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Discovery of novel 5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine derivatives as γ-secretase modulators (Part 2)

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ABSTRACT

γ-Secretase modulators (GSMs), which lower pathogenic amyloid beta (Aβ) without affecting the production of total Aβ or Notch signal, have emerged as a potential therapeutic agent for Alzheimer's disease (AD). A novel series of 5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine derivatives was discovered and characterized as GSMs. Optimization of substituents at the 8-position of the core scaffold using ligand-lipophilicity efficiency (LLE) as a drug-likeness guideline led to identification of various types of high-LLE GSMs. Phenoxy compound (*R*)-**17** exhibited especially high LLE as well as potent in vivo $A\beta_{42}$-lowering effect by single administration. Furthermore, multiple oral administration of (*R*)-**17** significantly reduced soluble and insoluble brain $A\beta_{42}$, and ameliorated cognitive deficit in novel object recognition test (NORT) using Tg2576 mice as an AD model.

1. Introduction

Alzheimer's disease (AD) is characterized by impaired memory and loss of other cognitive functions, and is the most common form of dementia, accounting for 50–75% of all dementias.[1] A characteristic neuropathology of AD is the formation of amyloid plaques, which are composed of insoluble deposits of amyloid beta (Aβ). β-Secretase and γ-secretase are responsible for producing Aβ through a stepwise cleavage of amyloid precursor protein (APP).[2] Since γ-secretase catalyzes the degradation of many other substrates besides APP, such as Notch,[3] the inhibition of γ-secretase led to induction of mechanism-based toxicities in clinical trials.[4] As γ-secretase modulators (GSMs) lower pathogenic Aβ through cleavage shift without affecting Notch signal, GSMs have emerged as a potential therapeutic agent for AD.[5] A recent analysis of GSMs indicated that their increased lipophilicity could enhance their potency, but might reduce their drug-likeness.[6] We therefore used ligand-lipophilicity efficiency (LLE) as a drug-likeness guideline in a lead generation study of GSM, and discovered 5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine derivative **1**. Finally, increasing lipophilicity of the distal phenyl moiety helped in identification of **2** as a potent GSM (Fig. 1). Although oral administration of **2** significantly decreased brain $A\beta_{42}$ in mice, further reduction of the drug exposure level in both brain and plasma (2.332 μg/g in cortex and 6.450 μg/mL in plasma at 3 h after oral dosing) at the pharmacologically effective dose remains desirable.[7] Furthermore, increasing lipophilicity for **2** to enhance the $A\beta_{42}$-lowering effect could induce a promiscuous effect, which could induce off-target risks. Through LLE-based optimization, we accordingly pursued more potent compounds without increasing lipophilicity to discover a safer GSM that could reduce $A\beta_{42}$ at a lower drug exposure. The synthetic intermediate **3** in the course of previous modification maintained the equipotent GSM activity compared to **1**, as shown in Figure 1. Encouraged by the tolerance for introduction of an additional polar group at the 8-position of **1**, we explored the optimal functional group based on LLE. Herein we describe identification of (*R*)-**17** by the optimization study, and also report the $A\beta_{42}$-lowering effect and the behavioral pharmacological evaluation in Tg2576 mice.

2. Chemistry

Various functional groups were introduced into the 8-position of **1**, as shown in Scheme 1. Oxidation of the 8-position of **1** under basic conditions and subsequent methylation gave 8-methoxy analog **4**. Hydroxymethylation to the 8-position was achieved by

Abbreviations: GSM, γ-secretase modulator; Aβ, amyloid beta; AD, Alzheimer's disease; LLE, ligand-lipophilicity efficiency; NORT, novel object recognition test; APP, amyloid precursor protein; MLM, mice liver microsomes; CNS, central nervous system; WT, wild type.

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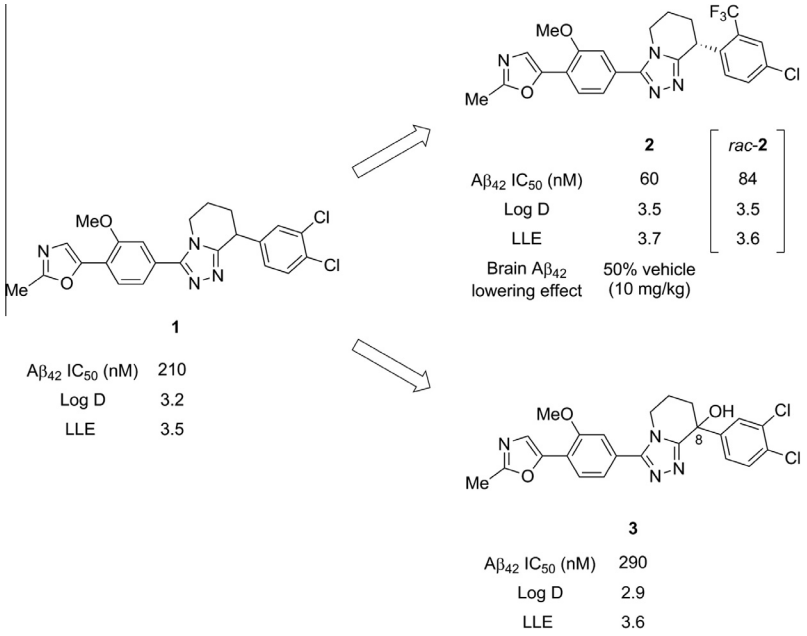
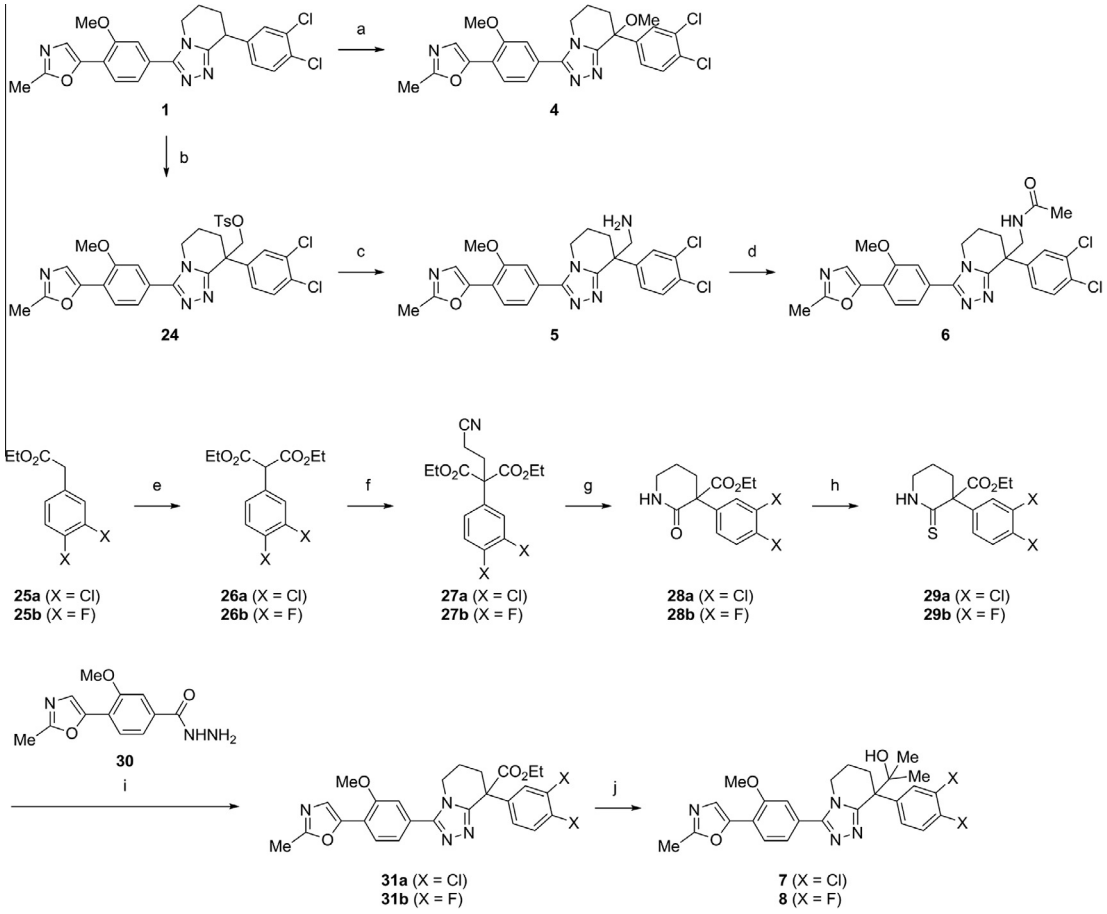


Figure 1. 5,6,7,8-Tetrahydro[1,2,4]triazolo[4,3-*a*]pyridines.



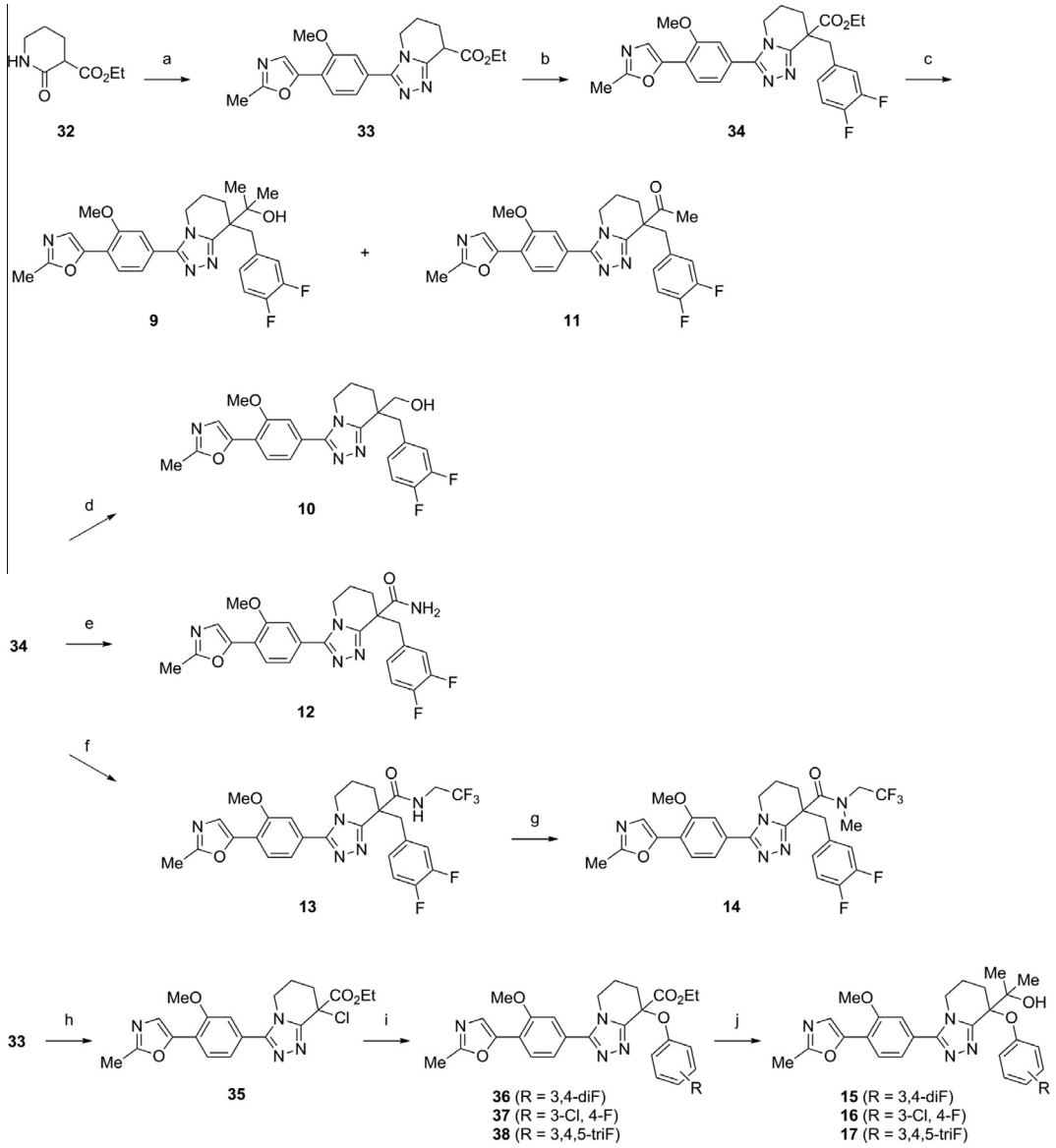
Scheme 1. Synthesis of the 8-position-substituted derivatives. Reagents and conditions: (a) NaH, air, then NaH, MeI, DMF, 31%; (b) (i) NaH, paraformaldehyde, DMF, 13%; (ii) TsCl, Py, 69%; (c) (i) NaN_3, DMSO; (ii) PPh_3, H_2O, THF, 49% (2 steps); (d) AcCl, Et_3N, THF, 72%; (e) NaH, diethyl carbonate, THF, 90–100%; (f) NaOEt, acrylonitrile, *t*-BuOH; (g) H_2, Raney cobalt, NH_3, EtOH, 41–73%; (h) Lawesson's reagent, toluene, 79–90%; (i) MeI, MeCN, then **30**, EtOH, 59–68%; (j) MeMgBr, THF, 36–41%.

nucleophilic alkylation with paraformaldehyde, and the following tosylation afforded **24**. The tosyl group was converted to the amino group in **5** through azidation and subsequent reduction. Acetylation of **5** smoothly proceeded to give amide derivative **6**. Tertiary alcohol analog **7** was then synthesized through a straightforward synthesis. Ethyl carboxylation of the α-position of **25a** followed

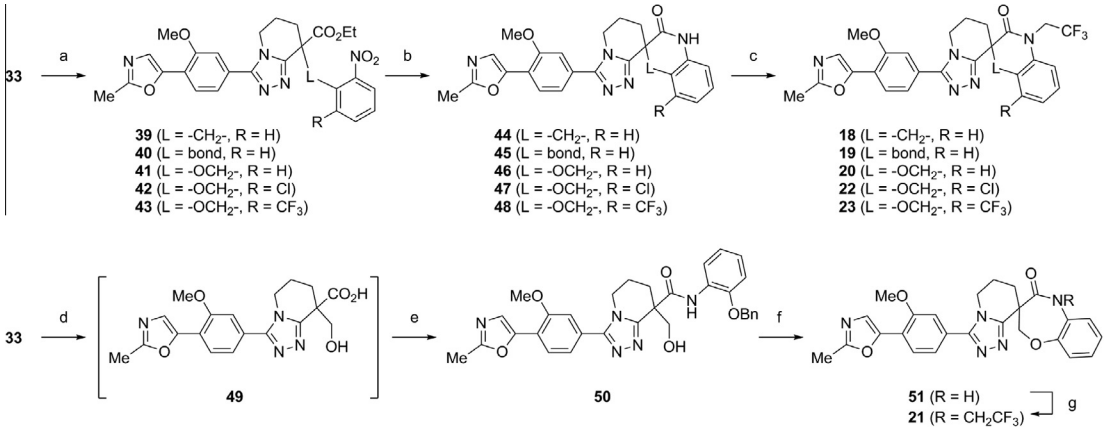
by the Michael reaction with acrylonitrile gave **27a**. Hydrogenation of cyano group of **27a** and subsequent intracyclization were achieved as a one-pot reaction to yield oxopiperidine **28a**, which was then converted to thioxo analog **29a** by use of Lawesson's reagent. The 5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine core of **31a** was constructed by activation of **29a** by iodomethane and the following cyclization reaction with hydrazide **30**.[7] Finally, the ester moiety of **31a** was converted to 2-hydroxypropan-2-yl group by treatment with a Grignard reagent to give **7**. Difluorophenyl analog **8** was prepared by a similar manner.

Benzyl derivatives **9**–**14** and phenoxy derivatives **15**–**17** were synthesized as illustrated in Scheme 2. Activation of oxopiperidine **32** by trimethyloxonium tetrafluoroborate and subsequent cyclization reaction with hydrazide **30** gave bicyclic compound **33**. Alkylation of the 8-position of **33** with benzyl bromide afforded **34**, which was reacted with the Grignard reagent to give 2-hydroxypropan-2-yl derivative **9** and ketone **11** as a by-product. Reduction of ester **34** with LAH gave the primary alcohol **10**. Direct conversion of the ester group of **34** to carboxamide group was achieved by treatment with formamide under basic conditions to afford **12**. Trifluoroethyl amide derivative **13** was obtained through hydrolysis of **34** and subsequent condensation with 2,2,2-trifluoroethylamine, and methylation of **13** gave tertiary amide **14**. The α-position of ester **33** was chlorinated with NCS to give **35**. Nucleophilic substitution at the quaternary carbon atom of **35** with phenols afforded **36**–**38**. Finally, tertiary alcohols **15**–**17** were prepared from the corresponding ester using the Grignard reagent.

Synthetic routes for spiro compounds **18**–**23** are illustrated in Scheme 3. Introduction of 2-nitrobenzyl moiety into **33** was accomplished by nucleophilic substitution with 2-nitrobenzyl bromide to afford **39**. Nitrophenyl derivative **40** was also obtained using 1-fluoro-2-nitrobenzene under similar conditions. After reduction of the nitro group, cyclized compounds **44** and **45** were smoothly obtained from the corresponding ester. The amide moiety was then alkylated with 2,2,2-trifluoroethyltriflate to give **18** and **19**, respectively. The 8-position of **33** was easily oxidized under basic conditions, and the introduced hydroxyl group was trapped by 2-nitrobenzyl bromide to give benzyloxy analog **41**.



Scheme 2. Synthesis of benzyl derivatives and phenoxy derivatives. Reagents and conditions: (a) (i) Me_3OBF_4, MeCN; (ii) **30**, MeCN, 38% (2 steps); (b) NaH, 3,4-difluorobenzylbromide, DMF, 87%; (c) MeMgBr, THF, 48% for **9**, 8% for **11**; (d) LAH, THF, 73%; (e) $HCONH_2$, NaOMe, MeOH, DMF, 85%; (f) (i) 1 M NaOH aq, MeOH, THF; (ii) 2,2,2-trifluoroethylamine, HATU, Et_3N, DMF, 31% (2 steps); (g) MeI, NaH, DMF, 25%; (h) NCS, NaH, DMF, 69%; (i) various phenols, K_2CO_3, DMF, 43–62%; (j) MeMgBr, THF, 58–66%.



Scheme 3. Synthesis of spiro compounds. Reagents and conditions: (a) NaH, 2-nitrobenzyl bromide, DMF for **39**, 60%; NaH, 1-fluoro-2-nitrobenzene, DMF for **40**, 46%; NaH, air, DMF, then NaH, 2-nitrobenzyl bromide analogs for **41**–**43**, 35–64%; (b) (i) H_2, Pd/C, MeOH, EtOH; (ii) EtOH for **44**, 43% (2 steps); Fe, AcOH for **45**, 39%; (i) H_2, PtO_2, MeOH; (ii) NaOH aq, THF; (iii) HATU, Et_3N, DMF for **46**, 57% (3 steps); (i) $NH_2NH_2{\cdot}H_2O$, $FeCl_3{\cdot}6H_2O$, activated charcoal, THE, MeOH; (ii) NaOH aq, THF, MeOH; (iii) HATU, Et_3N, DMF for **47** and **48**, 45–69%; (c) 2,2,2-trifluoroethyltriflate, NaH or Cs_2CO_3, DMF, 40–73%; (d) NaH, paraformaldehyde, DMF, then H_2O; (e) 2-benzyloxyaniline, HATU, DMF, 55% (2 steps); (f) (i) H_2, Pd/C, MeOH, THF; (ii) PPh_3, DEAD, THF, then PBu_3, ADDP; (g) 2,2,2-trifluoroethyltriflate, NaH, DMF, 22% (3 steps).

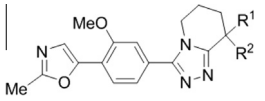
For benzoxazepine derivative **46**, HATU was used for intracyclization after preparing the aminobenzoic acid derived from **41**. The amide moiety of **46** was then alkylated with 2,2,2-trifluoroethyltriflate to give **20**. Benzoxazepine derivatives **22** and **23** were synthesized in a similar manner with that of **20**. Synthesis of the regioisomeric benzoxazepine analog **51** was achieved by the Mitsunobu reaction of the phenol derived by deprotection of **50**, which was prepared from **33** by hydroxymethylation of the 8-position and subsequent benzamide formation. Finally, 2,2,2-trifluoroethylation of amide moiety of **51** was conducted to give **21**.

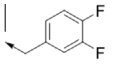
3. Results and discussion

All compounds were evaluated for inhibitory activity against $A\beta_{42}$ production in rat primary neuronal cells, lipophilicity (log*D* at pH 7.4),[8] and LLE (= $pIC_{50} - \log D$). We initially introduced various substituents to the 8-position of **1** as shown in Table 1. Methylation of the hydroxyl group of **3** slightly enhanced potency. However, the increased lipophilicity of **4** resulted in the decreased LLE value. While replacement of hydroxyl group of **3** with an aminomethyl group (**5**) or an acetamidomethyl group (**6**) did not offer positive effect on the potency or LLE, 2-hydroxypropan-2-yl derivative **7** showed an 8-fold enhancement of potency as well as a significant improvement of LLE. High LLE was also observed with 3,4-difluoro phenyl analog **8** as we reported in the previous work.[7] Furthermore, insertion of methylene linker (**9**) into the distal phenyl group of **8** greatly enhanced the LLE value, indicating that the LLE is controlled by not only the polar group at the 8-position, but also the position of the distal phenyl group. Since benzyl derivative **9** showed high clearance in mouse liver microsomes (93 μL/min/mg), we next modified both the 2-hydroxypropan-2-yl group and the benzyl moiety of **9**, which were suspected to be metabolically labile, in order to improve the microsomal stability, as shown in Figure 2.

We modified the 2-hydroxypropan-2-yl group of **9**, and assessed the effect on metabolic stability and LLE by the modification (Table 2). Although neither removing geminal dimethyl moieties from the 2-hydroxypropan-2-yl group (**10**) nor converting the hydroxyl group to a carbonyl group (**11**) affected the microsomal stability, carboxamide derivative **12** showed a significantly improved metabolic stability with slight decrease of LLE. In addition, more lipophilic trifluoroethyl amide analog **13** also showed low clearance in mouse liver microsomes, with higher LLE than

Table 1
$A\beta_{42}$-lowering effects, log*D* values, and LLE values of **1** and **3**–**9**



Compound	R^1	R^2	$A\beta_{42}$ IC_{50}[a] (nM)	Log*D*[b]	LLE[c]
1	H		240	3.2	3.5
3	OH		290	2.9	3.6
4	OMe		120	3.7	3.2
5			280	3.1	3.5
6			300	3.2	3.3
7			35	3.7	3.8
8			160	2.9	3.9
9			48	3.0	4.3

[a] IC_{50} values are means of triplicate measurements.
[b] Measured at pH 7.4.
[c] LLE = $pIC_{50} - \log D$.

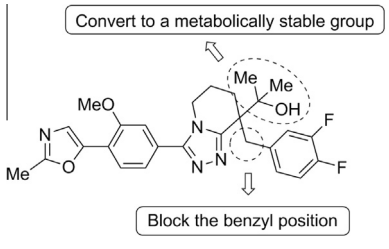
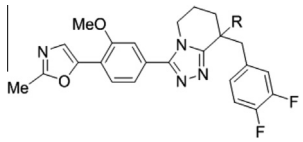
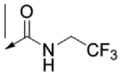
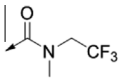


Figure 2. Approaches to improve metabolic stability of **9**.

Table 2
Aβ42-lowering effects, log*D* values, LLE values, and in vitro metabolic clearance of **10**–**14** in mouse liver microsomes



Compound	R	Aβ42 IC_{50}[a] (nM)	Log*D*[b]	LLE[c]	MLM[d] (μL/min/mg)
10		250	2.5	4.1	144
11		290	2.6	3.9	148
12		870	2.3	3.8	22
13		75	3.0	4.1	3
14		50	2.9	4.4	101

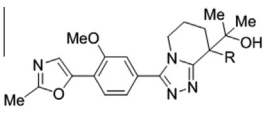
[a] IC_{50} values are means of triplicate measurements.
[b] Measured at pH 7.4.
[c] LLE = pIC_{50} − log*D*.
[d] In vitro metabolic clearance in mice liver microsomes.

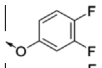
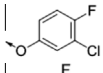
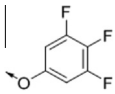
that of **12**. Introducing a methyl group into the amide moiety of **13** further enhanced Aβ42-lowering effect and LLE (**14**), though this modification significantly deteriorated the metabolic stability. We accordingly selected **13** for in vivo Aβ42-lowering test, and next turned our attention to modifying the benzyl moiety of **9**.

As shown in Table 3, we replaced the methylene at the benzyl position of **9**, a potential metabolic soft spot, with an oxygen atom (**15**). This modification significantly improved the microsomal stability while maintaining the high LLE value. Enhancement of Aβ42-lowering effect was then achieved by increasing minimal lipophilicity of the distal phenyl ring while retaining LLE (**16** and **17**). Since **16** and **17** maintained the good microsomal stability, we selected **16** and **17** for further in vivo examination.

We next pursued further improvement of LLE. Starting from trifluoroethyl amide analog **14**, which showed the highest LLE value, we designed spiro compounds in which the 8-position geminal

Table 3
Aβ42-lowering effects, log*D* values, LLE values, and in vitro metabolic clearance of **15**–**17** in mouse liver microsomes



Compound	R	Aβ42 IC_{50}[a] (nM)	Log*D*[b]	LLE[c]	MLM[d] (μL/min/mg)
15		88	2.8	4.3	−1
16		38	3.2	4.2	−7
17		41	3.2	4.2	−3

[a] IC_{50} values are means of triplicate measurements.
[b] Measured at pH 7.4.
[c] LLE = pIC_{50} − log*D*.
[d] In vitro metabolic clearance in mice liver microsomes.

substituents, the benzyl moiety and the methyl group on the amide moiety, were connected to fix the high-LLE conformation while improving microsomal stability, as depicted in Figure 3. In addition, undesirable flexibility of **14** as a drug for use in central nervous system (CNS) could be reduced by this modification.[9]

As shown in Table 4, the six-membered spiro derivative **18** showed both high LLE and low metabolic clearance. The spiro ring size of **18** was enlarged or reduced to seek a favorable conformation for LLE. While ring contraction resulted in slight decrease of the LLE (**19**), great enhancement of potency and LLE were observed with benzoxazepine derivative **20**, with moderate metabolic stability. Unfortunately, shifting the oxygen atom in the benzoxazepine ring of **20** to its benzyl position had a negative effect on the activity and LLE (**21**) in spite of improved metabolic stability, which suggests that the benzyl position of **20** is a main metabolized part. We accordingly introduced bulky substituents into the 6-position of benzoxazepine ring of **20** to metabolically shield its benzyl position by the steric hindrance. However, the metabolic stability could not be improved in either the 6-chloro (**22**) or the 6-trifluoromethyl (**23**) analog. As further reduction of metabolic clearance would be difficult while retaining high LLE, **22** and **23**, with potent Aβ42-lowering effect and very high LLE, were selected from spiro series for in vivo Aβ42-lowering test.

We next evaluated in vivo Aβ42-lowering effect of the selected compounds in wild-type mice (C57BL/6J), and the results are shown in Table 5. Trifluoroethyl amide analog **13**, with moderate in vitro Aβ42-lowering effect, was dosed at 30 mg/kg. On the basis of metabolic stabilities in mouse liver microsomes, 2-hydroxypropan-2-yl derivatives **16** and **17**, and spiro compounds **22** and **23** were orally administered at doses of 10 mg/kg and 30 mg/kg, respectively. Tested compounds except **13** (30 mg/kg) afforded positive Aβ42-lowering effects in mouse brain, and especially **16** (10 mg/kg) and **17** (10 mg/kg) exhibited the same or more potent activity than **22** (30 mg/kg) and **23** (30 mg/kg), most likely due to the much lower microsomal clearances of **16** and **17**. Consequently we selected 3,4,5-trifluorophenyl analog **17**, which exhibited the most potent Aβ42-lowering effect in mouse brain, and optically resolved **17** into compounds (*R*)-**17** and (*S*)-**17**.[10] The chiral configuration greatly affected the potency, and the eutomer (*R*)-**17** exhibited more than 460 times the Aβ42 inhibitory activity compared to the distomer (*S*)-**17**, as shown in Table 6. We also reported that compound (*R*)-**17** reduced both Aβ42 and Aβ40, but increased Aβ37, without affecting total Aβ level in cellular assay using Neuro2A cells, indicating that (*R*)-**17** would modulate γ-secretase activity by shifting the APP cleavage site.[11] In addition, (*R*)-**17** exhibited no activity against Notch signal (IC_{50} >10 μM). Potent in vivo Aβ42-lowering effect in the brain of (*R*)-**17** was confirmed at a dose of 6 mg/kg in normal mice (Table 6) with lower drug exposure level (0.677 μg/g in cortex and 3.307 μg/mL in plasma at 3 h after oral dosing) than those of **2**. Repeated oral treatment of (*R*)-**17** (3, 6 mg/kg) to APP-transgenic Tg2576 mice was then performed to further investigate the pharmacological profiles. We first confirmed that concentration of (*R*)-**17** in cortex 18 h after single oral administration to Tg2576 mice was below the measurable limit, and the multiple administration had little effect on the compound concentration in plasma or cortex (data not shown).

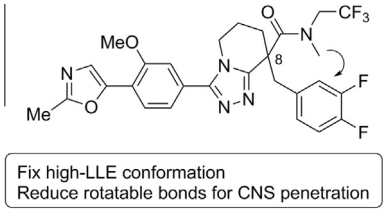
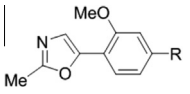
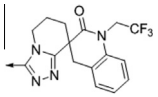
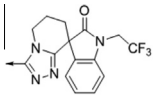
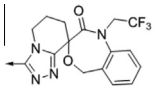
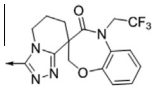
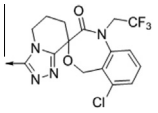
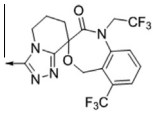


Figure 3. Design of spiro compounds from trifluoroethyl amide analog **14**.

Table 4
Aβ$_{42}$-lowering effects, log*D* values, LLE values, and in vitro metabolic clearance of **18**–**23** in mouse liver microsomes



Compound	R	Aβ$_{42}$ IC$_{50}$[a] (nM)	Log*D*[b]	LLE[c]	MLM[d] (μL/min/mg)
18		240	2.4	4.2	29
19		520	2.2	4	−10
20		140	2.2	4.6	62
21		420	2.5	3.9	11
22		30	2.8	4.8	107
23		31	2.9	4.6	83

[a] IC$_{50}$ values are means of triplicate measurements.
[b] Measured at pH 7.4.
[c] LLE = pIC$_{50}$ − log*D*.
[d] In vitro metabolic clearance in mice liver microsomes.

Table 5
In vivo Aβ$_{42}$-lowering effects in C57BL/6J mice

	Brain Aβ$_{42}$[a] (%)	Plasma Aβ$_{42}$[a] (%)
13 (30 mg/kg)	96	80
16 (10 mg/kg)	71	42
17 (10 mg/kg)	66	35
22 (30 mg/kg)	77	49
23 (30 mg/kg)	75	39

[a] Expressed as % vehicle at 3 h after p.o. administration, n = 4–6.

After 58-day oral administration of (*R*)-**17** at both doses, significant and dose-dependent reduction of insoluble brain Aβ$_{42}$ as well as soluble brain Aβ$_{42}$ was observed, as shown in Figure 4A and B. We also confirmed decreased level of Aβ$_{40}$ without effect on total Aβ (Fig. 4C and D). We used the novel object recognition test (NORT) to assess the effect of (*R*)-**17** on cognitive function on the 45th day of the course of the multiple administration. The preference ratio was significantly improved to the normal range compared to the vehicle-treated group (Fig. 5). Furthermore, continuous cognitive improvement was observed even after withdrawal of (*R*)-**17** administration for 7 days, as we had reported.[11] These studies indicate the chronic treatment (*R*)-**17** ameliorated cognitive deficit of Tg2576 mice by lowering pathogenic Aβ, and (*R*)-**17** might have a disease-modifying effect.

4. Conclusion

In conclusion, we optimized 5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine derivatives using LLE as a drug-likeness guideline. Introduction of the 2-hydroxypropan-2-yl group to the 8-position of the core scaffold and insertion of methylene linker into the distal phenyl group at the 8-position effectively enhanced LLE to give **9**. Improvement of the insufficient microsomal stability of **9** in mouse liver microsomes was achieved by converting the 2-hydroxypropan-2-yl group to trifluoroethyl amide, or replacing the benzyl moiety with a phenoxy group, while retaining the high LLE. In addition, spiro compounds that showed extremely high LLE were discovered by the design from the trifluoroethyl amide analog to reduce the flexibility and fix its active conformation. Through in vivo Aβ42-lowering effect screening, we identified highly potent GSM (*R*)-**17**. Chronic treatment with (*R*)-**17** significantly reduced brain Aβ$_{42}$ and then successfully ameliorated cognitive impairment in Tg2576 mice as an AD model. These results suggest that GSM (*R*)-**17** should be a promising candidate for AD therapy. Further pharmacological profiling of (*R*)-**17** is in progress and will be reported in due course.

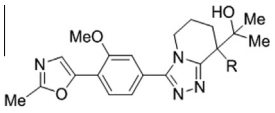
5. Experimental procedure

Melting points were determined on a Yanaco micro melting point apparatus or a Büchi melting point apparatus B-545 and were uncorrected. Proton nuclear magnetic resonance (1H NMR) spectra were recorded on a Varian Mercury-300 (300 MHz), a Bruker AVANCE III (300 MHz) or a Bruker Advance III plus (400 MHz) spectrometer. Chemical shifts are given in parts per million (ppm) with tetramethylsilane as an internal standard. Abbreviations are used as follows: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, m = multiplet, dd = doublet of doublets, dt = doublet of triplets, td = triplet of doublets, qd = quartet of doublets, br s = broad singlet. Coupling constants (*J* values) are given in hertz (Hz). Elemental analyses were carried out by Takeda Analytical Laboratories Ltd, and were within 0.4% of the theoretical values unless otherwise noted. LC–MS analysis was performed on a Waters Liquid Chromatography–Mass Spectrometer System (LC–MS) or a Shimadzu LC–MS, 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. Reagents and solvents were obtained from commercial sources and used without further purification. Chromatographic purification was carried out on silica gel columns [(Merck Kieselgel 60, 70–230 mesh or 230–400 mesh, Merck) or (Chromatorex NH-DM 1020, 100–200 mesh, Fuji Silysia Chemical, Ltd)] or on Purif-Pack (Si or NH, Moritex). Our method employed in determining purity utilized a Shimadzu UFLC instrument with an L-column 2 ODS 2 μm 2.1 × 30 mm. All tested compounds analyzed were >95% pure unless otherwise indicated.

5.1. 8-(3,4-Dichlorophenyl)-8-methoxy-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine (4)

NaH (60% in oil, 0.014 g, 0.35 mmol) was added to a mixture of **1** (0.16 g, 0.35 mmol) in DMF (2 mL) at 0 °C. After the mixture was stirred at 0 °C under air for 30 min, NaH (60% in oil, 0.014 g, 0.35 mmol) was added to the reaction mixture. After the mixture was stirred at 0 °C under N_2 for 30 min, MeI (22 μL, 0.35 mmol) was added to the reaction mixture. After being stirred at room temperature for 1 h, the reaction mixture was diluted with EtOAc and brine, and extracted with EtOAc. The extract was washed with brine, dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by preparative HPLC (L-Column 2 ODS, eluted with H_2O in acetonitrile containing 0.1% TFA) to give a solid, which was recrystallized from EtOAc/hexane to give **4**

Table 6
Aβ42-lowering effects, log*D* values, LLE values, and in vitro metabolic clearance in mouse liver microsomes, and in vivo Aβ42-lowering effects of (*R*)-**17** and (*S*)-**17** in C57BL/6J mice



Compound	R	Aβ42 IC_{50}[a] (nM)	Log*D*[b]	LLE[c]	MLM[d] (μL/min/mg)	In vivo Aβ42[e] (%)	
						Brain	Plasma
(*R*)-**17**		26	3.2	4.4	−7	60	36
(*S*)-**17**		>10000	3.2		−20	N.T.[f]	N.T.[f]

[a] IC_{50} values are means of triplicate measurements.
[b] Measured at pH 7.4.
[c] LLE = $pIC_{50} - \log D$.
[d] In vitro metabolic clearance in mice liver microsomes.
[e] Expressed as % vehicle at 3 h after p.o. administration, 6 mg/kg, $n = 6$.
[f] Not tested.

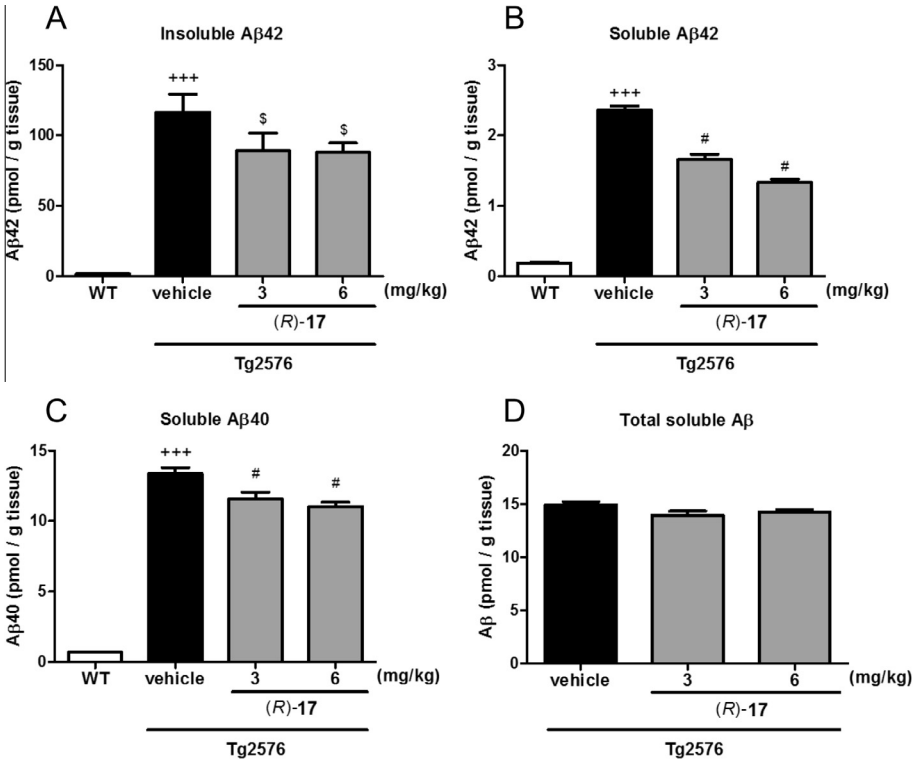


Figure 4. Effect of (*R*)-**17** on insoluble Aβ42 (A), soluble Aβ42 (B), soluble Aβ40 (C), and soluble total Aβ (D) in Tg2576 mouse cortex. (*R*)-**17** was administrated for 58 days and at 3 h prior to decapitation, $n = 10–15$. The bars represent means ± SEM. ^{+++}p <0.001, Aspin–Welch test versus wild-type (WT) mice treated with vehicle. $^{\$}p$ <0.025, Shirley–Williams test versus Tg mice treated with vehicle. $^{\#}p$ <0.025, Williams test versus Tg mice treated with vehicle.

(0.053 g, 31%) as a colorless solid, mp 166–169 °C. ^{1}H NMR ($CDCl_3$) δ: 1.89–2.07 (2H, m), 2.34–2.52 (2H, m), 2.57 (3H, s), 3.31 (3H, s), 3.98–4.11 (4H, m), 4.24–4.33 (1H, m), 7.29–7.36 (1H, m), 7.44–7.49 (1H, m), 7.50–7.53 (2H, m), 7.59 (1H, d, $J = 1.9$ Hz), 7.86 (1H, d, $J = 8.0$ Hz). MS *m*/*z*: 485.1 $(M+H)^+$. Anal. Calcd for $C_{24}H_{22}Cl_2N_4O_3$: C, 59.39; H, 4.57; N, 11.54. Found: C, 59.33; H, 4.56; N, 11.51.

5.2. {8-(3,4-Dichlorophenyl)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridin-8-yl}methyl 4-methylbenzenesulfonate (24)

NaH (60% in oil, 0.032 g, 0.79 mmol) was added to a mixture of **1** (0.30 g, 0.66 mmol) in DMF (5 mL) at 0 °C. After the mixture was stirred at room temperature under N_2 for 1 h under, paraformaldehyde

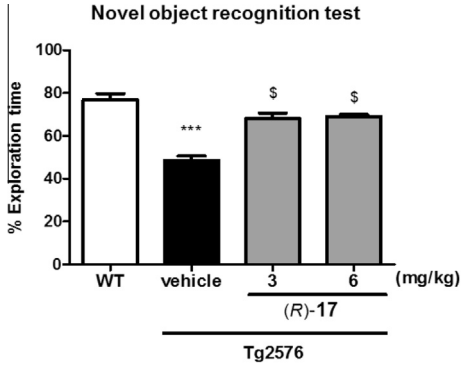


Figure 5. Effect of (*R*)-**17** on novel object recognition test in Tg2576 mouse on the 45th day of 58-day oral administration, $n = 10–15$. The bars represent means ± SEM. $^{***}p < 0.001$, *t*-test versus WT mice treated with vehicle. $^{\$}p < 0.025$, Shirley–Williams test versus Tg mice treated with vehicle.

(0.040 g) was added to the reaction mixture. After being stirred for 30 min, the reaction mixture was diluted water and extracted with EtOAc. The extract was washed with water and brine, dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by silica gel column chromatography (MeOH/EtOAc) to give {8-(3,4-Dichlorophenyl)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridin-8-yl}methanol (0.043 g, 13%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 1.76–1.93 (1H, m), 2.03–2.20 (2H, m), 2.30–2.40 (1H, m), 2.56 (3H, s), 3.83–3.89 (1H, m), 3.95–4.03 (2H, m), 4.05 (3H, s), 4.13–4.26 (2H, m), 7.09 (1H, dd, $J = 8.5$, 2.2 Hz), 7.29 (1H, dd, $J = 8.1$, 1.5 Hz), 7.33 (1H, d, $J = 2.2$ Hz), 7.41 (1H, d, $J = 8.5$ Hz), 7.50 (1H, d, $J = 1.6$ Hz), 7.51 (1H, s), 7.83 (1H, d, $J = 8.0$ Hz). MS *m*/*z*: 485.2 $(M+H)^+$. Anal. Calcd for $C_{24}H_{22}Cl_2N_4O_3$: C, 59.39; H, 4.57; N, 11.54. Found: C, 59.33; H, 4.64; N, 11.77.

To a mixture of the methanol derivative (0.18 g, 0.37 mmol) in pyridine (3 mL) was added 4-methylbenzenesulfonyl chloride (0.084 g, 0.44 mmol) at 0 °C. After the mixture was stirred at room temperature for 4 h, 4-methylbenzenesulfonyl chloride (0.084 g, 0.44 mmol) was added to reaction the mixture. After being stirred at room temperature for 2 h, and at 50 °C overnight, the reaction mixture was diluted with water, and the precipitate was collected by filtration. The solid was purified by silica gel column chromatography (MeOH/EtOAc) to give **24** (0.16 g, 69%) as a colorless amorphous. ^{1}H NMR ($CDCl_3$) δ: 1.73–1.94 (1H, m), 2.08–2.21 (1H, m), 2.26–2.39 (1H, m), 2.44 (3H, s), 2.55 (3H, s), 2.64–2.76 (1H, m), 3.94–4.15 (5H, m), 4.43–4.49 (1H, m), 4.51–4.57 (1H, m), 7.21–7.26 (2H, m), 7.26–7.30 (2H, m), 7.31–7.35 (1H, m), 7.43 (1H, d, $J = 1.6$ Hz), 7.47 (1H, d, $J = 2.2$ Hz), 7.50 (1H, s), 7.57–7.64 (2H, m), 7.83 (1H, d, $J = 8.2$ Hz). MS *m*/*z*: 638.9 $(M+H)^+$.

5.3. 1-{8-(3,4-Dichlorophenyl)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridin-8-yl}methanamine (5)

A mixture of **25** (0.15 g, 0.24 mmol) and sodium azide (0.046 g, 0.71 mmol) in DMSO (2 mL) was stirred at 100 °C for 2 days. Water was added to the reaction mixture, and the precipitate was collected by filtration. After the solid was dissolved in THF (2 mL)/water (2 mL), diphenylphosphino-polystyrene (1.99 mmol/g, 0.24 g) was added to the mixture. After being stirred at 60 °C overnight, the reaction mixture was filtered, and the filtrate was and concentrated in vacuo. The residue was recrystallized from EtOAc/hexane to give **5** (0.056 g, 49%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 1.77–1.91 (1H, m), 2.01–2.13 (1H, m), 2.23 (1H, td, $J = 13.2$, 2.6 Hz), 2.30–2.40 (1H, m), 2.56 (3H, s), 3.19 (1H, d, $J = 13.2$ Hz), 3.42 (1H, d, $J = 13.4$ Hz), 3.96–4.08 (4H, m), 4.12–4.22 (1H, m), 7.14 (1H, dd, $J = 8.4$, 2.3 Hz), 7.29 (1H, dd, $J = 8.2$, 1.4 Hz), 7.36–7.44 (2H, m), 7.50 (2H, s), 7.83 (1H, d, $J = 8.0$ Hz). MS *m*/*z*: 484.3 $(M+H)^+$.

5.4. *N*-({8-(3,4-Dichlorophenyl)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridin-8-yl}methyl)acetamide (6)

To a mixture of **5** (0.077 g, 0.16 mmol) and triethylamine (26 μL, 0.19 mmol) in THF (1 mL) was added acetic anhydride (16 μL, 0.17 mmol) at 0 °C. After being stirred at room temperature for 2 h, the reaction mixture was diluted with water, and the precipitate was collected by filtration. The solid was purified by silica gel column chromatography (MeOH/EtOAc) to give **6** (0.060 g, 72%) as a colorless amorphous. ^{1}H NMR ($CDCl_3$) δ: 1.74–1.86 (1H, m), 1.89 (3H, s), 1.97–2.12 (2H, m), 2.33–2.44 (1H, m), 2.56 (3H, s), 3.58 (1H, dd, $J = 13.6$, 3.7 Hz), 3.94–4.10 (4H, m), 4.16–4.27 (1H, m), 4.32 (1H, dd, $J = 13.6$, 8.4 Hz), 7.03–7.13 (2H, m), 7.21 (1H, d, $J = 2.5$ Hz), 7.31 (1H, dd, $J = 8.0$, 1.4 Hz), 7.40 (1H, d, $J = 8.2$ Hz), 7.52 (2H, s), 7.85 (1H, d, $J = 8.0$ Hz). MS *m*/*z*: 526.3 $(M+H)^+$.

5.5. Diethyl(3,4-dichlorophenyl)propanedioate (26a)

To a solution of ethyl 2-(3,4-dichlorophenyl)acetate (5.0 g, 21 mmol) in THF (60 mL) were added NaH (60% in oil, 1.7 g, 43 mmol) and diethyl carbonate (13 mL, 110 mmol) at room temperature. After being refluxed for 2 h, the reaction mixture was quenched with satd NH_4Cl aq and extracted with EtOAc. The extract was washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc/hexane) to give **26a** (6.6 g, 100%) as a pale yellow oil. ^{1}H NMR ($CDCl_3$) δ: 1.25–1.33 (6H, m), 4.11–4.28 (4H, m), 4.54 (1H, s), 7.24–7.27 (1H, m), 7.43 (1H, d, $J = 8.1$ Hz), 7.51 (1H, d, $J = 2.4$ Hz).

5.6. Diethyl(3,4-difluorophenyl)propanedioate (26b)

Compound **26b** was prepared from **25b** by a manner similar to that described for **26a** in 90% yield as a pale yellow oil. ^{1}H NMR ($CDCl_3$) δ: 1.27 (6H, t, $J = 7.1$ Hz), 4.16–4.29 (4H, m), 4.55 (1H, s), 7.08–7.20 (2H, m), 7.26–7.35 (1H, m).

5.7. Ethyl 3-(3,4-dichlorophenyl)-2-oxopiperidine-3-carboxylate (28a)

NaOEt (20% in ethanol, 0.84 mL) was added to a solution of **26a** (6.6 g, 21 mmol) in *t*-BuOH (20 mL) under N_2 at room temperature. After the mixture was stirred for 30 min, acrylonitrile (1.4 mL, 21 mmol) was added to the reaction mixture at room temperature. After being stirred at room temperature overnight, the reaction mixture was quenched with satd NH_4Cl aq and extracted with EtOAc. The extract was washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc/hexane) to give **27a** as crude. A mixture of the crude compound **27a** and Raney cobalt (30 g) in 2 M ammonia ethanol solution (60 mL) was hydrogenated under balloon pressure at room temperature overnight. The catalyst was removed by filtration through Celite and the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc/hexane) to give **28a** (2.8 g, 41% from **26a**) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 1.27 (3H, t, $J = 7.0$ Hz), 1.56–1.91 (2H, m), 2.23–2.34 (1H, m), 2.61–2.74 (1H, m), 3.28–3.51 (2H, m), 4.20–4.30 (2H, m), 6.41 (1H, br s), 7.21 (1H, dd, $J = 8.5$, 2.5 Hz), 7.41 (1H, d, $J = 8.5$ Hz), 7.46 (1H, d, $J = 2.5$ Hz).

5.8. Ethyl 3-(3,4-difluorophenyl)-2-oxopiperidine-3-carboxylate (28b)

Compound **28b** was prepared from **26b** by a manner similar to that described for **28a** in 73% yield as an off-white solid. ^{1}H NMR ($CDCl_3$) δ: 1.26 (3H, t, J = 7.2 Hz), 1.58–1.90 (2H, m), 2.23–2.34 (1H, m), 2.61–2.72 (1H, m), 3.28–3.50 (2H, m), 4.25 (2H, q, J = 7.2 Hz), 6.47 (1H, br s), 7.06–7.30 (3H, m). MS m/z: 284.3 $(M+H)^+$.

5.9. Ethyl 3-(3,4-dichlorophenyl)-2-thioxopiperidine-3-carboxylate (29a)

Lawesson's reagent (0.90 g, 2.2 mmol) was added to a suspension of **28a** (1.0 g, 3.2 mmol) in toluene (20 mL) under Ar at room temperature. After being stirred at 110 °C for 2 h, the reaction mixture was cooled to room temperature and concentrated in vacuo. The residue was purified by NH silica gel column chromatography (EtOAc/hexane) to give **29a** (0.83 g, 79%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 1.29 (3H, t, J = 7.1 Hz), 1.46–1.64 (1H, m), 1.77–1.90 (1H, m), 2.26–2.36 (1H, m), 2.63–2.75 (1H, m), 3.28–3.54 (2H, m), 4.20–4.33 (2H, m), 7.22–7.28 (1H, m), 7.41 (1H, d, J = 8.5 Hz), 7.50 (1H, d, J = 2.5 Hz), 8.56 (1H, br s). MS m/z: 332.1 $(M+H)^+$.

5.10. Ethyl 3-(3,4-difluorophenyl)-2-thioxopiperidine-3-carboxylate (29b)

Compound **29b** was prepared from **28b** by a manner similar to that described for **29a** in 90% yield as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 1.29 (3H, t, J = 7.1 Hz), 1.45–1.62 (1H, m), 1.77–1.91 (1H, m), 2.26–2.35 (1H, m), 2.63–2.75 (1H, m), 3.28–3.53 (2H, m), 4.26 (2H, q, J = 7.1 Hz), 7.07–7.19 (2H, m), 7.23–7.32 (1H, m), 8.57 (1H, br s).

5.11. Ethyl 8-(3,4-dichlorophenyl)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine-8-carboxylate (31a)

MeI (0.47 ml, 7.5 mmol) was added to a solution of **29a** (0.83 g, 2.5 mmol) in MeCN (10 mL) at room temperature. After being stirred at 50 °C for 4 h, the reaction mixture was concentrated in vacuo and diluted with EtOH (10 mL). To the mixture was added **30** (0.62 g, 2.5 mmol) at room temperature. After being stirred at 90 °C overnight, the reaction mixture was quenched with satd $NaHCO_3$ aq and extracted with EtOAc. The extract was washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography (MeOH/EtOAc) to give **31a** (0.78 g, 59%) as an off-white amorphous. ^{1}H NMR ($CDCl_3$) δ: 1.25 (3H, t, J = 7.1 Hz), 1.89–2.20 (2H, m), 2.32–2.44 (1H, m), 2.55 (3H, s), 2.80–2.91 (1H, m), 4.04 (3H, s), 4.08–4.36 (4H, m), 7.26–7.30 (2H, m), 7.42 (1H, d, J = 8.5 Hz), 7.48–7.51 (3H, m), 7.83 (1H, d, J = 8.0 Hz). MS m/z: 527.1 $(M+H)^+$.

5.12. Ethyl 8-(3,4-difluorophenyl)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine-8-carboxylate (31b)

Compound **31b** was prepared from **29b** by a manner similar to that described for **31a** in 68% yield as a light brown amorphous. ^{1}H NMR ($CDCl_3$) δ: 1.25 (3H, t, J = 7.2 Hz), 1.89–2.21 (2H, m), 2.31–2.45 (1H, m), 2.55 (3H, s), 2.79–2.92 (1H, m), 4.00–4.06 (3H, m), 4.08–4.36 (4H, m), 7.08–7.18 (2H, m), 7.24–7.37 (2H, m), 7.51 (2H, s), 7.84 (1H, d, J = 7.9 Hz). MS m/z: 495.2 $(M+H)^+$.

5.13. 2-{8-(3,4-Dichlorophenyl)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridin-8-yl}propan-2-ol (7)

Methylmagnesium bromide (1 M in THF, 2.4 mL, 2.4 mmol) was added to a solution of **31a** (0.25 g, 0.47 mmol) in THF (2.5 mL) under Ar at room temperature. After being stirred under Ar at 0 °C for 1 h, and at room temperature overnight, the reaction mixture was quenched with satd NH_4Cl aq and extracted with EtOAc. The extract was washed with satd $NaHCO_3$ aq and brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by NH silica gel column chromatography (EtOAc/hexane) and recrystallization from EtOAc/hexane to give **7** (0.099 g, 41%) as a colorless solid, mp 186–188 °C. ^{1}H NMR ($CDCl_3$) δ: 1.15 (3H, s), 1.35 (3H, s), 1.75–1.92 (1H, m), 2.01–2.20 (2H, m), 2.55 (3H, s), 2.68–2.79 (1H, m), 3.94–4.15 (5H, m), 5.25 (1H, br s), 7.23–7.29 (1H, m), 7.36–7.41 (2H, m), 7.46–7.51 (2H, m), 7.60–7.63 (1H, m), 7.82 (1H, d, J = 8.2 Hz). MS m/z: 513.2 $(M+H)^+$.

5.14. 2-{8-(3,4-Difluorophenyl)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridin-8-yl}propan-2-ol (8)

Compound **8** was prepared from **31b** by a manner similar to that described for **7** in 36% yield as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 1.15 (3H, s), 1.35 (3H, s), 1.77–1.93 (1H, m), 2.00–2.23 (2H, m), 2.55 (3H, s), 2.67–2.81 (1H, m), 3.92–4.18 (5H, m), 5.26 (1H, br s), 7.03–7.18 (1H, m), 7.22–7.32 (2H, m), 7.35–7.44 (1H, m), 7.47–7.53 (2H, m), 7.82 (1H, d, J = 8.0 Hz). MS m/z: 481.1 $(M+H)^+$.

5.15. Ethyl 3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine-8-carboxylate (33)

A mixture of **32** (35 g, 200 mmol), trimethyloxonium tetrafluoroborate (95%, 32 g, 200 mmol) in MeCN (600 mL) was stirred at room temperature for 16 h. To the reaction mixture was added **30** (50 g, 200 mmol). After being refluxed for 24 h, the reaction mixture was concentrated in vacuo. After satd $NaHCO_3$ aq was added the residue, the insoluble material was removed by filtration, and the filtrate was extracted with EtOAc. The extract was washed with brine, and dried over Na_2SO_4 and concentrated in vacuo. The residue was crystallized from MeOH/EtOAc to give **33** (29 g, 38%) as a brown solid. ^{1}H NMR ($CDCl_3$) δ: 1.32 (3H, t, J = 7.1 Hz), 1.94–2.42 (4H, m), 2.55 (3H, s), 3.95–4.35 (8H, m), 7.23 (1H, dd, J = 8.0, 1.6 Hz), 7.44 (1H, d, J = 1.6 Hz), 7.49 (1H, s), 7.82 (1H, d, J = 8.0 Hz). MS m/z: 383.3 $(M+H)^+$.

5.16. Ethyl 8-(3,4-difluorobenzyl)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine-8-carboxylate (34)

To a suspension of NaH (60% in oil, 0.34 g, 8.5 mmol) in DMF (5 mL) was added **33** (3.0 g, 7.8 mmol) at 0 °C. After the mixture was stirred for 30 min at 0 °C, 3,4-difluorobenzyl bromide (1.5 g, 9.4 mmol) was added at 0 °C. After being stirred for 1 h at 0 °C, the reaction mixture was diluted with EtOAc, washed with satd NH_4Cl aq and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography (MeOH/EtOAc) to give **34** (3.5 g, 87%) as a colorless amorphous. ^{1}H NMR ($CDCl_3$) δ: 1.37 (3H, t, J = 7.0 Hz), 2.16–2.30 (1H, m), 2.34–2.60 (5H, m), 2.70–2.83 (1H, m), 3.97–4.16 (4H, m), 4.19–4.31 (1H, m), 4.39 (2H, q, J = 7.0 Hz), 7.21–7.28 (1H, m), 7.46 (1H, d, J = 1.4 Hz), 7.50 (1H, s), 7.83 (1H, d, J = 8.2 Hz). MS m/z: 509.2 $(M+H)^+$.

5.17. 2-{8-(3,4-Difluorobenzyl)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridin-8-yl}propan-2-ol (9) and 1-{8-(3,4-difluorobenzyl)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridin-8-yl}ethanone (11)

Methylmagnesium bromide (1 M in THF, 13 mL, 13 mmol) was added to a solution of **34** (1.6 g, 3.2 mmol) in THF (16 mL) at 0 °C. After being stirred at 0 °C for 1 h, the reaction mixture was quenched with satd NH_4Cl aq and extracted with EtOAc. The extract was washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by NH silica gel column chromatography (EtOAc/hexane) to give **9** (0.74 g, 48%) as a colorless amorphous, and **11** (0.12 g, 8%) as a colorless solid. **9**: ^{1}H NMR ($CDCl_3$) δ: 1.09 (3H, s), 1.42 (3H, s), 1.61–1.73 (2H, m), 1.91–2.04 (2H, m), 2.55 (3H, s), 2.84 (1H, d, J = 13.5 Hz), 3.43–3.55 (1H, m), 3.66–3.78 (1H, m), 3.94 (1H, d, J = 13.5 Hz), 4.04 (3H, s), 5.15 (1H, br s), 6.54–6.75 (2H, m), 6.87–6.98 (1H, m), 7.06 (1H, dd, J = 8.0, 1.4 Hz), 7.38 (1H, d, J = 1.4 Hz), 7.50 (1H, s), 7.80 (1H, d, J = 8.0 Hz). MS m/z: 495.1 $(M+H)^+$. **11**: ^{1}H NMR ($CDCl_3$) δ: 1.51–1.60 (1H, m), 1.76–1.94 (2H, m), 2.41–2.52 (4H, m), 2.55 (3H, s), 3.21 (1H, d, J = 13.5 Hz), 3.70–3.83 (2H, m), 4.01–4.10 (4H, m), 6.80–6.88 (1H, m), 6.89–7.06 (2H, m), 7.20 (1H, dd, J = 8.0, 1.6 Hz), 7.47 (1H, d, J = 1.6 Hz), 7.50 (1H, s), 7.81 (1H, d, J = 8.0 Hz). MS m/z: 479.3 $(M+H)^+$.

5.18. {8-(3,4-Difluorobenzyl)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridin-8-yl}methanol (10)

A solution of **34** (0.18 g, 0.35 mmol) in THF (2 mL) was added to a suspension of LAH (0.054 g, 1.4 mmol) in THF (2 mL) at 0 °C, and the reaction mixture was stirred for 10 min at 0 °C. After sodium sulfate decahydrate (0.50 g) was added to the mixture at 0 °C, the mixture was filtered and concentrated in vacuo. The residue was purified by NH silica gel column chromatography (EtOAc/hexane) to give **10** (0.12 g, 73%) as a colorless amorphous. ^{1}H NMR ($CDCl_3$) δ: 1.51–1.74 (1H, m), 177–2.04 (3H, m), 2.55 (3H, s), 3.16 (1H, t, J = 13.7 Hz), 3.27 (1H, d, J = 13.7 Hz), 3.58–3.74 (2H, m), 3.95 (2H, t, J = 5.9 Hz), 4.03 (3H, s), 4.07–4.20 (1H, m), 6.85–6.94 (1H, m), 6.96–7.12 (2H, m), 7.17 (1H, dd, J = 8.1, 1.5 Hz), 7.41 (1H, d, J = 1.5 Hz), 7.49 (1H, s), 7.80 (1H, d, J = 8.1 Hz). MS m/z: 467.0 $(M+H)^+$.

5.19. 8-(3,4-Difluorobenzyl)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine-8-carboxamide (12)

A mixture of **34** (0.10 g, 0.20 mmol), formamide (79 μL, 2.0 mmol) and NaOMe (28% in MeOH, 0.2 mL) in DMF (1 mL) was stirred for 30 min at 70 °C. After being cooled to room temperature, satd NH_4Cl aq was added to the mixture. The precipitate was collected by filtration, washed with water and IPE to give **12** (0.080 g, 85%) as an off-white solid, mp 208–210 °C. ^{1}H NMR ($CDCl_3$) δ: 1.55–1.70 (1H, m), 1.74–2.08 (2H, m), 2.55 (3H, s), 2.64–2.75 (1H, m), 3.34 (1H, d, J = 13.5 Hz), 3.63 (1H, d, J = 13.5 Hz), 3.76–3.91 (1H, m), 4.00–4.14 (4H, m), 5.54 (1H, d, J = 3.0 Hz), 6.82–6.91 (1H, m), 6.92–7.08 (2H, m), 7.21 (1H, dd, J = 8.1, 1.5 Hz), 7.44 (1H, d, J = 1.5 Hz), 7.50 (1H, s), 7.61 (1H, d, J = 3.0 Hz), 7.82 (1H, d, J = 8.1 Hz). MS m/z: 480.4 $(M+H)^+$. Anal. Calcd for $C_{25}H_{23}F_2N_5O_3$: C, 62.62; H, 4.83; N, 14.61. Found: C, 62.43; H, 4.89; N, 14.39. Purity: 90%.

5.20. 8-(3,4-Difluorobenzyl)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-*N*-(2,2,2-trifluoroethyl)-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine-8-carboxamide (13)

To a mixture of **34** (0.20 g, 0.39 mmol) in THF (1 mL) / MeOH (1 mL) was added 1 M NaOH aq (0.39 mL, 0.39 mmol) at room temperature, and the mixture was stirred at room temperature for 3 h. The reaction mixture was concentrated in vacuo. Et_3N (0.066 mL, 0.47 mmol) and 2,2,2-trifluoroethylamine (0.037 mL, 0.47 mmol) in DMF (2 mL) were added to the residue. After HATU (0.18 g, 0.47 mmol) was added to the mixture at 0 °C, the mixture was stirred at 0 °C for 1 h. The reaction mixture was diluted with EtOAc, washed with brine and dried over dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc/hexane) and recrystallization from EtOAc/MeOH to give **13** (0.069 g, 31%) as a colorless solid, mp 247–249 °C. ^{1}H NMR ($CDCl_3$) δ: 1.65–1.86 (2H, m), 1.94–2.06 (1H, m), 2.57 (3H, s), 2.68–2.79 (1H, m), 3.30 (1H, d, J = 13.5 Hz), 3.60 (1H, d, J = 13.5 Hz), 3.71–3.92 (2H, m), 3.99–4.13 (5H, m), 6.78–6.86 (1H, m), 6.86–6.96 (1H, m), 6.97–7.09 (1H, m), 7.22 (1H, d, J = 8.0 Hz), 7.47 (1H, s), 7.52 (1H, s), 7.84 (1H, d, J = 8.0 Hz), 8.27 (1H, t, J = 6.3 Hz). MS m/z: 562.4 $(M+H)^+$. Anal. Calcd for $C_{27}H_{24}F_5N_5O_3$: C, 57.74; H, 4.49; N, 12.50. Found: C, 57.75; H, 4.31; N, 15.47.

5.21. 8-(3,4-Difluorobenzyl)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-*N*-methyl-*N*-(2,2,2-trifluoroethyl)-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine-8-carboxamide (14)

To a mixture of **13** (0.10 g, 0.18 mmol) in DMF (2 mL) was added NaH (60% in oil, 7.8 mg) at 0 °C, and the mixture was stirred at room temperature for 1 h. After methyl iodide (0.012 mL, 0.20 mmol) was added to the reaction mixture at 0 °C, the mixture was stirred at room temperature for 3 h. The reaction mixture was diluted with EtOAc, washed with brine and dried over dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc/hexane) and recrystallization from EtOAc/MeOH to give **14** (0.025 g, 25%) as a colorless solid, mp 113–116 °C. ^{1}H NMR ($CDCl_3$) δ: 1.63–1.76 (1H, m), 1.79–1.94 (1H, m), 1.98–2.11 (1H, m), 2.43–2.61 (4H, m), 2.92 (3H, s), 3.58–3.73 (2H, m), 3.84–4.02 (2H, m), 4.03–4.09 (4H, m), 4.32–4.51 (1H, m), 6.82–7.09 (3H, m), 7.18 (1H, d, J = 8.0 Hz), 7.44 (1H, s), 7.52 (1H, s), 7.84 (1H, d, J = 8.0 Hz). MS m/z: 576.4 $(M+H)^+$. Anal. Calcd for $C_{28}H_{26}F_5N_5O_3$: C, 58.43; H, 4.55; N, 12.17. Found: C, 58.26; H, 4.79; N, 11.87.

5.22. Ethyl 8-chloro-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine-8-carboxylate (35)

To a suspension of NaH (60% in oil, 0.12 g, 2.9 mmol) in DMF (10 mL) was added **33** (1.0 g, 2.6 mmol) under Ar at 0 °C. After the reaction mixture was stirred for 20 min at 0 °C, NCS (0.38 g, 2.9 mmol) was added to the mixture at 0 °C. After being stirred for 20 min at 0 °C, the reaction mixture was diluted with EtOAc and satd NH_4Cl aq, and extracted with EtOAc. The extract was washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by NH silica gel column chromatography (MeOH/EtOAc) to give **35** (0.75 g, 69%) as an off-white solid. ^{1}H NMR ($CDCl_3$) δ: 1.37 (3H, t, J = 7.1 Hz), 2.16–2.61 (6H, m), 2.70–2.85 (1H, m), 3.96–4.17 (4H, m), 4.19–4.30 (1H, m), 4.34–4.46 (2H, m), 7.20–7.29 (1H, m), 7.46 (1H, d, J = 1.4 Hz), 7.50 (1H, s), 7.83 (1H, d, J = 8.2 Hz). MS m/z: 417.1 $(M+H)^+$.

5.23. Ethyl 8-(3,4-difluorophenoxy)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine-8-carboxylate (36)

A mixture of **35** (0.50 g, 1.2 mmol), 3,4-difluorophenol (0.16 g, 1.3 mmol) and K_2CO_3 (0.50 g, 3.6 mmol) in DMF (5 mL) was stirred at 100 °C under N_2 for 30 min. After being cooled to room temperature, satd NH_4Cl aq was added to the reaction mixture, and the mixture was extracted with EtOAc. The extract was washed with brine, dried over $MgSO_4$. The residue was purified by NH silica gel column chromatography (EtOAc/hexane) to give **36** (0.27 g, 44%) as an off-white amorphous. ^{1}H NMR ($CDCl_3$) δ: 1.31 (3H, t, J = 7.1 Hz), 2.16–2.29 (1H, m), 2.34–2.52 (2H, m), 2.57 (3H, s), 2.65 (1H, dd, J = 11.1, 5.6 Hz), 4.03 (3H, s), 4.06–4.19 (1H, m), 4.25–4.46 (3H, m), 6.89–7.14 (3H, m), 7.26–7.29 (1H, m), 7.48 (1H, d, J = 1.1 Hz), 7.51 (1H, s), 7.84 (1H, d, J = 8.0 Hz). MS m/z: 511.2 $(M+H)^+$.

5.24. Ethyl 8-(4-chloro-3-fluorophenoxy)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine-8-carboxylate (37)

Compound **37** was prepared from **35** and 4-chloro-4-fluorophenol by a manner similar to that described for **36** in 62% yield as a colorless amorphous. ^{1}H NMR ($CDCl_3$) δ: 1.29 (3H, t, J = 7.0 Hz), 2.11–2.28 (1H, m), 2.34–2.51 (2H, m), 2.55 (3H, s), 2.61–2.71 (1H, m), 4.02 (3H, s), 4.05–4.17 (1H, m), 4.22–4.46 (3H, m), 6.96–7.03 (1H, m), 7.08 (1H, dd, J = 10.6, 2.9 Hz), 7.17–7.31 (2H, m), 7.46 (1H, d, J = 1.4 Hz), 7.50 (1H, s), 7.83 (1H, d, J = 8.0 Hz). MS m/z: 527.2 $(M+H)^+$.

5.25. Ethyl 3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-8-(3,4,5-trifluorophenoxy)-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine-8-carboxylate (38)

Compound **38** was prepared from **35** and 3,4,5-trifluorophenol by a manner similar to that described for **36** in 43% yield as a colorless amorphous. ^{1}H NMR ($CDCl_3$) δ: 1.31 (3H, t, J = 7.1 Hz), 2.17–2.31 (1H, m), 2.34–2.52 (2H, m), 2.57 (3H, s), 2.65 (1H, dd, J = 11.0, 5.5 Hz), 4.04 (3H, s), 4.07–4.19 (1H, m), 4.26–4.44 (3H, m), 6.96 (2H, dd, J = 9.1, 6.0 Hz), 7.29 (1H, d, J = 1.4 Hz), 7.49 (1H, d, J = 1.1 Hz), 7.52 (1H, s), 7.85 (1H, d, J = 8.0 Hz). MS m/z: 529.2 $(M+H)^+$.

5.26. 2-{8-(3,4-Difluorophenoxy)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridin-8-yl}propan-2-ol (15)

Compound **15** was prepared from **36** by a manner similar to that described for **7** in 58% yield as a colorless solid, mp 171–174 °C. ^{1}H NMR ($CDCl_3$) δ: 1.29 (3H, s), 1.61 (3H, s), 2.03–2.17 (3H, m), 2.37–2.49 (1H, m), 2.57 (3H, s), 3.94–4.09 (4H, m), 4.21–4.31 (1H, m), 4.86 (1H, br s), 6.52–6.60 (1H, m), 6.63–6.70 (1H, m), 6.83–6.96 (1H, m), 7.19–7.24 (1H, m), 7.48 (1H, d, J = 1.1 Hz), 7.53 (1H, s), 7.85 (1H, d, J = 8.0 Hz). MS m/z: 497.2 $(M+H)^+$. Anal. Calcd for $C_{26}H_{26}F_2N_4O_4$: C, 62.90; H, 5.28; N, 11.28. Found: C, 62.80; H, 5.39; N, 11.16.

5.27. 2-{8-(4-Chloro-3-fluorophenoxy)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridin-8-yl}propan-2-ol (16)

Compound **16** was prepared from **37** by a manner similar to that described for **7** in 66% yield as a colorless amorphous. ^{1}H NMR ($CDCl_3$) δ: 1.28 (3H, s), 1.59 (3H, s), 2.02–2.18 (3H, m), 2.38–2.49 (1H, m), 2.56 (3H, s), 3.91–4.07 (4H, m), 4.20–4.30 (1H, m), 4.84 (1H, br s), 6.52–6.66 (2H, m), 7.07–7.15 (1H, m), 7.23 (1H, dd, J = 8.1, 1.5 Hz), 7.47 (1H, d, J = 1.5 Hz), 7.51 (1H, s), 7.84 (1H, d, J = 8.1 Hz). MS m/z: 513.1 $(M+H)^+$. Anal. Calcd for $C_{26}H_{26}N_4O_4{\cdot}0.5H_2O$: C, 59.83; H, 5.21; N, 10.73. Found: C, 59.95; H, 5.37; N, 10.71.

5.28. 2-{3-[3-Methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-8-(3,4,5-trifluorophenoxy)-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridin-8-yl}propan-2-ol (17)

Compound **17** was prepared from **38** by a manner similar to that described for **7** in 59% yield as a colorless solid, mp 190–193 °C. ^{1}H NMR ($CDCl_3$) δ: 1.29 (3H, s), 1.59 (3H, s), 1.99–2.24 (3H, m), 2.38–2.49 (1H, m), 2.58 (3H, s), 3.97–4.09 (4H, m), 4.26–4.36 (1H, m), 4.90 (1H, br s), 6.49 (2H, dd, J = 9.1, 6.0 Hz), 7.24 (1H, d, J = 1.4 Hz), 7.47 (1H, d, J = 1.4 Hz), 7.53 (1H, s), 7.86 (1H, d, J = 8.0 Hz). MS m/z: 515.3 $(M+H)^+$. Anal. Calcd for $C_{26}H_{25}F_3N_4O_4$: C, 60.70; H, 4.90; N, 10.89. Found: C, 60.56; H, 5.00; N, 10.81.

5.29. Ethyl 3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-8-(2-nitrobenzyl)-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine-8-carboxylate (39)

Compound **39** was prepared from **33** and 2-nitrobenzyl bromide by a manner similar to that described for **34** in 60% yield as a pale yellow amorphous. ^{1}H NMR ($CDCl_3$) δ: 1.26 (3H, t, J = 7.1 Hz), 1.65–1.77 (1H, m), 1.94–2.04 (2H, m), 2.36–2.47 (1H, m), 2.55 (3H, s), 3.74–3.90 (1H, m), 3.97 (1H, d, J = 14.0 Hz), 4.04 (3H, s), 4.05–4.15 (1H, m), 4.17–4.27 (3H, m), 7.19 (1H, dd, J = 8.0, 1.4 Hz), 7.32–7.45 (2H, m), 7.46–7.49 (2H, m), 7.80 (1H, d, J = 8.0 Hz), 7.85 (1H, dd, J = 7.7, 1.6 Hz). MS m/z: 518.4 $(M+H)^+$.

5.30. Ethyl 3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-8-(2-nitrophenyl)-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine-8-carboxylate (40)

To a mixture of **33** (2.0 g, 5.2 mmol) and 1-fluoro-2-nitrobenzene (1.0 g, 7.3 mmol) in DMF (10 mL) was added NaH (60% in oil, 0.21 g, 5.2 mmol) at room temperature. After being stirred for 5 h at 40 °C, the reaction mixture was diluted with satd $NaHCO_3$ aq and extracted with EtOAc. The extract was washed with water and brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc/hexane) to give **40** (1.2 g, 46%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 1.24 (3H, t, J = 7.0 Hz), 1.80–1.95 (1H, m), 2.16–2.34 (1H, m), 2.56 (3H, s), 2.58–2.69 (1H, m), 3.16–3.25 (1H, m), 4.06 (3H, s), 4.16–4.32 (4H, m), 6.77–6.85 (1H, m), 7.31–7.38 (1H, m), 7.44–7.58 (4H, m), 7.87 (1H, d, J = 7.9 Hz), 8.11 (1H, dd, J = 7.7, 2.1 Hz). MS m/z: 504.5 $(M+H)^+$.

5.31. Ethyl 3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-8-[(2-nitrobenzyl)oxy]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine-8-carboxylate (41)

To a solution of **33** (1.0 g, 2.6 mmol) in DMF (13 mL) was added NaH (60% in oil, 0.12 g, 2.9 mmol) at room temperature. After being stirred for 1 h at room temperature, NaH (60% in oil, 0.12 g, 2.9 mmol) was added to the reaction mixture. After being stirred for 10 min at room temperature, 2-nitrobenzyl bromide was added to the reaction mixture. After being stirred for 1 h at room temperature, the mixture was diluted with sat. NH_4Cl aq and EtOAc. After addition of sat. $NaHCO_3$ aq, the mixture was extracted with EtOAc. The extract was washed with water and brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by NH silica gel column chromatography (EtOAc/hexane) to give **41** (0.48 g, 35%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ: 1.32 (3H, t, J = 7.2 Hz),

2.06–2.16 (1H, m), 2.19–2.48 (3H, m), 2.56 (3H, s), 3.95–4.08 (4H, m), 4.22–4.46 (3H, m), 5.04–5.30 (2H, m), 7.29 (1H, dd, J = 8.0, 1.5 Hz), 7.38–7.45 (1H, m), 7.46–7.53 (2H, m), 7.53–7.61 (1H, m), 7.65–7.71 (1H, m), 7.85 (1H, d, J = 8.3 Hz), 7.88–7.94 (1H, m). 534.6 $(M+H)^+$.

5.32. Ethyl 8-[(2-chloro-6-nitrobenzyl)oxy]-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine-8-carboxylate (42)

Compound **42** was prepared from **33** and 2-(bromomethyl)-1-chloro-3-nitrobenzene by a manner similar to that described for **41** in 64% yield as a colorless amorphous. ^{1}H NMR ($CDCl_3$) δ: 1.26 (3H, t, J = 7.2 Hz), 1.97–2.07 (1H, m), 2.19–2.41 (3H, m), 2.56 (3H, s), 3.91–4.07 (4H, m), 4.25–4.51 (3H, m), 4.98 (1H, d, J = 11.1 Hz), 5.62 (1H, d, J = 10.9 Hz), 7.29–7.41 (2H, m), 7.48–7.55 (2H, m), 7.60 (2H, d, J = 7.7 Hz), 7.86 (1H, d, J = 8.1 Hz). MS m/z: 568.1 $(M+H)^+$.

5.33. Ethyl 3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-8-{[2-nitro-6-(trifluoromethyl)benzyl]oxy}-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine-8-carboxylate (43)

Compound **43** was prepared from **33** and 2-(bromomethyl)-1-nitro-3-(trifluoromethyl)benzene by a manner similar to that described for **41** in 39% yield as a colorless amorphous. ^{1}H NMR ($CDCl_3$) δ: 1.34 (3H, t, J = 7.0 Hz), 2.10–2.41 (4H, m), 2.56 (3H, s), 3.91–4.10 (1H, m), 4.05 (3H, s), 4.27–4.48 (3H, m), 5.13 (1H, d), 5.50 (1H, d, J = 11.7 Hz), 7.31–7.40 (1H, m), 7.47–7.63 (3H, m), 7.77–7.94 (3H, m). MS m/z: 602.1 $(M+H)^+$.

5.34. 3′-[3-Methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-1,4,6′,7′-tetrahydro-2*H*,5′*H*-spiro[quinoline-3,8′-[1,2,4]triazolo[4,3-*a*]pyridin]-2-one (44)

A mixture of **39** (0.16 g, 0.31 mmol) and 10% Pd/C (50% water, 0.016 g) in MeOH (2 mL) was hydrogenated under balloon pressure at room temperature for 9 h. After the reaction mixture was diluted with EtOAc, the catalyst was removed by filtration and the filtrate was concentrated in vacuo. After the residue was dissolved with EtOH (5 mL), the mixture was stirred at 60 °C overnight and concentrated in vacuo. The residue was recrystallized from MeOH/IPE to give **44** (0.059 g, 43%) as a colorless solid, mp 251–253 °C. ^{1}H NMR (DMSO-d_6) δ: 1.64–1.85 (1H, m), 1.89–2.10 (3H, m), 2.49 (3H, s), 3.02 (1H, d, J = 16.5 Hz), 3.79 (1H, d, J = 16.8 Hz), 4.01 (3H, s), 4.06–4.17 (1H, m), 4.17–4.28 (1H, m), 6.88–7.03 (2H, m), 7.15–7.26 (2H, m), 7.41–7.51 (3H, m), 7.78 (1H, d, J = 8.0 Hz), 10.49 (1H, s). MS m/z: 442.3 $(M+H)^+$. Anal. Calcd for $C_{25}H_{23}N_5O_3 \cdot 0.5\,H_2O$: C, 66.65; H, 5.77; N, 15.55. Found: C, 66.63; H, 5.38; N, 15.42.

5.35. 3′-[3-Methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-6′,7′-dihydro-5′*H*-spiro[indole-3,8′-[1,2,4]triazolo[4,3-*a*]pyridin]-2(1*H*)-one (45)

To a solution of **40** (0.60 g, 1.2 mmol) in AcOH (10 mL) and EtOH (10 mL) was added iron (0.30 g, 5.4 mmol) at 90 °C. The reaction mixture was refluxed for 3 h and concentrated in vacuo. The residue was diluted with 2 M NaOH aq and extracted with EtOAc. The extract was washed with water and brine, dried over $MgSO_4$, and concentrated in vacuo. After addition of IPE to the residue, the precipitate was collected by filtration and washed with IPE to give **45** (0.20 g, 39%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 2.13–2.31 (2H, m), 2.38–2.52 (1H, m), 2.56 (3H, s), 2.65–2.85 (1H, m), 4.04 (3H, s), 4.16–4.41 (2H, m), 6.85 (1H, d, J = 7.5 Hz), 6.96–7.07 (1H, m), 7.10–7.16 (1H, m), 7.16–7.24 (1H, m), 7.28–7.34 (1H, m), 7.45 (1H, s), 7.51 (1H, s), 7.86 (1H, d, J = 7.9 Hz), 8.64 (1H, s). MS m/z: 428.4 $(M+H)^+$.

5.36. 3′-[3-Methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-1,5,6′,7′-tetrahydro-2*H*,5′*H*-spiro[4,1-benzoxazepine-3,8′-[1,2,4]triazolo[4,3-*a*]pyridin]-2-one (46)

A mixture of **41** (0.34 g, 0.64 mmol) and PtO_2 (0.020 g) in MeOH (6 mL) was hydrogenated under balloon pressure at room temperature for 2 h. The catalyst was removed by filtration and the filtrate was concentrated in vacuo to give the aniline, which was subjected to the next reaction without further purification. After the aniline was diluted with 1 M NaOH aq (2 mL) and THF (4 mL), the mixture was stirred at room temperature for 4 h, neutralized with 1 M HCl aq and concentrated in vacuo to afford the aminobenzoic acid, which was subjected to the next reaction without further purification. After the aminobenzoic acid was azeotropically dried with toluene, DMF (25 mL), Et_3N (0.44 mL, 3.1 mmol) and HATU (0.29 g, 0.77 mmol) were added to the residue. After being stirred at room temperature for 30 min, the mixture was diluted with water and extracted with EtOAc. The extract was washed with water and brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by NH silica gel column chromatography (MeOH/EtOAc) to give **46** (0.17 g, 57%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ: 2.09–2.53 (4H, m), 2.56 (3H, s), 4.04 (3H, s), 4.07–4.17 (1H, m), 4.19–4.29 (1H, m), 4.48 (1H, d, J = 14.6 Hz), 5.80 (1H, d, J = 14.0 Hz), 6.95 (1H, d, J = 7.1 Hz), 7.02–7.13 (1H, m), 7.15–7.22 (1H, m), 7.27–7.34 (2H, m), 7.45 (1H, d, J = 1.4 Hz), 7.51 (1H, s), 7.85 (1H, d, J = 8.0 Hz), 7.99 (1H, s). MS m/z: 458.5 $(M+H)^+$.

5.37. 6-Chloro-3′-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-1,5,6′,7′-tetrahydro-2*H*,5′*H*-spiro[4,1-benzoxazepine-3,8′-[1,2,4]triazolo[4,3-*a*]pyridin]-2-one (47)

To a mixture of **42** (0.70 g, 1.2 mmol) and $FeCl_3 \cdot 6H_2O$ (0.067 g, 0.25 mmol) in MeOH (20 mL) and THF (20 mL) were added activated charcoal (0.10 g) and a solution of hydrazine monohydrate (0.37 g, 7.4 mmol) in MeOH at 60 °C. The mixture was stirred at 60 °C for 1 h, and concentrated in vacuo. The residue was diluted with EtOAc, washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by NH silica gel column chromatography (MeOH/EtOAc) to give the aniline. Compound **47** was prepared from the aniline by a manner similar to that described for **46** in 45% yield as a colorless amorphous. ^{1}H NMR (DMSO-d_6) δ: 2.08 (2H, d, J = 4.5 Hz), 2.25–2.45 (2H, m), 3.33 (3H, s), 4.03 (3H, s), 4.17–4.39 (2H, m), 4.99 (1H, d, J = 14.7 Hz), 5.29 (1H, d, J = 14.7 Hz), 7.12–7.38 (3H, m), 7.42–7.62 (3H, m), 7.82 (1H, d, J = 7.9 Hz), 10.75 (1H, s). MS m/z: 492.4 $(M+H)^+$.

5.38. 3′-[3-Methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-6-(trifluoromethyl)-1,5,6′,7′-tetrahydro-2*H*,5′*H*-spiro[4,1-benzoxazepine-3,8′-[1,2,4]triazolo[4,3-*a*]pyridin]-2-one (48)

Compound **48** was prepared from the aniline by a manner similar to that described for **47** in 69% yield as a pale yellow amorphous. ^{1}H NMR ($CDCl_3$) δ: 2.28–2.54 (4H, m), 2.56 (3H, s), 4.05 (3H, s), 4.08–4.18 (1H, m), 4.18–4.31 (1H, m), 4.93 (1H, d, J = 15.1 Hz), 5.68 (1H, d, J = 15.1 Hz), 7.23–7.55 (6H, m), 7.86 (1H, d, J = 7.9 Hz), 8.94 (1H, s). MS m/z: 526.4 $(M+H)^+$.

5.39. 3′-[3-Methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-1-(2,2,2-trifluoroethyl)-1,4,6′,7′-tetrahydro-2*H*,5′*H*-spiro[quinoline-3,8′-[1,2,4]triazolo[4,3-*a*]pyridin]-2-one (18)

To a suspension of **44** (0.066 g, 0.15 mmol) in DMF (1 mL) was added NaH (60% in oil, 7.2 mg, 0.18 mmol) at 0 °C under N_2. After

the mixture was stirred at 0 °C for 30 min, 2,2,2-trifluoroethyl trifluoromethanesulfonate (26 μL, 0.18 mmol) was added to the mixture. After being stirred at room temperature for 1 h, the mixture was diluted with water. The precipitate was collected by filtration, and purified by silica gel column chromatography (MeOH/EtOAc) and preparative HPLC (L-Column 2 ODS, eluted with H_2O in acetonitrile containing 0.1% TFA) to give **18** (0.031 g, 40%) as an off-white solid. ^{1}H NMR ($CDCl_3$) δ: 1.66–1.82 (1H, m), 1.94–2.27 (3H, m), 2.56 (3H, s), 3.01 (1H, d, J = 16.2 Hz), 3.89–4.02 (1H, m), 4.04 (3H, s), 4.16–4.31 (2H, m), 4.36 (1H, d, J = 15.9 Hz), 4.93–5.14 (1H, m), 7.07–7.19 (2H, m), 7.22–7.29 (2H, m), 7.31–7.39 (1H, m), 7.46 (1H, d, J = 1.4 Hz), 7.50 (1H, s), 7.83 (1H, d, J = 8.0 Hz). MS m/z: 524.3 $(M+H)^+$. Purity: 91%.

5.40. 3′-[3-Methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-1-(2,2,2-trifluoroethyl)-6′,7′-dihydro-5′*H*-spiro[indole-3,8′-[1,2,4]triazolo[4,3-*a*]pyridin]-2(1*H*)-one (19)

Compound **19** was prepared from **45** by a manner similar to that described for **18** in 73% yield as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 2.16–2.32 (2H, m), 2.35–2.47 (1H, m), 2.56 (3H, s), 2.71–2.88 (1H, m), 4.03 (3H, s), 4.06–4.27 (2H, m), 4.30–4.43 (1H, m), 4.66 (1H, dq, J = 15.5, 9.0 Hz), 7.05 (1H, d, J = 7.9 Hz), 7.10–7.23 (2H, m), 7.30 (1H, dd, J = 7.9, 1.5 Hz), 7.38 (1H, td, J = 7.6, 1.7 Hz), 7.44 (1H, s), 7.51 (1H, s), 7.86 (1H, d, J = 7.9 Hz). MS m/z: 510.5 $(M+H)^+$.

5.41. 3′-[3-Methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-1-(2,2,2-trifluoroethyl)-1,5,6′,7′-tetrahydro-2*H*,5′*H*-spiro[4,1-benzoxazepine-3,8′-[1,2,4]triazolo[4,3-*a*]pyridin]-2-one (20)

Compound **20** was prepared from **46** by a manner similar to that described for **18** in 60% yield as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 1.99–2.37 (4H, m), 2.55 (3H, s), 3.97–4.09 (5H, m), 4.30–4.50 (1H, m), 4.73 (1H, d, J = 13.2 Hz), 4.97–5.15 (1H, m), 5.44 (1H, d, J = 13.2 Hz), 7.22 (1H, dd, J = 7.9, 1.5 Hz), 7.24–7.29 (1H, m), 7.30–7.52 (5H, m), 7.81 (1H, d, J = 7.9 Hz). MS m/z: 540.4 $(M+H)^+$.

5.42. 6-Chloro-3′-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-1-(2,2,2-trifluoroethyl)-1,5,6′,7′-tetrahydro-2*H*,5′*H*-spiro[4,1-benzoxazepine-3,8′-[1,2,4]triazolo[4,3-*a*]pyridin]-2-one (22)

Compound **22** was prepared from **47** by a manner similar to that described for **18** in 43% yield as a colorless amorphous. ^{1}H NMR ($CDCl_3$) δ: 1.97–2.38 (4H, m), 2.54 (3H, s), 3.94–4.17 (5H, m), 4.34–4.51 (1H, m), 4.96–5.13 (1H, m), 5.16 (1H, d, J = 13.6 Hz), 5.40 (1H, d, J = 13.6 Hz), 7.16–7.44 (5H, m), 7.49 (1H, s), 7.81 (1H, d, J = 7.9 Hz). MS m/z: 574.6 $(M+H)^+$.

5.43. 3′-[3-Methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-1-(2,2,2-trifluoroethyl)-6-(trifluoromethyl)-1,5,6′,7′-tetrahydro-2*H*,5′*H*-spiro[4,1-benzoxazepine-3,8′-[1,2,4]triazolo[4,3-*a*]pyridin]-2-one (23)

Compound **23** was prepared from **48** by a manner similar to that described for **18** in 54% yield as a colorless amorphous. ^{1}H NMR ($CDCl_3$) δ: 1.97–2.13 (1H, m), 2.14–2.40 (3H, m), 2.54 (3H, s), 3.94–4.18 (5H, m), 4.35–4.52 (1H, m), 5.01–5.19 (2H, m), 5.37 (1H, d, J = 13.9 Hz), 7.19 (1H, dd, J = 7.9, 1.5 Hz), 7.38 (1H, s), 7.49 (1H, s), 7.54–7.68 (3H, m), 7.81 (1H, d, J = 8.3 Hz). MS m/z: 608.6 $(M+H)^+$.

5.44. *N*-[2-(Benzyloxy)phenyl]-8-(hydroxymethyl)-3-[3-methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridine-8-carboxamide (50)

To a suspension of NaH (60% in oil, 0.035 g, 0.86 mmol) in DMF (3 mL) was added **33** (0.30 g, 0.78 mmol) at 0 °C under Ar. After the mixture was stirred at 0 °C for 15 min, paraformaldehyde (0.028 g) was added to the reaction mixture at 0 °C. After the mixture was stirred at 0 °C for 15 min, water (0.014 mL, 0.78 mmol) was added to the mixture at room temperature. The mixture was stirred at room temperature for 1 h, and dried over Na_2SO_4 (0.11 g, 0.78 mmol). To the reaction mixture were added 2-benzyloxyaniline (0.19 g, 0.94 mmol) and HATU (0.31 g, 1.2 mmol) at 0 °C. After being stirred at room temperature for 30 min, the mixture was diluted with EtOAc, washed with water and brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography (MeOH/EtOAc) to give **50** (0.24 g, 55%) as an off-white amorphous. ^{1}H NMR ($CDCl_3$) δ: 1.78–1.91 (1H, m), 2.01–2.21 (2H, m), 2.56 (3H, s), 2.75–2.86 (1H, m), 3.92–4.05 (5H, m), 4.07–4.21 (2H, m), 5.15 (2H, s), 6.87–7.05 (3H, m), 7.20–7.24 (2H, m), 7.29–7.38 (3H, m), 7.43–7.49 (2H, m), 7.50 (1H, s), 7.82 (1H, d, J = 8.0 Hz), 8.29 (1H, dd, J = 8.0, 1.9 Hz), 9.52 (1H, s). MS m/z: 566.1 $(M+H)^+$.

5.45. 3′-[3-Methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-5-(2,2,2-trifluoroethyl)-6′,7′-dihydro-5′*H*-spiro[1,5-benzoxazepine-3,8′-[1,2,4]triazolo[4,3-*a*]pyridin]-4(5*H*)-one (21)

A mixture of **50** (0.44 g, 0.78 mmol) and 10% Pd/C (50% water, 0.060 g) in MeOH (2 mL) and THF (1 mL) was hydrogenated under balloon pressure at room temperature for 6 h. The catalyst was removed by filtration and the filtrate was concentrated in vacuo to give the deprotected compound, which was subjected to the next reaction without further purification. To a mixture of the deprotected compound and triphenylphosphine (0.079 g, 0.30 mmol) was added diethyl azodicarboxylate (40% in toluene, 0.14 mL) at 0 °C under Ar. After the mixture was stirred at room temperature for 2 h, tributylphosphine (75 μL, 0.30 mmol) and 1,1′-(azodicarbonyl)dipiperidine (0.076 g, 0.30 mmol) were added to the mixture at room temperature. After being stirred at room temperature for 4 days, the mixture was concentrated in vacuo. The residue was purified by silica gel column chromatography (MeOH/EtOAc) to give **51** as an off-white oil, which was subjected to the next reaction without further purification. To a solution of **51** in DMF (1 mL) was added NaH (60% in oil, 8.1 mg, 0.20 mmol) at 0 °C under Ar. After the mixture was stirred at 0 °C for 20 min, 2,2,2-trifluoroethyl trifluoromethanesulfonate (38 μL, 0.25 mmol) was added to the mixture at 0 °C. After being stirred at room temperature for 20 min, the mixture was diluted with EtOAc, washed with water and brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica gel column chromatography (MeOH/EtOAc) and NH silica gel column chromatography (EtOAc/hexane) to give **21** (0.024 g, 5.7%) as a colorless amorphous. ^{1}H NMR ($CDCl_3$) δ: 2.08–2.35 (2H, m), 2.47–2.66 (5H, m), 3.97–4.09 (4H, m), 4.14 (1H, d, J = 6.4 Hz), 4.19–4.29 (1H, m), 4.35–4.47 (2H, m), 4.81 (1H, d, J = 6.4 Hz), 6.87 (1H, dd, J = 7.9, 1.5 Hz), 7.03–7.16 (2H, m), 7.22–7.26 (1H, m), 7.43 (1H, d, J = 1.5 Hz), 7.51 (1H, s), 7.84 (1H, d, J = 8.3 Hz), 8.08 (1H, dd, J = 7.9, 1.9 Hz). MS m/z: 540.1 $(M+H)^+$.

5.46. 2-[(8*R*)-3-[3-Methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-8-(3,4,5-trifluorophenoxy)-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridin-8-yl]propan-2-ol ((*R*)-17) and 2-[(8*S*)-3-[3-Methoxy-4-(2-methyl-1,3-oxazol-5-yl)phenyl]-8-(3,4,5-trifluorophenoxy)-5, 6,7, 8-tetrahydro[1,2,4]triazolo[4,3-*a*]pyridin-8-yl]propan-2-ol ((*S*)-17)

Compound **17** was separated by HPLC (column: CHIRALPAK AD 50 mmID × 500 mmL, manufactured by DAICEL CHEMICAL INDUSTRIES,

LTD, mobile phase: hexane/isopropyl alcohol 700:300) to give (*R*)-**17** with a shorter retention time and (*S*)-**17** with a longer retention time. (*R*)-**17**: mp 188 °C. ^{1}H NMR ($CDCl_3$) δ: 1.27 (3H, s), 1.57 (3H, s), 1.99–2.23 (3H, m), 2.37–2.46 (1H, m), 2.56 (3H, s), 3.96–4.09 (4H, m), 4.25–4.35 (1H, m), 4.91 (1H, s), 6.42–6.54 (2H, m), 7.24 (1H, dd, *J* = 8.0, 1.4 Hz), 7.45 (1H, d, *J* = 1.3 Hz), 7.52 (1H, s), 7.85 (1H, d, *J* = 8.1 Hz). ^{13}C NMR (DMSO-d_6) δ: 13.6, 20.3, 25.0, 26.0, 27.7, 44.1, 55.9, 75.6, 83.5, 108.0, 108.2, 111.0, 117.7, 120.6, 125.2, 127.0, 127.2, 146.2, 151.6, 152.6, 155.0, 160.4. MS *m*/*z*: 515.3 $(M+H)^+$. Anal. Calcd for $C_{26}H_{25}F_3N_4O_4$: C, 60.70; H, 4.90; N, 10.89. Found: C, 60.74; H, 4.93; N, 10.84. $[\alpha]_D^{20}$ +6.9 (*c* 1.00, MeOH). (*S*)-**17**: ^{1}H NMR ($CDCl_3$) δ: 1.27 (3H, br s), 1.57 (3H, br s), 1.97–2.24 (3H, m), 2.42 (1H, d, *J* = 9.6 Hz), 2.56 (3H, br s), 4.04 (4H, br s), 4.29 (1H, d, *J* = 9.3 Hz), 4.90 (1H, br s), 6.41–6.55 (2H, m), 7.17–7.24 (1H, m), 7.39–7.55 (2H, m), 7.84 (1H, d, *J* = 7.1 Hz). MS *m*/*z*: 515.3 $(M+H)^+$.

5.47. Aβ ELISA

$A\beta_{40}$, $A\beta_{42}$ or total Aβ was quantified by two-site sandwich ELISA.[11] BNT-77 ($A\beta_{11-28}$) was used as the capture antibody and horseradish peroxidase-coupled BA-27, BC-05 or BAN50 was used as the detector antibody for $A\beta_{40}$, $A\beta_{42}$ or total Aβ, respectively.

5.48. Primary rat neuronal cells assay

Cerebral cortical neurons in rat primary culture were prepared from rat fetal brain at 17 days of gestation (SD; JCL) using neural cell dispersion kit (SUMILON). The dissociated cell suspensions were plated at a density of 5.0×10^4 cells/well on poly-L-lysine coated 96-well plates (Sumitomo Bakelite). After incubation for 7 days in neurobasal medium (Invitrogen) including 1% penicillin–streptomycin (Invitrogen), 2% B-27 supplement (Invitrogen), and 2 mM L-glutamine (Invitrogen), the cells were treated with or without compounds for 3 days. Then the culture media were subjected to measurement of $A\beta_{40}$ and $A\beta_{42}$ by ELISA.

5.49. Plasma and brain Aβ assay

Hippocampi and plasma were isolated from C57BL/6J mice, 3 h after compound was orally administrated using a solubilizing vehicle (10% DMSO/10% Cremophor EL/30% PEG400/60% 0.2 M citrate solution) for **13**, **16**, **17**, **22** and **23**, or 0.5% methylcellulose for (*R*)-**17**. Samples were homogenized in ice-cold Tris-extraction buffer (50 mmol/L Tris–HCl, pH 7.2, 200 mmol/L sodium chloride, 2% protease-free bovine serum albumin, and 0.01% sodium merthiolate) containing protease inhibitor cocktails (Roche, Basel, Switzerland). After centrifugation at 15,000 rpm for 15 min, the supernatants were subjected to ELISA to measure amount of soluble Aβ.

5.50. In vitro metabolic clearance in mouse hepatic microsomes

Hepatic microsomes from mice were purchased from Xenotech, LLC (Lenexa, KS). An incubation mixture consisted of microsomal protein in 50 mmol/L KH_2PO_4–K_2HPO_4 phosphate buffer (pH 7.4) and 1 μmol/L each compound. The concentration of hepatic microsomal protein was 0.2 mg/mL. An NADPH-generating system containing 25 mmol/L $MgCl_2$, 25 mmol/L glucose-6-phosphate, 2.5 mmol/L β-$NADP^+$, and 7.5 unit/mL glucose-6-phosphate dehydrogenase was added to the incubation mixture with a 20% volume of the reaction mixture to initiate the enzyme reaction. After the addition of the NADPH-generating system, the reaction mixture was incubated at 37 °C. The reaction was terminated by the addition of MeCN equivalent to the volume of the reaction mixture. As a control, the mixture without 37 °C-incubation was also prepared. All incubations were made in duplicate. Test compound in the reaction mixture was measured by an HPLC system equipped with a UV detector or LC/MS/MS. For the determinations of the metabolic clearance, chromatograms were analyzed for parent compound disappearance from the reaction mixtures.

5.51. Pharmacokinetics study

Compound (*R*)-**17** (6 mg/kg) was orally administered to C57BL/6J mice (male, 9 w.o., fed, *n* = 3) using 0.5% methylcellulose. At 3 h after oral administration, blood and cortex samples were collected. The blood samples were centrifuged to obtain the plasma fraction. Cortex was homogenized in saline to obtain the brain homogenate. The plasma and brain homogenate samples were deproteinized with MeCN containing an internal standard. After centrifugation, the supernatants were decanted into microplates, and were diluted with 0.01 mol/L ammonium formate and acetonitrile (7:3, v/v) containing 0.2% formic acid. After another centrifugation, the supernatants were injected into an LC/MS/MS system to measure the compound concentrations.

5.52. Multiple treatment study

Compound (*R*)-**17** (3 and 6 mg/kg) was orally administered for 58 days using 0.5% methylcellulose in Tg2576 mice (female, 6 m. o.). On the 45th day, mice were provided a novel object recognition test and then brain from them were collected to quantify Aβ by ELISA 3 h after administration on the 58th day. Cortex was isolated from Tg2576 mice brain and immediately frozen on dry ice and stored at −80 °C. Cortex was homogenized in ice-cold tris-extraction buffer (50 mmol/L Tris–HCl, pH 7.2, 200 mmol/L sodium chloride, 2% (w/v) protease-free bovine serum albumin, and 0.01% (v/v) sodium merthiolate) containing protease inhibitor cocktails (Roche, Basel, Switzerland). After centrifugation at 15,000 rpm for 15 min, the supernatants were subjected to two-site sandwich ELISA to measure amount of soluble Aβ. For assessment of insoluble Aβ, the pellets were homogenized in guanidine extraction buffer (5 mol/L guanidine, 1 mol/L Tris–HCl (pH 7.2)) and centrifuged at 15,000 rpm for 15 min. The supernatants were diluted 19-fold with tris-extraction buffer and applied for Aβ ELISA.

5.53. Novel object recognition test (NORT)

The box and objects used were purchased from BrainScience Idea (Osaka, Japan). The box was 30 × 30 × 30 cm and gray colored. The two objects used were aluminum, silver-colored cylinder (object A and A′) and ceramic, white-colored trigonal pyramid (object B). In the acquisition test, the identical objects (objects A and A′) were symmetrically placed in the box. Each animal was placed in the box for 5 min followed by a 24 h retention interval in the home cage. Mice were placed at the corner of the box turning their heads toward the wall. After replacing one of the objects with a novel object (object B), the mouse was reintroduced into the box as a 5 min retention test. Time spent in exploring the objects were measured during both tests. Retention was represented by how long the animals explored the novel object versus the familiar object.

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