title compound as pale pink crystals (358 mg, 1.57 mmol, 60%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.67−3.77 (2H, m),

4.12–4.23 (2H, m), 4.25–4.34 (2H, m), 9.14 (2H, brs).

5.1.50.

7-Bromo-2-((2,6-diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-ene (27)

Compound **27** was prepared from compounds **8** and **26** in a manner similar to that described for compound **9a**. Pale yellow crystals. Yield 84%. MS (ESI/APCI) m/z 463.1 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.09–1.18 (6H, m), 3.32 (2H, d, J = 8.4 Hz), 3.50–3.65 (6H, m), 3.95 (4H, q, J = 6.9 Hz), 6.61 (2H, s), 7.10–7.19 (2H, m), 7.27 (2H, dd, J = 8.3, 6.0 Hz).

5.1.51.

1-(2-((2,6-Diethoxy-4'-fluorobiphenyl-4-yl)methyl)-5-oxa-2,6-diazaspiro[3.4]oct-6-en-7-yl)azetidine-3-carboxylic acid hydrochloride (25c)

The mixture of ethyl azetidine-3-carboxylate hydrochloride (129 mg, 0.78 mmol), compound **27** (300 mg, 0.65 mmol), sodium carbonate (254 mg, 2.40 mmol) and ^{n}BuOH (3 mL) was stirred 120 °C for 18 h. The mixture was concentrated under reduced pressure. The residue was purified by NH silica gel column chromatography (hexane/AcOEt = 85/15 to 50/50) to give the light brown oil. 2 M NaOH aqueous solution (1 mL, 2.00 mmol) was added to a solution of the oil in EtOH (2 mL) at room temperature. The mixture was stirred at 70 °C for 30 min. The mixture was acidified with 2 M HCl at room temperature. The precipitate was collected by filtration. The solid was recrystallized from AcOEt–hexane to give the title compound as white crystals (40.0 mg, 0.083 mmol, 13% over 2 steps).

MS (ESI/APCI) m/z 484.5 $[M + H]^+$. ^{1}H NMR (400 MHz, CD_3OD) δ 1.26 (6H, t, *J* = 6.9 Hz), 3.37 (2H, s), 3.53−3.63 (1H, m), 4.04 (4H, q, *J* = 6.8 Hz), 4.07−4.19 (4H, m), 4.32−4.43 (4H, m), 4.50 (2H, d, *J* = 11.8 Hz), 6.82 (2H, s), 7.03−7.13 (2H, m), 7.22−7.33 (2H, m). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.0, 34.1, 41.4, 54.2, 58.2, 64.4, 65.1, 78.5, 107.8, 114.7 (d, *J* = 21.3 Hz), 119.0, 130.1 (d, *J* = 2.9 Hz), 133.1, 133.2, 156.9, 161.4 (d, *J* = 242.8 Hz), 161.7, 174.0. Mp 150−151 °C. Anal. Calcd for $C_{26}H_{30}FN_3O_5 \cdot HCl \cdot 0.5H_2O$: C, 59.03; H, 6.10; N, 7.94. Found: C, 58.95; H, 6.02; N, 7.74.

5.1.52. Ethyl 4-bromo-3,5-diethoxybenzoate (30)

K_2CO_3 (89.0 g, 644 mmol) and iodoethane (60.1 mL, 751 mmol) were added to a solution of 4-bromo-3,5-dihydroxybenzoic acid (**29**) (50.0 g, 215 mmol) in DMF (300 mL). The mixture was stirred at 60 °C for 2 h. The mixture was quenched with water and extracted with AcOEt. The organic layer was separated, washed with brine twice. The obtained organic layer was passed through NH silica gel pad (AcOEt) and concentrated under reduced pressure. The residue was solidified with Et_2O. The solid was collected by filtration and washed with Et_2O and hexane to give the title compound as a white solid (55.8 g, 176 mmol, 82%). MS (ESI/APCI) m/z 318.0 $[M + H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.40 (3H, t, *J* = 7.1 Hz), 1.49 (6H, t, *J* = 7.0 Hz), 4.17 (4H, q, *J* = 7.0 Hz), 4.38 (2H, q, *J* = 7.1 Hz), 7.21 (2H, s).

5.1.53. Ethyl 2,6-diethoxy-4'-fluorobiphenyl-4-carboxylate (**31**)

Palladium acetate (1.98 g, 8.80 mmol) was added to a mixture of compound **30** (55.8 g, 176 mmol), (4-fluorophenyl)boronic acid (43.1 g, 308 mmol), tricyclohexylphosphine (20% toluene solution, 31.2

mL, 17.6 mmol) and K_3PO_4 (112 g, 528 mmol) in toluene (300 mL) and water (150 mL) at room temperature. The mixture was stirred at 90 °C overnight under Ar atmosphere. The mixture was quenched with water at room temperature and extracted with AcOEt. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/AcOEt = 95/5 to 70/30) to give the title compound as a green oil (50.7 g, 153 mmol, 87%). MS (ESI/APCI) m/z 333.1 $[M + H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.27 (6H, t, *J* = 6.9 Hz), 1.41 (3H, t, *J* = 7.1 Hz), 4.03 (4H, q, *J* = 6.9 Hz), 4.40 (2H, q, *J* = 7.1 Hz), 6.99–7.14 (2H, m), 7.28–7.41 (4H, m).

5.1.54. 2,6-Diethoxy-4'-fluorobiphenyl-4-carbaldehyde (8)

A solution of compound **31** (50.7 g, 153 mmol) in THF (200 mL) was added dropwise to an ice cold stirred suspension of $LiAlH_4$ (4.34 g, 114 mmol) in THF (200 mL). After stirring at 0 °C for 30 min, water (4.5 mL) was added dropwise to the reaction mixture followed by 1 M NaOH aqueous solution (4.5 mL). After stirring for 5 min, water (13.5 mL) was added thereto. After stirring at room temperature for 1 h, the mixture was filtered through celite and the filtrate was concentrated under reduced pressure. Sulfer trioxide-pyridine complex (48.6 g, 305 mmol) was added portionwise to a stirred solution of the residue and triethylamine (63.8 mL, 458 mmol) in DMSO (250 mL). After stirring at room temperature for 30 min, water (450 mL) was added to the mixture. The precipitated solid was collected by filtration. The obtained solid was crystallized from EtOH (225 mL) and water (115 mL). The crystals were filtered and dried under reduced pressure to give the title compound as a

pale yellow solid (36.9 g, 128 mmol, 84%). MS (ESI/APCI) m/z 289.0 $[M + H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.29 (6H, t, *J* = 7.0 Hz), 4.06 (4H, q, *J* = 6.9 Hz), 7.08 (2H, t, *J* = 8.9 Hz), 7.13 (2H, s), 7.34 (2H, dd, *J* = 9.0, 5.6 Hz), 9.94 (1H, s). ^{13}C NMR (101 MHz, DMSO-d_6) δ 14.9, 64.6, 106.8, 114.8 (d, *J* = 21.3 Hz), 124.5, 129.7 (d, *J* = 2.9 Hz), 133.0 (d, *J* = 8.1 Hz), 137.3, 157.3, 161.7 (d, *J* = 243.6 Hz), 193.0. Mp 91−92 °C. Anal. Calcd for $C_{17}H_{17}FO_3 \cdot 0.5H_2O$: C, 68.67; H, 6.10. Found: C, 68.77; H, 6.33.

5.1.55. 1-(2,6-Diethoxy-4'-fluorobiphenyl-4-yl)ethanol (15)

Methylmagnesium bromide (1 M THF solution, 9 mL, 9.00 mmol) was added to a solution of compound **8** (1.69 g, 5.86 mmol) in THF (40 mL) at room temperature. The mixture was stirred at the same temperature under N_2 atmosphere for 4 h. The mixture was quenched with saturated aqueous NH_4Cl solution at room temperature and extracted with AcOEt. The organic layer was separated, washed with water and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/AcOEt = 75/25 to 20/80) to give the title compound as a colorless solid (1.37 g, 4.50 mmol, 77%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.25 (6H, t, *J* = 7.0 Hz), 1.53 (3H, d, *J* = 6.5 Hz), 1.83 (1H, d, *J* = 3.2 Hz), 3.98 (4H, q, *J* = 7.0 Hz), 4.89 (1H, qd, *J* = 6.4, 3.1 Hz), 6.65 (2H, s), 7.00-7.09 (2H, m), 7.29-7.37 (2H, m). ^{13}C NMR (101 MHz, DMSO-d_6) δ 15.1, 26.4, 64.2, 68.8, 103.2, 114.5 (d, J = 20.5 Hz), 116.9, 130.9 (d, J = 3.7 Hz), 133.2 (d, J = 7.3 Hz), 149.3, 156.6, 161.2 (d, J = 242.1 Hz). Mp 88−89 °C. Anal. Calcd for $C_{18}H_{21}FO_3 \cdot 0.1H_2O$: C, 70.61; H, 6.98. Found: C, 70.42; H, 7.02.

5.1.56. 2,6-Diethoxy-4'-fluorobiphenyl-4-carboxylic acid (18)

2 M NaOH aqueous solution (70.9 mL, 142 mmol) was added to a solution of compound **31** (15.7 g, 47.3 mmol) in a mixed solvent of THF (100 mL) and MeOH (50 mL). The mixture was stirred at 60 °C for 2 h. The mixture was neutralized with 6 M HCl and extracted with AcOEt twice, dried over $MgSO_4$ and concentrated under reduced pressure. The solid was collected by filtration and washed with Et_2O and hexane to give the title compound as a pale yellow solid (12.8 g, 42.1 mmol, 89%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.28 (6H, t, *J* = 7.0 Hz), 4.05 (4H, q, *J* = 7.0 Hz), 7.08 (2H, t, *J* = 8.9 Hz), 7.31-7.40 (4H, m). ^{13}C NMR (101 MHz, DMSO-d_6) δ 14.9, 64.5, 106.7, 114.7 (d, J = 20.5 Hz), 123.0, 129.9 (d, J = 3.7 Hz), 131.9, 133.1 (d, J = 8.1 Hz), 156.8, 161.6 (d, J = 243.6 Hz), 167.5. Mp 184−185 °C.

5.2. In vitro antagonistic activity against SSTR5

Chinese hamster ovary (CHO) cells stably expressing human SSTR5 or mouse SSTR5 were suspended in assay buffer (Hank's balanced salt solution supplemented with 5 mM HEPES (pH7.5), 0.1% fatty acid-free BSA and 500 μM IBMX). The cells were incubated with various concentrations of test compounds for 15 min at room temperature. Then, the cells were stimulated with 1 or 3 nM (for human SSTR5 or mouse SSTR5, respectively) SST14 and 0.3 μM folskolin for 30 min at room temperature. Intracellular cAMP level was determined using HTRF cAMP dynamic 2 kit (Cisbio Bioassays) by following the manufactures' instructions. HTRF signal was measured using Envision

(PerkinElmer).

5.3. hERG inhibition assay

hERG/CHO cells stably expressing hERG channel were purchased from Millipore (UK) Ltd. (catalog number CYL3038). Cells were cultured at 32 °C, 5% CO_2 in Ham's F-12 medium supplemented with 10% fetal bovine serum, 500 μg/mL Geneticin (Invtogen). The hERG inhibition assay was performed on the IonWorks Quattro (Molecular Devices) system in population patch clamp (PPC) mode. The extracellular solution was phosphate-buffered saline (PBS) with calcium and magnesium (catalog number 14040, Invitrogen). The intracellular solution contained 140 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, and 20 mM HEPES, pH 7.3, with KOH. After perforation using 100 μg/mL amphotericin B (Sigma-Aldrich), hERG current was measured under the potential-clamp protocol (holding potential −80 mV, the first voltage 40 mV for 2 sec, the second voltage −50 mV for 2 sec). The peak tail current before addition of the compounds was measured as the pre hERG current. Test compounds were incubated on the cells for a period of 5 min. The peak tail current after addition of the compounds was measured as the post-hERG current. % hERG inhibition was calculated (n = 4) according to the following.

% hERG inhibition = 100 − (post-hERG current / pre-hERG current) × 100

5.4. Estimation of log *D* at pH 7.4

Log $D_{7.4}$, which is a partition coefficient between 1-octanol and aqueous buffer at pH 7.4, of the compounds was measured using the chromatographic procedure whose condition was developed based on a published method.[35] The instruments were a Waters Alliance 2795 HPLC system with a 2996 UV–vis detector (Milford, MA, USA).

5.5. Solubility determination

Compound (0.4 mg) was weighed into a filter vial (Chrom Tech, Inc., Minnesota, U.S.A.). A 0.4 mL of the aqueous buffer solution (pH 6.8) was added into the vial. The vial was incubated at 37 °C with shaking at 500 rpm for 18 h and filtrated. The compound concentration of the filtrates was determined by HPLC analysis.

5.6. In vitro metabolic clearance in human and mouse hepatic microsomes.

Human and mouse liver microsomes were purchased from Xenotech, LLC (Lenexa, KS). An incubation mixture consisted of microsomal protein in 50 mM KH_2PO_4–K_2HPO_4 phosphate buffer (pH 7.4) and 1 μM test compound. The concentration of microsomal protein was 0.2 mg/mL. An NADPH-generating system containing 5 mM $MgCl_2$, 5 mM glucose-6-phosphate, 0.5 mM β-$NADP^+$, and 1.5 units/mL glucose-6-phosphate dehydrogenase was added to the incubation mixture to initiate the enzyme reaction. The reaction was terminated 15 and 30 min after the initiation of the reaction by

mixing the reaction mixture with acetonitrile, followed by centrifugation. The supernatant was subjected to LC / MS / MS analysis. The metabolic velocity was calculated as the slope of the concentration-time plot.

5.7. Pharmacokinetic analysis in mouse cassette dosing

Test compounds were administered intravenously (0.1 mg/kg) or orally (1 mg/kg, suspended in 0.5% methylcellulose aqueous solution) 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.

5.8. Animal experiments

5.8.1. Oral Glucose Tolerance Test (Figure 5)

8-week-old male C57BL/6J mice were purchased from CLEA Japan, Inc. After feeding high fat diet (HFD; D12492, Research Diets, Inc.) for ten days, the weight and blood glucose of mice were measured 4 hours after fasting was started from the morning of the day of the experiment, and the mice were separated in groups based on the weight and blood glucose. Vehicle (0.5% methyl cellulose solution) or test drugs (0.5% methyl cellulose suspension) were orally administered 5.5

hours after fasting was started and glucose load (5 g/kg) was given orally 6.5 hours after fasting was started. Just before the glucose load, Blood Glucose (BG) and plasma insulin concentration were measured by collecting blood from the tail (pre), and BG and plasma insulin concentration were measured again by collecting blood from the tail 10 minutes, 30 minutes, 60 minutes and 120 minutes after the glucose load. All values were indicated with Mean ± Standard Deviation (SD), and in the statistical analysis, Dunnett's test was used for the case of equal variance and Steel's test was used for non-equal variance in the comparison of the vehicle group and test drug group.

5.8.2. Oral Glucose Tolerance Test (Figure 6)

8-week-old male C57BL/6J mice were purchased from CLEA Japan, Inc. After feeding high fat diet (HFD; D12492, Research Diets, Inc.) for ten days, the weight and blood glucose of mice were measured 12.5 hours after fasting was started from the evening of the day before the experiment, and the mice were separated in groups based on the weight and blood glucose. Vehicle (0.5% methyl cellulose solution) or test drugs (0.5% methyl cellulose suspension) were orally administered 15 hours after fasting was started and glucose load (5 g/kg) was given orally 16 hours after fasting was started. Just before the glucose load, Blood Glucose (BG) was measured by collecting blood from the tail (pre), and BG was measured again by collecting blood from the tail 10 minutes, 30 minutes, 60 minutes and 120 minutes after the glucose load. All values were indicated with Mean ± Standard Deviation (SD), and in the statistical analysis, Williams's test was used for the case of equal variance and Shirly-Williams's test was used for non-equal variance in the comparison of the vehicle group

and test drug group.

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