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Design, synthesis, and biological evaluation of a novel series of peripheral-selective noradrenaline reuptake inhibitors: Part 3

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Pharmaceutical Research Division, Takeda Pharmaceutical Company Ltd., 26-1, Muraokahigashi 2-chome, Fujisawa, Kanagawa 251-8555, Japan

Abstract

Peripheral-selective inhibition of noradrenaline reuptake is a novel mechanism for the treatment of stress urinary incontinence to overcome adverse effects associated with central action. Here, we describe our medicinal chemistry approach to discover a novel series of highly potent, peripheral-selective, and orally available noradrenaline reuptake inhibitors with a low multidrug resistance protein 1 (MDR1) efflux ratio by cyclization of an amide moiety and introduction of an acidic group. We observed that the MDR1 efflux ratio was correlated with the pK_a value of the acidic moiety. The resulting compound **9** exhibited favorable PK profiles, probably because of the effect of intramolecular hydrogen bond, which was supported by a its single-crystal structure. The compound **9**, 1-{[(6*S*,7*R*)-7-(4-chloro-3-fluorophenyl)-1,4-oxazepan-6-yl]methyl}-2-oxo-1,2-dihydropyridine-3-carboxylic acid hydrochloride, which exhibited peripheral NET-selective inhibition at tested doses in rats by oral administration, increased urethral resistance in a dose-dependent manner.

KEYWORDS: Peripheral-selective noradrenaline reuptake inhibitor; 7-Aryl-1,4-oxazepane derivatives; Stress urinary incontinence; MDR1 efflux ratio; Intramolecular hydrogen bond.

ABBREVIATIONS: SUI, Stress urinary incontinence; DAT, dopamine transporter; SERT, serotonin transporter; NET, norepinephrine transporter; MDR1, multidrug resistance protein 1; P-gp, P-glycoprotein.

*Corresponding author. Tel: +81-466-32-1105. Fax: +81-466-29-4454;

e-mail: tomoya.yukawa@takeda.com

1. Introduction

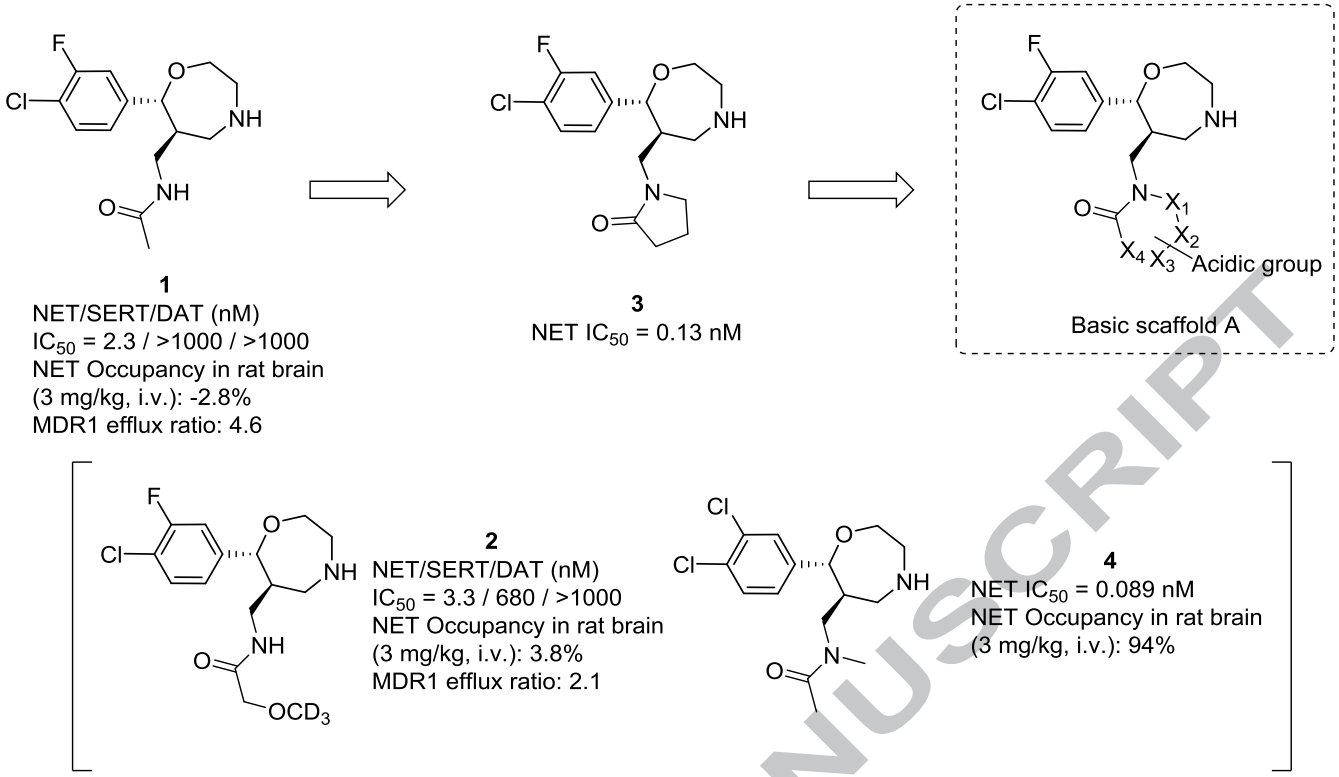
Peripheral-selective inhibition of noradrenaline reuptake has emerged as a new drug concept with the potential for the treatment of stress urinary incontinence (SUI). Biological evaluation of highly peripheral-selective noradrenaline reuptake inhibitors (NRIs) have been studied in our laboratory.[1,2] Peripheral-selective NRIs are assumed to have several advantages with respect to safety and efficacy over traditional central-acting NRIs as anti-SUI agents.[3]

We previously reported 7-phenyl-6-substituted oxazepane derivatives as peripheral-selective NRIs.[1,2] In these studies, our chemical modification focused on enhancing peripheral norepinephrine transporter (NET) selectivity and reducing the multidrug resistance protein 1 (MDR1) efflux ratio to mitigate drug–drug interaction (DDI) risks. P-glycoprotein (P-gp) is the major efflux transporter at the blood–brain barrier and is responsible for the efflux of a number of xenobiotic substances from the central nervous system.[4] DDI with P-gp inhibitor[5] may affect the balance of distribution of our compound to the central and peripheral nervous systems, resulting in a low peripheral NET selectivity. Derivatization of 7-phenyl-6-substituted oxazepane derivative **1** gave trideuterium methoxy derivative **2** as a potent and peripheral NET-selective inhibitor with a low MDR1 efflux ratio.[2]

In parallel with the aforementioned study, we investigated a new series of compounds for novel peripheral-selective NRIs. We initially explored a new scaffold exhibiting strong NET inhibitory activity. In our previous SAR study, we observed that a 3,4-disubstituted phenyl ring and an oxazepane ring were necessary for NET inhibitory activity. We also observed that the terminal amide moiety is a good enhancer for the NET inhibitory activity. For further study of the side chain containing the amide moiety, we investigated cyclization of the terminal amide moiety, and observed that pyrrolidone derivative **3** exhibited potent NET inhibitory activity compared with acyclic derivative **1**. We selected cyclic compound **3** as a lead compound for a new series of NRIs and investigated the peripheral NET selectivity. However, we also observed that the *N*-methylated derivative **4** exhibited high NET occupancy in the rat brain after intracavernous administration in our initial SAR. We speculated that the decrease in peripheral NET selectivity was due to the removal of one hydrogen bond donor (HBD),[6] and that the introduction of a hydrophilic group resulted in the recovery of high peripheral NET selectivity by increasing hydrogen bond aceptor (HBA) and/or HBD.[7] However, in general, adding HBA and/or HBD increases the MDR1 efflux ratio.[8] To identify peripheral NET-selective candidates with a low MDR1 efflux ratio, we selected an acidic group as a hydrophilic group. Acidic groups are known to increase peripheral selectivity[9] and acidic compounds commonly exhibit relatively low MDR1 efflux ratios.[10] In this study, we performed the synthesis of 7-aryl-6-substituted oxazepane derivatives with an acidic group on the designed amide-containing ring on the basis of basic scaffold A.

[Figure 1]

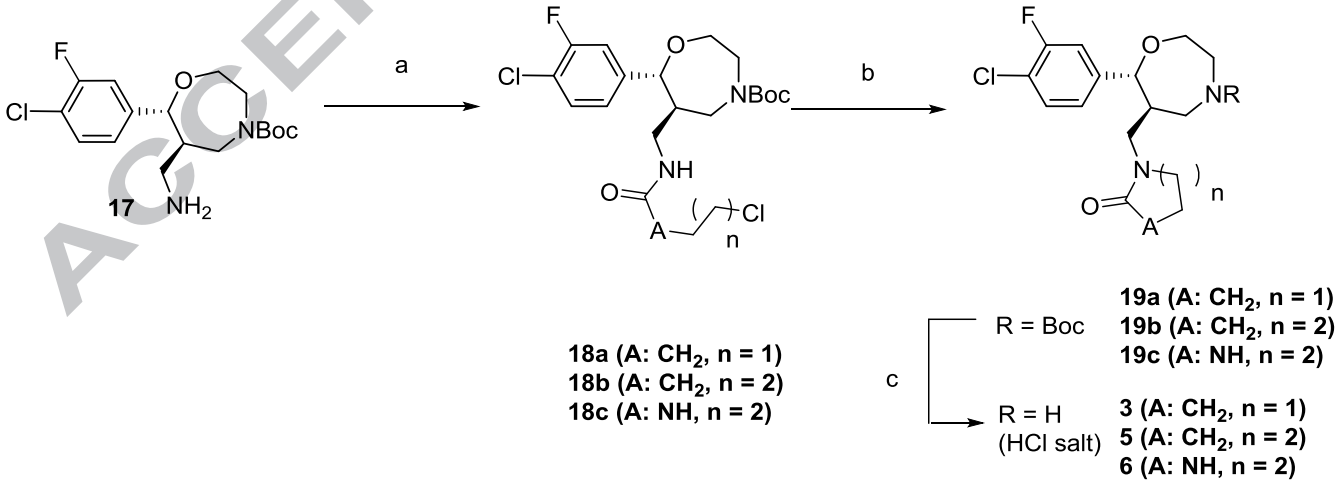
Figure 1. Design concept



2. Chemistry

The preparation of cyclic ring derivatives **3**, **5**, and **6** is shown in Scheme 1. (*6R*,*7R*)-Amine intermediate **17** was prepared according a previously reported procedure.[2] Condensation of amine **17** with acid chloride or isocyanate afforded alkyl chlorides **18a**–**18c**. Cyclization of **18a**–**18c** under basic conditions followed by treatment with hydrogen chloride gave **3**, **5**, and **6** as the HCl salts.

[Scheme 1]



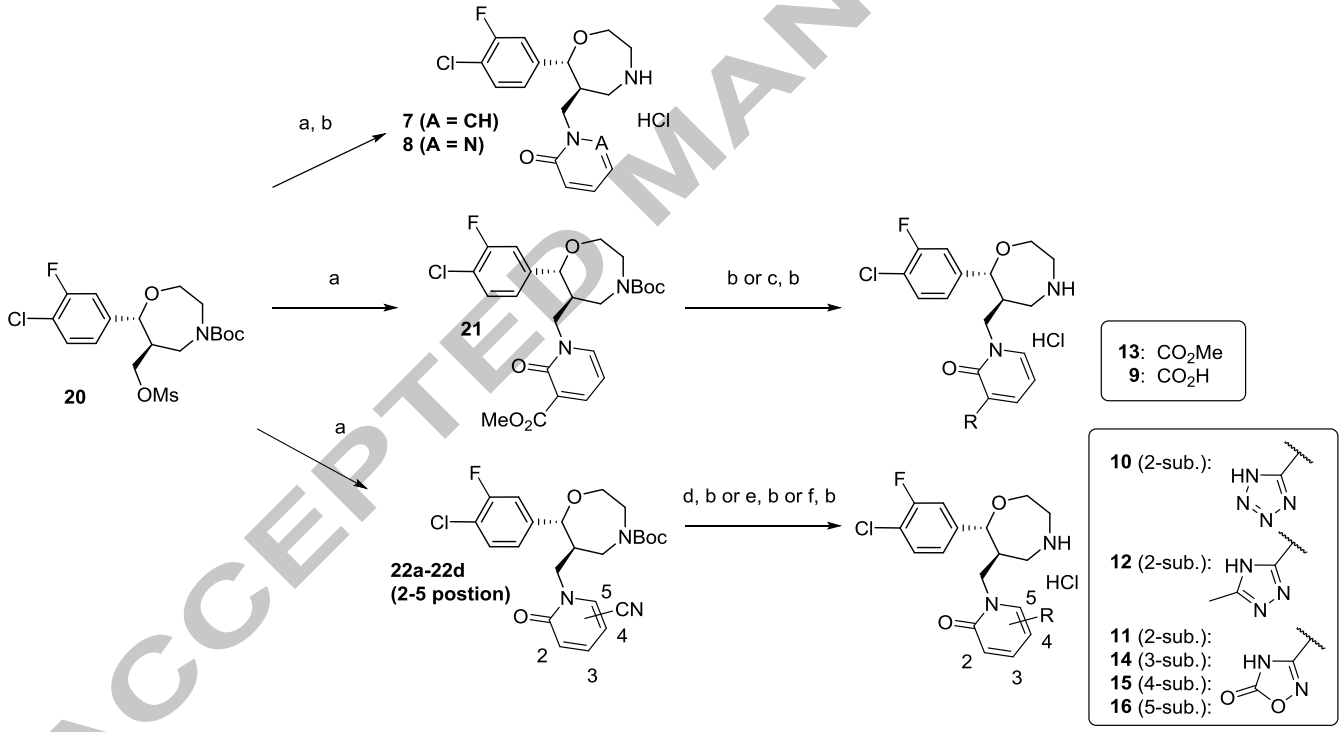
Scheme 1. Synthesis of Compounds **3**, **5**, and **6** [a]

[a] Reagents and conditions: (a) R^1COCl, Et_3N, THF, rt or R^2CNO, Et_3N, CH_3CN, rt; (b) NaH, THF or DMF, 0°C to rt; (c) 4 M HCl in EtOAc, rt.

The preparation of 2-pyridone or 3(2*H*)-pyridazinone derivatives **7**–**16** is illustrated in Scheme 2. Compound **7** or **8** was synthesized by the reaction of 2-pyridone or pyridazinone with mesylate **20**[2] and subsequent de-protection of the Boc group.

The pyridone possessing the ester or cyano groups were coupled with mesylate **20** to give *N*-substituted pyridones **21** or **22a**–**22d**. Removal of the Boc group of **21** resulted in ester **13**. The carboxylic acid **9** was prepared by modification of **21** followed by de-protection reaction. The tetrazole (**10**), 1,2,4-triazole (**12**), or 1,2,4-oxadiazolone (**11, 14**–**16**)-substituted derivatives were synthesized *via* cyano-substituted derivatives **22a**–**22d**.

[Scheme 2]



Scheme 2. Synthesis of Compounds **7**–**16** [a]

[a] Reagents and conditions: (a) corresponding pyridone, NaH, $LiBr_2$, DME, DMF, 80°C or K_2CO_3, DME, 80°C or pyridazinone, K_2CO_3, DMF, 80°C; (b) 4 M HCl in EtOAc or 11.7 M HCl in EtOH, EtOH, rt; (c) 2 M NaOH, EtOH, 30°C; (d) azidotributyltin, toluene, 100°C; (e) (i) *O*,*O*'-diethyl dithiophosphate, 4 M HCl in EtOAc, rt; (ii) $NaHCO_3$, Boc_2O, EtOAc, rt; (iii) MeI, K_2CO_3, acetone, rt; (iv) acetohydrazide, toluene, 120°C; (f) (i) NH_4Cl, $NaHCO_3$, DMSO, 80°C; (ii) CDI, DBU, THF, 70°C.

3. Results and discussion

The synthesized compounds were evaluated for their *in vitro* reuptake inhibitory activity against human NET, SERT, and DAT, expressed as IC_{50} values. In this study, we initially explored the terminal ring system and then investigated the acidic groups on the selected ring to identify highly peripheral NET selective compounds with a low MDR1 efflux ratio. As expected, all the cyclic amide derivatives (**3**–**8**) showed marked enhancement of NET inhibitory activities compared with acyclic amide derivative **1** (Table 1). The six-membered cyclic amide **5** and urea **6** showed decreased selectivity against SERT. In contrast, high selectivity against SERT and DAT was observed for the unsaturated amide series (pyridone derivative **7** and pyridazinone derivative **8**). Among the evaluated compounds, 2-pyridone derivative **7** demonstrated potent NET inhibitory activity and high selectivity against SERT and DAT inhibitions; we therefore selected pyridone (**7**) as the terminal ring system for further investigation.

[Table 1]

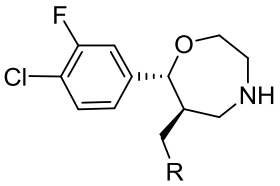
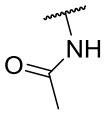
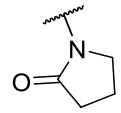
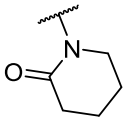
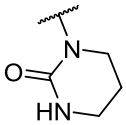
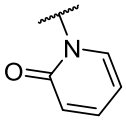
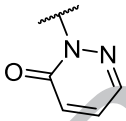


Table 1

Monoamine reuptake inhibitions.[a]

Compd.	R	Reuptake inh. IC_{50}[b] (nM), 95% CI[c]			NET/SERT	NET/DAT
		NET	SERT	DAT		
1		2.3 (1.9-2.7)	>1000	>1000	>440	>440
3		0.13 (1.10-0.16)	85 (68-110)	400 (200-770)	650	3100
5		0.1[d]	27 (21-35)	130 (74-220)	270	1300
6		0.08[d]	12 (10-15)	66 (55-79)	150	820
7		0.079 (0.070-0.090)	130 (110-160)	>1000	1700	>13000
8		0.065 (0.046-0.092)	79 (70-90)	240 (190-310)	1200	3600

[a] All compounds were isolated as HCl salt.

[b] These values were calculated from the results of two experiments.

[c] 95% confidence interval for each IC_{50} value.

[d] The IC_{50} value is not 95% confidence interval.

Next, we investigated the effects of introducing acidic groups to compound **7** (Table 2). Initially, we introduced carboxylic acid and bioisosteres of acidic groups at the 2-position of the ring. All the resulting acidic groups demonstrated potent NET inhibitory activities (**9–12**). Among them, carboxylic acid derivative **9** showed excellent selectivity against SERT and DAT. As we expected, the MDR1 efflux ratio was correlated with the pK_a value of the acidic parts; compounds **9–11**, which have pK_a values of less than 6, exhibited low MDR1 efflux ratios.[11] In addition, non-acidic methyl ester derivative **13** showed a high MDR1 efflux ratio, indicating that the introduction of acidic groups is suitable to our strategy. With several potent acidic motifs in hand, we next optimized of the substituent position on the pyridone ring using 1,2,4(4*H*)-oxadiazol-5-one (**14–16**). As a result, we observed that the ring position

was critical for NET inhibitory activity and that 2-substituted derivative **11** exhibited the most potent NET inhibitory activity, and highest selectivity against SERT, and DAT among 2-, 3-, 4-, and 5-substituted derivatives. Encouraged by these results, we next evaluated the pharmacokinetic (PK) profiles of **9**, **10** and **11**, and performed NET occupancy tests in rat brain (Table 3). Among the investigated derivatives, only carboxylic acid compound **9** exhibited favorable PK profiles, including good oral bioavailability in rats. In the single-crystal structure of **9**, intramolecular hydrogen bond between the carboxylic acid and the carbonyl group of pyridone (2.53 Å) was observed (Figure 2). It was suggested that compound **9** demonstrated good PK profiles as a consequence of this effect.[12] Compound **9** also demonstrated high peripheral NET selectivity[13] compared to that of **7**, consistent with our design hypothesis. Compound **9** was therefore selected as a candidate for *in vivo* evaluation.

[Table 2]

[Table 3]

[Figure 2]

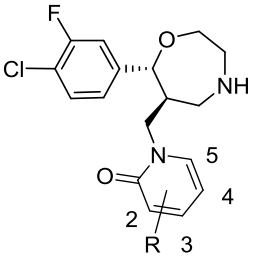
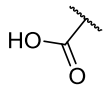
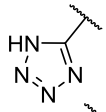
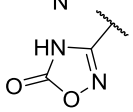
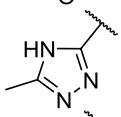
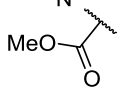
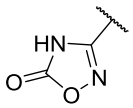


Table 2

Monoamine reuptake inh. and MDR1 efflux ratio.[a]

Compd.	R	position	Reuptake inh. IC_{50}[b] (nM), 95% CI[c]			MDR1 ratio (B to A / A to B)	pK_a
			NET	SERT	DAT		
9		2	1.1 (0.90-1.3)	>1000	>1000	2.1	2.58
10		2	1.4 (1.1-1.7)	730 (570-930)	>1000	1.7	3.88
11		2	1.0 (0.87-1.2)	380 (300-470)	>1000	1.9	5.45
12		2	0.64 (0.47-0.88)	67 (57-79)	>1000	6.8	9.68
13		2	0.21 (0.17-0.26)	54 (44-66)	>1000	7.8	
14		3	1.9 (1.6-2.3)	120 (100-140)	>1000	>0.8	5.89
15		4	25 (17-36)	>1000	>1000	1.6	5.72
16		5	100 (68-160)	>1000	>1000	0.6	6.35

[a] All compound were isolated as HCl salt

[b] These values were calculated from the results of two experiments.

[c] 95% confidience interval for each IC_{50} value.

Table 3

Pharmacokinetics parameters of compounds **7**, **9**, **10**, **11**.[a]

Compd.	$Cmax_{po}$ (ng/mL)	MRT_{po} (h)	AUC_{po} (ng*h/mL)	% F	NET occupancy in rat brain (%)[b]
9	93.8	1.9	197.3	41	14
10	16.2	2.0	44.0	8	12
11	17.2	1.5	38.9	12	28.4
7	47.9	2.1	107.4	20	83.4

[a] 0.1 mg/kg, i.v. and 1.0 mg/kg. p.o.

[b] 3 mg/kg, i.v.

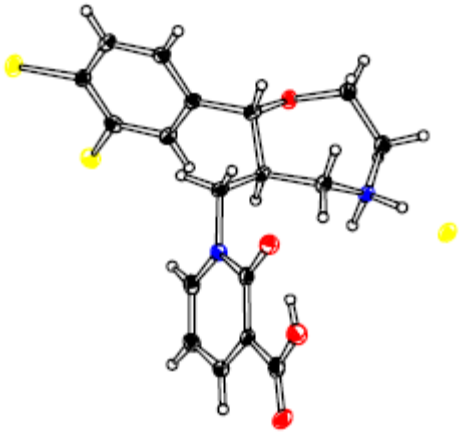


Figure 2. ORTEP drawing of compound **9**, thermal ellipsoids are drawn at 30% probability.

The leak point pressure of compound **9** was measured in rats to evaluate the urethral resistance-increasing effect. The ability of a compound to increase the leak point pressure predicts the compound's efficacy toward an anti-SUI effect. The leak point pressure was measured from 15 min to 4 h after administration. Compound **9** was administered orally at doses of 0.3, 1, 3, and 10 mg/kg. The results showed that, compared with the vehicle group, compound **9** increased the leak point pressure in a dose-dependent manner (Figure 3). The maximum leak point pressure elevation was comparable to that by esreboxetine, which is a centrally acting NRI.[1] Therefore, compound **9** was selected as a promising peripheral-selective NRI for further drug development.

[Figure 3]

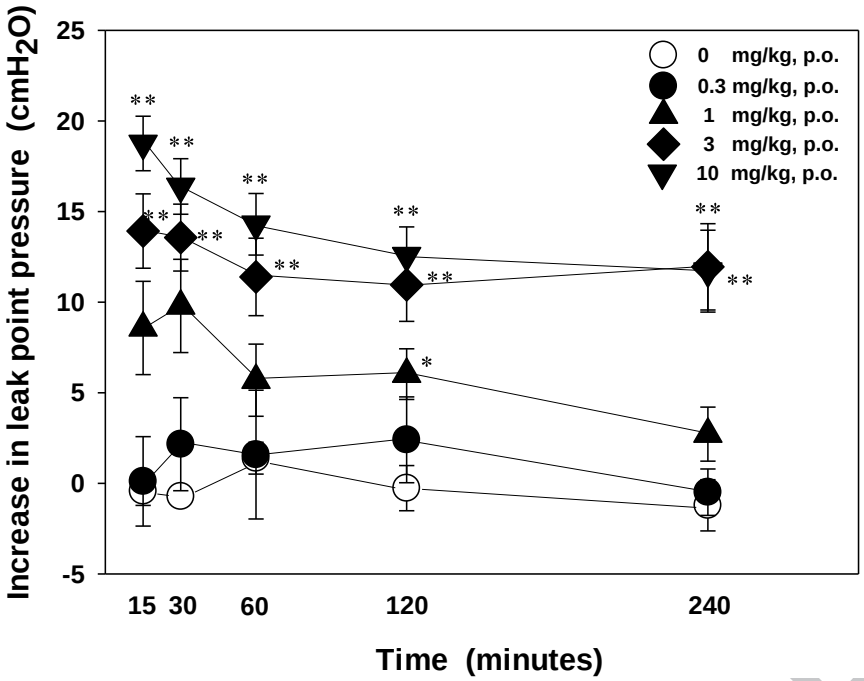


Figure 3. Time course study of the urethral resistance-increasing effects of compound **9** in rats. Data are expressed as the mean ± SEM in 5–12 rats. * $P < 0.05$, ** $P < 0.01$ vs. prevalue by paired ***t***-test with Holm's correction.

4. Conclusion

Novel peripheral-selective NRIs were designed and synthesized, and a compound with high peripheral NET selectivity was evaluated for its effects on urethral function. Cyclization of an amide moiety led to promising lead compound **3** with extremely enhanced NET inhibitory activity, following identification of pyridone as the best terminal ring system. Subsequently, investigation of the role of the acidic group led to the identification of carboxylic acid derivative **9** as a peripheral-selective NET inhibitor with high selectivity against SERT and DAT, low MDR1 efflux ratio and favorable PK profiles. Intramolecular hydrogen bond between the carboxylic acid group and the carbonyl group of pyridone, which was observed in the single-crystal structure, was assumed to contribute to the good PK profile. Compound **9** exhibited a significant increase in urethral resistance in rats in a dose dependent manner, with the maximum effects similar to those of the central acting and clinically effective esreboxetine.

5. Experimental Section

5.1. Chemistry

Melting points were determined with a Yanagimoto melting point apparatus or a Büchi melting point apparatus B-545 and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were obtained on a Varian Ultra-300, or a Bruker DPX-300 spectrometer (300 MHz for ^{1}H NMR, 75 MHz for ^{13}C NMR). Chemical shifts are given in δ values (ppm) using tetramethylsilane as the internal standard. Peak multiplicities are expressed as follows: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublet; ddd, doublet of doublet of doublets; dt, double triplet; td, triple doublet; quin., quintet; brs, broad singlet; m, multiplet. Elemental analyses were carried out by Takeda Analytical Laboratories Ltd. Reactions were followed by TLC on Silica gel 60 F 254 precoated TLC plates (E. Merck) or NH TLC plates (Fuji Silysia Chemical Ltd.). Chromatographic separations were carried out on silica gel 60 (0.063–0.200 or 0.040–0.063 mm, E. Merck) or basic silica gel (Chromatorex® NH, 100–200 mesh, Fuji Silysia Chemical Ltd.) using the indicated eluents. Yields are unoptimized. The HPLC analyses were performed using a Shimadzu UFLC instrument. Elution was done with a gradient of 5–90% solvent B in solvent A (solvent A was 0.1% TFA in water, and solvent B was 0.1% TFA in MeCN) through a L-column 2 ODS (3.0 × 50 mm, 2 μm) column at 1.2 mL min^{-1}. Area % purity was measured at 254 nm. Abbreviations for solvents are the following: Et_2O, diethyl ether; MeOH, methanol; EtOH, ethanol; EtOAc, ethyl acetate; THF, tetrahydrofuran; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; MeCN, acetonitrile; DME, 1,2-dimethoxyethane; $CDCl_3$, chroloform-*d*; Et_3N, triethylamine; Boc_2O, di-*tert*-butyl dicarbonate.

5.1.1. Typical procedure of obtaining compounds 3–16 by deprotection of Boc.

Typical procedure A: To a solution of Boc intermediate in EtOH (0.2 M) was added 14.7 M HCl in EtOH (10–100 eq.) at room temperature. The mixture was stirred at room temperature for 1–24 h and then concentrated *in vacuo*. The residue was crystallized or triturated with EtOAc-hexane to give target compound.

Typical procedure B: To a solution of Boc intermediate was added 4 M HCl in EtOAc (10–100 eq.) at room temperature. The mixture was stirred at room temperature for 1–24 h and then concentrated *in vacuo*. The residue was crystallized or triturated with EtOAc-hexane to give target compound.

5.1.2. (6*R*,7*R*)-*tert*-Butyl 7-(4-chloro-3-fluorophenyl)-6-((4-chlorobutanamido)methyl)-1,4-oxazepane-4-carboxylate (18a). A mixture of **17** (139 mg, 0.39 mmol), 4-chlorobutanoyl chloride (52 μL, 0.47 mmol) and Et_3N (108 μL, 0.78 mmol) in THF (3 mL) was stirred overnight at rt, poured into water and extracted with EtOAc. The combined organic layer was washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexane/EtOAc) to give **18a** (173 mg, 96%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.52 (9H, s), 2.05–2.44 (5H, m), 2.96–3.21 (2H, m), 3.24–3.42 (2H, m), 3.47–3.66 (3H, m), 3.95–4.17 (4H, m), 7.09–7.15 (1H, m), 7.19–7.25 (1H, m), 7.33–7.40 (1H, m). MS *m/z*: 363.1 $[M+H\text{-}Boc]^+$.

5.1.3. (6*R*,7*R*)-*tert*-Butyl 7-(4-chloro-3-fluorophenyl)-6-((2-oxopyrrolidin-1-yl)methyl)-1,4-oxazepane-4-carboxylate (19a). To a solution of **18a** (173 mg, 0.37 mmol) in THF (3 mL) was added 60% NaH in oil (35.9 mg, 0.75 mmol) at 0°C. The reaction mixture was stirred overnight at rt, poured into water and extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexane/EtOAc) to give **19a** (126 mg, 79%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.50 (9H, s), 1.54–1.76 (2H, m), 1.76–2.03 (1H, m), 2.06–2.48 (3H, m), 2.82–3.53 (5H, m), 3.56–3.86 (3H, m), 4.01–4.21 (2H, m), 7.01–7.08 (1H, m), 7.13 (1H, dd, J = 9.8, 1.9 Hz), 7.36 (1H, t, J = 8.0 Hz). MS *m/z*: 427.3 $[M+H]^+$.

5.1.4. 1-(((6*S*,7*R*)-7-(4-Chloro-3-fluorophenyl)-1,4-oxazepan-6-yl)methyl)pyrrolidin-2-one hydrochloride (3). Typical procedure B, Quant., Colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.62–1.89 (2H, m), 1.98–2.30 (2H, m), 2.62 (1H, dd, J = 14.0, 4.9 Hz), 2.80–3.04 (2H, m), 3.06–3.40 (4H, m), 3.71–4.12 (4H, m), 4.42 (1H, d, J = 9.8 Hz), 7.29 (1H, dd, J = 8.1, 1.7 Hz), 7.50 (1H, dd, J = 10.4, 1.7 Hz), 7.63 (1H, t, J = 7.9 Hz), 9.19–9.48 (2H, m). ^{13}C NMR (DMSO-d_6): δ 17.31, 30.15, 40.45, 41.92, 45.15, 46.36, 46.56, 63.27, 82.35, 115.86 (d, J = 21.5 Hz), 119.15 (d, J = 17.6 Hz), 124.80 (d, J = 3.3 Hz), 130.65, 141.97 (d, J = 6.1 Hz), 157.06 (d, J = 247.0 Hz), 174.63. MS *m/z*: 327.2 $[M+H]^+$. HPLC purity 100%. Anal. Calcd for $C_{16}H_{21}Cl_2N_2O_2F \bullet 0.6\ H_2O$: C,51.37; H,5.98; N,7.49. Found: C,51.26; H,5.82; N,7.29.

5.1.5. (6*R*,7*R*)-*tert*-Butyl 7-(4-chloro-3-fluorophenyl)-6-((5-chloropentanamido)methyl)-1,4-oxazepane-4-carboxylate (18b). A mixture of **17** (111 mg, 0.31 mmol), 5-chloropentanoyl chloride (48 μL, 0.37 mmol) and Et_3N (86 μL, 0.62 mmol) in THF (3 mL) was stirred overnight at rt, poured into water and extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexane/EtOAc) to give **18b** (148 mg, 100%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.51 (9H, s), 1.73–1.86 (4H, m), 2.16–2.34 (3H, m), 2.92–3.22 (2H, m), 3.27–3.41 (2H, m), 3.47–3.60 (3H, m), 3.93–4.18 (4H, m), 7.10–7.16 (1H, m), 7.18–7.25 (1H, m), 7.32–7.41 (1H, m). MS *m/z*: 477.3 $[M+H]^+$.

5.1.6. (6*R*,7*R*)-*tert*-Butyl 7-(4-chloro-3-fluorophenyl)-6-((2-oxopiperidin-1-yl)methyl)-1,4-oxazepane-4-carboxylate (19b). To a solution of **18b** (148 mg, 0.31 mmol) in THF (3 mL) was added 60% NaH in oil (29.7 mg, 0.62 mmol) at 0°C. The reaction mixture was stirred overnight at rt, poured into water and extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexane/EtOAc) to give **19b** (110 mg, 80%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.38–1.79 (13H, m), 2.07–2.54 (3H, m), 2.75–3.83 (9H, m), 4.02–4.16 (1H, m), 4.21 (1H, d, J = 9.1 Hz), 7.02–7.08 (1H, m), 7.13 (1H, dd, J = 9.8, 1.9 Hz), 7.36 (1H, t, J = 7.8 Hz). MS *m/z*: 441.4 $[M+H]^+$.

5.1.7. 1-(((6*S*,7*R*)-7-(4-Chloro-3-fluorophenyl)-1,4-oxazepan-6-yl)methyl)piperidin-2-one hydrochloride (5). Typical procedure B, Quant., Colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.42–1.68 (4H, m), 2.01–2.20 (2H, m), 2.65 (1H, dd, J = 13.8, 5.1 Hz), 2.78–3.33 (7H, m), 3.61 (1H, dd, J = 13.6, 9.4 Hz), 3.73–3.88 (1H, m), 3.98–4.11 (1H, m), 4.42 (1H, d, J = 10.2 Hz), 7.24–7.33 (1H, m), 7.51 (1H, dd, J = 10.6, 1.9 Hz), 7.63 (1H, t, J = 8.1 Hz), 9.18–9.63 (2H, m). ^{13}C NMR (DMSO-d_6): δ 20.56, 22.39, 31.88, 40.20, 45.19, 45.87, 46.44, 47.36, 63.52, 82.53, 115.82 (d, J = 21.5 Hz), 119.10 (d, J = 17.6 Hz), 124.81 (d, J = 3.3 Hz), 130.61, 142.08 (d, J = 6.1 Hz), 157.06 (d, J = 247.0 Hz), 169.26. MS *m/z*: 341.1 $[M+H]^+$. HPLC purity 100%.

5.1.8. (6*R*,7*R*)-*tert*-Butyl 7-(4-chloro-3-fluorophenyl)-6-((3-(3-chloropropyl)ureido)methyl)-1,4-oxazepane-4-carboxylate (18c). The mixture of **17** (400 mg, 1.11 mmol) and Et_3N (186 μL, 1.34 mmol) in CH_3CN (3.7 mL) was added of 3-chloropropyl isocyanate (120 μL, 1.17 mmol). The mixture was stirred at room temperature for 3 h, and concentrated *in vacuo* to give **18c** as a colerless oil. This product was subjected to the next reaction without further purification. MS *m/z*: 478.1 $[M+H]^+$.

5.1.9. (6*R*,7*R*)-*tert*-Butyl 7-(4-chloro-3-fluorophenyl)-6-((2-oxotetrahydropyrimidin-1(2*H*)-yl)methyl)-1,4-oxazepane-4-carboxylate (19c). To a solution of **18c** (531 mg, 1.11 mmol) in DMF (22 mL) was added 60% NaH in oil (48.8 mg, 1.22 mmol) at 0°C. The mixture was stirred at 0°C for 2 h and then rt for 3 h. The mixture was quenched with water and extracted with EtOAc. The organic layer was separated, washed with water and brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexane/EtOAc) to give **19c** (563 mg, quant.) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.41–1.91 (11H, m), 2.24–2.49 (1H, m), 2.66–3.90 (11H, m), 3.99–4.17 (1H, m), 4.23 (1H, d, J = 9.1 Hz), 4.55 (1H, brs), 7.07 (1H, d, J = 8.3 Hz), 7.16 (1H, dd, J = 9.8, 1.9 Hz), 7.31–7.41 (1H, m). MS *m/z*: 442.3 $[M+H]^+$.

5.1.10. 1-(((6*S*,7*R*)-7-(4-Chloro-3-fluorophenyl)-1,4-oxazepan-6-yl)methyl)tetrahydropyrimidin-2(1*H*)-one hydrochloride (6). Typical procedure B, 22%, Colorless solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.59–1.77 (2H, m), 2.52–2.61 (2H, m), 2.77–2.88 (2H, m), 2.93–3.39 (6H, m), 3.54–3.65 (1H, m), 3.75–3.87 (1H, m), 3.98–4.10 (1H, m), 4.40 (1H, d, J = 9.1 Hz), 6.34 (1H, brs), 7.30 (1H, d, J = 8.3 Hz), 7.51 (1H, dd, J = 10.6, 1.9 Hz), 7.58–7.66 (1H, m), 9.14–9.47 (2H, m). MS *m/z*: 342.2 $[M+H]^+$. HPLC purity 100%. HPLC purity 100%.

5.1.11. (6*R*,7*R*)-*tert*-Butyl 7-(4-chloro-3-fluorophenyl)-6-((2-oxopyridin-1(2*H*)-yl)methyl)-1,4-oxazepane-4-carboxylate. 60% NaH in oil (45.3 mg, 1.13 mmol) was added to a solution of pyridin-2(1*H*)-one (86 mg, 0.91 mmol) in DME (2 mL) and DMF (1 mL) at room temperature. After being stirred for 5 min, lithium bromide (131 mg, 1.51 mmol) was added to the reaction mixture. After being stirred at room temperature for 10 min, a solution of **20** (331 mg, 0.76 mmol) in DME (2 mL) was

added to the reaction mixture. The mixture was stirred at 80°C overnight. The mixture was diluted with water and extracted with EtOAc. The organic layer was separated, washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexane/EtOAc) to give (6*R*,7*R*)-*tert*-butyl 7-(4-chloro-3-fluorophenyl)-6-((2-oxopyridin-1(2*H*)-yl)methyl)-1,4-oxazepane-4-carboxylate (267 mg, 81%) as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ1.33–1.58 (9H, m), 2.55–2.96 (1H, m), 3.11–3.35 (1H, m), 3.45–3.80 (3H, m), 3.82–4.01 (2H, m), 4.02–4.26 (2H, m), 6.12 (1H, brs), 6.44 (1H, d, *J* = 9.1 Hz), 6.88–7.31 (4H, m), 7.37 (1H, t, *J* = 7.6 Hz), 7.78 (1H, brs). MS *m/z*: 437.2 $[M+H]^+$.

5.1.12. 1-(((6*S*,7*R*)-7-(4-Chloro-3-fluorophenyl)-1,4-oxazepan-6-yl)methyl)pyridin-2(1*H*)-one hydrochloride (7). Typical procedure A, Quant., Colorless solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.04–3.32 (5H, m), 3.64 (1H, dd, *J* = 13.2, 5.3 Hz), 3.77–3.88 (1H, m), 3.91–4.06 (2H, m), 4.54 (1H, d, *J* = 9.4 Hz), 6.17 (1H, td, *J* = 6.7, 1.3 Hz), 6.27–6.33 (1H, m), 7.29–7.40 (2H, m), 7.46 (1H, dd, *J* = 6.6, 1.7 Hz), 7.50–7.61 (2H, m), 9.24 (1H, brs), 9.80 (1H, brs). ^{13}C NMR (DMSO-d_6): δ41.40, 44.68, 46.39, 48.61, 64.11, 82.35, 105.56, 115.95 (d, *J* = 21.5 Hz), 119.07 (d, *J* = 17.6 Hz), 119.62, 125.07 (d, *J* = 3.3 Hz), 130.44, 139.18, 140.10, 141.50 (d, *J* = 6.6 Hz), 156.94 (d, *J* = 246.5 Hz), 161.62. MS *m/z*: 337.1 $[M+H]^+$. $[\alpha]^{25}_D$ +65.1 (c 0.25, MeOH). HPLC purity 100%. Anal. Calcd for $C_{17}H_{19}Cl_2N_2O_2F$•1.2 H_2O: C,51.71; H,5.46; N,7.09. Found: C,51.67; H,5.39; N,6.98.

5.1.13. (6*S*,7*R*)-*tert*-Butyl 7-(4-chloro-3-fluorophenyl)-6-((6-oxopyridazin-1(6*H*)-yl)methyl)-1,4-oxazepane-4-carboxylate. Pyridazin-3(2*H*)-one (41.1 mg, 0.43 mmol) and K_2CO_3 (73.9 mg, 0.53 mmol) were added to a solution of **20** (156 mg, 0.36 mmol) in DMF (4 mL). The mixture was stirred at 80°C for 4 h. The residue was diluted with water and extracted with EtOAc. The organic layer was separated, washed with 0.1 M HCl and brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexane/EtOAc) to give (6*S*,7*R*)-*tert*-butyl 7-(4-chloro-3-fluorophenyl)-6-((6-oxopyridazin-1(6*H*)-yl)methyl)-1,4-oxazepane-4-carboxylate (122 mg, 78%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ1.45 (9H, brs), 2.94 (1H, brs), 3.38–3.57 (1H, m),

3.64–3.80 (4H, m), 3.99–4.15 (3H, m), 4.28 (1H, d, J = 9.1 Hz), 6.72 (1H, dd, J = 9.4, 1.5 Hz), 6.98–7.09 (3H, m), 7.24–7.31 (1H, m), 7.62 (1H, dd, J = 3.8, 1.5 Hz). MS *m/z*: 438.2 $[M+H]^+$.

5.1.14. 2-(((6*S*,7*R*)-7-(4-Chloro-3-fluorophenyl)-1,4-oxazepan-6-yl)methyl)pyridazin-3(2*H*)-one hydrochloride (8). Typical procedure B, 86%, Colorless solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ3.11 (2H, td, J = 8.8, 4.0 Hz), 3.18–3.37 (3H, m), 3.73–3.89 (2H, m), 3.95–4.12 (2H, m), 4.56 (1H, d, J = 10.2 Hz), 6.86 (1H, dd, J = 9.4, 1.9 Hz), 7.27–7.33 (1H, m), 7.36 (1H, dd, J = 9.4, 3.8 Hz), 7.52 (1H, dd, J = 10.6, 1.9 Hz), 7.59 (1H, t, J = 8.1 Hz), 7.88 (1H, dd, J = 4.0, 1.7 Hz), 9.06–9.29 (1H, m), 9.65 (1H, brs). ^{13}C NMR (DMSO-d_6): δ41.80, 44.74, 46.29, 50.37, 63.78, 82.10, 115.97 (d, J = 21.5 Hz), 119.22, 125.09, 129.36, 130.46, 132.15, 137.09, 141.53 (d, J = 6.6 Hz), 156.91 (d, J = 246.5 Hz), 159.84. MS *m/z*: 338.2 $[M+H]^+$. HPLC purity 97%. Anal. Calcd for $C_{16}H_{18}Cl_2N_3O_2F$: C,51.35; H,4.85; N,11.23. Found: C,51.08; H,4.85; N,11.18.

5.1.15. (6*R*,7*R*)-*tert*-Butyl 7-(4-chloro-3-fluorophenyl)-6-((3-(methoxycarbonyl)-2-oxopyridin-1(2*H*)-yl)methyl)-1,4-oxazepane-4-carboxylate (21). K_2CO_3 (2.23 g, 16.1 mmol) and methyl 2-oxo-1,2-dihydropyridine-3-carboxylate (1.48 g, 9.67 mmol) was added to a solution of **20** (3.53 g, 8.06 mmol) in DME (70 mL). The mixture was stirred at 80°C overnight. The mixture was diluted with water and concentrated *in vacuo*. The residue was diluted with water and extracted with EtOAc. The organic layer was separated, washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexane/EtOAc) to give **21** (2.88 g, 72%) as a colorless amorphous powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ1.51 (9H, brs), 2.99 (1H, brs), 3.17 (2H, d, J = 13.6 Hz), 3.43–3.79 (3H, m), 3.87 (3H, s), 3.89–4.12 (4H, m), 6.23 (1H, brs), 7.06–7.24 (2H, m), 7.29–7.44 (1H, m), 7.97–8.16 (1H, m), 8.20 (1H, brs). MS *m/z*: 495.1 $[M+H]^+$.

5.1.16. Methyl 1-(((6*S*,7*R*)-7-(4-chloro-3-fluorophenyl)-1,4-oxazepan-6-yl)methyl)-2-oxo-1,2-dihydropyridine-3-carboxylate hydrochloride (13). Typical procedure B, 89%, Colorless solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ2.99–3.29 (5H, m), 3.63–3.87 (5H, m), 3.90–4.06 (2H, m), 4.51 (1H, d, J = 9.8 Hz), 6.26 (1H, t, J = 7.0 Hz), 7.28 (1H, dd, J = 8.3, 1.5 Hz), 7.48 (1H, dd, J = 10.6, 1.9 Hz), 7.52–

7.60 (1H, m), 7.74 (1H, dd, *J* = 6.6, 2.1 Hz), 7.94 (1H, dd, *J* = 7.2, 1.9 Hz), 8.62–9.34 (2H, m). MS *m/z*: 395.3 $[M+H]^+$. HPLC purity 96%. Anal. Calcd for $C_{19}H_{21}Cl_2N_2O_4F$: C,52.91; H,4.91; N,6.50. Found: C,52.93; H,4.98; N,6.36.

5.1.17. 1-(((6*R*,7*R*)-4-(*tert*-Butoxycarbonyl)-7-(4-chloro-3-fluorophenyl)-1,4-oxazepan-6-yl)methyl)-2-oxo-1,2-dihydropyridine-3-carboxylic acid. 2 M NaOH (44.9 mL, 89.8 mmol) was added to a solution of **21** (22.2 g, 44.9 mmol) in EtOH (220 mL). The mixture was stirred at 30 °C overnight. The mixture was concentrated *in vacuo*. The residue was diluted with Et_2O and extracted with water. The organic layer was removed, and the aqueous layer was acidified with 1 M HCl and extracted with water. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated *in vacuo* to give 1-(((6*R*,7*R*)-4-(*tert*-butoxycarbonyl)-7-(4-chloro-3-fluorophenyl)-1,4-oxazepan-6-yl)methyl)-2-oxo-1,2-dihydropyridine-3-carboxylic acid (22.1 g, quant.) as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ1.52 (9H, s), 2.85 (1H, brs), 3.06–3.27 (2H, m), 3.65 (1H, t, *J* = 11.0 Hz), 3.77–4.14 (6H, m), 6.54 (1H, t, *J* = 6.4 Hz), 7.18 (2H, dd, *J* = 19.6, 8.3 Hz), 7.42 (1H, t, *J* = 7.6 Hz), 8.45 (2H, d, *J* = 7.2 Hz), 14.13 (1H, brs). MS *m/z*: 481.1 $[M+H]^+$.

5.1.18. 1-(((6*S*,7*R*)-7-(4-Chloro-3-fluorophenyl)-1,4-oxazepan-6-yl)methyl)-2-oxo-1,2-dihydropyridine-3-carboxylic acid hydrochloride (9). 4 M HCl in AcOEt (112 mL, 449 mmol) was added to a solution of 1-(((6*R*,7*R*)-4-(*tert*-butoxycarbonyl)-7-(4-chloro-3-fluorophenyl)-1,4-oxazepan-6-yl)methyl)-2-oxo-1,2-dihydropyridine-3-carboxylic acid (21.6 g, 44.9 mmol) in EtOAc (20 mL). The mixture was stirred at room temperature for 2 h, and then concentrated *in vacuo*. The residue was crystallized from 10% water in MeCN to give **9** (15.9 g, 85%) as white crystals. ^{1}H NMR (300 MHz, $CDCl_3$) δ3.10–3.30 (5H, m), 3.77–3.88 (1H, m), 3.89–3.98 (1H, m), 4.03 (1H, dt, *J* = 13.9, 4.4 Hz), 4.16 (1H, dd, *J* = 13.4, 7.7 Hz), 4.58 (1H, d, *J* = 9.1 Hz), 6.65 (1H, t, *J* = 6.8 Hz), 7.26 (1H, dd, *J* = 8.3, 1.5 Hz), 7.46 (1H, dd, *J* = 10.6, 1.9 Hz), 7.50–7.58 (1H, m), 8.07 (1H, dd, *J* = 6.8, 1.9 Hz), 8.26 (1H, dd, *J* = 7.4, 2.1 Hz), 9.45 (2H, brs), 14.19 (1H, brs). ^{13}C NMR (DMSO-d_6): δ40.60, 44.93, 46.55, 49.98, 63.92, 82.12, 108.44, 115.87, 116.16, 116.72, 119.30 (d, *J* = 17.1 Hz), 125.10 (d, *J* = 3.3 Hz), 130.45, 140.90

(d, J = 6.1 Hz), 145.15 (d, J = 14.9 Hz), 156.84 (d, J = 247.0 Hz), 163.64, 164.41. MS *m/z*: 381.1 $[M+H]^+$. Anal. Calcd for $C_{18}H_{19}Cl_2N_2O_4F$: C,51.81; H,4.59; N,6.71. Found: C,52.09; H,4.58; N,6.71. $[\alpha]^{25}_D$ +96.2 (c 0.25, MeOH). HPLC purity 100%.

5.1.19. (6*R*,7*R*)-*tert*-Butyl 7-(4-chloro-3-fluorophenyl)-6-((3-cyano-2-oxopyridin-1(2*H*)-yl)methyl)-1,4-oxazepane-4-carboxylate (22a). 60% NaH in oil (274 mg, 6.85 mmol) was added to a solution of 2-oxo-1,2-dihydropyridine-3-carbonitrile (658 mg, 5.48 mmol) in DME (10 mL) and DMF (5 mL) at 0°C. After being stirred at 0°C for 20 min, lithium bromide (793 mg, 9.13 mmol) was added to the reaction mixture. After being stirred at room temperature for 1.5 h, a solution of **20** (2.00 g, 4.57 mmol) in DME (10 mL) was added to the reaction mixture. The mixture was stirred at 60°C overnight. The mixture was concentrated *in vacuo*. The residue was diluted with water and extracted with EtOAc. The organic layer was separated, washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexane/EtOAc) to give **22a** (1.76 g, 83%) as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.52 (9H, s), 2.90 (1H, brs), 3.06–3.28 (2H, m), 3.55–3.75 (2H, m), 3.81–4.12 (5H, m), 6.26 (1H, t, J = 6.2 Hz), 7.18 (2H, d, J = 7.9 Hz), 7.41 (1H, t, J = 7.9 Hz), 7.76 (1H, d, J = 6.4 Hz), 8.31 (1H, d, J = 5.3 Hz). MS *m/z*: 362.2 $[M+H\text{-Boc}]^+$.

5.1.20 (6*R*,7*R*)-*tert*-Butyl 7-(4-chloro-3-fluorophenyl)-6-((2-oxo-3-(1*H*-tetrazol-5-yl)pyridin-1(2*H*)-yl)methyl)-1,4-oxazepane-4-carboxylate. Azidotributyltin (356 μL, 1.30 mmol) was added to a solution of **22a** (200 mg, 0.43 mmol) in toluene (5 mL). The mixture was stirred at 100°C overnight, and then concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexane/EtOAc) to give (6*R*,7*R*)-*tert*-butyl 7-(4-chloro-3-fluorophenyl)-6-((2-oxo-3-(1*H*-tetrazol-5-yl)pyridin-1(2*H*)-yl)methyl)-1,4-oxazepane-4-carboxylate (87 mg, 40%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.56 (9H, s), 2.99 (1H, brs), 3.16 (1H, t, J = 11.5 Hz), 3.30 (1H, dd, J = 15.1, 4.9 Hz), 3.64 (1H, t, J = 11.3 Hz), 3.89–4.22 (5H, m), 4.45 (1H, d, J = 12.5 Hz), 6.62 (1H, t, J = 6.6 Hz), 6.75 (1H, t, J = 7.2 Hz), 6.94 (1H, d, J = 7.9 Hz), 7.38 (1H, d, J = 9.8 Hz), 8.68 (2H, d, J = 7.2 Hz), 14.45 (1H, brs). MS *m/z*: 505.3 $[M+H]^+$.

5.1.21 1-(((6*S*,7*R*)-7-(4-Chloro-3-fluorophenyl)-1,4-oxazepan-6-yl)methyl)-3-(1*H*-tetrazol-5-yl)pyridin-2(1*H*)-one hydrochloride (10). Typical procedure B, 77%, Colorless solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ3.10–3.42 (5H, m), 3.79–3.97 (2H, m), 4.00–4.15 (2H, m), 4.58 (1H, d, *J* = 9.8 Hz), 6.50 (1H, t, *J* = 7.0 Hz), 7.27–7.33 (1H, m), 7.44–7.54 (2H, m), 7.85 (1H, dd, *J* = 6.8, 1.9 Hz), 8.33 (1H, dd, *J* = 7.2, 1.9 Hz), 9.46 (1H, brs). ^{13}C NMR (DMSO-d_6): δ41.19, 44.95, 46.49, 49.42, 64.20, 82.58, 106.19, 113.85, 115.97 (d, *J* = 21.5 Hz), 119.20 (d, *J* = 17.6 Hz), 125.01 (d, *J* = 3.3 Hz), 130.41, 140.59 (2 C), 141.22 (d, *J* = 6.1 Hz), 142.55, 152.74 (d, *J* = 374.1 Hz), 158.91. MS *m/z*: 405.1 $[M+H]^+$. $[\alpha]^{25}_D$ +90.9 (c 0.25, MeOH). HPLC purity 100%. Anal. Calcd for $C_{18}H_{19}Cl_2N_6O_2F\bullet 2H_2O$: C,45.29; H,4.86; N,17.61. Found: C,45.48; H,4.72; N,17.53.

5.1.22. (6*R*,7*R*)-*tert*-Butyl 6-((3-carbamothioyl-2-oxopyridin-1(2*H*)-yl)methyl)-7-(4-chloro-3-fluorophenyl)-1,4-oxazepane-4-carboxylate. *O*,*O*'-Diethyl dithiophosphate (189 µL, 1.13 mmol) was added to a solution of **22a** (348 mg, 0.75 mmol) in 4 M HCl in AcOEt (5 mL, 20.0 mmol). The mixture was stirred at room temperature overnight and then concentrated *in vacuo*. The residue was diluted with sat. $NaHCO_3$ aq. (10 mL) and EtOAc (10 mL). The mixture was added Boc_2O (210 µL, 0.90 mmol). The mixture was stirred at room temperature for 4 h. The mixture was diluted with water and extracted with EtOAc. The organic layer was separated, washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexane/EtOAc) to give (6*R*,7*R*)-*tert*-butyl 6-((3-carbamothioyl-2-oxopyridin-1(2*H*)-yl)methyl)-7-(4-chloro-3-fluorophenyl)-1,4-oxazepane-4-carboxylate (225 mg, 60%) as an yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ1.52 (9H, brs), 2.82 (1H, brs), 3.05–3.23 (2H, m), 3.57–3.82 (2H, m), 3.87–4.13 (5H, m), 6.44 (1H, t, *J* = 6.6 Hz), 7.07–7.28 (2H, m), 7.35–7.44 (1H, m), 7.90 (1H, brs), 8.35 (1H, brs), 9.12 (1H, d, *J* = 7.2 Hz), 11.39 (1H, brs). MS *m/z*: 496.1 $[M+H]^+$.

5.1.23. (6*R*,7*R*)-*tert*-Butyl 6-((1,4-dioxane-2-carboxamido)methyl)-7-(4-chloro-3-fluorophenyl)-1,4-oxazepane-4-carboxylate. K_2CO_3 (94 mg, 0.68 mmol) and MeI (142 µL, 2.27 mmol) was added to a solution of (6*R*,7*R*)-*tert*-Butyl 6-((3-carbamothioyl-2-oxopyridin-1(2*H*)-yl)methyl)-7-(4-chloro-3-

fluorophenyl)-1,4-oxazepane-4-carboxylate (225 mg, 0.45 mmol) in acetone (4 mL). The mixture was stirred at room temperature overnight and then concentrated *in vacuo*. The residue was diluted with water and extracted with EtOAc. The organic layer was separated, washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The half amount of residue in toluene (5 mL) was added acetohydrazide (33.3 mg, 0.45 mmol). The mixture was stirred at 120°C overnight and then diluted with water and extracted with EtOAc. The organic layer was separated, washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexane/EtOAc) to give (6*R*,7*R*)-*tert*-Butyl 6-((1,4-dioxane-2-carboxamido)methyl)-7-(4-chloro-3-fluorophenyl)-1,4-oxazepane-4-carboxylate (92 mg, 79%) as a colorless gum. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.34–1.58 (9H, m), 2.45 (3H, s), 2.90 (1H, brs), 3.10–3.33 (2H, m), 3.55–4.15 (7H, m), 6.41 (1H, t, *J* = 5.3 Hz), 6.96–7.25 (2H, m), 7.30–7.43 (1H, m), 8.02–8.17 (1H, m), 8.37 (1H, d, *J* = 7.2 Hz), 12.42 (1H, brs). MS *m/z*: 518.1 $[M+H]^+$.

5.1.24. 1-(((6*S*,7*R*)-7-(4-Chloro-3-fluorophenyl)-1,4-oxazepan-6-yl)methyl)-3-(5-methyl-4*H*-1,2,4-triazol-3-yl)pyridin-2(1*H*)-one hydrochloride (12). Typical procedure B, 87%, Colorless solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ2.50 (3H, s), 3.08–3.39 (5H, m), 3.79–3.98 (2H, m), 4.02–4.20 (2H, m), 4.59 (1H, d, *J* = 9.4 Hz), 6.51 (1H, t, *J* = 7.0 Hz), 7.25–7.33 (1H, m), 7.44–7.55 (2H, m), 7.88 (1H, dd, *J* = 6.4, 1.9 Hz), 8.30 (1H, dd, *J* = 7.2, 1.9 Hz), 9.44 (1H, brs), 9.96 (1H, brs). MS *m/z*: 418.1 $[M+H]^+$. HPLC purity 100%.

5.1.25. (6*R*,7*R*)-*tert*-Butyl 7-(4-chloro-3-fluorophenyl)-6-((2-oxo-3-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)pyridin-1(2*H*)-yl)methyl)-1,4-oxazepane-4-carboxylate. $NaHCO_3$ (1.94 g, 23.1 mmol) and hydroxylammonium chloride (1.60 g, 23.1 mmol) was added to a solution of **22a** (1.33 g, 2.88 mmol) in DMSO (15 mL) and the mixture was stirred at 80°C for 8 h. The mixture was quenched with water and extracted with EtOAc. The organic layer was separated, washed with brine, dried over Na_2SO_4 and concentrated *in vacuo* to give a colorless oil. To the oil in THF (15 mL) was added 1,8-diazabicyclo[5.4.0]undec-7-ene (431 μL, 2.88 mmol) and di(1*H*-imidazol-1-yl)methanone (700 mg,

4.32 mmol). The mixture was stirred at 70°C for 5.5 h. The mixture was concentrated *in vacuo*. The residue was diluted with 1 M HCl aq. and extracted with EtOAc. The organic layer was separated, washed with water and brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexane/EtOAc) to give (6*R*,7*R*)-*tert*-butyl 7-(4-chloro-3-fluorophenyl)-6-((2-oxo-3-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)pyridin-1(2*H*)-yl)methyl)-1,4-oxazepane-4-carboxylate (1.46 g, 97%) as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.54 (9H, s), 2.79–2.94 (1H, m), 3.06–3.20 (1H, m), 3.25 (1H, dd, *J* = 14.5, 4.3 Hz), 3.54–3.68 (1H, m), 3.76 (1H, t, *J* = 11.7 Hz), 3.95–4.18 (4H, m), 4.45 (1H, d, *J* = 12.8 Hz), 6.48 (1H, t, *J* = 6.8 Hz), 7.08 (1H, d, *J* = 8.3 Hz), 7.17 (1H, t, *J* = 7.7 Hz), 7.34 (1H, d, *J* = 9.8 Hz), 8.27 (1H, d, *J* = 5.7 Hz), 8.66 (1H, d, *J* = 5.7 Hz), 10.44 (1H, brs). MS *m/z*: 421.1 $[M+H\text{-}Boc]^+$.

5.1.26. 3-(1-(((6*S*,7*R*)-7-(4-Chloro-3-fluorophenyl)-1,4-oxazepan-6-yl)methyl)-2-oxo-1,2-dihydropyridin-3-yl)-1,2,4-oxadiazol-5(4*H*)-one hydrochloride (11). Typical procedure A, 96%, Colorless crystal. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.05–3.33 (5H, m), 3.74–3.89 (2H, m), 3.96–4.11 (2H, m), 4.55 (1H, d, *J* = 9.8 Hz), 6.41 (1H, t, *J* = 7.0 Hz), 7.26–7.34 (1H, m), 7.49 (1H, dd, *J* = 10.6, 1.9 Hz), 7.52–7.60 (1H, m), 7.84 (1H, dd, *J* = 6.6, 2.1 Hz), 7.91 (1H, dd, *J* = 7.2, 1.9 Hz), 9.13 (1H, brs), 9.61 (1H, brs), 12.22 (1H, brs). ^{13}C NMR (DMSO-d_6): δ 40.95, 44.86, 46.55, 49.27, 64.11, 82.29, 105.62, 113.27, 116.03 (d, *J* = 21.5 Hz), 119.25 (d, *J* = 18.0 Hz), 125.05, 130.49, 142.26 (d, *J* = 198.6 Hz), 141.19 (d, *J* = 6.1 Hz), 155.15, 155.28, 158.44, 158.55, 159.33. MS *m/z*: 421.1 $[M+H]^+$. Anal. Calcd for $C_{19}H_{19}Cl_2N_4O_4F$•0.2 H_2O: C,49.51; H,4.24; N,12.16. Found: C,49.92; H,4.40; N,11.88. $[\alpha]^{25}_D$ +113.9 (c 0.25, MeOH). HPLC purity 100%.

Compounds **14**–**16** were prepared in same manner using **20**.

5.1.27. (6*R*,7*R*)-*tert*-Butyl 7-(4-chloro-3-fluorophenyl)-6-((4-cyano-2-oxopyridin-1(2*H*)-yl)methyl)-1,4-oxazepane-4-carboxylate (22b). 26%, Colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.52 (9H, s), 2.80 (1H, brs), 3.07–3.26 (2H, m), 3.53–3.74 (2H, m), 3.80–3.95 (2H, m), 3.96–4.15 (3H, m),

6.27 (1H, d, *J* = 6.4 Hz), 6.75 (1H, s), 6.99–7.25 (2H, m), 7.41 (1H, t, *J* = 7.8 Hz), 8.16 (1H, d, *J* = 6.4 Hz). MS *m/z*: 362.3 $[M+H\text{-Boc}]^+$.

5.1.28. (6*R*,7*R*)-*tert*-Butyl 7-(4-chloro-3-fluorophenyl)-6-((2-oxo-4-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)pyridin-1(2*H*)-yl)methyl)-1,4-oxazepane-4-carboxylate. 58%, Colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.55 (9H, s), 2.64 (1H, brs), 2.99–3.43 (2H, m), 3.45–4.10 (7H, m), 6.47 (1H, brs), 6.83 (1H, d, *J* = 7.2 Hz), 7.03 (1H, brs), 7.19 (2H, brs), 8.30 (1H, brs), 12.48 (1H, brs). MS *m/z*: 421.3 $[M+H\text{-Boc}]^+$.

5.1.29. 3-(1-(((6*S*,7*R*)-7-(4-Chloro-3-fluorophenyl)-1,4-oxazepan-6-yl)methyl)-2-oxo-1,2-dihydropyridin-4-yl)-1,2,4-oxadiazol-5(4*H*)-one hydrochloride (14). Typical procedure B, 84%, Colorless solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.00–3.33 (5H, m), 3.66 (1H, dd, *J* = 13.4, 5.5 Hz), 3.76–3.88 (1H, m), 3.93–4.05 (2H, m), 4.53 (1H, d, *J* = 9.8 Hz), 6.51 (1H, dd, *J* = 6.8, 1.9 Hz), 6.77 (1H, d, *J* = 1.9 Hz), 7.27–7.33 (1H, m), 7.51 (1H, dd, *J* = 10.6, 1.9 Hz), 7.54–7.61 (1H, m), 7.64 (1H, d, *J* = 7.2 Hz), 8.99 (1H, brs), 9.43 (1H, brs), 13.21 (1H, brs). MS *m/z*: 421.3 $[M+H]^+$.

5.1.30. (6*R*,7*R*)-*tert*-Butyl 7-(4-chloro-3-fluorophenyl)-6-((5-cyano-2-oxopyridin-1(2*H*)-yl)methyl)-1,4-oxazepane-4-carboxylate (22c). 59%, Colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.52 (9H, s), 2.70–2.86 (1H, m), 3.04–3.26 (2H, m), 3.56–4.15 (7H, m), 6.45 (1H, d, *J* = 9.5 Hz), 7.09–7.25 (2H, m), 7.31–7.47 (2H, m), 8.59–8.71 (1H, m). MS *m/z*: 362.1 $[M+H\text{-Boc}]^+$.

5.1.31. (6*R*,7*R*)-*tert*-Butyl 7-(4-chloro-3-fluorophenyl)-6-((2-oxo-5-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)pyridin-1(2*H*)-yl)methyl)-1,4-oxazepane-4-carboxylate. 79%, Colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.58 (9H, s), 2.50–2.73 (1H, m), 3.36–3.92 (7H, m), 3.99–4.30 (2H, m), 6.59 (1H, d, *J* = 9.8 Hz), 7.08–7.24 (2H, m), 7.36–7.48 (1H, m), 7.70–7.83 (1H, m), 8.41–8.58 (1H, m), 10.01–10.47 (1H, m). MS *m/z*: 421.1 $[M+H\text{-Boc}]^+$.

5.1.32. 3-(1-(((6*S*,7*R*)-7-(4-Chloro-3-fluorophenyl)-1,4-oxazepan-6-yl)methyl)-6-oxo-1,6-dihydropyridin-3-yl)-1,2,4-oxadiazol-5(4*H*)-one hydrochloride (15). Typical procedure B, Quant.,

Colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.02–3.50 (5H, m), 3.56–3.67 (1H, m), 3.78–3.90 (1H, m), 3.95–4.08 (2H, m), 4.60 (1H, d, *J* = 10.2 Hz), 6.42–6.50 (1H, m), 7.27 (1H, dd, *J* = 8.3, 1.5 Hz), 7.44–7.58 (2H, m), 7.63 (1H, dd, *J* = 9.4, 2.6 Hz), 8.06–8.23 (1H, m), 8.94–9.16 (1H, m), 9.45–9.62 (1H, m), 12.89–13.08 (1H, m). ^{13}C NMR (DMSO-d_6): δ 45.17, 46.41, 49.61, 59.74, 63.88, 82.41, 102.24, 116.04 (d, *J* = 21.5 Hz), 119.29 (d, *J* = 17.6 Hz), 120.20, 125.09, 130.52, 135.62, 139.94, 141.15 (d, *J* = 6.6 Hz), 154.65, 156.92 (d, *J* = 247.0 Hz), 159.51, 161.09. MS *m/z*: 421.1 $[M+H]^+$. HPLC purity 98%.

5.1.33. (6*R*,7*R*)-*tert*-Butyl 7-(4-chloro-3-fluorophenyl)-6-((6-cyano-2-oxopyridin-1(2*H*)-yl)methyl)-1,4-oxazepane-4-carboxylate (22d). 17%, Colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.42–1.53 (9H, m), 2.88–3.17 (1H, m), 3.32–3.83 (5H, m), 3.96–4.38 (4H, m), 6.53 (1H, d, *J* = 6.8 Hz), 6.58–6.70 (1H, m), 7.00–7.10 (2H, m), 7.10–7.21 (1H, m), 7.27–7.36 (1H, m). MS *m/z*: 362.0 [M+H-Boc]$^+$.

5.1.34. (6*R*,7*R*)-*tert*-Butyl 7-(4-chloro-3-fluorophenyl)-6-((2-oxo-6-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)pyridin-1(2*H*)-yl)methyl)-1,4-oxazepane-4-carboxylate. 20%, Colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.52 (10H, s), 2.22–2.42 (1H, m), 3.14–3.33 (1H, m), 3.38–3.51 (1H, m), 3.70–4.01 (2H, m), 4.08–4.25 (3H, m), 4.27–4.37 (1H, m), 6.49 (1H, d, *J* = 6.8 Hz), 6.67–6.79 (1H, m), 6.95–7.14 (2H, m), 7.33–7.46 (2H, m). MS *m/z*: 421.1 [M+H-Boc]$^+$.

5.1.35. 3-(1-(((6*S*,7*R*)-7-(4-Chloro-3-fluorophenyl)-1,4-oxazepan-6-yl)methyl)-6-oxo-1,6-dihydropyridin-2-yl)-1,2,4-oxadiazol-5(4*H*)-one hydrochloride (16). Typical procedure B, 96%, Colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.69–3.26 (5H, m), 3.55 (1H, dd, *J* = 14.2, 4.4 Hz), 3.77–3.91 (1H, m), 3.98–4.10 (1H, m), 4.29–4.43 (1H, m), 4.48 (1H, d, *J* = 9.8 Hz), 6.58–6.67 (2H, m), 7.16–7.24 (1H, m), 7.37–7.45 (1H, m), 7.48–7.59 (2H, m), 8.87–9.28 (2H, m). MS *m/z*: 421.1 $[M+H]^+$. HPLC purity 98%.

5.1.36. *N*-(((6*S*,7*R*)-7-(3,4-dichlorophenyl)-1,4-oxazepan-6-yl)methyl)-*N*-methylacetamide hydrochloride (4). Colorless solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.75–1.97 (3H, m), 2.15–2.30

(1H, m), 2.63–2.96 (7H, m), 3.13–3.25 (2H, m), 3.42–3.58 (1H, m), 3.82–4.05 (1H, m), 4.22 (1H, dd, *J* = 13.0, 8.9 Hz), 7.35 (1H, td, *J* = 7.9, 2.3 Hz), 7.55–7.67 (2H, m). MS *m/z*: 331.3 $[M+H]^+$. HPLC purity 96%.

5.1.37. X-ray structure analysis

Crystal data for compound **9**: $C_{18}H_{19}ClFN_2O_4^+ \cdot Cl^-$, *MW* = 417.26; crystal size, 0.13 x 0.09 x 0.09 mm; colorless, block; orthorhombic, space group $P2_12_12_1$, *a* = 6.93199(13) Å, *b* = 14.1848(3) Å, *c* = 18.7907(4) Å, $\alpha = \beta = \gamma = 90°$, *V* = 1847.67(6) $Å^3$, *Z* = 4, *Dx* = 1.500 g/cm^3, *T* = 100 K, μ = 3.502 mm^{-1}, λ = 1.54187 Å, R_1 = 0.035, wR_2 = 0.071, Flack Parameter[13] = 0.036(17).

All measurements were made on a Rigaku R-AXIS RAPID-191R diffractometer using graphite monochromated Cu-Kα radiation. The structure was solved by direct methods with SIR2008[14] and was refined using full-matrix least-squares on F^2 with SHELXL-97.[15] All non-H atoms were refined with anisotropic displacement parameters.

CCDC 1456967 for compound **9** contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via http://www.ccdc.cam.ac.uk/Community/Requestastructure/Pages/DataRequest.aspx?.

5.2. Monoamine reuptake inhibition

5.2.1. Preparation of human monoamine expressing cell

Human serotonin transporter cDNA was amplified from human brain cDNA library by PCR, and inserted into pCRII-TOPO vector (manufactured by Invitrogen). The base sequence was confirmed and modified, and subcloned to pcDNA3.1 vector (manufactured by Invitrogen), whereby a human serotonin transporter expression plasmid was constructed.

Human norepinephrine transporter cDNA was purchased from Invitrogen, and the base sequence was confirm and modified, and subcloned to pcDNA3.1 vector, whereby a human norepinephrine transporter expression plasmid was constructed.

Human dopamine transporter expression plasmid was prepared by following procedure. SRαpromoter contained in pTB1411 described in JP-A-H5-076385 was cleaved with restriction enzyme HindIII (manufactured by TAKARA BIO INC.), blunt-ended, further cleaved with restriction enzyme EcoRI (manufactured by TAKARA BIO INC.), and fragmented. On the other hand, pCI vector was cleaved with restriction enzyme BglII (manufactured by TAKARA BIO INC.), blunt-ended with T4DNA polymerase, and further cleaved with restriction enzyme EcoRI (manufactured by TAKARA BIO INC.). Into this site was inserted a SRαpromoter fragment to give pCI-SRa. Then, pCI-SRa was cleaved with restriction enzyme ClaI (manufactured by TAKARA BIO INC.) and blunt–ended. Into this site was inserted a 1.63Kb fragment obtained by cleaving pGFP-C1 (manufactured by TOYOBO) with restriction enzyme Bsu36I (manufactured by Daiichi Pure Chemicals Co., Ltd.) followed by blunt-ending, whereby pMSRαneo was prepared. Human dopamine transporter cDNA was amplified from human substantia nigra cDNA library by PCR, and inserted into the pCRII vector (manufactured by Invitrogen). The base sequence was confirmed, modified and subcloned to pMSRαneo, whereby a human dopamine transporter expression plasmid was constructed.

The monoamine transporter expression plasmids thus prepared were introduced into CHO-K1 cells using FuGENE6 (manufactured by Roche Diagnostics) and according to the attached protocol, whereby each expressing cell was established.

5.2.2. Human serotonin transporter inhibitory activity

CHO cells stably expressing a human serotonin transporter were used for the measurement of human serotonin transporter inhibitory activity. Unless particularly indicated, these CHO cells were cultured in a Ham/F12 medium (Invitrogen) containing 10% fetal bovine serum (MOREGATE). The cultured cells that reached almost confluent were rinsed with PBS (Invitrogen), detached with Trypsin/EDTA (Invitrogen), and collected by a centrifugal operation. The obtained cells were counted, and diluted to 3×10^5 cells per 1 mL medium, the mixture was dispensed to a 96 well white plate (Corning) at 100 μL per well, and cultured overnight in a CO_2 incubator. Then, an assay buffer (126 mM NaCl, 4.95 mM KCl, 1.26 mM KH_2PO_4, 1.26 mM $MgSO_4$, 10 mM HEPES, 2.32 mM $CaCl_2$, 5.52 mM Glucose, 0.5%

BSA) was prepared, the medium in the cell plate was removed and the assay buffer was added by 80 μL. A test compound was diluted with the assay buffer to a 10-fold concentration of the final concentration, and the mixture was dispensed to a 96 well polypropylene plate. The diluted test compound was dispensed to the cell plate by 10 μL. [^{3}H]-5-Hydroxytryptamine (GE Healthcare) was diluted with the assay buffer to 200 nM, and the mixture was dispensed to the cell plate by 10 μL. At 20 min from [^{3}H]-5-hydroxytryptamine addition, the assay buffer was removed by suction, and the plate was washed twice with 150 μL of PBS (Invitrogen) per well. Microscinti 20 (PerkinElmer) was dispensed to each well by 100 μL, and the mixture was stirred for about 30 min. The radioactivity was measured by TopCount (PerkinElmer). The inhibitory activity of each compound (10 μM) was calculated as a relative activity value based on the inhibitory activity of 10 μM Paroxetine (serotonin transporter inhibitor) as 100%. IC_{50} and 95% confidence interval were calculated by XLfit (n=2).

5.2.3. Human norepinephrine transporter inhibitory activity

CHO cells stably expressing human norepinephrine transporter were used for the measurement of human norepinephrine transporter inhibitory activity. Unless otherwise indicated, these CHO cells were cultured in Ham/F12 medium (Invitrogen) containing 10% fetal bovine serum (MOREGATE). The cultured cells that reached almost confluent were rinsed with PBS (Invitrogen), detached with Trypsin/EDTA (Invitrogen), and collected by a centrifugal operation. The obtained cells were counted, and diluted to 3×10^5 cells per 1 mL medium, and the mixture was dispensed to a 96 well white plate (Corning) at 100 μL per well, and cultured overnight in a CO_2 incubator. Then, an assay buffer (126 mM NaCl, 4.95 mM KCl, 1.26 mM KH_2PO_4, 1.26 mM $MgSO_4$, 10 mM HEPES, 2.32 mM $CaCl_2$, 5.52 mM Glucose, 0.5% BSA) was prepared, the medium in the cell plate was removed and the assay buffer was added by 80 μL. A test compound was diluted with the assay buffer to a 10-fold concentration of the final concentration, and the mixture was dispensed to a 96 well polypropylene plate. The diluted test compound was dispensed to the cell plate by 10 μL. [^{3}H]-Norepinephrine (GE Healthcare) was diluted with the assay buffer to 200 nM, and the mixture was dispensed to the cell plate by 10 μL. At 45 min from [^{3}H]-norepinephrine addition, the assay buffer was removed by suction, and the plate was washed

twice with 150 μL of PBS (Invitrogen) per well. Microscinti 20 (PerkinElmer) was dispensed to each well by 100 μL, and the mixture was stirred for about 30 min. The radioactivity was measured by TopCount (PerkinElmer). The inhibitory activity of each compound (10 μM) was calculated as a relative activity value based on the inhibitory activity of 10 μM DMI (norepinephrine transporter inhibitor) as 100%. IC_{50} and 95% confidence interval were calculated by XLfit (n=2).

5.2.4. Human dopamine transporter inhibitory activity

CHO cells stably expressing human dopamine transporter were used for the measurement of human dopamine transporter inhibitory activity. Unless otherwise indicated, these CHO cells were cultured in Ham/F12 medium (Invitrogen) containing 10% fetal bovine serum (MOREGATE). One day before the assay, the cultured cells that reached almost confluent were rinsed with PBS (Invitrogen), detached with Trypsin/EDTA (Invitrogen), and collected by a centrifugal operation. The obtained cells were counted, and diluted to 3×10^5 cells per 1 mL medium, and the mixture was dispensed to a 96 well white plate (Corning) at 100 μL per well, and cultured overnight in a CO_2 incubator. On the day of the test, an assay buffer (126 mM NaCl, 4.95 mM KCl, 1.26 mM KH_2PO_4, 1.26 mM $MgSO_4$, 10 mM HEPES, 2.32 mM $CaCl_2$, 5.52 mM Glucose, 0.5% BSA) was prepared, the medium in the cell plate was removed and the assay buffer was added by 80 μL. A test compound was diluted with the assay buffer to a 10-fold concentration of the final concentration, and the mixture was dispensed to a 96 well polypropylene plate. The diluted test compound was dispensed to the cell plate by 10 μL. [^{3}H]-Dopamine (GE Healthcare) was diluted with the assay buffer to 200 nM, cold dopamine was diluted to 10 μM, and the mixture was dispensed to the cell plate by 10 μL. At 60 min from [^{3}H]-dopamine addition, the assay buffer was removed by suction, and the plate was washed twice with 150 μL of PBS (Invitrogen) per well. Microscinti 20 (PerkinElmer) was dispensed to each well by 100 μL, and the mixture was stirred for about 30 min. The radioactivity was measured by TopCount (PerkinElmer). The inhibitory activity of each compound (10 μM) was calculated as a relative activity value based on the inhibitory activity of 100 μM Nomifensine (dopamine transporter inhibitor) as 100%. IC_{50} and 95% confidence interval were calculated by XLfit (n=2).

5.3. NET ex vivo binding assay in rat brain cortex

Adults female rats of Sprague-Dawley strain (CLEA Japan) were studied using experimental protocols approved by Takeda's Experimental Animal Care and Use Committee. CNS penetrating property was measured by investigating occupation rate of NET in the brain cortex after drug administration. After rats were anesthetized with intraperitoneal injection of urethane (Wako Pure Chemical Industries, Ltd.), the test compound was intravenously injected. Twenty minutes after the injection, the cortex in the left brain was taken off and used for *ex vivo* binding study with [^{3}H]-nisoxetine (NEN). Fifteen times volume of assay buffer [50 mmol/L Tris Cl (pH 7.5), 125 mmol/L NaCl, 5 mmol/L KCl] as the taken cortex weight was added, homogenized, and plasma membrane fraction was prepared so that protein concentration of the fraction was 4 mg/mL. Six hundred μL of the membrane fraction, 200 μL of the assay buffer and 100 μL of [^{3}H]-nisoxetine solution (25 nmol/L) were mixed, and 100 μL of desipramine (10 μmol/L) or assay buffer was added, and the mixture was incubated at room temperature for 1 hour. The mixture was suctioned through GF/B glass filter (Whatman), and the glass filter was washed with assay buffer. Radio activity with the glass filter was measured using scintillation counter (Aloka, LSC6100). The NET occupation rate was calculated with specific binding of the vehicle group as 100%.

5.4. Evaluation of urethral resistance-increasing effects in rat

After rats were anesthetized with isoflurane (Abbott Japan) inhalation, the spinal cord was transected at the T8-T9 level after laminectomy. After closing the wound site on the back, the urinary bladder was exposed through an abdominal incision. Two polyethylene catheters (PE-100; Intramedic, Becton Dickson and Company) with a fire-flared tip were inserted into the bladder from the dome and secured with a ligature for bladder filling and pressure recording, and then, the abdomen was closed. Rats were placed in Bollman restraint cage (KN-326/3, Natsume Seisakusho). A pressure transducer (DX-100; Nihon Koden) connected to an amplifier (1257; NEC), an analog-to-digital converter (MP-100 BIOPAK systems) and a computer equipped with a data converting software (AcqKnowledge; BIOPAK systems) was used to record the intravesical pressure. Data was acquired at the rate of 100 Hz.

The bladder was then filled continuously with saline (0.1 mL/sec) containing Evans blue dye (Wako Pure Chemical) *via* the catheter using infusion pump. The intravesical pressure was raised by infusion until leakage of the Evans blue solution was observed from the urethral orifice. The peak of recorded intravesical pressure was recognized as the lowest intravesical pressure inducing urinary leakage, and the value was referred as the leak point pressure (LPP). As urine leakage theoretically occurs when intravesical pressure exceeds total urethral resistance, LPP is considered to show the total urethral resistance. Measurements were repeated, and the mean of last three LPPs was calculated. The vehicle and the test compound were orally administered, and LPP was again measured 15, 30, 60, 120 and 240 minutes after the administration. Data were analyzed with paired ***t***-test, and P values <0.05 was considered to be significant.

5.5. Permeability study across human MDR1 stably expressing cells

The transcellular transport was examined using human multidrug resistance 1 (MDR1) stably expressing LLC-PK1 cells. After incubating the test compound (10 μmol/L) for 2 h at 37°C, the apical to basolateral permeation coefficient value and the basolateral to apical permeation coefficient value as well as ratio of the permeation coefficient (efflux ratio) were calculated with determining the concentration of test compound by LC/MS/MS. The apparent permeability (Papp) of test compounds in the receiver wells was determined and the efflux ratio (ER) for MDR1 membrane permeability test was calculated using the following equation:

Efflux ratio = Papp(BtoA)/Papp(AtoB); (1)

where Papp(AtoB) and Papp(BtoA) are ratio of permeation amount in A to B and B to A, respectively.

5.6. Phamacokinetic study in rats

The test compound was administered intravenously (0.1 mg/kg) or orally (1 mg/kg) to 8 week old male Crl:CD(SD)(IGS) rats (cassette dosing, n=3 each). The blood samples were collected 5, 10, 15, 30 min, 1, 2, 4 and 8 h after intravenous administration and 15, 30 min, 1, 2, 4 and 8 h after oral administration. The blood samples were centrifuged to prepare the plasma samples. The plasma samples

were pretreated with protein precipitation method. The concentrations of compounds were determined by LC/MS/MS.

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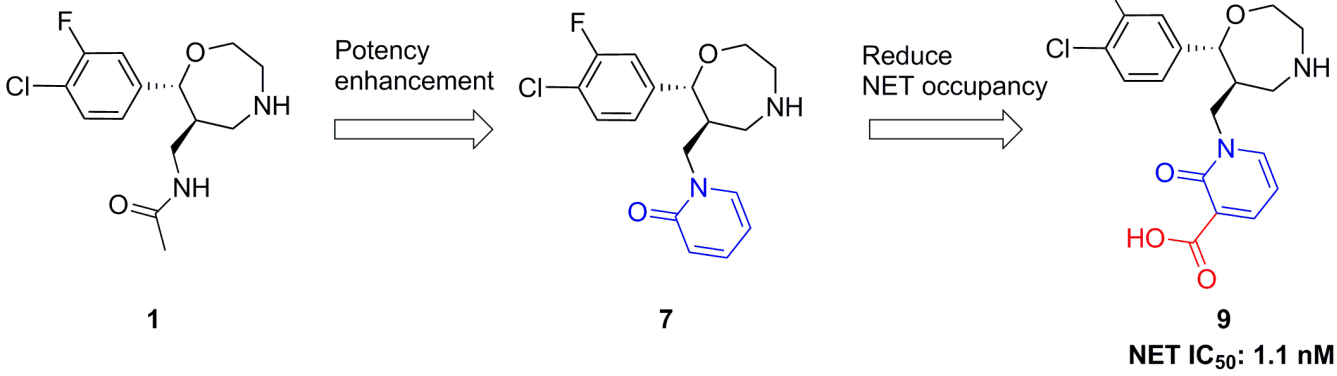
Graphical abstract



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NET IC_{50}: 1.1 nM